Exploration of the opioidergic system in the olfactory bulbectomized rat model of depression

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Declaration

I hereby declare that the work presented in this thesis was carried out in accordance with the regulations of the National University of Ireland, Galway. The research is original and entirely my own with the following assistance:

- **Chapter 2:** Systematic review and meta-analysis were performed and assisted by Prof. John P. Kelly.
- **Chapter 3:** Surgical operation was assisted by Dr. Hayley Doherty, Dr. Zara McAleavey, Ms. Patricia Calcagno and Ms. Morgane Clarke. Behavioural testing was assisted by Dr. Natalie Ann DeSanctis, Ms. Patricia Calcagno and Ms. Morgane Clarke. Chronic dosing was performed by Dr. Hayley Doherty.
- **Chapter 4:** Surgical operation was assisted by Prof. John P. Kelly, Ms. Patricia Calcagno and Ms. Morgane Clarke. Behavioural testing was assisted by Ms. Mehnaz Ferdousi and Ms. Patricia Calcagno. Count and automated forced swim scoring was performed by Ms. Stephanie Bourke. qRT-PCR was performed by Ms. Patricia Calcagno.
- **Chapter 5:** Surgical operation was assisted by Dr. Natalie Ann DeSanctis, Ms. Patricia Calcagno and Ms. Sonali Gupta. Behavioural testing was assisted by Dr. Natalie Ann DeSanctis, Dr. Zara McAleavey and Ms. Patricia Calcagno.

The thesis or any part thereof has not been submitted to the National University of Ireland, Galway, or any other institution in connection with any other academic award. Any views expressed herein are those of the author.

Signed: _____ Date: _____

Abstract

In recent years, there has been renewed interest in targeting the central opioid system as a novel strategy for the treatment of psychiatric disorders such as depression, due to its involvement in the regulation of mood, stress, social functioning and cognition. The use of preclinical modelling to investigate the role of the opioid system in depression is integral to further our understanding of the potential value of this system as a therapeutic strategy in the treatment of depression. The olfactory bulbectomised (OB) rat has a number of the physiological, endocrine, behavioural, and neuroinflammatory features which are relevant to clinical depression, many of which are attenuated with chronic, but not acute, antidepressant treatment. As such, the aim of this project was to investigate the role of the central opioid system in the OB rat model of depression. The reliability and reproducibility of the behavioural responses in the OB rat model were investigated via a meta-analysis, followed by characterisation of its behavioural effects with particular respect to social cognition and housing. The pharmacological impact on the behavioural deficits in the OB rat were investigated with chronic administration of conventional antidepressants, as well as opioid modulating drugs. Alterations to the central opioid system following exposure to acute (forced swim) and chronic (OB) stressors, alone and in combination, were also investigated.

The first results Chapter was a systematic review and meta-analysis of the OB rat which confirmed its value as a model of depression, with robust and reproducible behavioural responses across laboratories in a range of behaviours, in particular the hyperactivity in the open field (OF), the most commonly used behavioural endpoint. The second results chapter was a characterisation of the OB rat model, evaluating the effects of housing and examining for the first time the 3-chamber test of social cognition in the model. There was no effect of housing either singly or in pairs on the behaviour in the OF in sham-operated rats, but the characteristic hyperactivity in the OB rat was blunted when two OB rats were housed together. OB rats were also shown to habituate to the OF on re-exposure, with exposure to prior behavioural tests also affecting the response in OB animals. With regards to the 3-chamber sociability test, OB rats spent less time exploring the novel conspecific animal in the 3-chamber sociability test, with a distinct habituation to the test arena over time being observed, regardless of housing, when compared to sham-operated counterparts; these deficits in social cognitive functioning was not attenuated by chronic administration with the

antidepressants desipramine and fluoxetine. Chronic administration with opioid modulating drugs, either that target a single receptor, or using combination regimens, were shown to have differential effects on behaviour but overall also failed to attenuate this social cognitive deficit. Exposure to acute (forced swim) and chronic (olfactory bulbectomy) stressors in the rat caused reductions in expression primarily to the kappa opioid receptor (KOP) and delta opioid receptor (DOP) systems, with the primary regions affected being the hippocampus and prefrontal cortex; two regions involved in the processing of emotion and cognitive function.

In conclusion, the OB rat is a robust and well-established animal model, with its behavioural responses shown to be easily replicated across laboratories, and mirror a number of symptoms in MDD. The opioid system has been shown to be altered after removal of the olfactory bulbs, with the KOP and DOP systems being the primary subsystems effected. The impact of an acute stressor, via a forced swim, was also shown to further alter the opioid system in OB rats, delineating that the opioid system in this model is sensitive to additional stressors. The deficit in social cognitive functioning in OB rats in this project contributes a novel feature to the model; a feature which resembles that of the social cognitive dysfunction seen in MDD. This deficit of social cognition in the OB model was refractory to normalisation with both conventional antidepressants, and opioid modulating compounds, suggesting that it is a deeply engrained deficit in the model, representing an important addition to the behavioural alterations associated with this model.

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“I shall be telling this with a sigh

Somewhere ages and ages hence:

Two roads diverged in a wood, and I–

I took the one less traveled by,

And that has made all the difference.”

The Road not Taken by Robert Frost, 1915

List of Publications and Conference Proceedings

Peer-reviewed book publications

McHugh KL & Kelly JP. (2018). Modulation of the central opioid system as an antidepressant target in rodent models. In: Progress in Brain Research. **Chapter 2**, Vol.239, pp 49-87. Editor: O'Mara S. (DOI: 10.1016/bs.pbr.2018.07.003).

Peer-reviewed/published conference proceedings/abstracts

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McHugh KL, Clonan C, Curtin R, Duffy M, Flynn K, Keenan A, Kelly C, McCaffrey O, O'Flaherty M, Kelly JP. (2019). A systematic review and meta-analysis of the olfactory bulbectomized (OB) rat model of depression. *Presented at the annual summer meeting for the British Association for Psychopharmacology (BAP), Manchester, England.* Poster.

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McHugh KL, Calcagno P, Clarke M, Eyerman D, Sanchez C, Roche M, Finn DP, Kelly JP. (2017). A comparative assessment of single versus paired-housing on behavioural responses in the olfactory bulbectomised (OB) rat model of depression. *Presented at*

the Neuroscience Ireland Annual General Meeting 2017. Galway, Ireland. August 2017. Poster

McHugh KL, Doherty H, Deaver D, Roche M, Finn DP, Kelly JP. (2016). An assessment of social cognition in the olfactory bulbectomized rat model of depression. *Presented at the annual British Pharmacological Society (BPS) conference, Pharmacology 2016, London, England. December 2016.* Poster

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McHugh KL & Kelly JP. (2017). The role of the central opioidergic system in the olfactory bulbectomised rat model of depression – 2 Year Review. *Presented at Science Foundation Ireland Bi-annual review of Strategic Partnership Programme ALKS-TCD-NUI Collaboration, Trinity College Dublin, Ireland. December 2017.* Oral

McHugh KL, Calcagno P, Clarke M, Eyerman D, Sanchez C, Roche M, Finn DP, Kelly JP. (2017). A comparative assessment of single versus paired-housing on behavioural responses in the olfactory bulbectomised (OB) rat model of depression. *Presented at the Royal Academy of Medicine in Ireland (RAMI) Section of Biomedical Sciences Annual Meeting 2017. Dublin, Ireland. June 2017.* Poster

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McHugh KL & Kelly JP. (2016). Exploration of the central opioidergic system in the olfactory bulbectomised rat model of depression. *Presented at Departmental Research Day, Galway, Ireland. December, 2016.* Oral

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Morgane Clarke, **McHugh KL** & Kelly JP. (2016). Exploration of the central opioidergic system in the olfactory bulbectomized rat model of depression. *Presented at ALKS Site "Face-To-Face", Alkermes Inc., Athlone, Westmeath, Ireland. March 2016.* Oral

McHugh KL & Kelly JP. (2015). Exploration of the central opioidergic system in the olfactory bulbectomized rat model of depression. *Presented at annual Departmental Research Day, Galway, Ireland. December 2015.* Oral

Table of Contents

List of Figures

List of Tables

List of Abbreviations

(+)-S-20499	((+)-8(4[*N*-(5-methoxychroman-3yl)-8-azaspirol [4,5] decane-7,9-dione)
[(-)-BPAP]	(-)-1-(benzofuran-2-yl)-2-propylaminopentane
5'-AMN	5'-(2-aminomethyl) naltrindole
5'MABN	*N*-((Naltrindol-5-yl) methyl) pentanimidamide
5-HIAA	5-Hydroxyindoleacetic Acid
5-HT	5-Hydroxytryptamine/Serotonin
8-OH-DPAT	8-hydroxy-2-(di-n-propylamino)tetralin
A_{2A}	Adenosine 2A receptor
AChE	Acetylcholinesterase inhibitor
ACTH	Adrenocorticotropic hormone
ADL5859	*N*,*N*-diethyl-4-(5-hydroxyspiro[chromene-2,4'-piperidine]-4-yl) benzamide
ANOVA	Analysis of Variance
ANTI	5'-acetamidinoethylnaltrindole
Anti-SMAP	Serotonin-modulating anticonsolidation protein
AOB	Accessory olfactory bulb
APA	American Psychiatric Association
ATPM-ET	(-)-3-*N*-ethylaminothiazolo[5,4-b]-*N*-cyclopropylmethylmorphinan hydrochloride
AZD2327	4-[(*R*)-(3-aminophenyl)[4-(4-fluorobenzyl)-piperazin-1-yl]methyl]-*N*,*N*-diethylbenzamide
B.C	before Christ
BA24	Brodmanns area 24
BA9	Brodmanns area 9
BDI	Becks Depression Inventory
BDNF	Brain-derived neurotrophic factor
BIIE0246	(*S*)-N^2-[[1-[2-[4-[(*R*,*S*)-5,11-dihydro-6(6*h*)-oxodibenz[*b*,*e*]azepin-11-yl]-1-piperazinyl]-2-oxoethyl]cyclopentyl]acetyl]-*N*-[2-[1,2-dihydro-3,5(4*H*)-dioxo-1,2-diphenyl-3-*H*-1,2,4-triazol-4-yl]ethyl]-argininamid
BIP-1	5-(((4-benzo[α]isothiazol-3-yl) piperazin-1-yl)methyl)-6-chloroindolin-2-one

BNST	Bed nucleus of the stria terminalis
BNTX	(E)-7-Benzylidenenaltrexone
BW3373U86	(+)-[1(S^*),2α,5β]-4-[[2,5-dimethyl-4-(2-propenyl)-1-piperazinyl] (3-hydroxyphenyl)methyl]-*N*,*N*-diethyl-benzamide dihydrochloride
Ca^{2+}	Calcium ion
CAMARADES	Collaborative Approach to Meta-Analysis and Review of Animal Data from Experimental Studies
cAMP	Cyclic adenosine monophosphate
CB_1	Cannabinoid receptor type 1
CD3+	Cluster of differentiation 3 cells
CGP 36742	3-Aminopropyl-*n*-butyl-phosphinic acid
CGP 51176	3-amino-2(*R*)-hydroxypropyl-cyclohexylmethyl-phosphinic acid
ChAT	Choline acetyltransferase
CI	Confidence Interval
CMS	Chronic Mild Stress
CNS	Central Nervous System
CRF	Corticotropin-releasing factor
CRF_1	Corticotropin releasing factor receptor type 1
CRH	Corticotropin-releasing hormone
CRH_1	Corticotropin-releasing hormone receptor type 1
CSF	Cerebrospinal fluid
CTAP	$_D$-Phe-Cys-Tyr-$_D$-Trp-Arg-Thr-Pen-Thr-NH_2
CTOP	$_D$-Phe-Cys-Tyr-$_D$-Trp-Orn-Thr-Pen-Thr-NH_2
D_1	Dopamine receptor 1
D_2	Dopamine receptor 2
DA	Dopamine
DADLE	[$_D$-Ala^2,$_D$-Leu^5]-Enkephalin
DAGO	Tyr-$_D$-Ala-Gly-MePhe-Gly-ol-enkephalin
DALY	Disability-adjusted life-year
DAMGO	[$_D$-Ala^2, *N*-$MePhe^4$, Gly-ol]-enkephalin
DAT	Dopamine transporter
DG	Dentate gyrus

DHM	[^{3}H]dihydromorphine
DIPPA	2-(3,4-dichlorophenyl)-*N*-methyl-*N*-[(1*S*)-1-(3-isothiocyanatophenyl)-2-(1-pyrrolidinyl)ethyl] acetamide
DMSO	Dimethyl Sulfoxide
DOP	δ-opioid receptor
DOV 216,303	(+/−)-1-(3,4-dichlorophenyl)-3-azabicyclo-[3.1.0]hexane hydrochloride
DPDPE	[$_{D}$-Pen^{2},$_{D}$-Pen ^{5}]enkephalin
DRN	Dorsal raphe nuclei or nucleus
DSI	Desipramine
DSLET	$_{D}$-ser^{2}-Leu^{5}-enkephalin-Thr^{6}
DSM-V	Diagnostic and Statistical Manual of Mental Disorders (V)
DSP-1053	6-(2-{4-[4-Bromo-3-(2-methoxyethoxy)benzyl]piperidin-1-yl}ethyl)-2,3-dihydro-4*H*-chromen-4-one benzenesulfonate
DYN	Dynorphin
E-2078	[*N*-methyl-Tyr1, *N*-methyl-Arg7, $_{D}$-Leu8] dynorphin A(1-8) ethylamide
ECT	Electroconvulsive Therapy
ELISA	Enzyme-linked immunosorbant assay
EMD 386088	5-chloro-2-methyl-3-(1,2,3,6-tetrahydro-4-pyridinyl)-1*H*-indole hydrochloride
ENK	Enkephalin
EPA	Ethyl-eicosapentaenoate
EPM	Elevated Plus Maze
ERK	Extra-cellular signal-regulated kinase
F	Female
F-98214-TA	(*S*)-(-)-4-[(3-fluorophenoxy)-phenyl]methyl-piperidine
FLX	Fluoxetine
FST	Forced Swim Test
GABA	γ-aminobutyric acid
GABA$_{A}$	Gamma aminobutyric acid type A receptor
GABA$_{B}$	Gamma aminobutyric acid type B receptor
GBD	Global Burden of Disease
GDP	Guanosine diphosphate

GNTI	6'guanidinyl-17-(cyclopropylmethyl)-6,7-dehydro-4,5α-epoxy-3,14-dihydroxy-6,7-2′,3′-indolomorphinan dihydrochloride
GPCR	G-protein-coupled receptor
GTP	Guanosine triphosphate
H_2O	Water
HAM-D	Hamilton-D rating scale
HCL	Hydrochloric acid
HE	Hyperemotionality
HMG coA	3-hydroxy-3-methyl-glutaryl-coenzyme A
HPA	Hypothalamic-pituitary-adrenal
HPRA	Health Products Regulatory Authority
HSE	Health Service Executive
ICD	International Classification of Diseases
ICI 118,551	(2*R*,3*R*)-rel-3-isopropylamino-1-(7-methylindan-4-yloxy)-butan-2-ol hydrochloride
IDO	Indoleamine 2,3-dioxygenase
IFN-α	Interferon-α
IFN-γ	Interferon-γ
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-10	Interleukin-10
ILAR	Institute for Laboratory Animal Research
ISHH	In situ hybridization histochemistry
JDTic	(3*R*)-7-hydroxy-N-[(2*S*)-1-[(3*R*,4*R*)-4-(3-hydroxyphenyl)-3,4-dimethylpiperidin-1-yl]-3-methylbutan-2-yl]-1,2,3,4 tetrahydroisoquinoline-3-carboxamide
JNJ-31020028	*N*-(4-{4-[2-(diethylamino)-2-oxo-1-phenylethyl]piperazin-1-yl}-3-fluorophenyl)-2-pyridin-3-ylbenzamide
JNK	c-Jun N-terminal kinase
JOM-13	Tyr-c[$_D$-Cys-Phe-$_D$-Pen]OH
K^+	Potassium ion channel
KNT-127	1,2,3,4,4a,5,12,12a-octahydro-2-methyl-4aβ,1β ([1,2]benzenomethano)-2,6-diazanaphthacene-12aβ,17-diol

KO	Knock-out
KOP	κ-opioid receptor
LC	Locus coeruleus
LE	Long-Evans
LH	Learned Helplessness
LPS	Lipopolysaccharide
LSD	Lysergic acid diethylamide
LY2444296	(*S*)-3-fluoro-4-(4-((2*(*3-fluorophenyl)pyrrolidin-1-yl)methyl)phenoxy) benzamide
LY2456302	(*S*)-3-fluoro-4-(4-((2(3,5-dimethylphenyl)pyrrolidin-1 yl)methyl)phenoxy) benzamide
M	Male
MADRS	Montgomery-Asberg Depression Rating Scale
MAOI	Monoamine Oxidase Inhibitors
MAPK	Mitogen-activated protein kinase
***m*-CF_3-PhSe**	*m*-trifluoromethyl-diphenyl diselenide
MCH	Melanin converting hormone
MCL0042	1-[2-(4-fluorophenyl)-2-(4-methylpiperazin-1-yl)ethyl]-4-[4-(1-naphthyl)butyl]piperazine
mCPP	meta-Chlorophenylpiperazine
MDD	Major depressive disorder
MDE	Major depressive episode
MFB	Medial forebrain bundle
MGlu	Metabotropic glutamate
MGS0039	1*R*,2*R*,3*R*,5*R*,6*R*)-2-Amino-3-(3,4-dichlorobenzyloxy)-6-fluorobicyclo[3.1.0]hexane-2,6-dicarboxylic acid
MK-801	Dizocilpine
MOB	Main olfactory bulb
MOP	μ-opioid receptor
MPEP	2-Methyl-6-(phenylethynyl)pyridine
MRI	Magnetic resonance imaging
MTEP	3-((2-Methyl-4-thiazolyl)ethynyl)pyridine
MWM	Morris Water Maze
NA	Noradrenaline

nAChRs	Nicotinic acetylcholine receptors
NC3R	National Centre for Replacement, Refinement and Reduction of Animals in Research
NET	Noradrenaline transporter
NIH 11082	(-)-(1R,5R,9R)-5,9-dimethyl-2'-hydroxy-2-(6-hydroxyhexyl)-6,7-benzomorphan hydrochloride
NIH	National Institute of Health
NIHY	Novelty-induced Hypophagia
NK-2	Neurokinin-2
NMDA	N-methyl-D-aspartate
NOR	Novel Object Recognition
NorBNI	Nor-binaltorphimine dihydrochloride
NOS	Nitric oxidise synthase
NS	Not stated
NUIG	National University of Ireland, Galway
NPY	Neuropeptide Y
OB	Olfactory Bulbectomy
OF	Open Field
***Oprd1*$^{-/-}$**	DOP knockout mice
***Oprk1*$^{-/-}$**	KOP knockout mice
***Oprm1*$^{-/-}$**	MOP knockout mice
p38	p38 stress kinase
PA	Passive Avoidance
PDYN	Pro-dynorphin
PENK	Pre-proenkephalin
PET	Positron Emission Tomography
POMC	Pro-opiomelanocortin
PVN	Hypothalamic paraventricular nucleus
PYY$^{3-36}$	Peptide YY 3-36
qRT-PCR	Real-time quantitative polymerase chain reaction
R278995/CRA0450	1-[8-(2,4-dichlorophenyl)-2-methylquinolin-4-yl]-1, 2, 3, 6-tetrahydropyridine-4-carboxamide
RB 38A	(*R*,*S*)HONH-CO-CH_2-CH($CH_2C_6H_5$)-CONH-CH($CH_2C_6H_5$)-COOH

RB 38B	(*S*,*S*)HONH-CO-CH_2-CH($CH_2C_6H_5$)-CONH-CH($CH_2C_6H_5$)-COOH
RB101	(*N*-[(*R*,*S*)-2-benzyl-3-[(*S*)(2-amino-4-methyl-thio)-butyldithio]-1-oxopropyl]-l-phenylalanine benzyl ester
RDC2944	RDC002944-01
REM	Rapid Eye Movement
RLB	Radioligand binding
RS 67333	1-(4-amino-5-chloro-2-methoxy-phenyl)-3-(1-butyl-4-piperidinyl)-1-propa-none
RSPCA	Royal Society for the Prevention of Cruelty to Animals
rtPA	Recombinant tissue plasminogen activator
SA-4503	1-(3,4-dimethoxyphenethyl)-4-(3-phenylpropyl)piperazine dihydrochloride
SB-269970	(*R*)-1-[3-hydroxyphenyl)sulfonyl]-2-[2-(4-methyl-1-piperidinyl)ethyl]pyrrolidine
SD	Standard Deviation
SDS	Social Defeat Stress
SEM	Standard Error of the Mean
SERT	Serotonin transporter
SI	Social Interaction
SNC80	(+)-4-[(α*R*)-α-((2*S*,5*R*)-4-Allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-N,Ndiethylbenzamide
SNK	Student-Newman Keuls
SNRI	Noradrenaline Reuptake Inhibitors
SPD	Sprague Dawley
SPT	Sucrose/Saccharin Preference Test
SSA-426	(*S*)-2-((4-(1*H*-Indol-3-yl)-5,6-dihydropyridin-1(2*H*)-yl)methyl)-8-methyl-2,3-dihydro-[1,4]dioxino-[2,3-*f*]quinoline
SSR149415	(2*S*,4*R*)-1-[5-chloro-1-[(2,4-dimethoxyphenyl)sulfonyl]-3-(2-methoxyphenyl)-2-oxo-2,3-dihydro-1*H*-indol-3-yl]-4-hydroxy-*N*,*N*-dimethyl-2-pyrrolidinecarboxamide
SSRI	Serotonin Reuptake Inhibitors
SYRCLE	SYstematic Review Centre for Laboratory Animal Experimentation

TASP02333278	(4*R*)-1-[5-chloro-1-[(2,4-dimethoxyphenyl)sulfonyl]-3-(2-methoxyphenyl)-2-oxo-2,3-dihydro-1*H*-indol-3-yl]-4-fluoro-*N*,*N*-dimethyl-*L*-prolinamide
TASP0382650	*N*-(cis-4-{[6-(dimethylamino)-2-methylpyrimidin-4-yl]amino}cyclohexyl)-3,4,5-trifluorobenzamide
TASP0390325	2-[2-(3-chloro-4-fluorophenyl)-6-[3-(morpholin-4-yl)propoxy]-4-oxopyrido[2,3-d]pyrimidin-3(4*H*)-yl]-*N*-isopropylacetamide hydrochloride
TASP0489838	3-methoxy-*N*-[1-({7-[(2-methoxyethoxy)methyl]naphthalen-2-yl}methyl)piperidin-4-yl]benzamid
TCA	Tricyclic antidepressants
THC	Tetrahydrocannabinol
TNF-α	Tumor-necrosis factor alpha
TPH	Tryptophan hydroxylase
TRIM	1-(2-Trifluoromethylphenyl) imidazole
TST	Tail Suspension Test
U-69593	(+)-(5α,7α,8β)-*N*-Methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-benzeneacetamide
UFP-502	H-Dmt-Tic-NH-CH(2)-Bid
V1b	Vassopressin 1b receptor
VIP	Vasoactive intestinal peptide receptor
W	Wistar
WAY-100635	*N*-[2-]4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-(2 pyridinyl)cyclohexanecarboxamide
WAY-163909	(7*bR*, 10*aR*)-1,2,3,4,8,9,10,10*a*-octahydro-7*bH*-cyclopenta-[*b*][1,4]diazepino[6,7,1hi]indole
WHO	World Health Organisation
WKY	Wistar Kyoto
WS-50030	7-{4-[3-(1*H*-inden-3-yl)propyl]piperazin-1-yl}-1,3-benzoxazol-2(3*H*)-one
WT	Wild-type
Y_1	Neuropeptide Y receptor type 1
Y_5	Neuropeptide Y receptor type 5
YLD	Years of life living with a disability

YLL	Years of life lost due to mortality
ZM 241385	4-(2-[7-amino-2-(2-furyl) [1,2,4]-triazolo[2,3-a][1,3,5]triazin-5-yl amino]ethyl) phenol
$ZnSO_4$	Zinc Sulphate
β-FNA	β-Funaltrexamine

Chapter 1:

General Introduction

1.1 Depression

Depression is a progressive mood disorder that affects an individual's thoughts and emotions in a harmful and/or damaging manner, coinciding with a culmination of negative outcomes to that individual's behaviour and cognition. Symptoms include feelings of despair, guilt, sadness, low self-worth, self-harm, and suicide, as well as altered physiological responses such as sleep disturbance, fatigue, loss of motivation and weight fluctuation (Diagnostic and Statistical Manual of Mental Disorders-V (DSM-V)). The World Health Organisation (WHO) states that depression is estimated to effect 322 million people worldwide, which is around 4.4% of the world's population (Figure 1.1) (WHO, 2017).

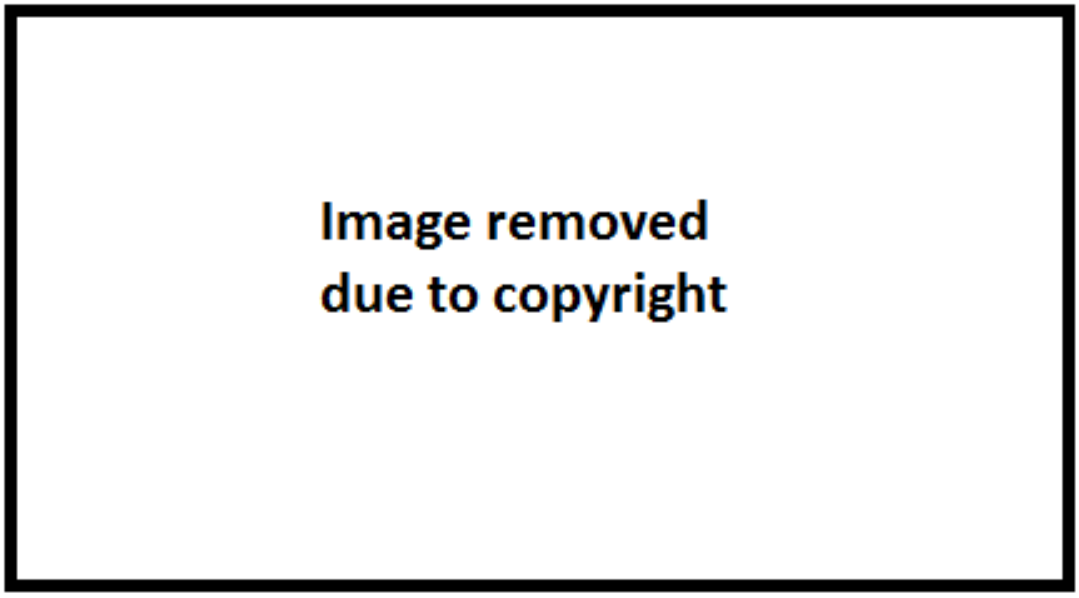


Figure 1.1 Cases of depressive disorder by millions per WHO defined region worldwide. (WHO, 2017). Data taken from the Global Burden of Disease (GBD) Study 2015, WHO.

Depression is believed to be a leading contributor, if not *the* single largest leading cause, of disability worldwide (WHO, 2017). The WHO conducts a global burden of disease study each year, using the metrics of disability-adjusted life-year (DALY) which is calculated as years of life lost due to mortality (YLLs) and years of life living with a disability (YLDs) (Vos et al., 2016). As such, one DALY equates to one year of healthy life that has been lost (Vos et al., 2016). In Ireland in 2017, the number of DALYs lost due to depressive disorders was estimated at 33,810.28 DALY's or 3.03% of the total DALYs worldwide (GBD, 2017, Institute of Health Metrics and Evaluation, 2017). Between the years 1990 and 2007, the global number of YLDs due

to depressive disorders increased by 33.4%, so that depressive disorders were the third leading cause of disability in 2007 (James et al., 2018). Similarly, between the years 2007 and 2017, a further increase of 14.3% in the global number of YLDs attributed to depressive disorders was seen, with depressive disorders once again ranked at the third highest contributor of disability worldwide (James et al., 2018). As such, it is clear to see that depression has been on the rise in the last few decades. Having said this, it is important to note the fact that between these timeframes, the Diagnostic and Statistical Manual of Mental Disorders (DSM) which is used by health practitioners to diagnose psychiatric disorders such as depression, would have brought out two new revised editions, which would have reclassified depression as a psychiatric illness with more specific endpoints whilst also covering a wider range of severities within the disorder and as such may have produced a higher criteria inclusion ratio. This must be taken into account with regards with huge increase in individuals diagnosed with the disease within this timeframe, in conjunction with the fact that mental illness as a whole was beginning to be regarded as more socially accepted by society, with the previous stigma's associated with depression being more liberally rejected and allowing individual's suffering from the disease to more willingly and actively seek help.

In 2015, depression accounted for 4.3% of the global burden of disease (WHO, 2013). It is thought that this global increase in depressive disorders is due to an increase in the general population worldwide and as such an increase in the incidence of the disease (WHO, 2017, James et al., 2018). In addition, this increase in depressive disorders is also thought to be due to demographic changes with the increase in the aging population, where the incidence of depression worldwide peaks at ages 55-60 years old (Figure 1.2) (WHO, 2017, James et al., 2018). This is indeed interesting as in Ireland, depression is seen to peak at a much younger age where adversity in early life or at a young age is regarded as a promienent risk factor (Rochford et al., 2018). According to the Health Service Executive (HSE), the inpatient admissions data for individuals aged under 18 years old showed that 37% of admissions were for depression (HSE, 2014). This global peak in depression at 55-60 years old may be explained by *aging* itself, with issues of retirement, loss of loved ones, serious illness or medical problems such as chronic pain but most importantly loneliness all seen to be attributing factors. Indeed, the central statistics office in Ireland highlighted that in 2014 in Ireland suicide rates due to mental illness, for both males and females, were

shown to peak at 45-54 years of age (Rochford et al., 2018). Lastly, with regards to gender, depression is much more prevalent in females than in males, and this is shown to be evident across all ages, and also across all regions (Figure 1.2) (WHO, 2017, James et al., 2018).

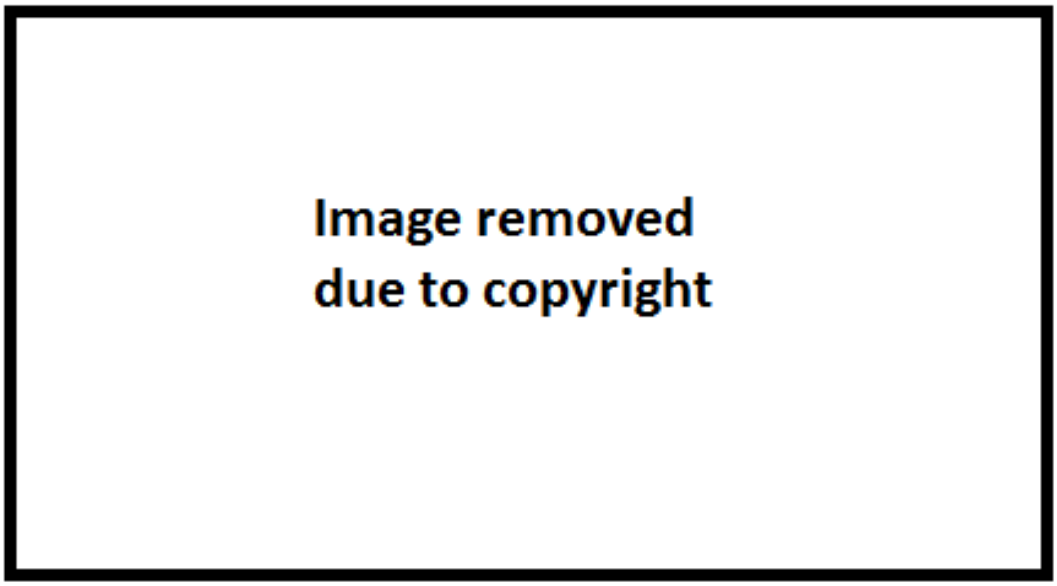


Figure 1.2 The percent global prevalence of depressive disorders by population and region (top) and by age and sex (bottom). (WHO, 2017). Data taken from the Global Burden of Disease Study 2015, WHO.

The prevalence of depression in society has a strong impact on economic burden and progression, as symptoms associated with depression include a lack in motivation and/or energy, as well as social withdrawal and secession. As such, this has consequential negative effects to both functionality in the workplace, and in turn growth to the economy. In a study conducted by the World Economic Forum and Harvard School of Public Health that was making predictions for the period between 2010 and 2030, the global impact of mental illness on lost economic productivity worldwide will reach $16.3 trillion over twenty years (Bloom et al., 2011, WHO, 2013). The cost of mental illness itself is also expected to more than double between this time-frame, with the global cost of mental health conditions expected to reach $6 trillion dollars for the year 2030 (Bloom et al., 2011). It is also important to note the fact that depression is often comorbid with other pre-existing medical conditions such as substance abuse, panic disorder, obsessive-compulsive disorder, anorexia nervosa and borderline personality disorder (DSM-V, 2013). In severe cases, depression can lead to suicide, with individuals that have been diagnosed with the psychiatric

disorders major depression and/or schizophrenia being found to be 40-60% more likely to have a premature death than the rest of the population (WHO, 2013).

In conclusion, it is clear to see that individuals who suffer from psychiatric disorders such as depression have a predisposition to undergo and suffer from a greater degree of disability throughout their lifetime, as well as mortality. In conjunction with this, mental illnesses such as depression have a profound impact on both the economic and healthcare systems in-turn worldwide. As such, advances need to be made to try to stem the growth and alleviate the medical burden of the condition to the individual, as well as the financial burden to society.

1.2 Diagnostic criteria for major depressive disorder (MDD)

The first recorded account of depression was in 400 B.C by the Greek Philosopher Hippocrates who believed that the human body had four separate fluids and that these were associated with a humour or disposition (Dewhurst, 1992). An individual who had 'black bile' as their fluid type was described as having 'melancholia', or being sad and gloomy in disposition (Dewhurst, 1992). Currently, depressive disorders are diagnosed using the criteria from two main guides, namely the Diagnostic and Statistical Manual of Mental Disorders (DSM) and the International Classification of Diseases (ICD). Originally, these two classification bodies worked in isolation from each other, but in more recent editions there have been attempts at harmonisation.

The Diagnostic and Statistical Manual of Mental Disorders (DSM) was first published in 1952 by the American Psychiatric Association (APA) and is used by clinicians worldwide as a reference for defining a psychiatric disorder and clinically diagnosing individuals with dysfunction in mental health. The fifth edition of DSM was published in 2013 and it is updated from previous editions to include more specificity on each explicit disease state and its revised subtypes. DSM-V now includes; specific criteria for diagnosis, diagnostic and associative features of the disease, prevalence of the disease, development and course of disease progression, onset of the disease, risk factors and lastly, cultural-related and gender-related issues associated with the disease (DSM-V). In DSM-V, major depressive disorder (MDD) is described as a major depressive episode (MDE) that lasts at least two weeks in duration and involves changes in behavioural and negative affect/functioning, causing distress and despair, that last the duration of the day, nearly every day, for that two-week period (DSM-V). Five of more of the following nine symptoms must have been

present in the two weeks – for most of the day, nearly every day - with at least one of those five symptoms being either **1) depressive mood**, and/or, **2) lack of interest or pleasure:**

1) ***Depressed mood***
2) ***Lack of interest/pleasure***
3) Dramatic weight loss/gain (loss/increase of appetite not due to dieting)
4) Sleep disturbance (insomnia/hypersomnia)
5) Psychomotor agitation (restlessness/unconscious repetitive behaviours)
6) Fatigue/loss of motivation
7) Feelings of low self-worth and guilt
8) Lack of concentration
9) Thoughts of self-harm and suicide

These symptoms must produce impairment in both social and work-related functioning and must not be attributed to another medical condition, or as a result of substance administration and inhalation. There are three main types of severity banding for MDD; mild, moderate and severe, and this is based upon the number of criterion symptoms present, as well as the degree of severity of each symptom (DSM-V). Whether an MDE is experienced as a single or recurrent episode also factors into further diagnosis and treatment of the patient, with an episode considered to be recurrent if the individual has not met the strict criteria for a period of at least two consecutive months and has been symptom free in this two month period.

MDD differs from episodes of normal sadness or grief, as individuals diagnosed with clinical depression have persistent feelings of sadness, and a complete inability to have expectation or anticipation for eventual happiness. Grief, in general, will fade with time and is usually attached to associated memories with the person who has passed away. MDD, in contrast, pertains to more persistent ruminations of despair and self-loathing (DSM-V). The functionality of the individual in their environment can be diversely impaired, with mild cases making the individual appear to act normal around family and close acquaintances, and severe cases including the individual being unable to attend to oneself and appearing catatonic in nature (DSM-V). MDD patients claim more pain/physical illness within the healthcare system, and

have increased social withdrawal, separation from physical activity, and loss of their-role in society in general (DSM-V).

The International Classification of Diseases (ICD) and Related Health Problems was first published in 1948 by the WHO and was considered a coded systematic guide that contained all the current statistics on health, including global morbidity and mortality rates. The eleventh edition of ICD was partially released in 2018 and will be officially presented at the World Health Assembly in 2019, to be released at the beginning of 2022 (Reed et al., 2019). This new version differs from previous version ICD-10 in that it is fully electronic, allows for integration and update between medical and healthcare-related systems, as well as allowing for global access with less chance of falsified versions being published. Over the years, ICD has extended its guide to include definitions of disease and disorders, as well as criteria to be met in disorders, in order to simplify classification of diseases and collaborate with DSM guidelines. With regards to the mental disorders section of ICD-10, simplifications in defining disorders, re-categorising and coding mental disorders, and their subtypes, has become key in order to aid primary health care providers in diagnosing these disorders; and not just merely psychiatric specialists (Reed et al., 2019). Similar to DSM-V, in ICD-10, a depressive episode is described as experiencing a depressed mood, a loss of interest/pleasure and reduced energy leading to fatigue and decreased interest in external activities. Two of these main symptoms must be present, in addition to two of the symptoms mentioned below, in order for a person to have been clinically diagnosed with having experienced a mild depressive episode;

1) ***Depressed mood***
2) ***Loss of interest/pleasure***
3) ***Increased Fatigue***
4) Lack of concentration
5) Diminished confidence/self esteem
6) Feeling of guilt and low self-worth
7) Bleak/pessimistic outlook on the future
8) Thoughts of self-harm and suicide
9) Loss of appetite

Similar to DSM-V, these symptoms must again last at least two weeks in duration and vary very little from day-to-day. Again, similar to DSM-V, depression is sub-divided into mild, moderate and severe episodes with moderate episodes and severe episodes intensifying in the number of symptoms experienced, and by the degree of magnification to which they are experienced. Social and work-related functioning are also considered to be markedly diminished. A recurrent episode is defined as a separate episode that meets the criteria for a mild depressive episode (4 symptoms, two weeks in duration, experienced nearly every day) and occurs several months later without any mood/affective dysregulation having been experienced in-between.

Depression has been described as a heterogeneous disorder in that multiple individuals can be diagnosed with depression but can differ in symptomology. For example, one patient could have insomnia and a depressed mood, whereas another could have a loss of pleasure with feelings of low self-worth and guilt (Zimmerman et al., 2015). Indeed, Zimmerman et al. (2015) state that 'there are 227 possible ways to meet symptom criteria for major depressive disorder'. As such, depression is hard to classify, with each case being particular and distinct to the individual sufferer. This variability and heterogeneity in the disease has also made it hard to form or produce tools in which to test depression and its severity. Psychological rating score scales exist such as the Hamilton-D rating scale (HAM-D), the Montgomery-Asberg Depression Rating Scale (MADRS) and the Becks Depression Inventory (BDI). All three rating scales were developed between 1960 and 1980. Each scale/inventory involves an observer rating the individual on scores based upon a series of symptomology-related questions and theirperceived efficacy of antidepressant medication (HAM-D and MADRS), or involves self-report screening and rating of symptomology, also via a series of questions and psychotherapy-based scoring scales (BDI) (Demyttenaere and De Fruyt, 2003). Although these rating scale tools help in delineating depression on a case-by-case manner, limitations of using such scales include false positives and bias with self-report and screening, lack in discrimination between depressive subtypes, the true ability of psychometric tests to detect antidepressant efficacy, emphasis on somatic symptoms verses psychological symptoms, and finally, eventual incorrect diagnosis by the practitioner (Demyttenaere and De Fruyt, 2003, Zimmerman et al., 2015).

1.3 Pathophysiology of depression

Described as a non-definitive disease or disorder, the underlying heterogeneity of MDD makes it hard to delineate the fundamental pathophysiology or cause of depression (Zimmerman et al., 2015). Having said this, both environmental risk factors and biological risk factors have been shown to contribute to disease initiation and susceptibility, with the overall consensus being that aetiology of depression is a combination of biological and environmental risk factors.

1.3.1 Genetic and Biological Risk Factors

It has been shown that first-degree relatives of individuals with depression have an increased risk of developing MDD; twofold to fourfold higher than the general public (Sullivan et al., 2000). As such, MDD can be described as a familial disorder, whereby heritability of the disease is a high risk factor (Sullivan et al., 2000). Individuals with genetic or familial MDD also have a higher chance of experiencing early-onset depression, as well as experiencing recurrent episodes of depression (Klein et al., 2013). It has been shown that biological risk or genetic risk of MDD is approximately 31%-42% (Hasler, 2010, Sullivan et al., 2000). With regards to loci or genes that make an individual susceptible to MDD *per se*, a specific gene or various factoring genes, have yet to be conclusively identified (Shadrina et al., 2018).

A separate biological risk factor that must be taken into account is that of sex/gender as there is a considerable difference in the prevalence of MDD between males and females. Depression is more prevalent among females (5.1%) than it is among males (3.6%) (WHO, 2017), with a recent meta-analysis conducted by Salk et al. (2017) that examined gender differences in depression in 160 papers, showing that women were conclusively shown to exhibit higher levels of MDD and depression symptoms than men. Females are more at risk of depressive episodes as a result of stressors such as social networking, serious illness and death, whereas men are more at risk of depressive episodes as a result of marital and workplace issues (Hasler, 2010, Kendler et al., 2001). It has been shown that females with MDD have a higher risk of suicide attempt (Boyd et al., 2015, Freeman et al., 2017), but that males with MDD have a more successful suicide completion rate (Freeman et al., 2017), and are less likely to use general health services that are available to them (Kovess-Masfety et al., 2014).

1.3.2 Non-Genetic and Environmental Risk Factors

Non-genetic or environmental risk factors that lead to a predisposed susceptibility to MDD include aversive childhood experiences, stressful or traumatic life events, poverty, unemployment, marital issues, sexual abuse, and/or substance abuse (but not withdrawal) (Hasler, 2010, Wilson et al., 2014). Experienced frequently and repeatedly, these stressful episodes or events have been shown to be potent risk factors for leading to the development of consequential mental illnesses such as depression. Hasler (2010) describes them as 'individual-specific environmental effects' and suggests that they contribute to 60-70% of this variance in vulnerability or predisposition to MDD. These environmental risk factors are not definitive but can help to inform the physician in a specific case-by-case manner which is very important in MDD. With this in mind, some individuals who are exposed to repeated stressful experiences or severe events have an innate resilience to succumbing to the consequences of environmental risk factors such as stress (Laird et al., 2019). These individuals are known as resilient and are a subdivision of the population. An individual's life experience, choices, activities and goals are the most important non-genetic risk factors for the emergence of MDD (Sullivan et al., 2000).

1.4 Theories of Depression

There are several theories behind the pathophysiology of depression, including the monoamine hypothesis, neurotrophic hypothesis, neuroinflammatory hypothesis and the hypothalamic-pituitary-adrenal (HPA) axis hypothesis. Each theory involves the up-regulation or down-regulation of various mediating factors within the brain that are believed to contribute to the emergence of the disease, leading to altered neurotransmission and reduced synaptic plasticity, followed by eventual cell loss and apoptosis, inducing a depressed system and depressive symptoms. Interesting to note, and as will be discussed, is the fact that there is considerable overlap and crosstalk between the factors involved in these different theories.

1.4.1 Monoamine Hypothesis

The monoamine hypothesis has been considered the principle theory amongst the pathophysiological theories of depression. The monoamine hypothesis was formulated in the 1960s after the serendipitous discovery that two different classes of drugs known as the monoamine oxidase inhibitors (MAOIs) and the tricyclic antidepressants

(TCAs), were shown to have mechanism of actions that elevated mood in depressed patients (Kelly, 2010). Iproniazid, a drug inhibiting the enzyme monoamine oxidase, an enzyme responsible for the breakdown of the monoamine neurotransmitters noradrenaline (NA) and serotonin (5-HT), was developed initially to treat tuberculosis, but was shown to cause an increase in mood and positive symptoms in patients with depression (Loomer et al., 1957, Ramachandraih et al., 2011, Schildkraut, 1965, Thompson et al., 2015). Shortly after, imipramine, a drug closely related to chlorpromazine and as such developed to treat antipsychotic symptoms, was shown to have a lack of antipsychotic properties, but elevated mood in the patients that received it, particularly in depressed patients (Kelly, 2010, Kuhn, 1958). Further analysis by Axelrod et al. (1961) identified that imipramine interfered with the reuptake of NA, a finding that was in tandem with those of iproniazid. In tandem with these discoveries was the finding that the anti-psychotic and blood pressure drug reserpine, was shown to cause a reduction in the catecholamine's 5-HT and NA and as such, was postulated to be associated with the symptoms of depression seen in patients to whom it has been prescribed (Axelrod et al., 1961, Katzung et al., 2009, Ramachandraih et al., 2011). The discoveries made about iproniazid and imipramine were to form the basis of the catecholamine theory of depression, in that although the mechanism of actions of each compound were uniquely different, both compounds worked by producing an elevation of the catecholamine's 5-HT and NA. As a result, both the serotonergic system and the noradrenergic system became potential and plausible targets for antidepressant therapy. Research focused on these neurotransmitters as targets for antidepressant treatment by targeting the stimulation, prevention of breakdown or inhibition of reuptake of serotonin and noradrenaline, with the introduction of the MAOIs and TCAs as antidepressants (Ramachandraih et al., 2011). However, these compounds were seen to be associated with a large range of side effects. In the late 1960s, research began to advocate a significant role for serotonin in MDD above the other monoamines, with this development followed by the introduction of the serotonin/noradrenaline reuptake inhibitors (SSRIs/SNRIs), which blocked the reuptake of these neurotransmitters (Hillhouse and Porter, 2015). These compounds were each seen to have less adverse effects and were better tolerated than the two previous classes of antidepressants (Hillhouse and Porter, 2015). Figure 1.3 depicts the mechanism of action of antidepressants that target the monoamine system as a result of the monoamine hypothesis.

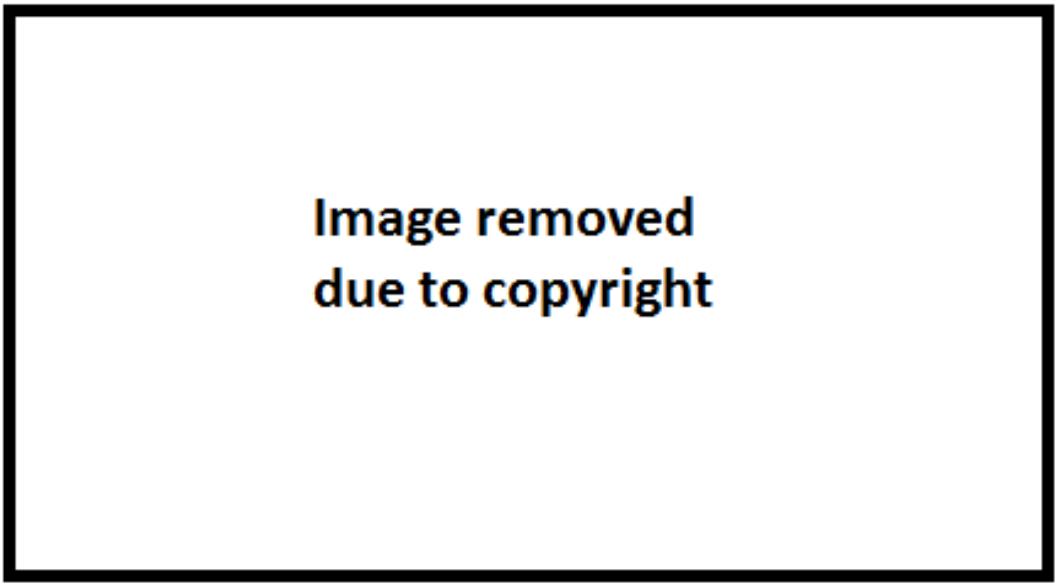


Figure 1.3 The mechanism of action of antidepressants at the monoamine receptors. The monoamine neurotransmitter or molecule (*black triangle*) is synthesised and released from the vesicle (*blue circle*) at the presynaptic neuron into the synaptic cleft. It then binds to the monoamine receptor (*white triangle*) on the postsynaptic neuron. The monoamine neurotransmitter that is left over is then taken back up into the presynaptic neuron by the reuptake transporter (*pink parallel lines*), where it is broken down and the process starts again. The TCA, SSRI and SNRI (*red diamond*) antidepressants function by blocking the reuptake transporter and so leaving more monoamine in the synaptic cleft to bind to the receptor and function. The MAOI (*red diamond*) antidepressants prevent the monoamines from being broken-down in the presynaptic cleft, again leaving more monoamine to function. MAOI=monoamine oxidase inhibitor, SNRI=selective noradrenaline reuptake inhibitor, SSRI=selective serotonin reuptake inhibitor, TCA=tricyclic antidepressants. Image taken from The Open University, (2019) and adapted to include antidepressant drugs.

Serotonergic neurons originate in the dorsal or median raphe nuclei or nucleus (DRN or MRN) whereas, noradrenergic neurons originate in the locus coeruleus (LC), both regions within the midbrain. Both systems project to a number of regions within the brain including the nucleus accumbens, the hippocampus, the amygdala and the pre-frontal cortex, and as such, they integrate functioning responsible for mood, emotion, cognition, stress and memory (Thompson et al., 2015).

The MRN, as well as the DRN, belong to the rostral raphe group. These nuclei are located in the mesencephalon and the rostral pons and project to several regions in the forebrain, unlike the caudal raphe group which projects mainly to the brainstem and spinal cord (Hornung, 2003) (Figure 1.4).

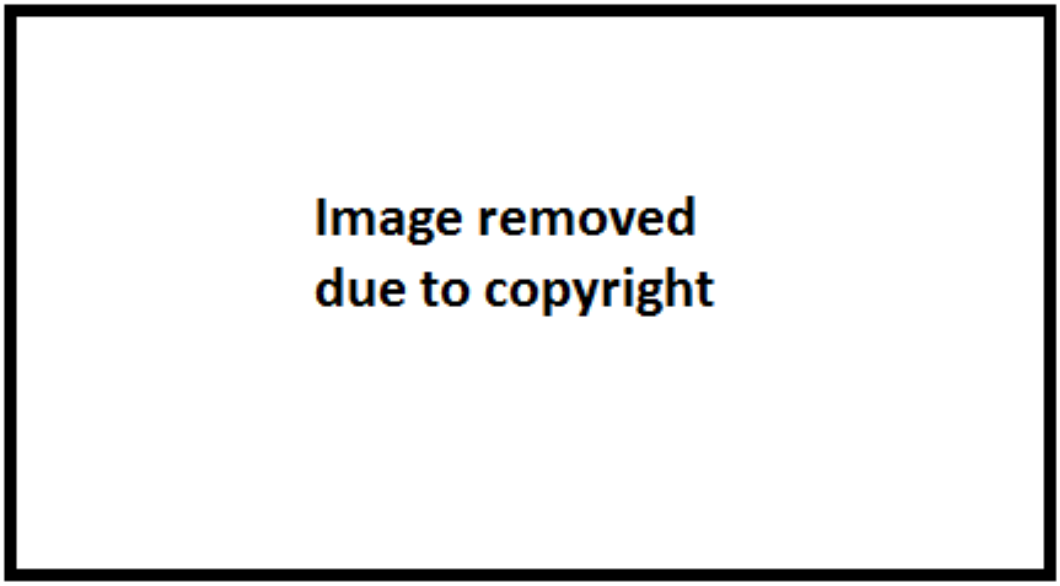


Figure 1.4 The raphe nuclei and the serotinergic pathway and regions that they project to. Image taken and adapted from Nestler et al. (2015).

The MRN and DRN account for 85% of all serotinergic neurons in the human brain (Hornung, 2003). Histological quantificantion has shown that the MRN makes up 20-30% of the total serotonin neuronal population (Dorocic et al., 2014), with over 80% of the neurons in the MRN synthesising 5-HT (Baker et al., 1991, Hornung et al., 2003). Axons of the MRN serontinergic neurons ascend into the forebrain and are largely located in the frontal cortex and hippocampal areas of the brain; two regions that are very important in emotional processing (Beliveau et al., 2015, Hornung et al., 2003). As such, deletion in the serotinergic innervations from these regions could have detrimental effects to emotional functioning. Indeed, in studies where tryptophan, the pre-cursor for serotonin, is experimentally depleted, this depletion increases the risk of developing symptoms of depression in individuals who have a family history of the disorder or who are in remission (Neumeister et al., 2002; 2004). In conjunction with this, some patients with MDD have shown alterations in central serotonin receptors, with reductions in the number of 5-HT_{1A} receptors across multiple brain regions (Drevets et al., 1998; 1999, Hasler, 2010; Katzung et al., 2009, Savitz et al., 2009). Post-mortem evidence also indicates an increase in 5-HT_2 receptor binding in the pre-frontal cortex of non-medicated patients with MDD (Stockmeier 2003, Yates et al., 1990), a receptor that has an inverse relationship with serotonin concentration; only shown to be increased when the synaptic availability of serotonin itself is reduced (Meyer, 2007).

Although there has been no great breakthrough in the alleviation of depression or antidepressant therapy since the discovery of the involvement of the monoamines, these neurotransmitters have become the institution for investigation of depression, with the vast majority of currently marketed antidepressants affecting monoaminergic neurotransmission in order to alleviate symptoms of the psychiatric disease. As such, the discovery of the association between the monoamine system and depression created the initial grounds for most of the research and findings on the disorder which has been established to-date (Thompson et al., 2015). Nevertheless, there is a current gap in the progression of antidepressant treatments as these current monoamine-derived therapies do not work for around 30% of MDD patients who are treated with them (Trivedi et al., 2006). As a result, this leads to patients with MDD having to attempt and try multiple pharmacological treatments and therapies which may continually remain uneffecacious, eventually causing treatment-resistant depression (Spijker and Nolen, 2010). As such, there is a major therapeutic need for new and novel strategies that target novel systems to try and alleviate the symptoms of MDD by other means.

1.4.2 Neurotrophic Hypothesis

The neurotrophic hypothesis is based upon the fact that depression is associated with overall cell atrophy and loss of neurogenesis in the hippocampus. The dentate gyrus is a region within the hippocampus that is associated with synaptic plasticity and adult neurogenesis, i.e. the birth of neurons. Neurogenesis and synaptic plasticity are mediated by neurotrophic factors that promote the survival of neurons and regulate the processing of cells (Groves, 2007). Brain-derived neurotrophic factor (BDNF) is a growth factor thought to be involved in the response to stress (Autry and Monteggia, 2012). BDNF is expressed on serotonergic neurons in the raphe nucleus and is also responsible for the regulation of dopaminergic neurons, as such it is a growth factor that has strong associations with the monoaminergic system (Guillin et al., 2001; Autry and Monteggia, 2012). Exposure to acute and chronic stress has been shown to decrease BDNF levels in humans (Duman and Monteggia, 2006, Hosang et al., 2014), and has also been shown to reduce levels of BDNF in animal models where stress plays a major component (Barrientos et al., 2003, Duman and Monteggia, 2006, Nibuya et al., 1995, Smith et al., 1995, Zhang et al., 2019). Moreover, post-mortem studies have revealed decreased levels of BDNF in suicide patients with MDD

(Dwivedi et al., 2003, Youssef et al., 2018), and have also revealed an overall loss of hippocampal volume (Videbech and Ravnkilde, 2004), as well as a reduction in neuron soma size and neuropil in MDD brains post-mortem (Duman and Li, 2012, Stockmeier et al., 2004). Though difficult to measure *in vivo*, BDNF plasma concentrations have been found to be reduced in depressed patients (Karege et al., 2005a; 2005b, Shimizu et al., 2003). Taking all of this into consideration, it is evident that there is considerable evidence for altered BDNF functioning in depression.

Interestingly, antidepressants appear to reverse the reduction in BDNF, aiding in the promotion of hippocampus function and neurogenesis (Figure 1.5). Patients treated with antidepressants have a higher level of BDNF expression when compared to untreated patients (Gervasoni et al., 2005, Shimizu et al., 2003). Low levels of BDNF in the plasma of untreated MDD patients has been shown to be retrieved or reversed with antidepressant treatment (Gervasoni et al., 2005, Shimizu et al., 2003). In conjunction with this, post-mortem levels of BDNF in the hippocampus have been shown to be increased in patients who had received antidepressants, when compared to MDD patients who were untreated (Chen et al., 2001). A number of classes of antidepressant drugs have also been shown to increase BDNF levels in the hippocampus pre-clinically (Nibuya et al., 1995). As such, BDNF has become a potential biomarker for depression, and has begun to be used when predicting the efficacy of antidepressant drugs (Autry and Monteggia, 2012). Nevertheless, not all findings are conclusive across studies. BDNF expression, infusion and knockout (KO) can be very region-specific, with BDNF-KO often increasing 'depressive-like' behaviours pre-clinically or showing no change in behaviour (Groves, 2007). There is also a lack of consistency in antidepressant efficacy in alleviating BDNF reductions in some antidepressants when tested across laboratories (Groves, 2007). Although there is considerable evidence that BDNF is reduced in MDD, and that antidepressant treatment can 'normalise' this alteration, it is still unclear how this knowledge can be translated into more effective treatments that primarily target BDNF. As a result, BDNF is still confined to the status of a biochemical marker for the disease and for antidepressant response (Duman and Li, 2012, Groves, 2007).

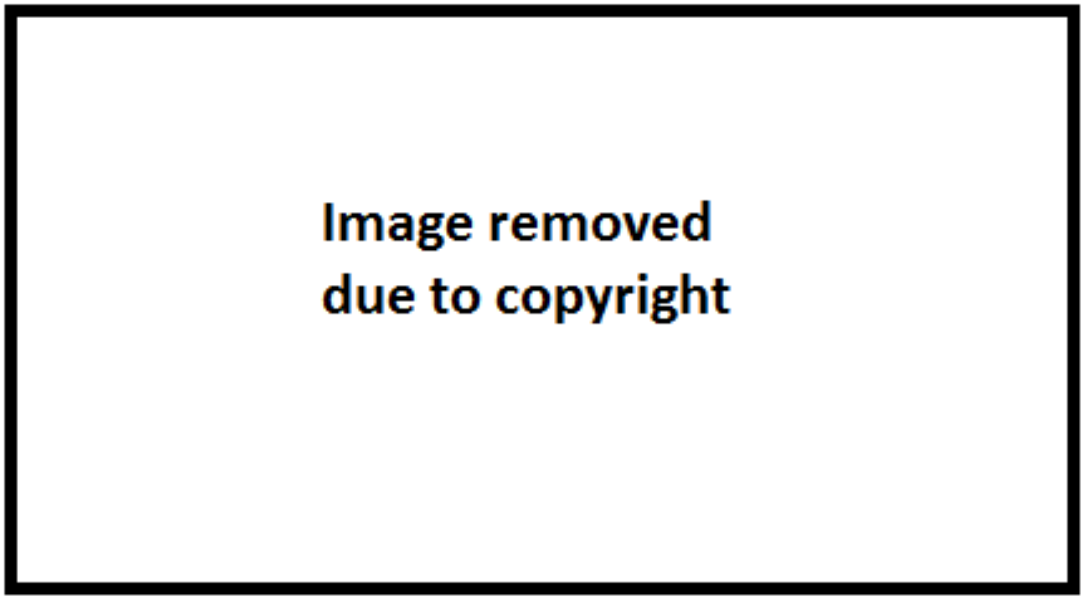


Figure 1.5 Diagram displaying the effect of stress on BDNF activity in the hippocampus which is reversed with antidepressant therapy. Stress causes a reduction in BDNF levels which leads to a reduction in neurogenesis and plasticity in the hippocampus, a region responsible for emotion/mood, leading to a decrease in mood. Antidepressant therapy has been shown to reverse this process. BDNF=brain-derived neurotropic factor, TrkB receptor=tyrosine kinase receptor B. Image taken from Groves et al. (2007).

1.4.3 HPA Axis Hypothesis

Corticotropin-releasing factor (CRF) (also known as corticotropin-releasing hormone (CRH)) is released from the hypothalamus in response to stress. This hormone prompts the release of adrenocorticotropic hormone (ACTH) from the pituitary gland which encourages the release of cortisol from the adrenal gland (Hasler, 2010, Nemeroff et al., 1984). This physiological cycle is known as the HPA axis and is thought to be dysfunctional and hyperactive in depression due to overactivation as a result of acute and repeated exposure to stress (Binder and Nemeroff, 2010). DSM-V states that until recently, 'hypothalamic-pituitary-adrenal axis hyperactivity had been the most extensively investigated abnormality associated with MDEs, with HPA axis hyperactivity appearing 'to be associated with melancholia, psychotic features and risks for eventual suicide''. Indeed, clinical research shows that patients with MDD have elevated concentrations of CRF-like immunoreactivity in cerebrospinal fluid (CSF) in comparison to healthy controls, as well as schizophrenia and dementia patients (Banki et al., 1987, Nemeroff et al., 1984). Austin et al. (2003) looked at CRF-immunoreactivity in noradrenaline and serotonin-containing pontine nuclei of MDD suicide male post-mortem, and found increased levels of CRF in the LC, median raphe

and caudal dorsal raphe (Austin et al., 2003). Secretion of saliva cortisol levels have been shown to be increased in acutely depressed MDD patients upon waking, with the measurement of cortisol levels often used as a test or marker for hyperactivity of the HPA axis in depression (Bhagwagar et al., 2005). Persistent abnormalities to the HPA-axis system like this could have other consequential effects to the overall health of the individual other than just vulnerability to depression (Bhagwagar et al., 2005). Having said all this, some researchers have found no differences in CRF activity in MDD patients when compared to controls (Charlton et al., 1988, Hucks et al., 1997). Hucks et al. (1997) found no differences in the number or affinity of CRF receptors in the frontal and motor cortexs in antidepressant-free and antidepressant-treated depressed suicide victims, when compared to controls. No difference in CRF-immunoreactivity was found between depressed suicide victims (treated and untreated) in the frontal, temporal, motor or parietal cortex, when compared to matched healthy controls (Charlton et al., 1988). Strategies that modulate the HPA axis as an antidepressant therapy such as corticotropin-releasing hormone 1 (CRH_1) receptor antagonists have not proven to be successful (Menke, 2019), with the HPA axis used more as a marker of the disease with its physiological effects consequential symptoms, rather than being targeted to become an active antidepressant strategy or therapy (Hasler, 2010, Menke, 2019).

To conclude, although a substantial amount of evidence exists for abnormalities to the HPA axis system in MDD, these endocrine markers are often found to be inconsistent in their findings. Similar to the use of BDNF as a tool in MDD, perhaps these changes to the HPA system in depression can be considered a trait of vulnerability in the disease (Nemeroff et al., 1984), or as Bhagwagar et al. (2005) describes them as ‘a neuroendocrine “scar” of previous episodes of illness’.

1.4.4 Neuroinflammatory Hypothesis

The neuroinflammatory system is made up of proinflammatory and anti-inflammatory cytokines that activate the immune system response to antigens and regulate this response so as not to overwork and cause damage to the body but rather to protect it (Irwin and Miller, 2007). In some cases however, the inhibitory effect of this circuit becomes impaired or compromised, leading to prolonged activation of the immune response, which can then become neurotoxic in nature rather than neuro-protective. This is postulated as the case with depression, where elevations of pro-inflammatory cytokines (which promote inflammation) are the basis of the neuroinflammatory theory of depression, first proposed by Smith (1991).

Proinflammatory cytokines, including interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor-necrosis factor alpha (TNF-α), are all seen to be elevated in plasma samples in patients with MDD (Anisman et al., 1999, Kim et al., 2007, Musselman et al., 2001, Sluzewska et al., 1996, Tsoa et al., 2006, Tuglu et al., 2003) and in animal models of depression (Zhang et al., 2019, Sukoff Rizzo et al., 2012). Treatment with antidepressants reduces this elevation in proinflammatory markers (Tuglu et al., 2003, Hannestad et al., 2011). Cytokines also induce a syndrome called 'sickness behaviour', which resembles and overlaps specific features of depression such as reduction in appetite and social behaviour, cognitive impairments and fragmented sleep (Dantzer et al., 2008, Irwin and Miller, 2007). Injection of the cytokine interferon-α (IFN-α) increases inflammatory markers and is seen to induce this 'sickness behaviour' (Capuron et al., 2004, Wichers et al., 2007). IFN-α is also used to treat cancer, and it has been shown that treatment with IFN-α for cancer can result in the development of depression, cognitive impairment and sleep disturbances (Capuron et al., 2004, Irwin and Miller, 2007, Wichers et al., 2005, Wichers et al., 2007). Musselman et al. (2001) found that IL-6 was elevated in cancer patients with depression, to a higher degree than cancer patients without depression, and in comparison to healthy control (Musselman et al., 2001). Treatment with antidepressants, such as paroxetine and escitalopram, have been shown to reverse the behavioural and depressive-like emotional symptoms of IFN-α treatment (Capuron et al., 2002, Ehret and Sobieraj et al., 2014). Indeed, inflammatory markers have been shown to have a potent effect on the HPA axis, inducing the release of CRH, which we know from the previous section has been shown to be elevated in MDD and is seen as a biomarker of the disease (Capuron et al., 2003, Irwin and Miller, 2007). The

monoaminergic system is also seen to be closely linked to cytokine activity, with IL-1 shown to activate noradrenergic systems, and IL-1, IL-6 and TNF-α shown to activate serotonin pathways (Dunn et al., 2005). Another link between cytokines and the monoamines, is that interferon-γ (IFN-γ), a cytokine that activates the enzyme indoleamine 2,3-dioxygenase (IDO) in macrophages in response to viral infections, degrades tryptophan, the precursor of serotonin, which we have shown earlier is a major contributor to the monoamine hypothesis of depression.

In conclusion, it is evident that there is a profound association between proinflammatory cytokines and MDD. As we have pointed out, cytokines are increased in MDD patients, and cytokine therapies which are used to treat cancer and viral diseases induce behavioural changes that personify depression and its symptoms. Lastly, many pathways known to be involved in the pathophysiology of depression are affected/influenced by the inflammatory system and its markers, i.e. the endocrine system (HPA axis hypothesis) and monoamine system (monoamine hypothesis) (Irwin and Miller, 2007, Capuron et al., 2003). Whether this proinflammatory response is a secondary effect of depression, or the initial trigger of the disease is yet to be determined.

1.5 Antidepressant Treatment

As mentioned in Section 1.4.1, the discovery of ipronizaid, a MOAI and imipramine, a TCA, to elevate mood in depressed patients via mechanisms that produce an increase in monoamines levels, lead to the monoamine hypothesis of depression, and as such an introduction of these classifications of drugs being used to alleviate the symptoms of depression. This discovery was followed by a period (1960-1980s), that resulted in a mass production of antidepressant drugs that were MAOI, followed by TCA, based in mechanism. Although the MAOIs originated as the first-line drugs used to treat depression in the 1960s, they were accompanied by many side-effects due to their initial irreversible nature and lack of selectivity (Fiedorowicz and Swartz, 2004, Katzung et al., 2009). The TCAs then followed shortly after, with each tricyclic drug differing merely by one methyl group (Katzung et al., 2009). The TCAs, such as desipramine and imipramine, were shown to be as efficacious as the MAOIs but were also gradually shown to be associated with a number of safety concerns. Their high affinity for histamine and acetylcholine receptors meant that they became associated with a number of adverse reactions including constipation, weight gain, drowsiness

and dry mouth (Feighner, 1999, Hirschfeld, 1999, Katzung et al., 2009). With this in mind, the TCAs are often only prescribed as a final resort and when individuals appear unresponsive to main-line treatments, i.e. treatment-resistant (Hirschfeld, 1999, Katzung et al., 2009, Stotz et al., 1999). In conjunction with this, the low tolerability of both the TCAs and the MAOIs coincided with a high potential for toxicity upon acute overdosing (Hirschfeld, 1999, Kelly, 2010).

In the late 1960s, emphasis began to be put on 5-HT over the other monoamines as a result of the findings that concentrations of 5-HT were reduced in the brains of MDD patients post-mortem (Hillhouse and Porter, 2015, Shaw et al., 1967). Pharmaceutical companies began developing compounds that would selectively block the reuptake of 5-HT through inhibition of its transporters; leaving higher levels of the neurotransmitter within in the synaptic cleft (Ferguson, 2001, Hillhouse and Porter, 2015, Katzung et al., 2009). In 1974 Eli Lilly developed the compound LY110140, now known as fluoxetine (Prozac®), and published results to show that this compound, which acts by selective inhibition of 5-HT, provided promising results for a potential strategy for the treatment of mental disorders such as depression (Hillhouse and Porter, 2015, Wong et al., 1974). Fluoxetine became the first SSRI approved by the FDA and prescribed in the United States in 1987, and was followed by the development and approval to the market of other SSRIs such as sertraline, citalopram, paroxetine and escitalopram (Hillhouse and Porter, 2015). Drugs that selectively block NA neurotransmission (SNRIs) have also been developed, such as venlafaxine, and are regarded as having similar efficacy (Kelly, 2010). Figure 1.6 depicts the timeline for the discovery and development of the antidepressant treatments discussed above.

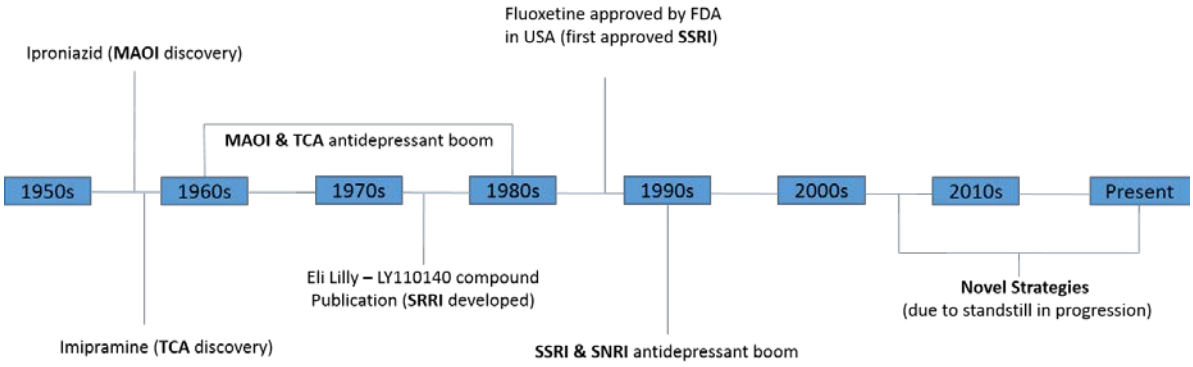


Figure 1.6 Timeline of the discovery and development of antidepressants for the treatment of depression from the period 1950-present. MAOI=monoamine oxidase inhibitors, SNRI=selective noradrenaline reuptake inhibitor, SSRI=selective serotonin reuptake inhibitor, TCA=tricyclic antidepressants.

The SSRI are more commonly prescribed over other classes of antidepressant drugs as they are associated with features of lower toxicity, less sedation, more tolerability, ease of use, and are regarded as financially of cost benefit to the economy (Cipriani et al., 2018). As a result, the SSRIs are currently the most clinically prescribed and efficacious antidepressants to-date (Anderson, 1998; 2000, Cipriani et al., 2018, Ferguson, 2001, Katzung et al., 2009). Figure 1.7 depicts the ten most commonly prescribed antidepressants in Ireland in 2004 and 2013.

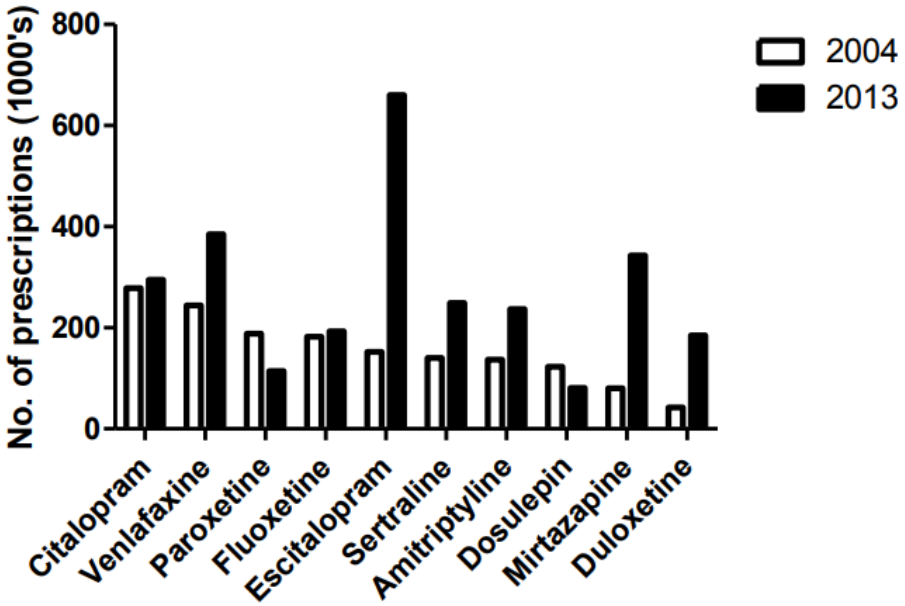


Figure 1.7 The ten most commonly prescribed antidepressants in Ireland (per 1000) in the year 2004, as compared to the ten most popular in 2013. The drugs prescribed were the same for 2004 and 2013, with the exception of trazadone (2004) being replaced by duloxetine (2013). Data provided by Garvey and Kelly (2015).

Having said all this, this class of antidepressants do not come without their own shortcomings, with SSRIs reported to cause sexual dysfunction, weight gain and sleep disturbance (Ferguson, 2001).

The most prominent feature of all classes of antidepressant treatment is the 'lag' between the neurochemical effects and the emergence of a therapeutic response. Indeed, pharmacological evidence indicates that although these drugs have immediate biochemical action, it takes up to six weeks for any of the behavioural changes to occur (Frazer and Benmansour, 2002, Harmer et al., 2009). The direct mechanism behind this anomaly has yet to be fully unravelled but it has been suggested that perhaps these monoamines are regulators of another adaptive target and that it is the secondary downstream effects that cause the eventual adaptive improvement in symptoms of this disease state, but that this particular mechanism takes time to work (Blier and Abbott, 2001).

1.5.1 Limitations of Treatment

As discussed in the previous section, although the first antidepressants represented a major step forward in the treatment of MDD, they were soon realised to be associated with a number of adverse effects, including low tolerability. The current monoamine-derived treatment modalities also have considerable drawbacks. These drawbacks include slow onset of action, and also continued adverse reactions, but by far the most prominent limitation is the lack of efficacy across the depressed population. Failure to take the medication prescribed has been proven to be a factor (DiMatteo et al., 2000), and this may be due to the lag in duration to experience a therapeutic response. However, regardless of this, current antidepressant drugs only work for around 30% of patients they are prescribed to (Al-Harbi, 2012). It has also been reported that less than 50% of patients achieve remission (Frank et al., 1991, Kennedy et al., 2001). In a study conducted by Stassen et al. (2007) on 2,848 patients with MDD, seven different antidepressants were examined for their time to onset of action or improvement, and their time to onset of response in the patients. Stassen et al. (2007) found that on average it took 13 ± 1 days to achieve improvement or action, and 19 ± 1 days to achieve a response in the patient. It was also shown that of the 2,848 MDD patients examined and administered an antidepressant for 10-12 weeks, only 28% of these patients achieved remission (Stassen et al., 2007). As a result, depressed patients are often prescribed more than one antidepressant treatment, with an initial

pharmacological therapy, followed by a second pharmacological treatment if the first has not been seen to be efficacious. With this in mind, exposures to multiple pharmacological strategies mean that many patients become treatment-resistant, with alterative techniques having to be used to try and alleviate their symptoms, such as somatic approaches like deep brain stimulation and electroconvulsive therapy (Al-Harbi, 2012). Added to this is the fact that many drugs are not given as simple monotherapy, but rather in conjunction with combining pharmacological treatment and as such, each drug can affect the mechanism and metabolism of each other (Spijker and Nolen, 2010). As such, contraindications need to be understood and factored into therapeutic treatment by physicians and psychiatrists.

Although monoaminergic strategies provided the pathway for the antidepressant treatments, there has been a stem in the growth of formulating new modification strategies, particularly with regards pharmacological compounds. Crucially, there is an overall need for better animal models of the disease within which to test novel antidepressant efficacy, but importantly, that have an overall relatable translatability to the clinical condition (Albelaira et al., 2013, Krishnan and Nestler, 2011, Wang et al., 2017); this will be discussed in the next section. Novel strategies are now being used to try to combat this standstill in the development of novel treatment for depression, with researchers beginning to target other systems that were previously shown to have antidepressant efficacy in both preclinical and clinical models (Brown and Lucki, 2019, O'Leary et al., 2015). Table 1.1 Novel treatments and interventions being tested in depression models for their antidepressant activity.Table 1.1 depicts the novel treatments and intervention strategies being investigated to alleviate the symptoms of depression in animal models of depression, by mechanisms that do not target the monamine system.

Target	Treatment	Mechanism	Reference
Opioid System	Buprenorphine	Mu opioid receptor agonist (antagonism at the kappa opioid receptor)	Burke et al. 2019a; 2019b; Falcon et al. 2015; 2016; Smith et al., 2019
	SNC80	Delta opioid receptor agonist	Henry et al. 2018; Nozaki et al. 2014; Saitoh et al. 2008; Takahashi et al. 2008
Glutamatergic System	Ketamine	Non-competitive NMDA antagonist	Fitzgerald et al. 2019; Holubova et al. 2016
Exercise	Exercise	Chronic exercise (wheel running, treadmill exercise)	Chambliss et al. 2004; Shin et al. 2017; Van Hoomissen et al. 2011
Vasopressin	SSR149415	Vassopressin 1b receptor antagonist	Breuer et al. 2009; Iijima and Chaki, 2007
Anti-inflammatory	Minocycline	Microglia suppressant	Borre et al. 2012; Burke et al. 2014
Cannabinoid	THC	Cannabinoid receptor agonist	Elbatsh et al. 2012
Electrical Stimulation Therapy	Deep brain stimulation	Vagus nerve/deep brain stimulation	Follesa et al. 2007; Gebhardt et al. 2013; Hamani et al. 2010; Jiménez-Sánchez et al. 2016

Table 1.1 Novel treatments and interventions being tested in depression models for their antidepressant activity. NMDA= N-methyl-D-aspartate, THC=Tetrahydrocannabinol.

These include systems such as the opioid system and the glutamatergic systems which have been shown to be altered in patients with MDD and which have been shown to improve symptoms in MDD when targeted with novel compounds (Brown and Lucki, 2019, Ehrich et al., 2015, Murrough et al., 2013a; 2013b, Thase et al., 2019, Zajecka et al., 2019).

1.5.2 Ketamine as a potential antidepressant therapy

As mentioned above, targeting the glutamatergic system has begun to be examined for its role as an antidepressant target in MDD. Indeed, the glutamatergic system has been suggested to have a role in the pathophysiology of depression (Murrough, 2012). As mentioned previously, it has been suggested that the reduction in the monoamines may

not be the cause of depression but may actually be a secondary mechanism or downstream result of the initial trigger. With this in mind, it has been postulated that blocking the N-methyl-D-aspartate (NMDA) receptor, leads to an increased secretion in glutamatergic neurotransmission, which is the main excitatory neurotransmitter in the brain, which then leads to increased synaptic signalling and connectivity, and as such an increase in monoaminergic functioning (McCloud et al., 2015, Murrough, 2012). Ketamine is a non-competeitive NMDA antagonist has been shown to improve the symptoms of MDD, and in particular treatment-resistant depression (Covvey et al., 2012, Machado-Vieira et al., 2009, Murrough et al., 2013a; 2013b, Phillips et al., 2019, Zarate et al., 2006). Indeed, it has been shown that ketamine has rapidly acting antidepressant action, with treatment seen to alleviate the depressive symptoms in treatment-resistant MDD patients within 24 hours (Murrough et al., 2013a; 2013b), and in some cases within 110 minutes after infusion (Zarate et al., 2006). In fact in a study by Zarate et al. (2006), 29% of the MDD patients treated with ketamine met the criteria set out for remission the day following ketamine infusion. This fast onset of action has major connotations for future depression reaserch and treatment. Having potential antidepressants that work within two hours of treatment verses taking up to six weeks for a behavioural response to be seen, would have major positive implications with regards patient response and care. Nevertheless, there are a number of disadvantages to ketamine treatment, including the fact that ketamine has must be administered intravenously in order for its effects to be seen which in itself can be quite invasive to the patient. Second, its effects are only short-lived or transient. Zarate et al. (2006) showed that symptoms had returned to baseline in 65% of patients one week after acute administration. As a result, repeated administration is needed and this can have a number of side effects that must be continuously monitored including tolerability (Covvey et al., 2012). As a result, concerns around ketamine treatment remain relating to frequency of administration and long-term safety (Gosek et al., 2012). Nevertheless, the fast-acting and acute improvement of MDD symptoms cannot be argued and its potential as a novel antidepressant target is now well-established. Analogues of ketamine are currently being developed to combat its tolerability concerns.

1.6 Animal Models of Depression

Firstly, it must be stated that it would never be possible for any animal model, especially one that is psychiatric or emotive in nature such as depression, to be able to fully replicate all of the criteria within the human state of the disease. Having said this, an animal model of disease should actively try to encompass as many of the criteria in the human condition as possible, in order to be considered a useful animal model for disease pathology. Frazer and Morilak (2005) identified a number of questions that need to be addressed when selecting an animal model of depression:

- Does the researcher want to replicate the whole syndrome?
- Does the researcher want to mirror specific behavioural effects?
- Does the researcher want to mirror specific molecular effects?
- Or, how does the model aid in establishing the predictive efficacy of current and novel therapeutic treatment? (Frazer and Morilak, 2005)

Indeed some diseases and disorders, such as hypertension and diabetes, are much easier to model in animals than others such as psychiatric diseases. In disorders such as hypertension and diabetes, there is a clearer trigger and measurable output. For example elevated blood pressure, or high blood glucose respectively. Psychiatric disorders can be slightly more complex than this, with emotive outputs much more difficult to represent and measure in animals. The first attempts to classify animal models of psychiatric conditions was made by McKinney and Bunney (1969), who proposed that the method by which animal models of psychiatric disorder are assessed for their efficacy should follow the criteria of:

- Being ‘reasonably analogous’ to the human disorder in terms of symptoms and manifestations of the disease
- Exhibiting behavioural responses or changes that can be monitored
- Exhibiting behavioural responses or changes that are reversed by treatments that are efficacious in the human condition
- Producing models, and behavioural endpoints, that are reproducible across laboratories

This was further developed and refined by Willner (1984) such that all animal models are now assessed under the following three criteria:

1) **Face Validity:** the ability of the model to replicate behavioural features of the disease
2) **Construct Validity:** the ability of the model to replicate the pathophysiological changes in the disease
3) **Predictive Validity:** the ability of model to predict the efficacy of anti-depressants

Rodents are primarily used in animal research for several reasons; they are easy to work with, and relatively inexpensive to produce and house (Parker et al., 2014). In conjunction with this, rodents have relatively similar anatomical structuring to humans, particularly with regards their central nervous system (CNS) (Figure 1.8). This being said, we cannot broadly assume that the networks and ultimate physiological responses that are evoked by rodents, exactly duplicate those which are made by humans, but more that they provide a tool in which to investigate the pathways and anatomical structures that are effected in disease states.

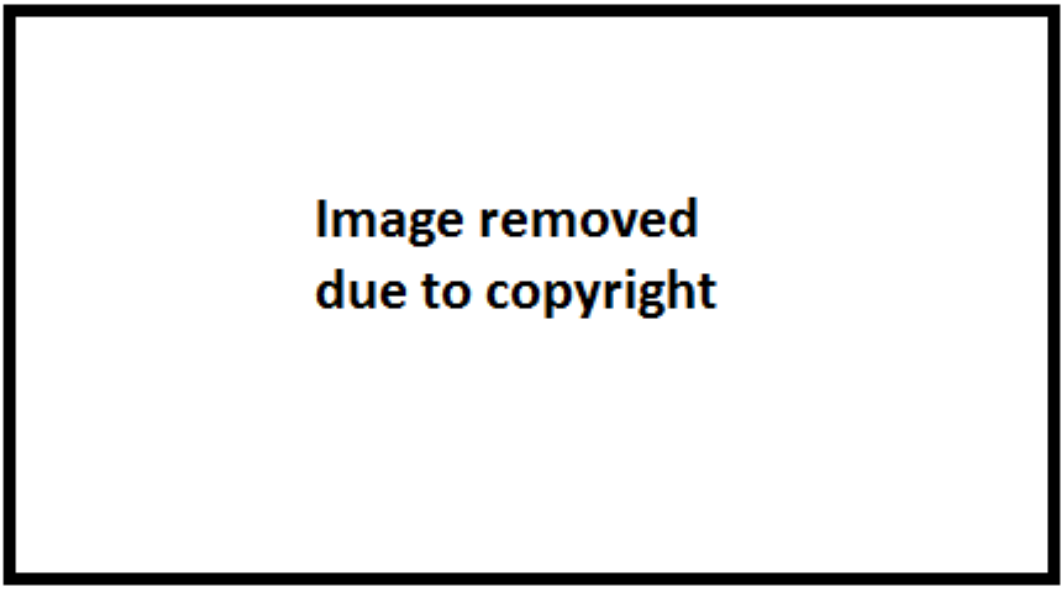


Figure 1.8 Comparison of the similarities between the regions and structure of an adult human brain and an adult rat brain. Image taken from the Genetic Science Learning Centre, (2013).

Depression is difficult to replicate in animals and rodents as it is a disorder with affective, somatic and cognitive dysfunction (Frazer and Morilak, 2005). The idea of knowing whether an animal is “depressed” is indeed complex, as you cannot ask an

animal how they are feeling (Abelaira et al., 2013). Depression is also difficult to replicate as it is a disease of comorbidity, and so often shares symptoms of other diseases, such as that of anxiety disorder (DSM-V). Having said this, and as discussed previously, depression itself is heterogeneous in nature and intrinsically depressed patients do not always display all the criteria of the illness.

The study of depression in preclinical research can be split into two categories: 'tests', or, 'models' (Abelaira et al., 2013, Wang et al., 2017). With regards to 'tests', animals are exposed to a test whereby a behavioural endpoint is assessed. Tests usually involve exposure to environmental stressors, and can have the addition of pharmacological manipulations (Albelaira et al., 2013). The forced swim test (FST) and the tail suspension test (TST) are two examples of these tests and will be discussed in further detail later in the General Introduction, but the concept behind these tests is that increases in immobility in both tests signifies a lack of escape, and hence a hopelessness or learned helplessness in the animal. This learned helplessness is seen to resemble a loss of interest or hopelessness, and as such is thought to mirror the loss in motivation/interest in MDD patients as outlined as one of the criteria for the disorder in DSM-V (DSM-V, Porsolt et al., 1977). Table 1.2 displays the symptomatic criteria in MDD and the tests of 'depressive-like' and 'anxiety-like' behaviour that animals, and animal models of depression are examined in; defining how these symptoms relate to each other.

With regards 'models', this encompasses exposure to environmental factors or modifying the physiological make-up of the animal, which in turn causes alterations to behavioural and molecular composition that replicate the pathophysiological changes seen in depression (Abelaira et al., 2013, Wang et al., 2017). With this in mind, animal models can be classified under the following categories:

- Genetic
- Environmental
- Lesion-induced
- Drug-induced

Each of these types of animal models involves exposure to one of the following: stress, injury, cytokines, endotoxins or genetic modification; each of which is thought to be involved in the pathophysiology of depression. With regards rodent models of

depression, a commonly used genetic model is the Wistar Kyoto rat (WKY) which has been selected bred and has inherent phenotypes that resemble the depressive-like behaviour and neurochemistry in MDD (Nam et al., 2014, Tejani-Butt et al., 2003). Two commonly used environmental models are that of the chronic mild stress (CMS) model, and the learned helplessness (LH) model, which involve exposure to repeated stress-induced paradigms that are thought to resemble exposure to traumatic and stressful events that are hypothesised to be involved in the pathophysiology of depression. The olfactory bulbectomised (OB) model of depression is a commonly employed lesion model that involves removal of the olfactory bulbs that causes a number of neurochemical and behavioural adaptations that reflect alterations that are seen in depressed patients (Kelly et al., 1997).

MDD Symptom	Animal Symptom	Behavioural/Methodological Test
Primary		
Depressed mood	N/A	Not achievably measurable
Lack of interest/pleasure	Anhedonia	Sucrose/saccharin preference test; female urine sniffing
Dramatic weight loss/gain (loss/increase in appetite not due to dieting)	Weight loss	Measure bodyweight
Sleep disturbance (insomnia/hypersomnia)	Sleep disturbance	Measure sleep fragmentation; REM sleep
Psychomotor agitation (restlessness/unconscious repetitive behaviours)	Atypical locomotor activity	Open field test; home-cage activity
Fatigue/loss of motivation	Behavioural despair, lack of escape	Force swim test; tail suspension test
Feelings of low self-worth and guilt	Grooming?	Grooming?
Lack of concentration	Cognitive deficits	Morris water maze; passive avoidance test; Y-Maze; T-Maze; novel object recognition
Thoughts of self-harm and suicide	N/A	Not achievably measurable
Secondary		
Social deficits	Decreased social interaction	Social interaction test; 3-chamber sociability test
Anxiety	Anxiety-like behaviour	Open field test; elevated plus maze; marble burying

Table 1.2 Symptoms of depression and the corresponding symptoms in animals as measured by specific behavioural tests. The criteria for diagnosis of MDD are separated into primary and secondary symptoms as guided by DSM-V. N/A=not applicable, REM=rapid eye movement. Taken and adapted from Czeh et al. (2016).

Drug-induced or pharmacological models include the IFN-α model, and the lipopolysaccharide (LPS) model but these models are less frequently used (Czeh et al., 2016). The face, construct and predictive validity for four of the animal models mentioned above, are summarised in Table 1.3.

	Face Validity	Construct Validity	Predictive Validity	Reference
Chronic Mild Stress (CMS)	↑ anhedonia, ↑ immobility, ↓ cognitive functioning, ↓ sexual activity	↑ sleep disturbances, ↑ HPA axis activity, ↑ cytokines, ↓ monoaminergic activity, Structural hippocampal alterations	Yes (acute and chronic administration)	Dalla et al., 2010, Delagado et al., 2011, Elizalde et al., 2008, Fracchia et al., 1992, Grønli et al., 2012, Henningsen et al., 2009, Luo et al., 2014, Réus et al., 2012, Song et al., 2006, Wang et al., 2018, Wei-Wei et al., 2014
Learned Helplessness (LH)	↑ anhedonia, ↑ immobility, no change in cognitive functioning	↑ HPA axis activity, ↓ BDNF Expression, ↑ cytokines, ↓ monoaminergic activity, Structural hippocampal alterations	Yes (acute)	Adrien et al., 1991, Arakawa et al., 2012, Cheng et al., 2018, Dwivedi et al., 2005, Fogel et al., 2011, Hajszan et al., 2009, Li et al., 2016, Schulz et al., 2010, Song et al., 2006, Vollmayr et al., 2001; 2004, Zhou et al., 2017
Olfactory Bulbectomy (OB)	↑ anhedonia, ↑ immobility ↑ locomotor activity ↓ cognitive functioning, ↓ sexual activity	↑ sleep disturbances, ↑ HPA axis activity, ↑ cytokines, ↓ monoaminergic activity, Structural hippocampal alterations	Yes (acute and chronic administration, dependant upon behavioural endpoint)	Refer to Thesis Intro: Section 1.7
Wistar Kyoto (WKY)	↑ anhedonia, ↑ immobility	↑ sleep disturbances, ↓ BDNF Expression, ↓ monoaminergic activity, Structural hippocampal alterations	Yes (acute)	Burke et al., 2016, Dugovic et al., 2000, Hauser et al., 2011, Nam et al., 2014, Paré and Redi, 1993, Scholl et al., 2010, Smaga et al., 2017, Solberg et al., 2001, Tejani-Butt et al., 2003, Tizabi et al., 2010

Table 1.3 Face, construct and predictive validity for the Chronic Mild Stress, Learned Helplessness, Olfactory Bulbectomy and Wistar Kyoto animal models of depression. Anhedonia, as tested in the sucrose preference test; a reduction in sucrose preference meaning an increase in anhedonia. Motor activity as tested in the open field test. Immobility as tested in the forced swim test (FST) or tail suspension test (TST). HPA axis activity, as tested by measuring the level of corticosterone. BDNF expression was examined in the region of the hippocampus. Cytokines explored include IL-1, IL-6 and TNF-α. Monoamine activity including expression, turnover and metabolism of 5-HT, NA and DA neurotransmitters. Structural hippocampal alterations as measured by volume and spine synapses. Antidepressant efficacy as measured by acute and/or chronic administration. BDNF=brain-derived neurotropic factor, HPA=hypothalamic-pituitary-adrenal, WKY=Wistar Kyoto ↑=increased, ↓=decreased. Idea for table taken and adapted from Abelaira et al. (2013).

The rationale for choosing these models over other models is that they appear more commonly throughout the literature on rodent models of depression, and appear to be well regarded and utilised (Abelaira et al., 2013, Czeh et al., 2016, Willner, 1984, Wang et al., 2017). Overall, each model mentioned produces positive findings with regards face, construct and predictive validity, however, each of these animal models of depression is not without its limitations.

The CMS model involves the exposure of an animal to a series of relatively stressful events that are repeated each day and that last up to eight weeks in length (Katz et al., 1981, Katz, 1982). These include water deprivation, alterations in the light-dark cycle, changes to cagemates, and social-isolation (Willner, 2016). In this manner, it utilises the exposure to stress in the animal, to resemble the exposure to stress that is thought to be involved in the pathophysiology of MDD (Willner, 2016). The CMS model has been shown to have very good construct, face and predictive validity, with deficits in this model proven to be attenuated with chronic antidepressant treatment, resembling the timeframe that it takes for behavioural changes to occur in patients with MDD on antidepressant medication (Elizalde et al., 2018, Wang et al., 2018, Wei-Wei et al., 2014). A primary drawback of the CMS model is that it involves a long time to implement and as such is labour intensive (Abelaira et al., 2013, Wang et al., 2017). It also demands the need for a lot of space in which to house the animals for a prolonged period of time during implementation of the stressors (Abelaira et al., 2013). The largest drawback of the CMS model is that it is difficult to establish and as such, replication across laboratories can be very sensitive to variations in design (Hill et al., 2012, Krishman and Nestler, 2011, Stepanichev et al., 2014). Hill et al. (2012) highlights the fact that factors such as the duration of procedure and the housing of the animals are important implementation tools, with many laboratories carry out the procedure over differing lengths of time than the classic eight week exposure period (Hill et al., 2012). As such, the CMS model is often associated as a model that has poor reproducibility (Krishman and Nestler, 2011, Wang et al., 2017, Stepanichev et al., 2014).

The LH model involves subjecting the animal to inescapable stressful stimuli such as electric shock or loud noise, following which the animal develops a state of ‘helplessness’ displayed by a reduction in the motivation to escape (Stepanichev et al., 2014). The LH model is thought to mimic depression in that the individual experiences a perceived loss of control of the situation believing that they have no control over the

underlying outcome (Fogel et al., 2011). This fosters an attitude of helplessness in depressed patients, along with a lack of motivation and hope. The LH model has also been shown to have good face, construct and predictive validity as an animal model of depression. Similar to the CMS model, the LH model is associated with the use of different protocols across different laboratories, and a lack of consistency in the length of time that the procedure is employed for. This then creates difficulty in consistent reproducibility of findings across laboratories. In conjunction with this, a major drawback of this model is the fact that the 'depressive-like' behavioural symptoms cease shortly after the footshocks are halted, meaning the behavioural measures can only be conducted over a relatively short timeframe (Abelaira et al., 2013).

The WKY model is a genetic model that was originally developed as a normotensive control for the hypotensive rat, but was shown to display a 'depressive-like' phenotype, with an increase in immobility in the FST and an acquired helplessness in an LH paradigm (Paré, 1994). It quickly evolved as a model of depression, and has been shown to present good face, construct and predictive validity in many behavioural examinations. Nevertheless, differences have been found in HPA axis activity in this model, with Solberg et al. (2001) reporting increases in corticosterone plasma levels of WKY rats, and Nam et al. (2014) reporting no changes. An important feature of the WKY model, is the aspect and use of appropriate controls. Classically, Sprague-Dawley (SPD) rats are used as a positive control for WKY rats as they are the most commonly used rats in scientific research and as such act as the appropriate control (Scholl et al., 2010, Tejani-Butt et al., 2003). However, given the fact that WKY rats originate from the Wistar line, this species can also be utilised as the positive control (Scholl et al., 2010, Tejani-Butt et al., 2003). With this in mind, variations in behavioural results in the WKY model can depend upon which strain *is* used as the control (Scholl et al., 2010). The WKY model has also been proven to show variability in behavioural output dependent upon source and breeding (Nam et al., 2014, Paré and Kluczynski, 1997, Will et al., 2003), and as such, is particularly sensitive to environmental stressors, especially animal-handling (Tejani-Butt et al., 2003). Lastly, SSRI's such as paroxetine (Tejani-Butt et al., 2003), fluoxetine (Griebel et al., 1999, Will et al., 2003) and citalopram (Pollier et al., 2000) have been shown to have no effect behaviourally or neurochemically in this model and as such, an unresponsiveness or insensitivity of WKY rats to 5-HT modification has been hypothesised (Tejani-Butt et al., 2003). This is a major confound of predictive validity

in the WKY model, as SSRI's are considered among first-line treatment in MDD. Nevertheless, it has been suggested that as a result of this resistance to SSRI antidepressants, perhaps the WKY model may function more appropriately as a model for treatment-resistant depression, rather than MDD *per se* (Nam et al., 2014).

Indeed, the therapeutic efficacy of antidepressants is a big factor that should be taken into consideration with regards strong predictive validity in animal models of depression, and particularly with regards the duration of drug treatment and improvement, i.e. the antidepressant action and effect (Frazer and Morilak, 2005). Chronic rather than acute treatment with antidepressants should be the mainstay in animal models of depression, as this more fully resembles the course and time it takes for currently marketed antidepressants to work in the human condition. Having said this, it is unfortunately more often that acute antidepressant treatment that is more regularly examined. Acute treatment with antidepressants has been shown to work in the four models mentioned in Table 1.2, with the exception of the WKY model that does not respond to acute or chronic SSRI treatment at all and as such is considered a model of treatment-resistant depression (Nam et al., 2014, Tejani-Butt et al., 2003). On the otherhand, chronic treatment with antidepressants has only been seen to be consistently effective in the CMS (Song et al., 2006, Wang et al., 2018, Wei-Wei et al., 2014) and OB model (Breuer et al., 2009, Thakare et al., 2017, Yang et al., 2014). The LH model has shown more mixed results. For example, chronic administration with deprenyl, a MAOI, attenuated helplessness in the LH model, but had no effect on anxiety-like behaviour in the open field (OF) test (Schulz et al., 2010). In a separate study, chronic administration with imipramine and fluoxetine, a TCA and an SSRI respectively, has been shown to retrieve cognitive function in the LH model (Song et al., 2006). As a result, it is important to note that attenuation or retrieval of a behavioural response by chronic treatment with antidepressant drugs may be dependant upon the behaviour/endpoint that is being examined. For example, although both chronic and acute antidepressant treatment has been examined in the OB model (Breuer et al., 2009, Saitoh et al., 2008, Thakare et al., 2017, Wang et al., 2012, Yang et al., 2014), researchers tend to focus on chronic treatment in this model as the classic hyperactive phenotype displayed in this model in the OF is only attenuated with chronic but not acute antidepressant treatment (Kelly et al., 1997). For example, Wang et al. (2012) examined acute treatment with the SSRI fluoxetine in the OB rat and found it had no effect in attenuating the FST or OF behaviours in this model. Similarly,

Breuer et al. (2009) examined the effects of acute and chronic antidepressant treatment with several antidepressant drugs on OF behaviour in the OB rat and found that chronic but not acute treatment attenuated hyperactivity in the OF in these animals. As a result, chronic, rather than acute, treatment with antidepressant drugs is the main focus of predictive validity in the OB model, and this more appropriately resembles the treatement course that is seen in the clinical condition.

1.7 Olfactory Bulbectomy model

The olfactory bulbectomy model, involves bilateral removal of the olfactory bulbs that causes adaptations to immune, endocrine, neuroinflammatory and monoamine functioning, altering behavioural aspects that reflect or resemble behavioural abnormalities in MDD (Cairncross et al., 1979, Czeh et al., 2016, Kelly et al., 1997, Song and Leonard, 2005). The downstream neurodegeneration and behavioural changes that occur as a result of removal of the olfactory bulbs are not due to a simple loss in olfaction. These alterations are believed to be due to a reorganisation of neuroanatomical limbic structures within the brain, that lead to behavioural aberrations and adaptations in neurophysiology that resemble the symptoms and neurochemical changes seen in depression (Morales-Medina et al., 2017, Song and Leonard, 2005). Interestingly, abnormal olfactory functioning has been shown to be present in patients with MDD, with the loss in olfaction being correlated with the degree of symptoms (Kohli et al., 2016, Pause et al., 2001).

The highest attribute or quality of the OB model is the fact that the behavioural changes associated with the model, are attenuated with chronic, but not acute antidepressant treatment (Harkin et al., 2003, Kelly et al., 1997, Morales-Medina et al., 2017). This has a strong resemblance to the clinical condition in both course of development of the disorder, and duration of action of antidepressant treatment; in that it takes time for the behavioural changes to develop and that it takes time for the therapeutic effects of the antidepressant treatment to reverse these changes. As a result, the OB model has shown strong face, construct, and predictive validity (Kelly et al., 1997, Song and Leonard, 2005) and this be discussed in the following sections.

1.7.1 Anatomy and Physiology

The olfactory bulbs are bilateral projections of the telencephalon, and rostral to the prefrontal cortex, that comprise of ~4% of the mass of a rodent brain (Kelly et al.,

1997, Ribeiro et al., 2014). The olfactory bulbs are comprised of two organs that send messages to a serious of regions associated with limbic functioning; these are the main olfactory bulb (MOB), and the accessory olfactory bulb (AOB). The main olfactory bulb is comprised of two tiers for processing; the outer layer which contains glomeruli and external tufted cells which are dopaminergic and γ-aminobutyric acid (GABA)-ergic, and the inner layer which contains mitral cell projections and neurons that are cholinergic, serotonergic, and noradrenergic, and also contain enkephalins (Kelly et al., 1997) (Figure 1.9).

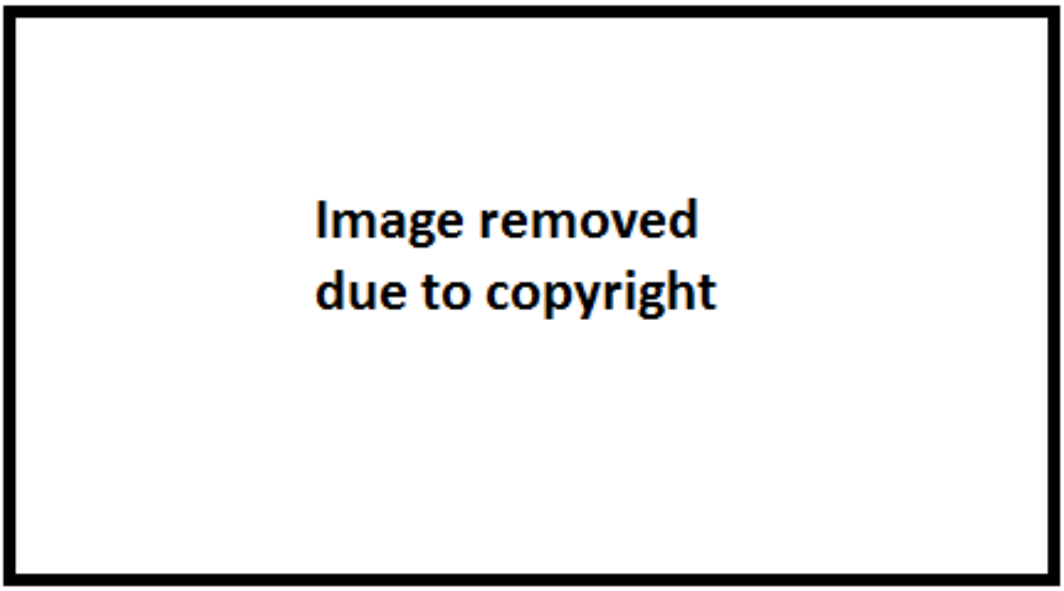


Figure 1.9 Outline of the connections between the olfactory bulbs and the limbic regions in a rodent brain. The main olfactory bulb (*in green*) is comprised of two tiers for processing; the outer layer which contains glomeruli, and the inner layer which contains mitral cells. These cells project down the olfactory tract, with the mitral cells then projecting to the amygdala, entorhinal cortex and the posterior pyriform cortex, and with the glomeruli cells projecting to the anterior piriform cortex. The AOB (*in blue*) projects to the dorsal/lateral olfactory tract and the amygdala, which projects neurons to the hypothalamus. Arrow key in top right-hand corner points to dorsal, ventral, posterior and anterior. AOB=accessory olfactory bulbs, AON=anterior olfactory nucleus, LOT=lateral olfactory tract, OB=olfactory bulbs, OT=olfactory tubercle. Image taken from Suárez et al. (2012).

These cells project to the olfactory tract, with the mitral cells then projecting to the amygdala, entorhinal cortex and the posterior pyriform cortex, and with the tufted cells projecting to the anterior piriform cortex (Kelly et al., 1997). The AOB projects to the dorsal olfactory tract and the amygdala, with particular respect to the bed nucleus of the stria terminalis (BNST) (Kelly et al., 1997). The BNST projects to the

hypothalamus, with the medial forebrain bundle (MFB) in the hypothalamus, which receives inputs from limbic regions such as the hippocampus, nucleus accumbens and prefrontal cortex (Kelly et al., 1997). Bilateral olfactory lesioning leads to a number of structural changes to several brain regions due to the disruption of the pathways that connect these regions, with denervation and anterograde degeneration to the neurons involved (Harkin et al., 2003, Nesterova et al., 2008). Distinct features of the model have been shown to resemble a number of the anatomical changes that are seen in depression. Indeed, OB removal leads to an enlargement in the lateral and third ventricles (Wrynn et al., 2000), an alteration that is also seen in the brains of patients with MDD (Dolan et al., 1985, Kellner et al., 1986). In a study by Wrynn et al. (2000), OB lesioning resulted in decreased signal intensity in the frontal cortex, the caudate and the amygdala of rats when examined by magnetic resonance imaging (MRI). MDD patients have been shown to have altered blood flow and glucose metabolism in the frontal cortex, caudate and amygdala, when compared to healthy controls, signifying changes to synaptic transmission and signalling (Drevets 1998). The volume of the hippocampus is also reduced in OB animals after lesioning (Yurttas et al., 2017), another feature that has been shown in the brains of depressed patient's post-mortem (Videbech and Ravnkilde, 2004). In conjunction with this, dendritic spine density has been shown to be reduced in the hippocampus in rodents after olfactory ablation (Norrholm and Ouimet, 2001), with Morales Medina et al. (2013) also reporting a reduction in the branching of CA1 neurons in the hippocampus after olfactory bulbectomy (Morales-Medina et al., 2013).

With regards the physiological features of the OB model, reports of a reduction in the bodyweight of OB animals after surgery has been shown in many studies (Aswar et al., 2012, Saitoh et al., 2008, Kalshetti et al., 2015a, Kelly et al., 1997), and a reduction in appetite and alterations in bodyweight gain is a feature of MDD (DSM-V). Patients with depression have been shown to have abnormal heart rate variability when compared to healthy individuals (Carney et al., 2001), and this is thought to be linked with an increased cardiac mortality in patients with MDD. Interestingly, this feature was also observed by Kalshetti et al., (2015a) and Roche et al. (2007), in that OB animals that were exposed to an acute stressful stimuli, had a significantly decreased heart rate when compared to sham-operated animals, indicative of the autonomic dysfunction seen in the clinical scenario. Sleep is also altered after OB

removal, with OB animals shown to have increased REM sleep in comparison to sham rats, resonate of the sleep disruptions seen in depression (Wang et al., 2012).

1.7.2 Species variations in OB animals

At this stage, it is important to note that there are variations between species in the OB model (Hendriksen et al., 2015). Firstly, it must be acknowledged that in the human condition of the psychiatric disorder of depression, the olfactory bulbs are not surgical removed. Although a reduction in olfactory function can bee seen in humans with MDD (Kohli et al., 2016, Pause et al., 2001), this lack of surgical change is nevertheless, a limitation of the OB animal model of depression. As this project will focus on the OB rat, it was important to include this section. The model is primarily used in rats and mice, although it has also been performed in hamsters (Roche et al., 2008). Indeed, the OB model was first performed in rats by Marks et al. (1971) to assess the effects of anosmia on learning performance and by far the most utilised species for the OB model is rats. Nevertheless, the use of mice is also common. Both OB rats and mice display increases in locomotor activity in the OF test, which can be attenuated with chronic antidepressant treatment (Roche et al., 2008), and deficits in passive avoidance (PA), a test that is used to examine learning and cognitive function (Harkin et al., 2003). However, unlike rats, this can be dependent upon strain (Harkin et al., 2003). C57Bl/6j mice and DBA/2j mice display hyperactivity in the OF test as seen in rats, but only C57B1/6j mice display a deficit in the PA test, which was shown to be attenuated with chronic antidepressant treatment; an effect that was not present in DBA/2j mice (Otmakhova et al., 1992). Indeed, OB mice have also been shown to have differential findings with antidepressant modification. Chronic treatment with amitriptyline, a TCA, restores performance in the PA test in rats, but has no effects in C57B1/6j mice (Jarosik et al., 2007, van Riezen et al., 1977). Enhanced rearing was decreased in C57B1/6j mice after antidepressant treatment, but had no effect in DBA/2j mice (Otmakhova et al., 1992). Neurochemical evidence is also inconsistent between species particularly with regards BDNF expression in the hippocampus, with C57B1/6j mice showing increased BDNF expression in the hippocampus (Hellweg et al., 2007), and OB in Sprague-Dawley rats displaying decreases in BDNF expression (Hendriksen et al., 2012) as it found in patients with MDD. Similarly, in the clinical condition, depressed patient have an increase in the density of 5-HT_2 receptors in the

brain (Stockmeier 2003, Yates et al., 1990), an effect which is seen after OB removal in rats (Earley et al., 1994), but not in all mice strains (Gurevich et al., 1993).

In conclusion, variations to behaviour and neurochemistry exist in the OB model dependent upon the species it is performed in. Although both rats and mice have been used, there are more differences across strains in OB mice than in OB rats. Although in the following sections the effects of bilateral OB removal will focus on evidence in rats, evidence in mice may also be alluded to.

1.7.3 Endocrine System

There are many features of the endocrine system that are seen to be elevated or altered as a result of OB surgery, and these features are seen to resemble changes that occur to the HPA and neurotropic systems in MDD. As mentioned previously, cortisol is a marker of stress induction and is seen to be increased in patients with MDD (Bhagwagar et al., 2005). In animals, the equivalent of this stress hormone is corticosterone and this is shown to be increased after OB removal. Olfactory bulbectomy causes increased corticosterone levels in serum in OB animals, when compared to levels in sham-operated animals (Jindal et al., 2015b, Rinwa and Kumar, 2013, Song et al., 2009, Thakare et al., 2017, Yang et al., 2014). Similarly, increased plasma corticosterone concentrations are seen in OB rats in comparison to sham-operated rats (Marcilhac et al., 1999). Interestingly, Cairncross et al. (1977) showed that sham-operated rats that were exposed to stress had a significant increase in corticosterone levels, and that rats that underwent OB surgery alone, without any subsequent stress exposure, were shown to have the same level of corticosterone elevation as the stress-exposed sham-operated rats, delineating a significant increase in HPA-axis activity as a result of OB surgery. CRH mRNA levels are also shown to be significantly increased in the hypothalamus of OB rats when compared to sham-operated animals (Park et al., 2013, Song et al., 2009, Yang et al., 2014), another biomarker of stress that has been shown to be elevated in depression. However, Marcilhac et al. (1999) found no difference in ACTH plasma concentrations between sham-operated and OB animals (Marcilhac et al., 1999). Overall, the OB model shows good construct validity and displays a dysregulation in HPA axis activity and endocrine functioning similar to that which is seen in MDD.

1.7.4 Inflammatory System

The inflammatory system has been shown to be activated in the OB model, similar to that which is seen in MDD. Increased levels of pro-inflammatory markers TNF-α and IL-6 in the cerebral cortex and hippocampus have been observed in OB rats when compared to sham-operated counterparts (Rinwa and Kumar, 2013, Thakare et al., 2017), with Yang et al. (2014) finding increases in TNF-α and IL-1β in the hippocampus of OB rats. Rinwa and Kumar (2013) also showed that this increase in these inflammatory markers after OB surgery caused a reduction in cell density in the CA1 of the hippocampus and cerebral cortex. Interestingly, Almeida et al. (2017) looked at the effects of the OB procedure on inflammation after two, four and eight weeks post-surgery. Apoptosis, is the process of cell death and patients with MDD have been shown to be more susceptible to stress, which induces abnormal changes to populations of neurons undergoing cell death, with increased activity of apoptosis being hypothesised (Eilat et al., 1999, McKernan et al., 2009). The OB model has been shown to induce neuronal cell death, with increases in the number of cells showing pyknosis in the temporal cortex and hippocampus (Nesterova et al., 2008), and increases in the number of cells showing karyolysis, cytolysis, and vacuolization also shown in the hippocampus (Nesterova et al., 2008). Although the primary focus of this thesis is the OB model in rats, changes to the inflammatory system have also been seen in OB mice. In the hippocampus, IL-1, IL-6, TNF-α and IL-10 were shown to be significantly elevated at all three timepoints in OB mice when compared to sham-operated rats, and in the frontal cortex, IL-1 and IL-6 were also elevated at all three timepoints, with TNF-α being elevated at two and four weeks post-surgery, and IL-10 being shown to be elevated at two weeks post-surgery (Almeida et al., 2017). Similarly, Bobkova et al. (2016) also examined apoptotic neurons in the temporal cortex, and the CA1 and CA3 of the hippocampus of sham-operated and OB mice, and found that OB mice had a significant increase in the proportion of apoptotic cells in comparison to sham-operated mice, with significant increases of karyolysis, cytolysis, pyknosis and vacuolization. To conclude, there is evidence for an increase in the inflammatory markers and cell apoptosis in the OB model, another neurochemical feature that resembles alterations in the inflammatory system in MDD.

1.7.5 Monoamine Evidence

Removal of the olfactory bulbs causes a number of changes to neurotransmitters systems, with particular respect to monoaminergic functioning and specific brain regions such as the hippocampus, amygdala and prefrontal cortex. Indeed, the OB model has been described as a model of hypo-serotonergic depression (Zhou et al., 1998, Harkin et al., 2003), due to the major involvement of serotonin in this model. Olfactory bulbectomy results in reductions in dopamine (DA), 5-HT and NA levels in the hippocampus and cerebral cortex (Thakare et al., 2017). OB rats have been found to have decreases in 5-HT in the amygdala (Marcilhac et al., 1999, Saitoh et al., 2008), hippocampus (Saitoh et al., 2008) and frontal cortex (Saitoh et al., 2008), with increases in the level of 5-HT in the hypothalamus (Saitoh et al., 2008), or no changes in this monoamine in the hypothalamus (Marcilhac et al., 1999). In contrast, Wang et al. (2012) showed that OB rats have a decrease in 5-HT levels in the medulla, but no significant difference in the midbrain, cortex, hippocampus, thalamus, hypothalamus or striatum when compared to sham-operated rats. 5-hydroxyindoleacetic acid (5-HIAA) is the metabolite of 5-HT, and this has been shown to be decreased in the nucleus accumbens (Connor et al., 1999), and the hippocampus and amygdala of OB rats (Saitoh et al., 2008). Connor et al., (1999) looked at the effect of air-puff stress on 5-HIAA concentrations in the nucleus accumbens of OB rats and found that OB rats exhibited increased levels of 5-HIAA in comparison to shams after exposure to stressful stimuli, again exhibiting an susceptibility to the serotonergic pathway in this model after exposure to stress (Connor et al., 1999).

As mentioned previously, serotonergic neurons are synthesised in the DRN, and are projected to several limbic and forebrain regions (Harken et al., 2003). Shin et al. (2017) found that 5-HT-postive cells, TPH-positive cells and the expression of 5-HT_{1A} receptors were all decreased in the dorsal raphe of OB rats. Saitoh et al. (2008) also looked at tryptophan hydroxylase (TPH) immunoreactivity (the rate-limiting enzyme in the synthesis of 5-HT) in the raphe, an area involved in the activation and inhibition of 5-HT, and found that these TPH-positive cells were decreased in the median raphe of OB rats when compared to sham-operated counterparts. Serotonergic hyper-innervation of the frontal cortex in OB animals has been shown (Zhou et al., 1998), and this is a feature that is seen upon brain imaging of MDD patients (Agren and Reibring, 1994). Up-regulation of the density of serotonergic nerve terminals in this region in MDD is thought to reflect a local compensatory response to the neuronal

degeneration shown in the dorsal raphe (Agren and Reibring, 1994, Zhou et al., 1998). An increase in the density of 5-HT_{2A} receptors is also seen after OB removal (Sato et al., 2010), a distinct feature in patients with MDD (Stockmeier 2003, Yates et al., 1990).

Although changes to the monoamine system after OB removal have focused on the serotonergic pathway, OB removal has also been shown to decrease NA (Kalshetti et al., 2015b, Pawar et al., 2018, Pistovcakova et al., 2008, Redmond et al., 1999, hang et al., 2016) and DA (Jancsár and Leonard, 1984, Ruda-Kucerova et al., 2015) concentrations. Therefore, overall the OB model displays alterations in monoaminergic functioning, resembling changes to this system in MDD.

1.7.6 Molecular Evidence

As mentioned previously, BDNF is a neurotropic factor that is involved in the response to stress and has been shown to be reduced in patients with MDD (Hosang et al., 2014). BDNF is considered a feature of depression for a number of reasons. Neurogenesis is maintained by neurotrophins such as BDNF which support and regulate cell proliferation, migration, survival and death (Groves et al., 2007). With this in mind, BDNF supports the survival of neurons and has strong neuroprotective properties. When BDNF binds to the tyrosine kinase B (TrkB) receptor, it activates the mitogen-activated protein kinase (MAPK) pathway, increasing a protein known as bcl-2 which is involved in capase-regulated apoptosis and neuronal survival (Yuan and Yankner, 2000). Bcl-2 regulates apoptosis by inhibition of pro-apoptotic members of its own family (Yuan and Yankner, 2000). BDNF therefore indirectly assisits in the regulation of apoptosis, with a reduction in BDNF decreasing the antiapoptotic functioning of bcl-2 (Groves et al., 2007). This then effects overall neurogenesis in the dentate gyrus of the hippocampus, leading to a reduction in hippocampal function and in turn emotion and mood. Therefore, BDNF is a key component in the process of neurogenesis and cell survival in the hippocampus, and as such the overall functioning of mood, key features which are seen to be dysfunctional and reduced in MDD (Bremner et al., 2000, Duman and Li, 2012, Duman and Monteggia, 2006, Hosang et al., 2014). Second, it is believed that the restoration of BDNF in depressed patients plays a critical role in the mechanism of antidepressant efficacy (Groves et al., 2007, Wolkowitz et al., 2011). BDNF KO in the dentate gyrus in rats has been shown to attenuate the actions of antidepressant efficacy (Adachi et al., 2008). In a study by

Wolkowitz et al. (2011), it was shown that baseline BDNF serum levels were correlated with antidepressant success and response, in that MDD patients with higher BDNF serum levels at baseline (prior to treatment) had a better response after antidepressant treatment than MDD patients with a lower BDNF baseline. In this manner, the authors suggested that BDNF is a feature of depression in that it can help to predict responders to antidepressant therapy, but also that this neurotropic factor facilitates the mechanism by which antidepressants work (Wolkowitz et al., 2011).

With regards BDNF in the OB model, Li et al. (2015) found reduced expression of BDNF protein levels in the CA3 of the hippocampus in OB rats, but no differences in the CA1 or dentate gyrus (DG) when compared to controls. BDNF protein levels were seen to be decreased in the hippocampus (Jindal et al., 2015b, Thakare et al., 2017, Yang et al., 2014) and cerebral cortex in OB rats in comparison to sham-operated rats (Thakare et al., 2017). In contrast, Jastrzębska-Więsek et al. (2018) found no difference in BDNF protein levels in the PFC or hippocampus between sham-operated and OB rats. BDNF expression has been shown to be reduced in patients with MDD (Youssef et al., 2018), and although there is mixed findings with regards BDNF expression as a result of OB removal, overall the model has been shown to represent this feature of the disease.

Other neurotransmitters are disrupted by removal of the olfactory bulbs. Several studies have found increases in the concentration of GABA after OB surgery (Dennis et al., 1993, Jancsár and Leonard, 1984, Ruda-Kucerova et al., 2015). Glutamate levels have been shown to be increased in the nucleus accumbens (Ruda-Kucerova et al., 2015), and medial prefrontal cortex (Jiménez-Sánchez et al., 2016), with reduction in levels being observed in the olfactory tract (Collins, 1984).

Interestingly, the OB ablation has also been shown to effect the cholinergic system, a neurotransmitter system that is also shown to be effected in patients with MDD (Saricicek et al., 2012). Saricicek et al. (2012) found that *in vivo* patients with acute MDD were shown to have a lower availability of the β_2-subunit-containing nicotinic acetylcholine receptors (nAChRs) in a number of brain regions including the thalamus, striatum, cerebellum, frontal and parietal cortex, and the anterior cingulate cortex, but that there was no differences in the number of nAChRs in the frontal cortex of MDD patients post-mortem when compared to healthy controls. Similarly, Stanley (1984) found no differences in the number of binding sites for muscarinic receptors in the frontal cortex in the post-mortem brains of MDD patients, whereas Meyerson et

al. (1982) found an elevation in the number of binding sites for muscarinic receptors in MDD suicide patients, and Gibbons et al. (2009) found a reduction in binding in this region in MDD patients, all when compared to healthy controls. As a result, it is difficult to underpin the exact role of the cholinergic system in MDD as the finding are mixed across studies, but it is often regarded as being dyfuctional, imbalanced or disregulated in depression rather than there being *deficits* in this system per sé (Dagytė et al., 2011). On a separate note, as the cholinergic system plays an important role in arousal, cognition, and memory, and there are changes in the OB model in this neurotransmitter system, the OB model is often used as a model of Alzheimer's disease due to the cholinergic changes and cognitive deficits that are observed (Bobkova et al., 2016, Feng et al., 2017, Stepanichev et al., 2016). Stepanichev et al. (2016) showed that OB removal induces a loss of cholinergic neurons in the medial septum of mice, which is the main input of cholinergic function to the hippocampus. Bobkova et al. (2016) has also shown a decrease in choline acetyltransferase (ChAT) immunopositive neurons in the forebrain after OB ablation; the enzyme responsibility for the synthesis of acetylcholine. In tandem with this, Feng et al. (2017) has shown an increase in the acetylcholinesterase (AChE) inhibitor; the enzyme that metabolises acetylcholine. As a result, the OB model may also have potential as a model for disorders such as dementia.

The opioid system has also been shown to be effected by olfactory bulbectomy, with μ-opioid (MOP) binding shown to be decreased in the amygdala, piriform cortex, olfactory tubercle and olfactory peduncle, and increased in the hypothalamus after removal of the olfactory bulbs (Hirsch, 1980). Holmes (1999) showed that OB surgery caused an increase in pre-proenkephalin (PENK) mRNA expression in the olfactory tubercle at two and four weeks post-surgery, but with no effects being seen at one week post-surgery. Interestingly, Primeaux and Holmes (2000) also reported an increase in PENK mRNA expression in the olfactory tubercle and piriform cortex of bulbectomised rats, as well as an increase in enkephalin (ENK)-like immunoreactivity in the olfactory tubercles. Holmes (1999) also examined PENK expression in the nucleus accumbens after OB surgery, but found no significant differences between sham-operated and OB rats. As the opioid system has been shown to be altered in MDD (Hsu et al., 2015, Hurd, 2002), this is another feature of the OB model that may warrant more investigation as it could mirror the neurochemical disruptions observed in this system in MDD.

1.7.7 Behavioural Evidence

Firstly, the OB model is often depicted as a model of agitated depression (Kelly et al., 1997). OB animals have been shown to display an aggressive behaviour, associated with irritability and agitation, similar to the agitated symptoms which are seen in MDD (Leonard and Tuite, 1981, van Riezen et al., 1977). Interestingly, the degree of irritability has been shown to be correlated with the degree of 'bulbectomy'; in that the deeper the lesion encroaches on the olfactory peduncle, the more reactive the animal is likely to be (Leonard and Tuite, 1981, Sieck and Gordon, 1972, Sieck, 1973). Having said this, this aggression in the rat can be pronouncedly reduced following daily handling by the researcher, in conjunction with group-housing conditions such as the employment of two sham-operated animals with two OB animals (Kelly et al., 1997, Leonard and Tuite, 1981).

Hyperemotionality (HE) is a test in which rodents can be examined for their response to unexpected stressful stimuli, as assessed by acts such as poking with a rod, attempted capture with a glove, or sudden puffs of air (Leonard and Tuite, 1981). Scored on an arbitrary scale, a rat can be assessed for their level of HE or 'irritability' *per se*. OB removal has been shown to significantly increase HE or 'emotional' behaviour in animals, when compared to sham-operated counterparts (Devadoss et al., 2010, Jiménez-Sánchez et al., 2016, Pandey et al., 2010; 2014). This hyperactivity in behaviour in the syndrome is also seen with regards locomotor activity, when animals are placed into a novel brightly lit environment. The OF is a test that is used to look at 'anxiety-like' behaviour in a novel aversive environment, whilst also assessing locomotor activity. Rodents when placed into the OF are less likely to spend more time exploring the centre of the OF, as they are not fond of open spaces. If an animal is seen to spend more time in the centre, this is seen as 'anxiolytic-like' behaviour. In OB animals, a characteristic hyperactivity, or, increased movement is seen when these animals are placed into the OF. This hyperactivity can only be attenuated with chronic antidepressant modification (Kelly et al. 1997), and is seen to be comparative to the psychomotor agitation and restless unconscious repetitive behaviours in MDD. Evidence for this hyperactivity in OB animals has been widely shown (Almeida et al., 2017, Burke et al., 2015; 2019b, Kelly and Leonard, 1999, Kalshetti et al., 2015a; 2015b, Li et al., 2015, Linge et al., 2013, Morales-Medina et al., 2012a; 2012b; 2012c,; 2013, Rinwa and Kumar, 2013, Shin et al., 2017, Smaga et al., 2017, Stepanichev et al., 2016, Thakare et al., 2017, Van Riezen et al., 1977, Wang et al., 2012, Yang et al.,

2014). OB rats also exhibit increased grooming and rearing in the OF in comparison to sham-operated animals, again exemplifying more 'anxiety-like' behaviour (Kalshetti et al., 2015a; 2015b, Ling et al., 2013, Morales-Medina et al., 2012a,; 2012b; 2012c; 2013, Thakare et al., 2017). OB animals also spend less time in the more brightly lit centre of the OF, again indicative of 'anxiety-like' behaviour (Linge et al., 2013). An important feature of this hyperactive deficit in OB animals is the set-up of the aversive arena, with aluminium reflective walls or black polythene walls being necessary to illicit this increase in locomotor function in this syndrome (Kelly and Leonard, 1997).

Another test of anxiety-like behaviour is the elevated plus maze (EPM). The EPM consists of a maze in the shape of a cross with two open or exposed arms and two walled or enclosed arms. The amount of time and the number of entries animals make into the open and closed arms measures 'anxiety-like' behaviour with the more time the animal spends in the open arms being seen as anxiolytic. OB rats spent less time in the open arms than sham-operated rats (Saitoh et al., 2008, Stepanichev et al., 2016) and have a decrease in the amount of open arm entries in comparison to sham-operated rats (Saitoh et al., 2008). However, some studies, in contrast, have found no difference in the number or percentage of open arm entries made by OB rats when compared to shams (Holubova et al., 2016, Stepanichev et al., 2016).

The FST and TST, as mentioned previously, are tests of 'depressive-like' behaviour and have been examined in the OB model. In the FST, an animal is placed into a beaker of water and left to swim for a period of 5-15 minutes, with an increase in the time an animal spends immobile seen to be indicative of helplessness; symptomatic of this behavioural trait in MDD. Both tests are also used as 'screens' for testing antidepressant activity (Abelaira et al., 2013). OB surgery in rats has been shown to significantly increase immobility time in the FST when compared to sham-operated animals (Li et al., 2015, Linge et al., 2013, Morales-Medina et al., 2012a; 2012b; 2012c; 2013, Rinwa and Kumar, 2013, Shin et al., 2017, Smaga et al., 2017, Thakare et al., 2017, Wang et al., 2012, Yang et al., 2014).

Anhedonia, or the loss of interest/pleasure, is another symptom of MDD and is a behavioural trait that has been shown to be altered as a result of OB ablation. The sucrose/saccharin preference test (SPT) is used as a test for 'anhedonic-like' behaviour. Animals are water restricted for a short period of time and are then given two water bottles to choose from overnight; one containing water and the other

containing sucrose/saccharin. A decrease in sucrose consumption in the SPT is identified as 'anhedonia-like' behaviour, with the animal displaying a loss of pleasure. OB animals have been shown to have reduced sucrose preference when compared to sham-operated counterparts, and as such are said to exhibit anhedonia as a behavioural symptom (Jiménez-Sánchez et al., 2016, Kalshetti et al., 2015b, Li et al., 2015, Linge et al., 2013, Shin et al., 2017). Some studies have found that OB animals have reduced sucrose consumption rather than a preference *per se* (Stepanichev et al., 2016, Zhang et al., 2016).

Social functioning is an important feature of everyday life and is a characteristic that is seen to be impaired in individuals with MDD (Hirschfield et al. 2000, Kan et al., 2004, Lee et al., 2005). Social interaction and deficits in sociability can be measured in animals via the social interaction (SI) test, which involves placing an animal into an environment with another novel or unfamiliar animal (conspecific) that they have not met before. Animals are free to explore each other, and the arena, and several different parameters can be scored with interaction time being summarised as a measure of 'anxiety-like' behaviour and sociability; two deficits in MDD. Social interactions such as social contacts, play, mounting, probing, grooming and crawling over and under the other rat can be measured. OB animals have been shown to have a deficit in social interaction, with decreased interaction time (Jiménez-Sánchez et al., 2016, Morales-Medina et al., 2012a; 2012b; 2012c, Pandey et al., 2008; 2010; 2014, Rajkumar et al., 2009) and a reduction in the number of social contacts (Morales-Medina et al., 2012a; 2012b; 2012c) in the SI test.

Cognitive dysfunction is another aspect of the behavioural abnoramlities in MDD, and similarly has been shown to be altered after OB removal. Indeed, OB animals have shown cognitive deficits in a number of behavioural tests for cognition and memory. These include a reduction in latency to enter the negative chamber in the PA test (Borre et al. 2012a; 2012b; 2012c, Douma et al. 2011, Hendriksen et al., 2012, van Riezen et al., 1977), increased time to learn to reach the platform in the Morris water maze (MWM) test (Holubova et al., 2016, Morales- Medina et al., 2013, van Rijzingen et al., 1995), a reduction in alterations between arms in the T-Maze test (Borre et al. 2012c, Hendriksen et al., 2012, Zueger et al., 2005), Y-Maze test (Stepanichev et al., 2016) and failure to discriminate between the novel and familiar object in the novel object recognition (NOR) task (Douma et al., 2011).

Sexual behaviour has also been shown to be disrupted after OB surgery, with sexual dysfunction also being seen in patients with MDD (Kennedy and Rizvi, 2009). After OB surgery, male rats have been shown to have a decrease in sexual activity, with a reduction in ejaculation and in androgen receptor binding; a hormone involved in male reproductively activity, in both the amygdala and hypothalamus which was shown to be correlated with the reduced ejaculation (Lumia et al., 1987). Latency to mount is increased in OB rats when compared to sham-operated counterparts (Aswar et al., 2012). Edwards et al. (1990) showed that OB male rats had no preference between a sexually receptive and non-receptive rat, and OB males spent equal amounts of time interacting with each female and a neutral compartment, in comparison to control rats who preferred and spent the most time with the sexually receptive female.

In conclusion, the OB model displays a number of symptoms that resemble the physiological and behavioural changes that are seen in MDD.

1.7.8 Antidepressant evidence

A well-established feature of the OB model of depression is its strong predictive validity, with behavioural deficits in the model being shown to be attenuated with chronic, rather than acute, antidepressant treatment (Kelly et al., 1997). Indeed, this characteristic of the OB model strengthens its translational validity over other models, by reflecting the timecourse it takes for antidepressant therapy to effect behaviour in the clinical scenario; an important feature of preclinical depression research. The 'core' features associated with the OB model are that of hyperactivity in the OF test, or indeed an increase in locomotor activity when measured in the homecage (Kelly et al., 1997, Song and Leonard, 2005). In addition, OB animals present with an overall agitation or irritability in their behaviour (Van Riezen et al., 1977). These features of the model are often considered as the phenotypic behaviour that is expected after surgerical procedure and prior to antidepressant intervention, and as a result, are 'core' features that a successful OB candidate should present with. As such, these measures are often regarded as a control before intervention and as such are indicative that animals are 'depressive-like' in behaviour.

A substantial number of marketed antidepressants, as well as experimental compounds, have been examined in the OB model. A summary of results for the effects of chronic dosing with a number of classes of antidepressants in the OB model is shown in Table 1.3. Three tests that have been consistently used to assess

antidepressant efficacy in this model are that of the OF, FST and PA tests (Kelly et al., 1997, Song and Leonard, 2005); with the behavioural effects of each test in this model having been shown to be reversed with chronic antidepressant treatment, and by a number of classes of antidepressant drugs (Van Riezen et al., 1977, Kelly and Leonard, 1994; 1999, Thakare et al. 2017). OB animals are considered 'reactive' in nature, such that on presentation of stressful stimuli such as the OF and FST, their rate of initial reactivity is heightened and causes an adaptation in their ability to habituate to an environment (Kelly et al., 1997, Song and Leonard, 2005). It is believed that in the OF, the most commonly examined paradigm in this model, chronic antidepressant treatment in OB animals evoke their effects by increasing the rate at which these syndrome animals habituate to the arena (Mar et al., 2000). Although a number of studies have examined the effects of *acute* administration with different antidepressants, the results are less consistent. For example, Wang et al., (2012) found that acute treatment with fluoxetine, an SSRI, had no significant effect on hyperactivity in the OF, or immobility in the FST, but abolished increased REM sleep in OB animals. Acute administration of pramipexole and 7-OH-DPAT, two dopamine agonists (Breuer et al., 2009), imipramine, a TCA (Breuer et al., 2009; 2007), and escitalopram, an SSRI (Breuer et al., 2007) had no significant effect on locomotor activity in the OF test. Indeed, chronic treatment with the conventional TCAs, SSRIs, and the MAOI class of antidepressants have all been shown to reverse a number of behavioural and neurochemical deficits in the OB model (Table 1.4 Summary of sub-chronic and chronic antidepressant treatment in the OB model of depression.Table 1.4). Breuer et al. (2007) also showed that upon cessation of chronic antidepressant treatment (dosing for 14 days), OB rats treated with the SSRI citalopram continued to have a reduction in hyperactivity in the OF comparable to sham-operated rats up to 6 weeks after treatment, and OB rats treated with the TCA imipramine continued to have locomotor activity comparable to sham-operated rats up to 10 weeks after treatment. Again this is translatable to the timecourse of action of antidepressant treatment in the clinical scenario of depression. Taking all of this into consideration, as the behavioural and neurochemical symptoms within the OB model have been shown to be reversed by a number of marketed antidepressants where chronic regimens are used, the OB model can be most appropriately regarded as a model of standard depression, as opposed to a model of treatment-resistant depression, as the current antidepressants therapies used to treat MDD effectively attenuate any deficits seen with OB syndrome

animals. With this in mind, the OB model is often recognised as a pertinent paradigm for assessing novel antidepressant targets (Harkin et al., 2003, Kelly et al., 1997).

Drug	Class	Dose	Species	Dosing Period	Effect	Reference
Mianserin	TCA	5, 10 mg/kg	Rat	7 days	↓ Locomotor activity, ↑ Cognitive Functioning	van Riezen et al. 1977*****
		10 mg/kg i.p	Rat	28 days	↑ Cognitive Functioning, ↓ Hyperemotionality, ↓ Corticosterone	Jesberger and Richardson, 1986
Desipramine	TCA	10 mg/kg oral	Rat	14 days	↓ Locomotor activity, ↓ Immobility time	Kelly and Leonard, 1999
		10 mg/kg i.p	Rat	7 days	↑ OA time and entries, ↓ Hyperemotionality	Saitoh et al. 2008
Lofepramine	TCA	20 mg/kg oral	Rat	14 days	↓ Locomotor activity	Kelly and Leonard, 1999
Amitriptyline	TCA	10 mg/kg i.p	Rat	14 days	↑ Social interaction,↑ Sucrose Preference, ↓ Locomotor activity, ↓ Hyperemotionality	Pandey et al. 2010
		10 mg/kg i.g	Rat	14 days	↓ Locomotor activity, ↓ Immobility time, ↓ TNF-α and IL-1, ↓ Corticosterone	Yang et al. 2014
		10 mg/kg i.p	Rat	14 days	↓ Locomotor activity	Burke et al. 2015
		3, 10 mg/kg	Rat	7 days	↓ Locomotor activity, ↑ Cognitive Functioning	van Riezen et al. 1977*****
		3, 10, 20 mg/kg i.p	Rat	28 days	↑ Cognitive Functioning, ↓ Hyperemotionality	Jesberger and Richardson, 1986
Iprindole	TCA	25 mg/kg i.p	Rat	28 days	↑ Cognitive Functioning, ↓ Hyperemotionality, ↓ Corticosterone	Jesberger and Richardson, 1986
Tianeptine	TCA	5 mg/kg i.p	Rat	21 days	↓ Immobility time, ↓ Locomotor activity	Kelly and Leonard, 1994
Imipramine	TCA	10 mg/kg s.c	Rat	14 days	↓ Locomotor activity	Roche et al. 2008
		20 mg/kg oral	Rat	14 days	↓ Locomotor activity, ↓ rearing, ↓ grooming	Aswar et al. 2012
		20 mg/kg i.p and oral	Rat	7 and 14 days	↓ Locomotor activity	Breuer et al. 2007
		10 mg/kg i.p	Rat	7 and 14 days	↓ Locomotor activity	Breuer et al. 2009
Paroxetine	SSRI	10 mg/kg i.p	Rat	14 days	↓ Locomotor activity, ↑ Social interaction	Pandey et al. 2009
Sertraline	SSRI	5 mg/kg i.p	Rat	21 days	↓ Immobility time, ↓ Locomotor activity, ↓ 5-HIAA	Kelly and Leonard, 1994

Drug	Class	Dose	Species	Dosing Period	Effect	Reference
Escitalopram	SSRI	5, 10 mg/kg oral	Rat	14 days	↓ Locomotor activity	Breuer et al. 2007
		10 mg/kg oral	Rat	14 days	↑ Social interaction, ↓ Locomotor activity, ↓ Hyperemotionality	Pandey et al. 2014
Fluoxetine	SSRI	30 mg/kg i.p	Rat	14 days	↓ Locomotor activity, ↓ rearing, ↓ grooming	Aswar et al. 2012
		20 mg/kg i/p	Rat	14 days	↓ Locomotor activity, ↓ Immobility time, ↑ BDNF, ↑ DA, ↑ NA, ↑ 5-HT, ↓ TNF-α and IL-1, ↓ Corticosterone	Thakare et al. 2017
		15 mg/kg s.c	Rat	14 days	↑ 5-HT	Marcilhac et al. 1999
Moclobemide	MAOI	15 mg/kg i.p	Rat	14 days	↓ Locomotor activity, ↓ rearing, ↓ grooming, ↓ mounting latency, ↑ OA time	Aswar et al. 2012

Table 1.4 Summary of sub-chronic and chronic antidepressant treatment in the OB model of depression. The drug name, class of antidepressant, dose given, route of administration, duration of dosing, and behavioural and neurochemical effects are shown in the table above. Locomotor activity as measured in the open field. Immobility time as measured in the FST or TST. Cognitive functioning as measured in the MWM, PA or T-maze. Social interaction as measured in the SI test. Hyperemotionality as measured in the HE test. OA time and entries as measured in the EPM test. BDNF=brain-derived neurotropic factor, DA=dopamine, i.g=intragastrically, IL-1=interleukin 1β, i.p=intraperitoneal, MAOI=monoamine oxidase inhibitor, NA=noradrenaline, OA=open arm, oral=oral gavage, s.c=subcutaneous, SSRI=selective serotonin reuptake inhibitors, TCA=tricyclic antidepressants, TNF-α=tumor-necrosis factor alpha, 5-HIAA=5-hydroxyindoleacetic acid, 5-HT=serotonin. *van Riezen et al. (1977) did not specify the route of administration.

1.7.9 Anosmia in the OB model

Olfaction is an important sensory function in humans, but even more so in animals, especially with regards social interaction. Originally, researchers questioned whether it was a mere loss in olfaction due to the removal of the olfactory bulbs that was causing the multiple biochemical and behavioural disparities shown in the OB model. However, research has shown that the change in molecular and behavioural functioning in these animals is not due to a simple loss in olfaction, but that a more in-depth reorganisation of the limbic system, particularly of the cortical-hypothalamic-hippocampal-amygdala circuits, is at play (Czeh et al., 2016).

The procedure of rendering an animal anosmic has been used as a positive control for changes seen in the OB model. Zinc Sulphate ($ZnSO_4$), a naturally occurring metallic salt compound, has been shown to cause degeneration to the olfactory epithelium, and as such a loss of smell or anosmia, when employed via intranasal application (Mayer and Rosenblatt, 1993, McBride et al., 2003, Thor et al., 1976, van Riezen et al., 1977). McBride et al. (2003) showed that intranasal irrigation with 5% $ZnSO_4$ caused a significant increase in the time it took mice to accurately detect vapour cues in an odour discrimination task, when compared to saline-treated controls. Mayer and Rosenblatt (1993), showed that two days after treatment with $ZnSO_4$, mothers of new born litters had an absence of preference for their pup's odours over that of scented wood-shavings. The behaviour of animals exposed to $ZnSO_4$ has been compared with that of animals that have gone through OB removal, and their behavioural outputs are very different. In contrast to OB animals, animals exposed to $ZnSO_4$ display normal taste aversion (Grigson et al., 1997), no differences in the MWM (van Rijzingen et al., 1995), no difference in sexual activity (Cain and Paxinos, 1974), no differences in locomotor activity (Andiné et al., 1995, Borre et al., 2014, Mar et al., 2000, van Riezen et al., 1977), no difference in PA acquisition (Borre et al., 2014, van Riezen et al., 1977), no difference in % alternation in the T-Maze (Borre et al., 2014), and no difference in irritability or aggressive behaviour (Cain and Paxinos, 1974, van Riezen et al., 1977), when compared to sham-operated animals. Unlike the OB model, after $ZnSO_4$ lavage there are also very few neurochemical changes that are reported with regards the systems that are effected in depression (Borre et al., 2014, Mucignat-Caretta et al., 2004).

Implementation of $ZnSO_4$ is not without its challenges, and as a result some studies report the fact that many animals are not proven to be rendered significantly

anosmic when tested in olfactory discrimination tasks (Slotnick et al., 2000). Another major drawback of the implementation of $ZnSO_4$ is that is has been shown to cause injury and high mortality rates in many animals and as such has become a tool that is now rarely employed (Andiné et al., 1995, Crusio and van Abeelen 1987, Mc Bride et al., 2003, Schoots et al., 1978, Slotnick et al., 2000, Thor et al., 1976).

Taking all of this into consideration, anosmia is not a straight-forward procedure and its lack of consistency in duration of action and application across studies needs to be considered for its true value and validity as a positive control. In conjunction with this, the high injury and mortality rates associated with the procedure present a number of ethical confounds (Schoots et al., 1978, Slotnick et al., 2000). Most importantly, the behavioural changes that are exhibited upon OB removal, and that resonant symptomatic changes seen in depression, are not exhibited in $ZnSO_4$-treated animals.

1.7.10 Limitations of the OB model

The sections above show that the OB model is an animal model that exhibits very good face, construct and predictive validity as an animal model of depression. Nevertheless, like all animal models of depression to-date, it also has its own limitations. Firstly, removal of the olfactory bulbs is an irreversible procedure (Wang et al., 2017). As such, and as has been noted in this laboratory, the OB model can be associated with a relatively high mortality rate, 5-10%, but this is a statistic that many published papers fail to include and report. In tandem with this, is the fact that OB surgery cannot be verified and validated until the completion of the study. Upon verification, if both olfactory bulbs are not completely removed, or, if an animal in contrast has excessive bulb removal, i.e. cortical damage, this animal cannot be included in any further analysis. Thus, an overall representation of the *n* numbers of a group cannot be fully clarified until an animal study is completed. With this in mind, many laboratory groups that utilise the OB model enhance their *n* numbers upon the commencement of surgery and at the stage of ethical approval, bearing in mind that their *n* numbers depend upon successful surgery. Lastly, the exact mechanism behind the hyperactive profile in OB animals being attenuated with chronic, but not acute, antidepressant treatment has not been fully elucidated.

1.8 The Opioid System

The endogenous opioid system is comprised of three G-protein-coupled receptors (GPCR) which are commonly known as the:

- Mu (μ/MOP) opioid receptor
- Delta (δ/DOP) opioid receptor
- Kappa (κ/KOP) opioid receptors

Each receptor has a corresponding endogenous peptide classified as the endorphin peptides, and the selective peptide for each receptor are known as:

- Beta-endorphin (β-endorphin) (selective for MOP)
- The enkephalins (ENK) (Met and Leu enkephalin; selective for DOP, but also with high affinity for MOP)
- Dynorphin (DYN) (selective for KOP)

Although there are over twenty endorphins that have been discovered, these three endorphins are the most studied and have become the primary focus of opioid ligand research. The precursor proteins for each of these endogenous peptides are known as:

- Pro-opiomelanocortin (POMC) for β-endorphin
- Pro-enkephalin (PENK) for the enkephalins
- Pro-dynorphin (PDYN) for dynorphin

POMC also acts as the precursor protein for ACTH, and so POMC plays a role in the synthesis and regulation of stress. Endomorphin-1 and endomorphin-2 are also two naturally occurring ligands that are highly selective for binding to the MOP (Zhang et al. 2006, Cravezic et al. 2011, Fichna et al. 2007), but their synthesis *in vivo* has yet to be established, and as such their status as endogenous peptides *per se* has yet to be fully recognized (Alexander et al., 2017). A fourth receptor exists known as the nociceptin/orphanin FQ (NOP) opioid receptor which also has its own endogenous peptide known similarly as nociceptin/orphanin FQ (N/OFQ). NOP is considered “opioid-like” or “opioid-related”, as it contains a similar structural homology to the other three opioid receptors but it’s pharmacology differs in that none of the endorphins bind to this receptor with any great affinity, and nor does the peptide

N/OFQ bind to the other three opioid receptor subtypes (Alexander et al., 2017). For the purpose of this project we focused on the main three opioid receptors (MOP, KOP and DOP) and their targets, and so the following sections will no longer discuss the effects of the NOP.

Each opioid receptor subtype is spread throughout both the central and peripheral nervous system, with many receptor subtypes overlapping each other in regions (Lutz and Kieffer, 2013, Peciña et al., 2019, Valentino and Volkow, 2018) (Figure 1.7). Opioid receptors are able to form both homodimers and heterodimers between each receptor subtype, as well as among some other GPCRs (such as the cannabinoids and adrenoceptors) and as such modulate ligand binding, trafficking and signalling, via secondary pathways (Berrocoso et al., 2009, Henriksen and Willoch, 2007). With this in mind, the endorphins are thought to act as neuromodulators in the brain (Berrocoso et al., 2009) and infamously, the opioid system is associated with the modulation of pain (Holden et al., 2005). Indeed, manipulating the opioid system, primarily the MOP, has been shown to have very effective analgesic properties (Holden et al., 2005, Pasternak and Pan, 2011), with MOP agonists such as morphine currently used as first-line treatment in the alleviation of pain in contemporary medicine (Holden et al., 2005, Le Merrer et al., 2009, Pasternak and Pan, 2011). The opioid system also has a number of other physiological roles in the body including the regulation of stress, respiration, gastrointestinal transit, endocrine function and the regulation of affective states (Bodnar, 2013, Lutz and Kieffer, 2013, Peciña et al., 2019). Co-distributed with the monoamines in limbic regions associated with stress, cognition and emotion, it is posited that the opioid system may regulate the functioning and effects of the monoamine systems (Berrocoso et al., 2009). Indeed, the monoamines conduct their function by exchanges with GPCRs on the post-synaptic cell membrane, and as the opioid receptor family are GPCR in structure, their role in the stimulation or inhibition of monoaminergic neurotransmission via glutamate and GABA release, is likely (Berrocoso et al., 2009, Lutz and Kieffer, 2013). Located in limbic and paralimbic regions in the brain, and in general, associated with inducing a euphoric effect when activated, the opioid receptors and their peptides have the potential as a prospective system to alleviate the dysfunction seen in affective disorders, including that of depression (Brown and Lucki, 2019, Lutz and Kieffer et al., 2013, Peciña et al., 2019).

1.8.1 Anatomy of the central opioid system

As mentioned briefly above, the opioid receptors and endogenous peptides are widely distributed throughout the brain, particularly in limbic areas. Preclinical research has shown that the binding sites for these main opioid receptors overlap in the majority of regions that they are shown to be expressed in, with the degree of expression per receptor per region shown to be the differential factor (Le Merrer et al., 2009). Nevertheless, there are specific species differences in the distribution of opioid receptors in the brain so this should always be taken into consideration when extrapolating results from one species to another. In general, the distribution and expression of the opioid system in the human brain has been shown to resemble that of the rat brain, with the exception that there is less DOP binding and more KOP binding in the human brain, than in that of the rat brain (Peciña et al., 2019, Pfeiffer et al., 1982).

Pfeiffer et al. (1982) was one of the earliest researchers to examine the regional distribution of binding sites of all three opioid receptor systems in the human brain post-mortem. Pfeiffer and colleagues (1982) found significantly high levels of MOP binding in the thalamus, hypothalamus, cortical structures and cingulate gyrus, with increased levels of KOP binding found in the amygdala, hypothalamus and the cortex, and finally, with DOP binding being shown to be the lowest, accounting for less than 20% of total binding capacity in deeper brain structures. Positron Emission Tomography (PET) advanced the examination of opioid receptor density and binding in the human brain *in vivo*, and the usefulness of this technique will be discussed in a later section in this chapter. PET studies in the healthy human brain have shown high concentrations of MORs in the thalamus and basal ganglia, with medium concentrations in the frontal and parietal cortex, and decreased concentrations in the cerebellum and occipital lobe (Frost et al., 1985). PET scanning has also revealed a high binding availability of DOPs in the human central nervous system in the basal ganglia and putamen (Smith et al., 1999), as well as in the neocortex and caudate nucleus (Madar et al., 1996), with intermediate binding found in the amygdala (Madar et al., 1996) and frontal cortex (Smith et al., 1999), and decreased binding availability seen in the cerebellum and the thalamus (Madar et al., 1996, Smith et al., 1999). A selective KOP tracer analog has only recently been discovered for use in PET in humans, and as such has revealed high levels of KOP binding in the amygdala, anterior cingulate cortex, insula, frontal cortex, and ventral pallidum (Vijay et al., 2016). Hiller

and Fan (1996), found peak KOP density in the parahippocampal gyrus at the amygdaloid formation. Sex differences have also been shown with regards opioid binding with higher MOP binding shown in women than in men in multiple brain regions (Zubieta et al., 1999), and with higher KOP binding found in men as compared to women (Vijay et al., 2016).

Preclinical findings have furthered the knowledge of the distribution of the opioid system in limbic regions. Figure 1.10 depicts the distribution and expression of the MOP, KOP and DOP in the rat brain.

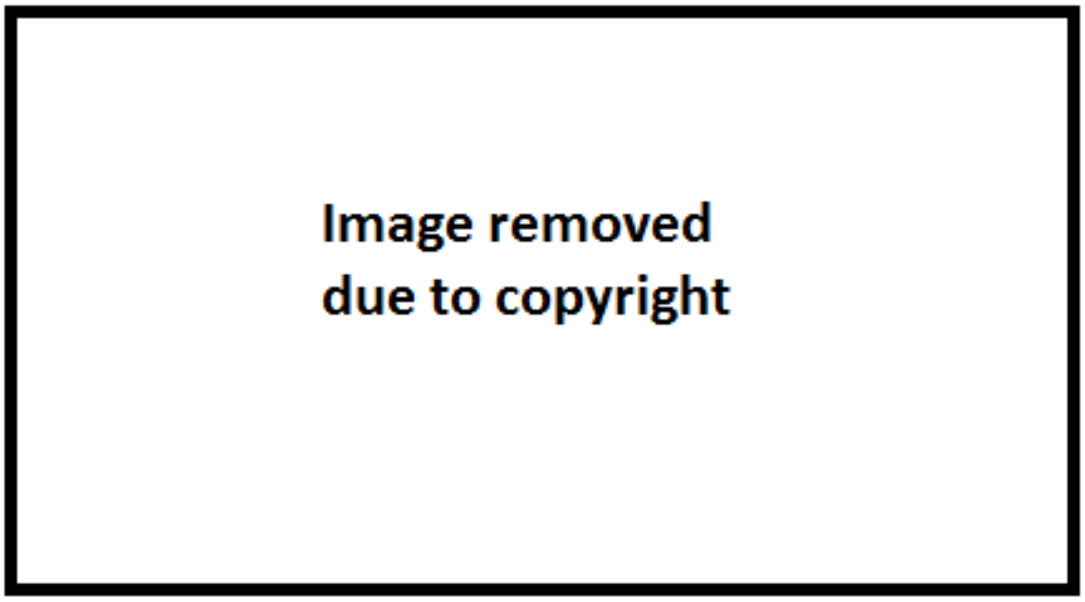


Figure 1.10 Distribution of the MOP, KOP and DOP in different limbic regions in the rat brain. MOP concentrations are represented in red, DOP in yellow, and KOP in blue. A shaded oval represented high concentration of the opioid receptor subtype, with an empty oval representing low concentration. The opioid receptor subtypes are shown to overlap in many limbic regions, with the differing factor being their level of expression in each region. Amy=amygdala, DOP=delta opioid receptor, DRN=dorsal raphe nucleus, Hipp=hippocampus, KOP=kappa opioid receptor, LC=locus coeruleus, MOP=mu opioid receptor, NAc=nucleus accumbens, PFC=prefrontal cortex, VTA=ventral tegmental area. Image taken from Lutz and Kieffer (2013).

Rodent studies have revealed that the MOP is the most abundantly expressed opioid receptor in the brain, with the highest concentrations seen in the amygdala, thalamus and mesencephalon, including regions such as the hippocampus (Brown and Lucki, 2019, Le Merrer et al., 2009, Mansour et al., 1987). The KOP receptor has been shown to be expressed in the hypothalamus, pituitary gland, olfactory tubercle, frontal cortex (Talbot et al., 2005), nucleus accumbens and caudate putamen, with its precursor protein PDYN present throughout the majority of brain structures (Le Merrer et al.,

2009). Particularly high expression of the precursor protein PDYN has been shown in the nucleus accumbens, with a high number of cell bodies found in the hypothalamus (Le Merrer et al., 2009). Finally, preclinical studies have revealed that the DOP receptor is the most extensively expressed opioid receptor in the olfactory bulbs, olfactory tract, amygdala and striatum (Le Merrer et al., 2009, Mansour et al., 1987, Pradhan et al., 2011), with a high density of receptors also found in the prefrontal cortex and hippocampus (Lutz and Kiefer, 2013, Mansour et al., 1987). PENK is the most widely distributed opioid precursor peptide in the brain, with particularly high concentrations found in the thalamus (Le Merrer et al., 2009).

In general, opioid receptor expression (mRNA) in each region, is co-localised and matched with the circulation of opioid binding sites (protein) in each region (Henriksen and Willoch, 2007, Le Merrer et al., 2009). Having said this, DYN is the endogenous ligand for the KOP, and there are areas of the brain where there is differential expression of DYN and KOPs (Taylor and Manzella, 2016). For example, PDYN mRNA and KOPs are co-localised in the striatum, nucleus accumbens, olfactory tubercles, hypothalamus and hippocampus, while high concentrations of KOPs alone are seen in the basolateral amygdala, endopiriform nucleus and stria terminalis (Taylor and Manzella, 2016). In both humans and rodents, the MOP and KOP are co-distributed in most structures, with the localisation of DOPs being found to be much more specific (Le Merrer et al., 2009, Pfeiffer et al., 1982). The distribution of the DOP precursor protein PENK overlaps with the distribution of the MOP and is shown to be well-localised among MOPs, and this is plausible as β-endorphin also has affinity for the MOP (Drolet et al., 2001, Le Merrer et al., 2009).

1.8.2 Opioid receptor signalling

The MOP, KOP and DOP consist of 7-transmembrane spanning proteins that are GPCR in mechanism (Law et al., 2000). The opioid receptors have been shown to activate or couple to inhibitory G-proteins and as such, are known as G_i/G_o in mechanism; inhibiting the enzyme adenylyl cyclase (Law et al., 2000, McDonald, 2005). As GPCRs, the opioid receptors are activated by the binding of their endogenous peptide to the receptor (Figure 1.11). The nucleotide guanosine diphosphate (GDP) is exchanged to guanosine triphosphate (GTP), which causes the Gαβγ unit to dissociate and split into the Gα and Gβγ subunits, which then act on intracellular effector pathways (Al-Hasani and Bruchas, 2011). Firstly, the Gα subunit,

moves to directly interact with the potassium (K^+) ion channel, rectifying the modulation and output of the potassium channel, $K_{ir}3$ (Al-Hasani and Bruchas, 2011). The Gβγ subunit inhibits the incoming calcium efflux by closing the voltage sensitive calcium (Ca^{2+}) channels (McDonald, 2005). The hydrolysis of GTP back to GDP, along with stimulation of the potassium channels, leads to hyperpolarization, inhibiting cyclic adenosine monophosphate (cAMP) production via a reduction in the enzyme adenylyl cyclase (Al-Hasani and Bruchas, 2011, McDonald, 2005). This leads to a reduction in neuronal cell excitability, causing a decrease in transmission and a reduction in neurotransmitter release (McDonald, 2005). Inhibition of cAMP causes the activation of MAPK signalling, which activates kinases such as extra-cellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 stress kinase (p38), which are involved in gene expression, cell proliferation and division, apoptosis and tissue regeneration, responding to stimuli such as stress and inflammation (Al-Hasani and Bruchas, 2011).

Activation of the opioid receptors can also cause another downstream signal transduction pathway that involves phosphorylation of the receptor, followed by internalisation and desensitization (Al-Hasani and Bruchas, 2011, Lefkowitz, 1998) (Figure 1.11). When the endogenous ligand or agonist has bound to the receptor, GPCR kinases can also bind to the free Gβγ subunit and phosphorylate the receptor in this 'active' (ligand-bound) state (Rang and Dale, 2012). As a result, intracellular proteins known as arrestins can now be recruited and bind to the phosphorylated receptor, blocking the interaction between the receptor and the G proteins and sustaining a persistent stimulation (Lefkowitz, 1998). Arrestins can then internalize the receptor deeper into the cell and into endosomes, whereby the receptor is in essence desensitised, to be later dephosphorylated and recycled to the plasma membrane, or to be trafficked to lysosomes for degradation of the opioid receptor (Lefkowitz, 1998). This second potential mechanism is much slower than the Ca^{2+}/K^+ ion channels pathway, and is believed to be a critical molecular feature in opioid tolerance, and in turn dependence, occurring after chronic exposure to the agonist, or as a result of sustained peptide release (Al-Hasani and Bruchas, 2011). Interestingly, the binding of arrestins to the Gβγ subunit was initially believed to decrease the functioning of the opioid receptor, in essence making it 'inactive' and in turn ceasing the functioning of MAPK signalling. However, in recent years it has been indicated that recruitment of

transducer molecules such as β-arrestins can continue to activate these cytoplasmic signalling complexes (Lefkowitz, 1998, Lefkowitz and Shenoy, 2005).

Overall the effect of activation of the opioid receptors at the cellular level is inhibitory. However, activation of the opioid receptor can also cause increased excitatory activity along pathways by a process called disinhibition, whereby they inhibit the activity of inhibitory neurons and pathways, causing an increase in excitatory functioning (Rang and Dale, 2012). The coupling of opioid peptides to these Ca^{2+}/K^{+} ion channels has been shown in a number of regions, many of which are important in the role of limbic and monoaminergic functioning, including the hippocampus, locus coeruleus and ventral tegmental area (Al-Hasani and Bruchas, 2011). Therefore, at the cellular level all three receptors act the same, it is their region-specific distribution that causes differing effects.

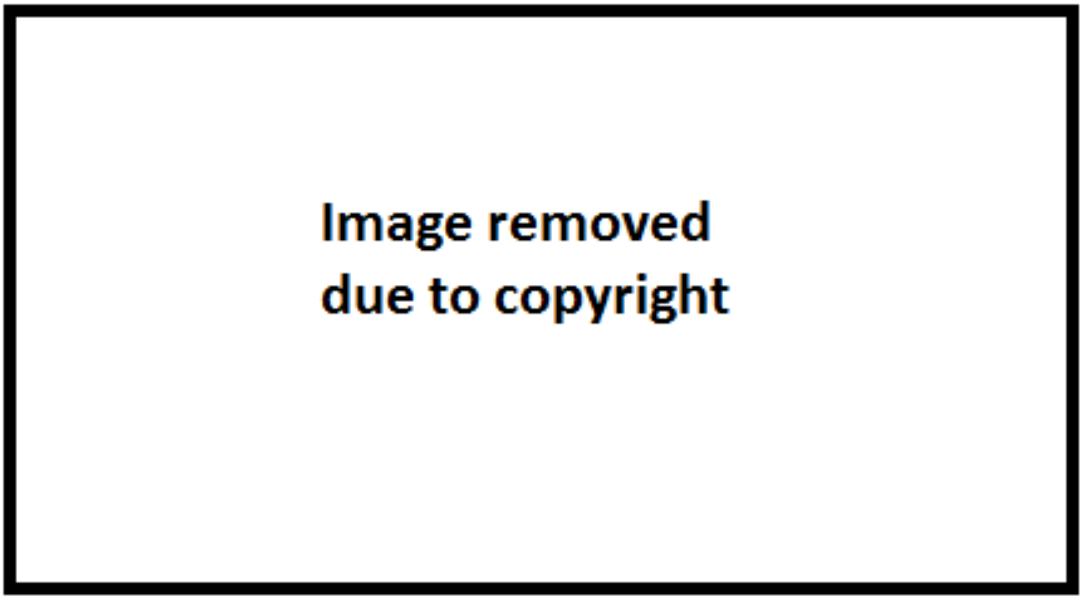


Figure 1.11 Opioid receptor signalling. (1) The opioid receptors are activated by the binding of their endogenous peptide/opioid agonist to the receptor. Guanosine diphosphate (GDP) is exchanged to guanosine triphosphate (GTP), which causes dissociate of the Gα subunit from the Gβγ subunits. The Gα subunit rectifies the modulation and output of the potassium (K^+) ion channel, $K_{ir}3$, and also inhibits cyclic adenosine monophosphate (cAMP) production. The Gβγ subunit closes the voltage sensitive calcium (Ca^{2+}) ion channels. This leads to a reduction in neuronal cell excitability and a reduced neurotransmitter release. (2) Activation of the opioid receptors can also cause another downstream signal transduction pathway whereby when the endogenous ligand or agonist has bound to the receptor, G-protein-coupled receptor (GPCR) kinases can also bind to the free Gβγ subunit and phosphorylate the receptor in this 'active' (ligand-bound) state. (3a) Intracellular proteins known as arrestins can now be recruited and bind to the phosphorylated receptor, blocking the interaction between the receptor and the G proteins. (3b) Arrestins can then internalize the receptor deeper into the cell, sustaining a persistent stimulation, whereby the receptor is in essence desensitised. (4) In pathway 1, inhibition of cAMP causes the activation of mitogen-activated protein kinase (MAPK) signalling, and in pathway 2, binding of certain transducer molecules such as arrestins can also activate MAPK signalling. This activates downstream mechanisms activating kinases such as extra-cellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 stress kinase (p38), responsible for cellular mechanism such as proliferation, division and apoptosis. (5) The internalized receptor can be later dephosphorylated and recycled back to the plasma membrane, or can be trafficked to lysosomes for degradation. Ca^{2+}=calcium (Ca^{2+}) ion channels, cAMP=cyclic adenosine monophosphate, ERK=extra-cellular signal-regulated kinase, GDP=guanosine diphosphate, Gi=Gα subunit, GPCR=G-protein-coupled receptor, GTP=guanosine triphosphate, JNK=c-Jun N-terminal kinase, K^+=potassium channel, MAPK=mitogen-activated protein kinase, p38=p38 stress kinase. Image taken from Al-Hasani and Bruchas (2011).

1.8.3 Activation of the central opioid system with regards mood, stress and cognition

Activation of the opioid system by agonists, primarily in preclinical research, has revealed a distinct function for each opioid receptor subtype with regards the modulation of mood, stress and cognition, with each receptor having opposing effects on mood and hedonic homeostasis regulation (Lutz and Kieffer, 2013, Valentino and Volkow, 2018). Figure 1.12 depicts the effects of activation of each opioid receptor in pharmacological genetic models such as opioid knockouts, in terms of affect, stress and hedonic behaviour. This will be discussed in detail for each opioid receptor.

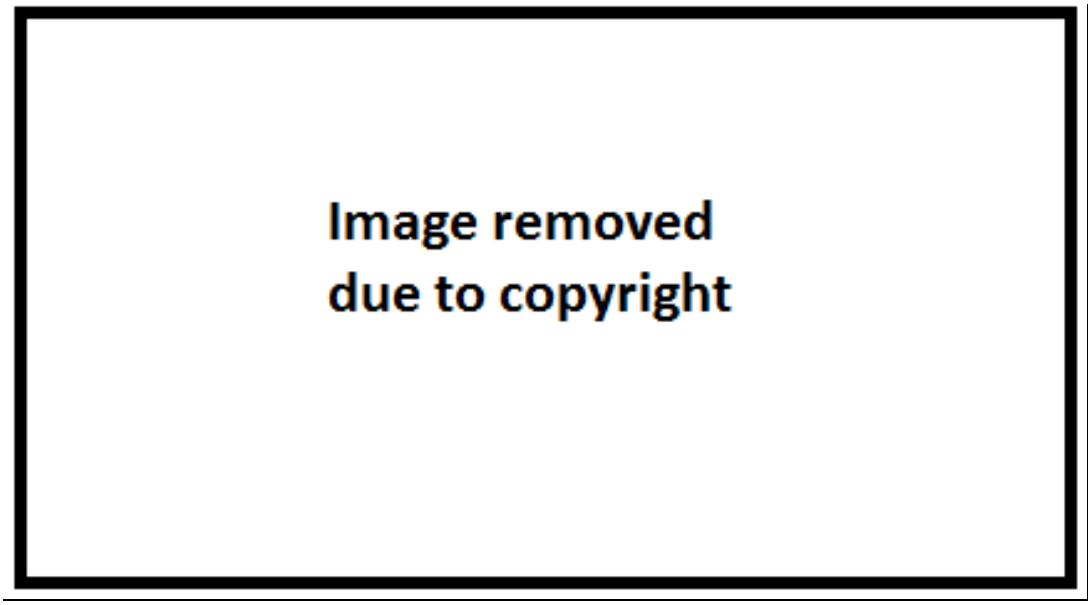


Figure 1.12 The effects of MOP, KOP and DOP agonism in terms of mood and hedonic behaviour. Hedonic homeostasis and mood are described in terms of a low-to-high continuum. In general, agonists at the MOP produce a euphoric effect and are associated with stress-coping mechanisms. Agonists at KOP have the opposite effect, promoting dysphoria, stress and negative affect. Lastly, agonism of the DOP receptor is associated with positive affect, and as such is seen to cause anxiolytic and antidepressant functioning. DOP=delta opioid receptor, KOP=kappa opioid receptor, MOP=mu opioid receptor. Image taken and adapted from Valentino and Volkow (2018).

1.8.3.1 MOP

Given its high concentration in the thalamus, MOPs have been shown to be pivotal in the modulation of analgesic effects, with activation of the MOP associated with euphoric effects in humans. MOPs have also been shown to be an important factor in emotional regulation, particularly with regards social functioning, anhedonia and anxiety (hippocampus and amygdala) (Browne and Lucki, 2019).

Activation of MORs has been implicated in social interaction in humans and in animals. Administration of MOP agonist morphine has been shown to promote attention to the face and eyes of others in humans, with administration of MOP antagonist naltrexone shown to reduce attention to these social cues (Chelnokova et al., 2016). Similarly, deletion of the MOP in *Oprm1*$^{-/-}$ KO mice reveals social interactions are less salient in these mice than in their controls (Cinque et al., 2012), with social interactions following exposure to stress, also shown to be unaffected, unlike their wild-type (WT) counterparts who show social avoidance (Komatsu et al., 2011). Indeed, *Oprm1*$^{-/-}$ KO pups separated from dam and littermates are shown to emit significantly less ultrasonic vocalisations that their WT littermates (Cinque et al., 2012). As a result, the MOP receptor is crucial to the functioning of social processes.

The MOP receptor has also been shown to have a role in stress-coping mechanisms. However, its exact function is quite complex. MOP agonists have been shown to have anxiolytic-like and antidepressant-like effects in humans (Emrich et al., 1982, Karp et al., 2014) and pre-clinically (Almatroudi et al., 2015, Berrocoso et al., 2013), and MOP activation also been implicated in the attenuation of fear memories and reduces fear-related freezing in conditioned animals (Szklarczyk et al., 2015). In tandem with this, blockade of the receptor with MOP antagonists has been shown to enhance the acquisition of fear in acute threat paradigms (Haaker et al., 2013, Brown and Lucki, 2019), also delineating a role for MOP blockade in cognition and learning. These results are in tandem with the knowledge that activation of the MOP causes euphoric effects. However, in contrast with this, deletion of the MOP has also exhibited beneficial results with regards resilience to stress. *Oprm1*$^{-/-}$ KO mice have been shown to exhibit a reduction in freezing behaviour on re-exposure to a foot-shock fear paradigm when compared to WT mice (Sanders et al., 2005). *Oprm1*$^{-/-}$ KO exposed to chronic stress by repeated FST have been shown to have a decrease in immobility in comparison to WT counterparts, along with an increase in open arms entries and time in the EPM, and decreased immobility in the TST (Ide et al., 2010). These genetic manipulations add a level of complexity to our understanding of MOP activation. Browne and Lucki (2019) state that these differential findings in MOP activation may be as a result of specific contextual threat, i.e. acute verses chronic constructs. Lutz and Kieffer (2013), state the same ideology, along with the idea that acute activation as a result of pharmacological manipulation may be antidepressant,

and that chronic activation as a result of genetic manipulation may develop a compensatory mechanism of high mood as a result MOP deletion.

MOR activation has also been shown to produce positive valence, in regards to hedonic and reward acting behaviour (Browne and Lucki, 2019). Positive valence can be defined as that which drives reactions to positive stimuli in both humans and animals, i.e. reward behaviour. Dopaminergic neurons originate in the ventral tegmental pathway, and administration of MOP agonists into this region has been shown to increase DA concentrations locally (Moreno et al., 2017), and as such effects the neural circuits in the mesolimbic reward pathway, increasing reward behaviour (Lutz and Kieffer, 2013). Morphine in humans has been shown to increase processing efficiency in a task for sucrose-sweets, interpreted as a measure to obtain the 'reward', and which was seen to be blocked with MOP antagonist naltrexone (Eikemo et al., 2017). With regards to preclinical research, injection of MOP agonists directly into many reward-seeking associated regions in the brain including the nucleus accumbens, amygdala and hypothalamus has been shown to increase food intake in a reward 'wanting' manner (Le Merrer et al., 2009).

Finally with regards to cognition, MOR activation has been shown to have mixed findings on enhancing cognitive function, and interestingly, sex-specific effects are seen (Browne and Lucki, 2019, van Steenbergen et al., 2019). In human studies, oral administration of morphine for two weeks in males improved the error rate in an attentional set-shifting task, but had no effect on memory or executive function (Quednow et al., 2008). In contrast, the MOP agonist dihydrocodeine has been shown to increase working memory and attention in humans (Szekely et al., 1986). PET imaging has revealed that MOPs have been shown to be distributed throughout the human brain in decision-making and cognitive-control networks (Gorgolewski et al., 2015, van Steenbergen et al., 2019, Yarkoni et al., 2011). In rodents, the MOP agonist [$_{D}$-Ala^{2}, N-MePhe^{4}, Gly-ol]-enkephalin (DAMGO) increased the total number of errors, including regressive and random errors, and also increased premature responses in male rats in an operant task, whereas female rats were only shown to have increased perseverative errors (Guajardo et al., 2017). Interestingly, administration of MOP agonist DAMGO into the LC increased the number of trials it took animals to complete the task, regardless of sex, when compared to vehicle-treated animals (Guajardo et al., 2017).

In conclusion, MOP agonists are crucial in social functioning, and pharmacologically are associated with an increase in antidepressant- and anxiety-like behaviour, positing a role in stress-coping mechanisms. MOP agonists may posit a role in the drive of motivated behaviours via decision making and reward, but their role in cognition is still to be fully elucidated.

1.8.3.2 DOP

Discovered in the 1970s, the DOP receptor was the first opioid receptor to be sequenced (Browne and Lucki, 2019, Lord et al., 1977). Located in regions such as the frontal cortex and amygdala, activation of the DOP system has been associated with strong analgesic effects similar to the MOP (Brown and Lucki, 2019), but has also been posited to have a strong role in the regulation of stress, anxiolytic behaviour and as such, positive affect.

The DOP system is thought to regulate autonomic function, and a major component of autonomic function is response to stress. The majority of structures that are involved in the neurobiology of stress receive ENK innervations (Drolet et al., 2001). In animals, exposure to stress has been shown to alter expression of the DOP system, and in tandem, DOP activation has been shown to reduce stress-induced behaviours (Drolet et al., 2001). Interestingly, acute vs. chronic stress exposure activates the DOP system in different ways. ENK mRNA is increased in the hypothalamus after acute restraint stress (Ceccatelli and Orazzo, 1993, Dumont et al., 2000). With regards chronic exposure to stress, DOP binding has been shown to be decreased after sleep deprivation (Fadda et al., 1991), and chronic restraint stress has been shown to decrease PENK mRNA in the nucleus accumbens of rats (Poulin et al., 2014).

On a separate note, deletion of aspects of the DOP system in mice has indicated that the DOP system could have potential as a target for affective disorders and states. Deletion of the DOP results in anxiogenic and depressive-like behaviour in mice, with an increase in immobility time in the FST, and a reduction in open arm entries and time in the EPM (Fillol et al., 2000). Konig et al. (1996) observed that *Enk1*$^{-/-}$ KO results in increased anxiety-like behaviour in mice. These results suggest that agonism of the DOP receptor could play an important role in mood disorders. However, it must be noted that upon deletion and knockdown of the DOP peptide or precursor protein,

results are much more variable. Knockdown of the peptide ENK has revealed an increase in exploration in the open arms in the EPM in mice, and has been shown to reduce freezing time (Poulin et al., 2013), in contrast to the findings stated by Konig et al. (1996) above. In divergence from these findings, KO of the precursor peptide PENK has been shown to have no effect on immobility time in the FST (Bilkei-Gorzo et al., 2007, Noble et al., 2008) or TST (Bilkei-Gorzo et al., 2007). Therefore, although deletion of various features of the DOP system have revealed differing effects, overall deletion of the DOP receptor indicates an enhancement in negative affect and anxiogenic behaviour, and so activation of the DOP system should cause an increase in positive effect. With this in mind, agonism of the DOP has been shown to successfully enhance anxiolytic and antidepressant-like effects. Preclinically, DOP agonists have been shown to reduce anxiety and depressive-like symptoms in the LH model (Hudzik et al., 2011, Tejedor- Real et al., 2005), the OB model (Gotoh et al., 2017), the FST (Henry et al., 2018, Jutkiewicz et al., 2006, Nozaki et al., 2014), and the TST (Henry et al., 2018, Yang et al., 2011). This is also the case in humans, where a recent study has shown that administration of the highly selective DOR agonist AZD2327 has been shown to illicit positive antidepressant-like properties when given to MDD patients (Richards et al., 2016). Nevertheless, activation of the DOP system is often associated with seizure and/or convulsive behaviours and as a result, this has had limiting effects on the promise that this system may have as a strategy for treating affective states (Berrocoso et al., 2009, Comer et al., 1993, Henry et al., 2018).

The effect of DOPs on hedonic behaviour has been much less examined and as a result is less clear (Lutz and Kieffer, 2013). With regards morphine and reward behaviour, *Oprd1*$^{-/-}$ KO mice exhibit decreased morphine place preference, but self-administration of the MOP agonist is still observed (Le Merrer et al., 2011). Self-administration of other substances have also been examined, with alcohol consumption being increased in *Oprd1*$^{-/-}$ KO mice (Roberts et al., 2001), but nicotine self-administration is decreased in *Oprd1*$^{-/-}$ KO mice (Berrendero et al., 2012). As a result, the role of the DOP system on reward behaviour is not quite direct and needs further investigation in order to be quantified.

In conclusion, activation of the DOP system causes an enhancement in anxiolytic and antidepressant-like behaviour in both animals and humans, and as such this receptor system has potential as a target for affective disorders (Brown and Lucki, 2019, Drolet et al., 2001). The development of therapeutic DOP agonists that lack the

stimulation of seizure behaviour will be advantageous to the use of this system for the treatment of mood and affect (Berrocoso et al., 2009).

1.8.3.3 KOP

In general, activation of the KOP has the opposite effects than that of activation of the DOP and MOP systems (Taylor and Manzella, 2016). Given its high concentration in the hypothalamus, the KOP system, with regards to both protein and mRNA, has been shown to be modulated by the induction of stress, and in tandem, activation of KOP signalling has been shown to cause dysphoria and aversion (Bailey and Husbands, 2018, Browne and Lucki, 2019). DYN, and its precursor PDYN, are released following both acute and chronic stress in laboratory animals in regions of the brain that are important in affective disorders (Taylor and Manzella, 2016). This has special relevance when modelling depression in laboratory animals where chronic stressors are more indicative of the human experience in terms of depression. PDYN immunoreactivity is increased in the hippocampus and nucleus accumbens in rats following acute immobilisation and inescapable footshock in an LH paradigm (Shirayama et al., 2004), with increased PDYN immunoreactivity also being observed in the hippocampus of LH rats following FST exposure (Shirayama et al., 2004). In a model of resident intruder defeat, DYN mRNA was found to be elevated in the dorsal and medial shell of the nucleus accumbens in vulnerable rats, when compared to their control and resilient counterparts (Bérubé et al., 2013). With regard to the KOP itself, fear conditioning increases KOP mRNA in the basolateral amygdala and decreases KOP mRNA in the striatum (Knoll et al., 2011). Therefore, stress regulates KOP activity, but this is dependent on the nature of the stressor; acute or chronic.

In a similar fashion, KOP activity has been shown to regulate stress. Given that activation of the KOP increases aversive behaviour, KOP agonists have been shown to assist depressive and passive behaviour (Pearson et al., 2006). This is line with the fact that KOP and PDYN KO mice display reductions in depressive-like behaviour in the FST (McLaughlin et al., 2003). With all of this in mind, KOP blockade has been shown to reduce and attenuate stress-induced behavioural outputs, proposing a potential target for the treatment and alleviation of affective disorders such as anxiety disorder and MDD. KOP antagonism produces antidepressant-like effects in the FST (Beardsley et al., 2005, Carr et al., 2010, Huang et al., 2016), and LH (Shiryamma et

al., 2004), whilst also inducing decreases in fear in rodents (Knoll et al., 2007), and reducing anxiety-like behaviour in WKY rats. As a result of these findings, KOP antagonists have begun to be examined in clinical trials for the treatment of affective and mood disorders, such as MDD, with initial phase III and IV trials positing positive results (Lowe et al., 2014, Reed et al., 2018).

Sociability is also an important feature in affect and has been shown to be dysfunctional in mood disorders such as MDD. Given that activation of the KOP system increases dysphoric effects, KOP agonists have been shown to decrease sociability and interaction in rodents. Vanderschuren et al. (1995) found that KOP agonists reduced social play in rodents, with another study by Dogra et al. (2016) showing that chronic KOP activation with U50,488 decreased sociability time in mice. This effect was blocked by KOP antagonist nor-binaltorphimine dihydrochloride (norBNI) (Dogra et al., 2016). As a result, blockade of the KOP receptor could have potential to promote sociability in disorders where social dysfunction is seen.

Lastly, the KOP system has also been shown to play a role in motor function, hedonic response and cognitive functioning, due to its close association with dopaminergic transmission (Browne and Lucki, 2019). KOPs are located presynaptically along the ventral striatum (Di Chiara and Imperto, 1988) and KOP activation causes a decrease in DA release along the mesolimbic pathway (Taylor and Manzella, 2016). Given that decreases in DA neurotransmission cause dysfunction to motor function, KOP agonists have been shown to decrease locomotor activity in rodents (Kuzmin et al., 2001, Smith et al., 2009), with KOP antagonists blocking these effects (McDougall et al., 1997, Smith et al., 2009). With regards hedonic behaviour and motivation, KOP activation has been shown to blunt excitatory glutamate release, and glutamate-mediated arousal in the locus coeruleus, causing a reduction in motivation (Valentino and Volkow, 2018). KOP agonists have been shown to attenuate cognitive dysfunction in rodents (Hiramatsu et al., 1988; 2009, Takahashi et al., 2018), through increases in cholinergic transmission, with blockade of the KOP by KOP antagonists then shown to reverse these effects (Hiramatsu et al., 1988; 2009, Takahashi et al., 2018). Micro–injection of KOP agonist Dynorphin A into the hippocampus, a region involved in learning and memory, has been shown to ameliorate memory impairments in rats (Hiramatsu et al., 2009). As a result, activation of the KOP system increases cognitive performance preclinically, suggesting a role for this system in cognitive disorders.

In conclusion, activation of the KOP system induces dysphoria through stress-related mechanisms, and as such as been shown to increase depressive-like and anxiety-like behaviour, and decrease social functioning. Activation of the KOP system also facilitates cognition functioning, but causes a reduction in motivational behaviour. Therefore, targeting the KOP system could have therapeutic effects in a number of disorders of psychiatric illness.

1.8.4 The opioid system and depression

Therapeutic use of the opioid system for the treatment of 'melancholia' or depression is known to predate the use of the current monoamine-derived strategies (Berrocoso et al., 2009, Tenore, 2008). Believed to first be documented by the Sumerians in Asia in 3400BC, the use of the opium poppy, *Papaver somniferum*, as a compound to alleviate symptoms of sadness was considered so effective, that the poppy itself became known as the 'joy plant' (Carod-Artal, 2013, Krikorian, 1975, Tenore, 2008). Around 400BC, Hippocrates the 'father of medicine', is also documented to have used opium for the treatment of many ailments, including 'melancholia' (Tenore, 2008). This later sparked an era at the end of the 18th century that became known as the 'opium cure', whereby opioid drugs were derived from the opium poppy (such as morphine and codeine), primarily for the treatment of pain, but also for the treatment of affective states, such as depression. Indeed, the use of opium to treat the dysphoric symptoms of depression were shown to be very efficacious, with Emil Kraepelin recommending the use of increasing doses of *tincture opii* (opium tinctures), followed by decreasing doses, for the treatment of severe MDEs in the early 19th century (Browne and Lucki, 2009, Weber and Emrich, 1988). Nonetheless, the application of the opioid system as a target for antidepressant therapy was overtaken by the introduction of the MAOI and TCA antidepressants in the 1950s which were shown to have a much lower addiction potential (Tenore, 2008). Nevertheless, due to the standstill in detecting novel antidepressant strategies and compounds, and the lack of efficacy in current therapies to treat the symptoms across all MDD cases, renewed interest has been reignited in utilising the opioid system as a novel target and approach for the treatment of depression (Brown and Lucki, 2019, Lutz and Kieffer et al., 2013, Peciña et al., 2019). The growing knowledge of the opioid subtypes, and the ability that now exists to selectively target their subtypes, has also furthered their potential as a strategy for the treatment of depression. The evidence, re-emergence and relevance

of the opioid system as a potential target for antidepressant therapy will be discussed in terms of:

- The anatomy of the opioid system in MDD
- The neurochemical association between the central opioid and monoaminergic systems
- Preclinical evidence of modulation of the central opioid system as an antidepressant target
- Clinical evidence of modulation of the central opioid system as an antidepressant target
- Limitations of current research

1.8.4.1 Anatomy of the opioid system in MDD

The opioid systems presence in brain regions associated with emotion and stress, has posited that it may play a key role in mood regulation and as a consequence in MDD. The main strategies for evaluating the opioid system in depression clinically have involved measuring opioid elements in the following:

- Blood (i.e. plasma)
- Cerebrospinal Fluid (CSF)
- Post-mortem brain
- *In vivo* brain

Table 1.5 summaries the main advantages and disadvantages associated with each of these sources, with subsequent sections evaluating the evidence for changes in the opioid system in depression.

	Blood	**CSF**	**Post-Mortem Brain**	***In Vivo* Brain**
Elements measured	β-endorphin	β-endorphin	β-endorphin and MOP DYN and KOP	MOP (binding potential)
Methodologies	Immunnoreactivity	Immunnoreactivity RIA	ISHH RLB qRT-PCR	PET Scanning
Advantages	Minimally invasive Multiple samples Inexpensive Can be continuously monitored	Accessible window on the CNS	Specific brain regions measured Examine circuitry	Specific brain regions measured Examine circuitry Living brain "State" changes can be measured Can be continuously monitored
Disadvantages	Certain constituents measured Peripheral measures only Susceptible to contamination	Certain constituents measured Expensive Quite invasive	Post-mortem measures only Terminal subjects Single time-point	Expensive Specificity of radioligands/probes Binding potential of target Special equipment and training required

Table 1.5 Advantages and disadvantages of methodologies used to clinically measure opioid activity in MDD to date. The main elements measured, methodologies used for measurements, along with the advantages and disadvantages of each biological technique are described. CNS=central nervous system, DYN=dynorphin, ISHH=*in situ* hybridization histochemistry, KOP=kappa opioid receptor, MOP=mu opioid receptor, PET=positron emission tomography, RIA=radioimmunoassay, RLB=radioligand binding, qRT-PCR=real-time quantitative polymerase chain reaction.

1.8.4.1.1 Blood measurements

β-endorphin, the endogenous ligand for the MOP has been the only element measured in the plasma of MDD patients, the rationale being that it reflects pituitary secretion, and over-activity of the HPA axis, as opposed to being a marker of central opioid function *per se* (Brambilla et al., 1996, Gispen-de-wied et al., 1987). Findings with plasma β-endorphin in depression have been equivocal, with some studies finding an increase (Gispen-de-wied et al., 1987, Risch 1982), while others have found a decrease (Darko et al., 1992), or no change at all (Alexopoulos et al., 1983, Brambilla et al., 1996). As a result, such variation makes it impossible to make a link between MDD and β-endorphin activity (Merenlenden-Wagner et al., 2009).

1.8.4.1.2 Cerebrospinal Fluid measurements

All CSF studies examined so far have examined β-endorphin levels and have shown no difference in CSF β-endorphin levels in MDD (Emrich et al., 1979, France and Urban, 1991, Gerner and Sharp, 1982, Naber et al., 1981, Pickar et al., 1982, Rubinow et al., 1981). Justifications for examining β-endorphin in CSF in MDD are similar to that of plasma, in that hormone secretion of ACTH and cortisol were seen to be correlated with β-endorphin activity via pituitary stimulation in psychiatric illness and thus this endogenous peptide was investigated (Nakao et al., 1980, Pickar et al., 1982, Rubinow et al., 1981). However, lack of change in β-endorphin levels manifest a tenuous link between this endogenous ligand and MDD.

1.8.4.1.3 Post-mortem measurements

When evaluating the evidence for post-mortem changes in the opioid system and MDD, the following challenges need to be considered:

- *Methodologically valid comparisons:* real-time quantitative polymerase chain reaction (qRT-PCR), radioligand binding (RLB) and *in situ* hybridization histochemistry (ISHH) have been used as measures of protein
- *Diagnostic differences:* some studies being MDD, others MDD suicide whilst others have been suicide without prior diagnosis
- *Choice of brain regions:* the principal areas investigated have been the amygdala, prefrontal or cingulate cortex

- *Choice of opioid elements:* measuring the endogenous ligand or the receptor: studies have primarily examined the KOP and its endogenous ligand PDYN, or the MOP and its endogenous ligand β-endorphin

The evidence of opioid changes in post-mortem brain in MDD/suicide are summarised in Table 1.6.

With regards the KOP system, increased PDYN mRNA has been found in MDD, in the amygdala, (Anderson et al., 2013, Hurd, 2002) and caudate nucleus (Hurd et al., 1997), with no changes being seen in prefrontal and cingulate cortex (Peckys and Hurd, 2001). However, only a single study to date has examined KOP expression in depression, with no changes being found in the prefrontal cortex and cingulate (Peckys and Hurd, 2001).

With regards the MOP system, reductions in β-endorphin mRNA have been found in the hypothalamus and paraventricular neurons (Bernstein et al., 2002), in the left frontal cortex, left caudate nucleus and left temporal cortex (Scarone et al., 1990). With regard to the MOP, some studies have found increases in the frontal cortex (Escriba et al., 2004, Gabilonda et al., 1995, Gross-Isseroff et al., 1990) and temporal cortical gyri (Gross-Isseroff et al., 1990), whilst others have found no differences in the Brodmanns area 9 (BA9), Brodmanns area 24 (BA24), caudate putamen (Scarr et al., 2012), prefrontal cortex (González-Maeso et al., 2002, Zalsman et al., 2005) and pre-post central gyri (Zalsman et al., 2005).

There is a lack of studies investigating the DOP in post-mortem MDD patients, presenting a gap in the current literature that should try to be addressed to future opioid-MDD post-mortem examinations.

Element	Effect	Region	Prognosis	Reference
KOP system				
PDYN	↑	Amygdala	MDD	Hurd, 2002
PDYN	↑	Amygdala	MDD	Anderson et al. 2013
PDYN	↔	Prefrontal cortex and cingulate	MDD	Peckys and Hurd, 2001
PDYN	↑	Caudate nucleus	Suicide	Hurd et al. 1997
KOP	↔	Prefrontal cortex and cingulate	MDD	Peckys and Hurd, 2001
MOP system				
β-endorphin	↓	Hypothalamus, paraventricular neurons	MDD	Bernstein et al. 2002
β-endorphin	↓	Left frontal cortex, left caudate nucleus, left temporal cortex	Suicide	Scarone et al. 1990
MOP	↔	BA9, BA24, Caudate putamen	MDD/MDD Suicide	Scarr et al. 2012
MOP	↔	Prefrontal cortex	MDD Suicide	González-Maeso et al. 2002
MOP	↑	Frontal cortex	MDD Suicide	Escriba et al. 2004
MOP	↑	Frontal cortex	Suicide	Gabilonda et al. 1995
MOP	↑	Frontal and temporal cortical gyri	Suicide	Gross-Isseroff et al. 1990
MOP	↔	Prefrontal cortex and pre-post central gyri	Suicide	Zalsman et al. 2005

Table 1.6 Activity of the MOP and KOP and their endogenous ligands in various brain regions in MDD, MDD suicide, and suicide victims not diagnosed prior to death post-mortem. BA9=Brodmann area 9, BA24=Brodmann area 24, KOP=kappa opioid receptor, MDD=major depressive disorder, MOP=mu opioid receptor, PDYN=prodynorphin.

1.8.4.2 *In Vivo* brain measurements

The advent of *in vivo* imaging techniques, primarily using the PET approach has been a defining technique in examining the central opioid system in depression. Radio-labelled tracers allow the binding potential of a neurotransmitter system to be measured, delineating a rate of "activation" or "deactivation" for the receptor and endogenous ligand under investigation, which in turn can be extrapolated to suggesting an increase or decrease in receptor-peptide affinity and/or expression (Kennedy et al., 2006, Prossin et al., 2011). "Activation" of receptor activity implies a reduction in binding potential and as such a reduction in receptor availability/increase in peptide expression (Kennedy et al., 2006). "Deactivation" of receptor activity implies an increase in binding potential and as such an increase in receptor availability/reduction in peptide expression (Kennedy et al., 2006). This "activation-deactivation" matrix can be examined with regards "state-changes" induced by mood and motivational challenges.

Patients can be examined at basal level or in a "neutral" state (brought about by relaxation, awareness of sensory stimuli in the scanner), in a "sadness-induced" state (brought about by recollection of a negative autobiographical experience) or in a socially employed feedback task (whereby emotions of "sadness" "rejection" "happiness" or "acceptance" are measured after patients being told of reciprocal "like" or "dislike" matches in a series of intimate match-profiles to their preferred sex). PET studies thus far have focused on the binding potential of the MOP in MDD and these are summarised in Table 1.7.

At basal level, an increase in MOP binding potential was observed in the amygdala, right hypothalamus, left ventral pallidum and bilateral anterior cingulate cortices (Prossin et al., 2016), with a decrease being observed in the thalamus (Kennedy et al., 2006). In the "sadness" state of a negative-affect induced paradigm, a decrease in MOP binding potential was observed in the left inferior temporal cortex (Kennedy et al., 2006), amygdala (Kennedy et al., 2006, Prossin et al., 2011, Prossin et al., 2016), ventral basal ganglia and right anterior insular cortex (Kennedy et al., 2006), hypothalamus and ventral palladium (Prossin et al., 2011, Prossin et al., 2016), subgenual anterior cingulate (Prossin et al., 2011), ventral tegmental area and bilateral medial thalamus (Prossin et al., 2016). In a "social emotion" state paradigm, whereby MOP binding potential was viewed during social acceptance, an increase in binding

potential was observed in the left nucleus accumbens (Hsu et al., 2015), with a decrease observed in the thalamus (Hsu et al., 2015). During social rejection, an increase in binding potential was observed in the amygdala (Hsu et al., 2015). During rejection, no change was observed in the right nucleus accumbens, left and right amygdala, midline thalamus or periaqueductal gray in MDD, as compared to a decrease in the binding potential in these regions in healthy controls (Hsu et al., 2015).

At present, only the MOP system has been investigated *in vivo* in patient with MDD. The implications of KOP and DOP activity have yet to be assessed *in vivo* in MDD, the main reason for this being that there is a lack of radio-labelled tracers that have select specificity for these receptors.

Effect	Binding Potential	State	Region	Reference
MOP system				
Deactivation	↑ BP	Basal	Amygdala, right hypothalamus, left ventral pallidum and bilateral anterior cingulate cortices	Prossin et al. 2016
Activation	↓ BP	Basal	Thalamus	Kennedy et al. 2006
Activation	↓ BP	Sadness**	Left inferior temporal cortex	Kennedy et al. 2006
Activation	↓ BP	Sadness	Bilateral amygdala, ventral basal ganglia and right anterior insular cortex	Kennedy et al. 2006
Activation	↓ BP	Sadness	Subgenual anterior cingulate, amygdala, hypothalamus and bilateral ventral pallidum	Prossin et al. 2011
Activation	↓ BP	Sadness	Amygdala, ventral tegmental area, hypothalamus, left ventral pallidum, and bilateral medial thalamus	Prossin et al. 2016
Deactivation	↑ BP	Acceptance	Left nucleus accumbens	Hsu et al. 2015
Activation	↓ BP	Acceptance	Thalamus	Hsu et al. 2015
Deactivation	↑ BP	Rejection	Amygdala	Hsu et al. 2015
Activation	↔*	Rejection	Right nucleus accumbens, left and right amygdala, midline thalamus and periaqueductal gray	Hsu et al. 2015

Table 1.7 Binding potential of the MOP system in human MDD at basal level or after an emotional induced challenge during PET scanning. The binding potential is measured in humans with MDD. BP=binding potential, ↓=decreasing, ↑=increasing, ↔=no change. *activated in HC's but not in MDD, **activated on sadness induction.

1.8.4.3 Critiques

The relevance of the plasma and CSF measure in MDD patients is questionable. Although it is adding to the body of literature on the opioid system in depression, it must be remembered that these measures also take into account for peripheral opioid expression and only certain constituents can be measured. In conjunction with this, CSF measurements can be quite invasive to undertaken, and blood measurements may be susceptible to contamination.

Post-mortem analysis has provided vital evidence to the research on the opioid system in MDD. Measuring receptor and peptide expression in the brain provides knowledge on the central opioid circuitry in this affective disorder, as well as providing evidence on which regions to focus on for future work. Having said this, there are methodological and diagnostic challenges of post-mortem studies, including the fact that patients can only be measured at a single timepoint. In conjunction with this, comprehensive knowledge of their mental health, and severity of such, prior to death is important, as this will be applied to the findings, and will also ascertain if findings can be comparable to others.

Imaging studies are by far the most advantageous methodology to date, allowing for measuring in the living brain, both continuously or on repeated exposures, and can also employ the use of a cognitive task or 'state' change to examine alterations to the brain circuitry. Indeed imaging studies perhaps represent the most relevant investigations for the future, but so far have been confined to the MOP. The selection of radio-labelled tracer is an important feature of PET imagine studies, as the tracer must be highly selective for the receptor or system being explored, so as not to bind to other receptors and represent unreadable results (Henriksen and Willoch, 2008). In addition to this, this methodology is particularly expensive, involving the use of specialised equipment by highly trained personnel. Having said this, future studies should try to utilise this technique as it provides evidence for the role of the opioid system in MDD, and can help to indicate the direction that further research should be going in.

1.8.5 Neurochemical associations between the central opioid and monoamine systems

As mentioned briefly above, the opioid system and monoamine systems are co-distributed in similar limbic regions throughout the brain. In this regard, opioid regulation of, an interaction with, the monoamine system has been suggested to facilitate a role in the regulation of mood and emotion, and as such in the mediation of affective disorders such as depression. The neurons for the serotonergic and noradrenergic systems that originate in the DRN and LC respectively, project to a number of brain regions that are important in depression, including the medial prefrontal cortex, hippocampus, amygdala and nucleus accumbens (Figure 1.13). The dopaminergic neurons originating in the ventral tegmental area also project to regions such as the nucleus accumbens and prefrontal cortex, and dysfunction of this mesolimbic circuit is implicated in anhedonia and a lack of motivation (Lutz and Kieffer, 2013).

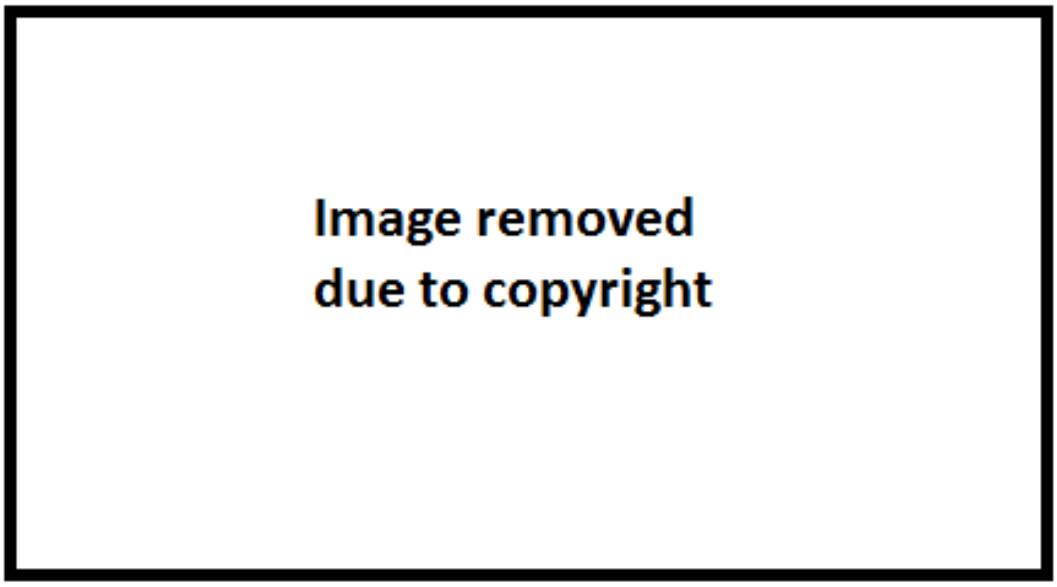


Figure 1.13 Monoaminergic nuclei (dopaminergic (DA), serotinergic (5-HT), noradrenergic (NA)) and the limbic regions they project to in the rodent brain. The dopaminergic neurons (*blue square*) originate in the ventral tegmental area (VTA) and project to the amygdala (Amy), nucleus accumbens (NAc) and the prefrontal cortex (PFC). The serotonergic neurons (*purple square*) originate in the dorsal raphe nucleus (DRN) and project to the PFC, hippocampus (Hipp), NAc, and the Amy. The noradrenergic neurons (*green square*) originate in the locus coeruleus (LC) and project to the bed nucleus of the stria terminalis (BNST). All of these region are involved in the regulation of mood, stress and cognition. DA=dopamine, NA=noradrenaline, 5-HT=serotonin. Image taken from Lutz and Kieffer (2013).

KOP receptors have been shown to be present on the cell bodies of noradrenergic neurons in the LC (Al-Hasani et al., 2013, Taylor and Manzella, 2016), the cell bodies of serotonergic neurons in the DRN (Pinnock, 1992, Schindler et al., 2012, Toa and Auerbach, 2002), and on the presynaptic terminals of DA neurons in the nucleus accumbens (Lutz and Kieffer, 2013, Svingos et al., 2001). Opioid receptors are co-localised with α_2-adrenoceptors in the LC, diencephalon and amygdala, with a_2-adrenoceptors being responsible for the synthesis and neural circuitry of noradrenergic transmission (Malonado, 1997). Direct administration of agonists of the various opioid receptor subtypes into specific loci has helped in identifying roles for the opioid receptor system in modulating monoamine release and signalling. In tandem, administration of drugs that target the monoaminergic system, with the inclusion of current monoamine-derived antidepressant therapies, have been shown to modulate opioidergic functioning; overall suggesting that the opioid system may be a plausible target for novel antidepressant therapy.

1.8.5.1 Opioidergic regulation of the monoamine system

A modulatory role for the MOP system on the serotonergic and dopaminergic system has been demonstrated. Administration of MOP agonists (DAMGO, endomorphin-1 and endomorphin-2) into the DRN results in an increase in extracellular 5-HT both locally and in the nucleus accumbens (Tao and Auerbach, 2002), whilst chronic administration of MOP agonists (endomorphin-1 and endomorphin-2) into the ventral tegmental area produces a decrease in 5-HT in the medial prefrontal cortex (Chen et al., 2001). Chronic administration with the MOP agonist morphine also produces a decrease in 5-HT_{2A} receptor binding in the frontal, temporal and parietal cortices, as well as in the subcortical regions (Adriaens et al., 2014). Acute administration of several MOP agonists (DAMGO, morphine, methadone and fentanyl) into the nucleus accumbens have been shown to increase extracellular DA locally (Di Chiara and Imperato, 1988, Hirose et al., 2005, Spanagel et al., 1990), whilst administration of MOP agonists (DAGO, DAMGO and endomorphin-1) into the ventral tegmental area produces an increase in extracellular DA in the nucleus accumbens (Spanagel et al., 1992, Terashvili et al., 2008) and in the ventral tegmental area (Chefer et al., 2009, Moreno et al., 2017). Administration of the MOP antagonist CTOP into the ventral tegmental area produces a decrease in DA release, (Spanagel et al., 1992). Acute

injection of MOP antagonist CTOP into the nucleus accumbens has no effect on extracellular DA in this region when given alone, but block the effects of MOP agonist-induced (DAMGO) increases in extracellular DA in the nucleus accumbens (Hirose et al., 2005). Furthermore, the role of the MOP has been developed further by demonstrating that the MOP-induced increase in DA when administered into the ventral tegmental area is not evident in MOP KO animals (Chefer et al., 2009). The effects of MOP administration on dopamine-striatal functioning has also been examined with direct injection of MOP agonist DAMGO into the dorsal striatum (medial, rostral and caudal) producing a decrease in extracellular DA in the caudal and rostral poles, with an increase in the medial pole (Campos-Jurado et al., 2016). Chronic administration of the MOP agonist endomorphin-1 into the lateral ventricle produces a decrease in DA in the ventral striatum (Chen et al., 2001). Acute administration of the MOP agonist morphine has been shown to decrease D_2 dopamine receptor mRNA in the striatum, with no change in D_1 receptor mRNA in this region (Georges et al., 1999). In conclusion, agonism of the MOP system increases DA transmission in many regions along the mesolimbic pathway, while causing increases in 5-HT in the nucleus accumbens and decreases in the serotonergic system in the prefrontal cortex.

The KOP system has also been shown to modulate monoaminergic release, again with particular respect to serotonergic and dopaminergic functioning. KOP agonists (CI-977 and U50,488) when directly injected into the DRN decrease 5-HT, an effect that is attenuated by the KOP antagonist norBNI (Pinnock, 1992, Tao and Auerbach, 2002). Moreover, direct injection of the KOP agonist U50,488 into the medium raphe nucleus and nucleus accumbens reduces 5-HT in both of these regions (Tao and Auerbach, 2002). In addition, acute systemic administration of U50,488 produces an increase in the serotonin transporter (SERT) uptake rate in synaptosomes, which is blocked by the KOP antagonist norBNI (Schindler et al., 2012). With regards dopaminergic neurotransmission, direct injection of KOP agonists (E-2078) and U-69593) into the nucleus accumbens produces a decrease in DA release locally (Spanagel et al., 1990, Spanagel et al., 1992), which is blocked by the KOP antagonist norBNI (Spanagel et al., 1990). This effect on DA release is also seen in the nucleus accumbens and the caudate, when KOP agonists (Bremazocine, U50,488 and tifluadom) were administered systemically (Di Chiara and Imperato, 1988). Whilst direct injection of the KOP agonist U-69593 into the ventral tegmental area had no effect on DA levels, administration of the KOP antagonist norBNI into the nucleus

accumbens produces an increase in DA release, but not when injected into the ventral tegmental area (Spanagel et al., 1992).

With regard to the DOP system, when DOP agonists (DPDPE, deltorphin II and DADLE) are injected directly into the DRN, they produce an increase in extracellular 5-HT locally (Tao and Auerbach, 2002), whilst the DOP antagonist naltrindole injected into the DRN produces a reduction in 5-HT (Tao and Auerbach, 2002). Such effects in 5-HT concentrations were absent when the DOP agonist DPDPE was injected into the medium raphe nucleus and nucleus accumbens (Toa and Auerbach, 2002). Chronic administration of the DOP agonist SNC80 increased concentrations of 5-HT in the frontal cortex, hippocampus and amygdala, and reduced 5-HT concentration in the hypothalamus in rats (Saitoh et al., 2008). Several DOP agonists (deltorphin II, DPDPE and DSLET), when injected into the nucleus accumbens, have been shown to increase extracellular DA (Hirose et al., 2005, Murakawa et al., 2004, Spanagel et al., 1990), with these effects being blocked by some, but not all, DOP antagonists (Hirose et al., 2005, Spanagel et al., 1990). Direct injection of DOP antagonists (BNTX and naltriben) alone into the nucleus accumbens had no effect on extracellular DA locally (Hirose et al., 2005).

1.8.5.2 Monoaminergic regulation of the opioid system

Monoaminergic regulation of the opioidergic system is also evident. Agonism of the dopaminergic system, in a manner that there is more extracellular DA available to function in the synaptic cleft, has been shown to elevate the DOP and KOP peptides. For example, chronic systemic administration of DA receptor agonist apomorphine increases DYN mRNA in the dorsolateral striatum (Gerfen et al., 1991). Acute administration of DA uptake inhibitor (cocaine and GR 12909), which block the dopamine transporter, have been shown to increase both DYN and ENK mRNA in the striatum (Hurd and Herkenha, 1992). PENK mRNA expression has also been shown to be increased in the striatum after acute and chronic administration of a DA uptake inhibitor (cocaine) (Przewlocka and Lasoń, 1995), with chronic administration also increasing PENK mRNA expression in the nucleus accumbens (Przewlocka and Lasoń, 1995).

Much of the evidence with regards serotonergic and noradrenergic regulation of the opioid system is founded upon abuse and opiate withdrawal, rather than on

activity of the opioid system *per se*, with more behavioural changes being shown upon administration of monoaminergic-acting compounds. The α_2-adrenoceptor agonist clonidine has been shown to attenuate opiate withdrawal in rats (Britton et al., 1984). Morphine-induced conditioned place preference in rats, was reduced by administration of the α_2-adrenoceptor agonist clonidine (Kosten, 1994). Inter-cranial infusion of selective β-noradrenergic receptor antagonists (ICI 118,551 and betaxolol) into the bed nucleus of the stria terminalis, alleviates opiate-withdrawal-induced conditioned place aversion in rats (Delfs et al., 2000). Intracranial infusion of morphine into the dorsal periaqueductal grey increases latency to escape from the T-maze, which is blocked by pre-treatment with the 5-HT_{1A} antagonist WAY-100635 (Roncon et al., 2013). Morphine intake in rats was reduced after intraperitoneal injection of anti-serotonin-modulating anticonsolidation protein (anti-SMAP) antibody (Mekhtiev et al., 2014), and neurochemical lesioning with the selective serotonin neurotoxin 5,7-DHT, causes a decrease in MOP and DOP binding in the hypothalamus (Allen et al., 1993).

Another approach that has been used is to examine the consequences of acute and chronic antidepressant exposure on central opioid receptor signalling. For example, chronic treatment with desipramine and sertraline resulted in a reduction in MOP binding in a range of brain regions including the amygdala, the CA1 and CA3 of the hippocampus, olfactory tubercle, and the occipital and temporal cortices (Chen and Lawrence, 2004). Chronic fluoxetine administration in rats resulted in selective reductions in PENK gene expression in the nucleus accumbens shell and caudate putamen, and a reduction in PDYN gene expression in the nucleus accumbens core and shell, caudate putamen and hypothalamus, that may in part explain its therapeutic efficacy (Oliva et al., 2005). Following the FST, PDYN mRNA is increased in the nucleus accumbens which is blocked by treatment with desipramine (Chartoff et al., 2009). It has been found that chronic administration of the TCA imipramine results in a reduction in DOP receptor density in the frontal cortex and striatum (Varona et al., 2003). Marketed antidepressants have also been shown to effect opioid-induced behaviour, or to interact with opioid compounds to alter behavioural functioning. Tianeptine is an antidepressant whose mechanism of action was first believed to be via 5-HT release (as opposed to inhibition of 5-HT reuptake), and more latterly via modifying glutamate release (Samuels et al., 2017). However, it has been recently found that tianeptine acts as a full MOP agonist, and a less potent DOP agonist

(Gassaway et al., 2014). Furthermore, the effects of tianeptine in reducing immobility time in the mouse FST and latency in the NIHY test were both abolished in MOP KO mice, suggesting a role for the MOP in the mechanism of action of tianeptine (Samuels et al., 2017). Combination of SSRIs (fluoxetine or citalopram) with a weak MOP agonist codeine produces a reduction in immobility time in mice when tested in the TST (Berrocoso and Mico, 2009). Mice injected with a KOP agonist U50,488, showed increased cocaine conditioned place preference, which was shown to be attenuated by pre-treatment with citalopram (Schindler et al., 2012). Desipramine, a TCA, has been shown to block the increase in PDYN mRNA in the nucleus accumbens (Chartoff et al., 2009) and in the dorsal bed nucleus of the stria terminalis and the lateral division of the amygdala (Chung et al., 2014) after exposure to the FST. Prior administration of the SSRI citalopram has also been shown to attenuate these stress-induced PDYN changes (Chung et al., 2014).

1.8.6 Preclinical evidence of modulation of the central opioid system as an antidepressant target

Given the close association between the monoamine and opioid system, in conjunction with the changes to the opioid system in MDD patients, activation of the opioid system has been examined in animal models of depression, and after exposure to and induction of stress-induced paradigms (Lutz and Kieffer, 2013). As discussed earlier, exposure to stress has been shown to alter the neurochemical functioning of the opioid system, particularly with regards exposure to ‘depressive-like’ and ‘anxiety-like’ stressful paradigms, or indeed by the implementation of animal models of depression (Drolet et al., 2001, Knoll and Carlezon, 2010, Taylor and Manzella, 2016). In tandem with this, activation and manipulation of the opioid system, by administration of opioidergic compounds, has been shown to alter behaviours in animal models of depression and tests of ‘depressive-like’ behaviour (McHugh et al., 2018). Indeed, activation of the MOP and DOP is associated with an elevation in mood and as such antidepressant-like effects, and in contrast, it is *blockade* of the KOP system that abolishes symptoms of dysphoria and potentiates antidepressant-like activity. With this in mind, opioid modulation has pertinent potential to act as a novel target for antidepressant therapy. Modulation of the opioid system in preclinical depression research has been examined and this will be discussed with regards:

- MOP agonists
- KOP antagonists
- DOP agonists – direct and indirect
- Combination therapies – targeting more than one opioid receptor

1.8.6.1 MOP

The additive-potential concerns associated with administration of selective MOP agonists, has slightly hindered their consideration as a viable target for the treatment of affective disorders. Having said this, opioid compounds that are classically used for the treatment of pain have been examined in rodent models of depression, and are often used to compare effects to the use of the more selective MOP-modulating drugs (Table 1.8). Berrocoso et al. (2013) looked at the effects of MOP agonists (morphine, codeine, methadone, and tramadol) on immobility time in the TST, with all drugs shown to cause a decrease in time spent immobile in mice, an effect seen with morphine in a separate study by Rosa et al. (2017). Intercranial infusion of MOP agonists (endormorphin-1 and endomorphin-2) into the left ventricle has also been shown to decrease immobility time in the FST and the TST in mice (Fichna et al., 2007), an effect that is blocked by the selective MOP antagonist beta-funaltrexamine (β-FNA), suggesting that it is mediated via the MOP (Fichna et al., 2007). Other MOP agonists have also been examined in the FST, such as met-enkephalin (Zhang et al., 2006), endomorphin-1 and -2 (Cravezic et al., 2011, Zhang et al., 2006), morphine (Zomkowski et al., 2005) and tramadol (Jesse et al., 2010), and these are all shown to reduce immobility time in this test of 'depressive-like' behaviour in animals. In LH animals, administration of MOP agonists (morphine, tramadol, methadone, leu-enkephalin and levorphanol) has been shown to reverse escape deficits (Berrocoso et al. 2013, Besson et al., 1996, Rojas-Corrales et al., 2002, Tejedor-Real et al., 1995), with the effects of morphine shown to be attenuated by naloxone (Besson et al., 1996). Interestingly, Zhang et al. (2006) also found that β-endorphin, endomorphin-1, and endomorphin-2, all increased BDNF mRNA levels in a number of brain regions including the hippocampus; a marker that is seen to be decreased in patients with depression.

In support of the knowledge that MOP agonist alleviate symptoms of stress and depression, MOP KO mice have been examined to ascertain the potential

antidepressant effects of MOP deletion. MOP KO mice have been shown to display an increase in immobility time in the FST, which can be normalised by acute treatment with the MOP antagonist naloxone (Filliol et al., 2000). MOP KO mice spend more time in the open arms of the EPM and display decreased immobility time in the FST and TST when compared to wild-type counterparts (Ide et al., 2010).

In summary, the vast majority of studies suggest that activation of MOPs results in antidepressant effects. However, the troubling adverse effects (respiratory depression, constipation, addictive potential and tolerance following repeated exposure) associated with the use of MOP agonists has meant that they have been less pursued as viable clinical option in the treatment of depression. Nevertheless, experimental compounds with stricter pharmacological properties and that utilise lower dosages could present promising targets for antidepressant therapy.

Compound	Mechanism	Test	Species	Dose(s); route; duration	Finding	Reference
Met-enkephalin, Leu-enkephalin	Agonist	FST LH	Rat Rat	100nmol; icv; acute 50 µg; icv; acute	Reduced immobility Reversal of escape deficit	Zhang et al. 2006 Tejedor-Real et al. 1995
Endomorphin-1, Endomorphin-2	Agonist	FST FST FST TST	Rat Rat Mouse Mouse	30, 90 nmol; icv; acute 50 µg; icv; acute 10 µg; icv; acute 1-30 µg; icv; acute	No effect Reduced immobility Reduced immobility Reduced immobility	Zhang et al. 2006 Cravezic et al. 2011 Fichna et al. 2007 Fichna et al. 2007
β-endorphin	Agonist	FST	Rat	3 nmol ; icv ; acute	Prolonged immobility	Zhang et al. 2006
Tyr-Pro-D-ClPhe-Phe-NH_2(A) Tyr-Pro-Ala-NH_2 (B)	Indirect agonist	FST	Mouse	A: 5 µg; icv; acute B: 10 µg; icv; acute	Prolongation of reduction in immobility induced by endomorphin-1 and endomorphin-2	Cravezic et al. 2011
Morphine	Agonist	TST TST LH LH FST	Mouse Mouse Rat Rat Mouse	5 mg/kg; sc; acute 20, 40 mg/kg; ip; acute 0.5-2 mg/kg; sc; 3d 0.25-8 mg/kg; sc; 3d 5-10 mg/kg; sc; acute	Reduced immobility Reduced immobility Reversal of escape deficit Reversal of escape deficit Reduced immobility	Rosa et al. 2017 Berrocoso et al. 2013 Tejedor-Real et al. 1995 Besson et al. 1996 Zomkowski et al. 2005
Tramadol	Agonist	FST TST Reserpine LH	Mouse Mouse Mouse Rat	40 mg/kg; oral; acute 32, 64 mg/kg; ip; acute 20-80 mg/kg; NS; acute 10, 20 mg/kg; ip; 3 d	Reduced immobility Reduced immobility Attenuation of effects of reserpine Reversal of escape deficit	Jesse et al. 2010 Berrocoso et al. 2013 Rojas-Corrales et al. 2004 Rojas-Corrales et al. 2002
Codeine	Agonist	TST	Mouse	20, 40 mg/kg; ip; acute	Reduced immobility	Berrocoso et al. 2013
Methadone	Agonist	TST LH	Mouse Rat	5 mg/kg; ip; acute 2, 4 mg/kg; ip; 3d	Reduced immobility Reversal of escape deficit	Berrocoso et al. 2013 Rojas-Corrales et al. 2002
Levorphanol	Agonist	TST LH	Mouse Rat	5 mg/kg; ip; acute 0.5, 1 mg/kg; ip; 3d	Reduced immobility Reversal of escape deficit	Berrocoso et al. 2013 Rojas-Corrales et al. 2002

Table 1.8 MOP manipulations and their effects on behaviour in rodent models and tests. D=days, FST=forced swim test, LH=learned helplessness, TST=tail suspension test, icv=intracerebroventricular, ip=intraperitoneal, iv=intravenous, NS=not specified, sc=subcutaneous.

1.8.6.2 KOP

As discussed previously, the KOP system is activated and increased on the induction of stress. This increase in activity in the KOP system has also been seen in animal models of depression such as in the LH (Shirayama et al., 2004), and WKY models (Pearson et al., 2006), and indeed as a result of stress-induced behavioural tests such as the FST or resident intruder defeat test (Bérubé et al., 2013, Chartoff et al., 2009). Therefore, when examining the consequences of behavioural manipulations that have a relevance to negative affect (e.g. stress or fear responses), there is evidence for a linkage between activation of the opioid system causing dysphoria, and in turn blockade of the opioid system presenting antidepressant and anxiolytic results. For example, KOP antagonist norBNI has also been shown to decrease 'depressive-like' symptoms in the LH model after intercranial infusion into the nucleus accumbens shell and the CA3 region of the hippocampus (Shirayama et al., 2004), a similar effect seen by Newton et al. (2002) after injection into the DG of the hippocampus. KOP antagonists (norBNI and DIPPA) reduced immobility in the FST in WKY model, a model thought to be appropriate for treatment resistant depression (Carr et al., 2010). Nor-BNI has also shown a reduction in immobility time in the FST in both rats (Beardsley et al., 2005, Mague et al., 2003, Zhang et al., 2007) and mice (Casal-Dominguez et al., 2013). Other KOP antagonists that have demonstrated 'antidepressant-like' activity in the FST include JDTic (Beardsley et al., 2005), GNTI and ANTI (Mague et al., 2003). Carr (2009) also showed that KOP antagonist DIPPA produces an increase in food consumption and a decrease in latency to feed in the NIHY test in WKY rats, as well as decreases in burying duration in a test of defensive marble burying test. Acute systemic administration of KOP agonist U50,488 produces an increase in tail withdrawal latency, which is blocked by acute systemic administration of KOP antagonist norBNI (McLaughlin et al., 2003). Direct injection of KOP antagonist JDTic into the basolateral amygdala (Knoll et al., 2011), and systemically (Knoll et al., 2007), has been shown to reduce anxiety in rats with increased open arm entries and percentage open arm duration in the EPM, an effect that is also seen with acute administration of norBNI (Knoll et al., 2007). By far the KOP antagonist norBNI has been the compound to be most examined in animal models of depression and tests of stress-induced anxiety. Moreover, norBNI has been shown to increase hippocampal BDNF in mice following i.c.v administration, an effect

that has also been observed with clinically active antidepressants (Russo-Neustadt et al., 2004). A summary of results of the effects of administration of KOP antagonists in animal models of depression and tests of 'depressive-like behaviour' is shown in Table 1.9.

Knockout of the KOP system on animals has presented mixed results with regards their effects on 'depressive-like' behaviour. McLaughlin et al. (2003) found reductions in immobility in the FST in *Oprk1*$^{-/-}$ mice, but Filliol et al. (2000) found no changes in KO mice when examined in this test. PDYN KO mice have also been reported to display a decrease in immobility in the FST (McLaughlin et al., 2003), no change in behaviour in the FST (Kastenberger et al., 2012), or indeed an increase in immobility (Wittmann et al., 2009). It must be remembered that the KOP system is believed to be differentially activated dependent on the nature of stress that is encountered, with potentially different signalling of KOPs occurring whether it is an acute or a chronic stressor that the animals are exposed to (Knoll and Carlezon, 2010). This also has particular resonance when assessing the preclinical effects of compounds affecting the KOP system, where both acute and chronic stress models are employed, as well as the potential for single and repeated administration of drugs to have differential consequences. For example, McLaughlin et al. (2003) showed that systemic administration of KOP antagonist norBNI had no effect on immobility in the FST on the first exposure, but decreased immobility on the second day of exposure to the FST (McLaughlin et al., 2003).

In summary, activation of the KOP system appears to be associated with dysphoric and anhedonic consequences, although the picture is complicated by the nature of whether acute or chronic stressors are investigated in rodent models. The strategy that KOP antagonists are capable of having "antidepressant" effects has been demonstrated in many models, but the long-term consequences of single administration of such KOP antagonists limits their clinical usefulness. The original KOP antagonists that were developed exhibited long-term effects lasting for several weeks after acute administration (Casal-Dominguez et al., 2013, Rorick-Kehn et al., 2014). These effects of slow onset of action and prolonged effects will limit their development into clinically useful drugs (Almatroudi et al., 2015). Having said this, shorter-acting KOP antagonists are now being actively developed and evaluated, that will retain the effects in animal models but that do not persist long-term.

Compound	Mechanism	Test	Species	Dose(s); route; duration	Finding	Reference
Nor-BNI	Antagonist	FST	Rat	1, 10 mg/kg; ip; acute	Reduced immobility	Beardsley et al. 2005
		FST	Rat	20 μg; icv 24h; before	Reduced immobility	Zhang et al. 2007
		FST	Rat	20 μg; icv; acute	Reduced immobility	Mague et al. 2003
		FST	Rat	5, 10 mg/kg; ip; acute	Reduced immobility in WKY strain	Carr et al. 2010
		FST	Mouse	1, 10 mg/kg; NS; acute	Reduced immobility	Casal-Dominguez et al. 2013
		FST	Mouse	10 mg/kg; ip; acute	Reduced immobility only with male C57Bl/6J	Laman-Maharg et al. 2018
		LH	Rat	2.5 μg; various regions; acute	Reversal of escape deficit	Shirayama et al. 2004
		SDS	Mouse	10 mg/kg; ip; acute	Reduced defeat behaviour	Grimwood et al. 2011
		Cocaine withdrawal	Rat	20 mg/kg; icv; prior to cocaine regime	Attenuation of ICSS and immobility deficits	Chartoff et al. 2012
		Morphine withdrawal	Mouse	2.5 μg; intra NAc; acute	Attenuation of immobility deficit	Zan et al. 2015
		EPM	Rat	10, 30 mg/kg; ip; acute	Reduced anxiety-like behaviour	Knoll et al. 2007
LY2444296	Antagonist	FST	Mouse	10, 30 mg/kg; sc; acute	Reduced immobility	Huang et al. 2016
		Cocaine withdrawal	Rat	3 mg/kg; ip; acute	Attenuation of immobility deficit	Valenza et al. 2017
JDTic	Antagonist	FST	Rat	0.3-3 mg/kg; ip; acute	Reduced immobility	Beardsley et al. 2005
		EPM	Rat	10 mg/kg; ip; acute	Reduced anxiety-like behaviour	Knoll et al. 2007
		EPM	Rat	0-10 μg; icv; acute	Reduced anxiety-like behaviour	Knoll et al. 2011
LY2456302	Antagonist	FST	Mouse	10 mg/kg; oral; acute	Reduced immobility	Rorick-Kehn et al. 2014
PF-04455242	Antagonist	FST	Mouse	32 mg/kg; sc; acute	Reduced immobility	Grimwood et al. 2011
		SDS	Mouse	3.2,10 mg/kg; sc; acute	Reduced defeat behaviour	Grimwood et al. 2011
GNTI	Antagonist	FST	Rat	10, 20 μg; icv; acute	Reduced immobility	Mague et al. 2003
ANTI	Antagonist	FST	Rat	0.3-3 mg/kg; ip; acute	Reduced immobility	Mague et al. 2003
DIPPA	Antagonist	FST	Rat	5, 10 mg/kg; ip; acute	Reduced immobility in WKY strain	Carr et al. 2010

Table 1.9 KOP antagonists and their effects on behaviour in rodent models and tests. EPM=elevated plus maze, FST=forced swim test, ICSS=intracranial self-stimulation, LH=learned helplessness, icv=intracerebroventricular, ip=intraperitoneal, iv=intravenous, NAc=nucleus accumbens, NS=route not specified, sc=subcutaneous, SDS=social defeat stress, WKY=Wistar Kyoto rats.

1.8.6.3 DOP

Strategies that activate the central DOP system include administration of direct DOP agonists, as well as indirect DOP agonists that act by enhancing the concentrations of the endogenous ligands. In this manner, we will discuss direct and indirect-acting DOP agonists. A summary of DOP agonists and there effects on rodent models of depression can be seen in Table 1.10.

With regards indirect-acting DOP agonists, inhibition of the breakdown of enkephalins has been proposed as a strategy for increasing DOP activity. There are two enzymes responsible for the breakdown of enkephalins, namely neutral endopeptidase and aminopeptidase N, and dual inhibition of these enzymes is considered necessary to be able to enhance central enkephalin levels (Noble and Roques, 2007). The dual enkephalinase inhibitor RB101 has demonstrated activity in the LH model (Tejedor-Real et al. 1998), as well as in the FST in both mice (Nieto et al., 2005) and rats (Jutkiewicz et al., 2006). These effects of RB101 in the FST in rats have been attributed to DOP activation, as they have been shown to be blocked by the DOP antagonist naltrindole (Nieto et al., 2005, Jutkiewicz et al., 2006), and could be prevented in DOP but not MOP KO mice (Nieto et al., 2005). RB101 has also been shown to produce an increase in locomotor activity in rats at a dose that is active in the FST, but did not cause any convulsions, an effect which is often seen with DOP agonists (Jutkiewicz et al., 2006). Another non-selective enkephalinase inhibitor, RB 38A reduced escape failures in the LH model in rats, whilst RB 38B, which only inhibits neutral endopeptidase, had less effects on reversing the deficits in the LH model (Tejedor-Real et al., 1995). Orpiorphin is a naturally occurring compound that inhibits the breakdown of enkephalins by peptidases. It has been shown to reduce immobility time in the FST in rats, which is blocked by the DOP antagonist naltrindole, suggesting that it mediates its effects via the DOP (Javelot et al., 2010). In addition, opriorphin reduces immobility time in the FST in mice (Yang et al., 2011).

With regards directly acting DOP agonists, a number of anti-depressant behavioural effects have been seen. A range of DOP agonists (DPDE, Deltorphin II, JOM-13 and UFP-502) were evaluated by Torregrossa et al. (2006) following central administration to rats and were all found to reduce immobility time in the FST; reflective of an increase in motivation to endure. Torregrossa et al. (2006), also found that DOP agonists (DPDPE and H-Dmt-Tic-NH-CH2-Bid) injected directly into the

right LV increased BDNF mRNA in the frontal cortex post- FST exposure, which was blocked with one DOP antagonist naltrindole. Acute administration of selective DOP agonist deltorphin II increased BDNF mRNA in the CA3 and DG of the hippocampus, but selective DOP agonist DPDPE had no effect on BDNF mRNA in these regions (Torregrossa et al., 2006). Similarly, all increases were blocked by pre-treatment with a DOP antagonist naltrindole (Torregrossa et al., 2006). Other DOP agonists have been shown to reduce immobility time in the FST in animals, including UFP-502 (Vergura et al., 2006) and UFP-512 (Vergura et al., 2008). Many of these early DOP agonists had the disadvantage of being peptide in nature, with the consequent lack of oral bioavailability that means they are only useful as preclinical tools without any clinical utility. Thus, non-peptide DOP agonists such as SNC80 and BW3373U86 have been created and also examined in rodent models. Acute administration of SNC80 has been shown to reduce immobility time in the FST in rats (Broom et al., 2002, Jutkiewicz et al., 2004), and in mice (Saitoh et al., 2004, Nozaki et al., 2014), with chronic treatment shown to increase time-spent and number of entries into the open arms of the EPM (Saitoh et al., 2008). SNC80 has also been shown to increase resilience in animals in a model of social defeat (Henry et al., 2018). Acute administration of BW373U86 reduces immobility time in rats in the FST (Broom et al., 2002, Torregrossa et al., 2005, Zhang et al., 2006), an effect which is not seen following repeated administration suggesting a tolerance developing to this effect (Broom et al., 2002, Torregrossa et al., 2005).

As mentioned earlier in this section, a property of the DOP agonists is the potential to induce convulsions that is seen with some of the agents such as SNC80 (Broom et al., 2002, Comer et al., 1993, Henry et al., 2018) and JOM-13 (Torregrossa et al., 2006). The extent of convulsions caused by SNC80 can be reduced by a slow administration of SNC80, which still manages to retain the reduction in immobility in the FST in rats (Jutkiewicz et al., 2005). Saitoh et al. (2011) found that KNT-127 reduced immobility time in mice in the FST at doses that did not induce convulsions, with a similar degree of reduced immobility observed following five days administration; suggesting a lack of tolerance to this effect (Nozaki et al., 2014). Other DOP agonists have been shown to have antidepressant effects without producing convulsion. For example, NIH 11082 reduced immobility time in mice in the TST without producing any convulsions (Naidu et al., 2007). ADL5859 reduces immobility time in the FST in mice (Huang et al., 2016) and does not produce seizures (Chung et

al., 2015). DOP agonist AZD2327 has demonstrated antidepressant-like activity in the LH model in rats, and without producing any convulsions (Hudzik et al., 2011).

DOP agonists have also been examined in the OB model and antidepressant effects were evident in the model after both short-term administration, as well as after chronic treatment (Saitoh et al., 2008). This early effect of SNC80 is not seen with clinically effective antidepressants in this model, where chronic treatment is necessary to reverse the OB-induced behavioural effects (Kelly et al., 1997). However, chronic administration of the DOP agonist KNT-127 attenuated the HE effects seen in the OB rat, an effect that was evident within 3 days of treatment, in contrast to fluoxetine which required 10 days of treatment (Gotoh et al., 2017). Subchronic treatment with SNC80 has also been shown to cause increases in the number of open arm entries and time spent in the open arms in OB rats in the EPM (Saitoh et al., 2008). Saitoh et al. (2008) also showed that subchronic treatment with SNC80 caused a significant increase in the concentration of 5-HT in the frontal cortex, hippocampus and amygdala in OB rats, an effect that is seen with currently marketed antidepressants (Saitoh et al., 2008).

Compound	Mechanism	Test	Species	Dose(s); route; duration	Finding	Reference
Directly Acting DOP agonists						
SNC-80	Agonist	FST	Rat	32 mg/kg; sc; acute	Reduced immobility	Broom et al. 2002
		FST	Rat	3.2-100 mg/kg; sc; 3.2 mg/kg; iv; acute 1, 3 mg/kg; sc; acute		Jutkiewicz et al. 2004
		FST	Mouse	3 mg/kg; ip; acute and 5d	Reduced immobility	Saitoh et al. 2004
		FST	Mouse	10 mg/kg; ip; acute	Reduced immobility	Nozaki et al. 2014
		FST	Mouse	10 mg/kg; ip; acute	Reduced immobility	Henry et al. 2018
		TST	Mouse	1-10 mg/kg; ip; 7d	Reduced immobility	Henry et al. 2018
		OB	Rat	10 mg/kg; ip; 3d	Reversal of emotionality; Reversal of anxiety-like behaviour	Saitoh et al. 2008
		SDS	Mouse		Increase in resilience	Henry et al. 2018
SNC-86	Agonist	FST	Rat	3.2-32 mg/kg; sc; 3.2 mg/kg; iv; acute	Reduced immobility	Jutkiewicz et al. 2004
SNC-162	Agonist	FST	Rat	100 mg/kg; sc; 3.2 mg/kg; iv; acute	Reduced immobility	Jutkiewicz et al. 2004
BW373U86	Agonist	FST	Rat	3.2, 10 mg/kg; sc; acute	Reduced immobility	Broom et al. 2002
		FST	Rat	10 mg/kg; sc; acute	Reduced immobility	Torregrossa et al. 2005
		FST	Rat	10-100 nmol; icv; acute	Reduced immobility	Zhang et al. 2006
Dynorphin	Agonist	FST	Rat	1-10 nmol; icv; acute	No effect	Zhang et al. 2006
DPDPE	Agonist	FST	Rat	155 nmol; icv; acute	Reduced immobility	Torregrossa et al. 2006
Deltorphin II	Agonist	FST	Rat	0.03, 0.1 nmol; icv; acute	Reduced immobility	Torregrossa et al. 2006
JOM-13	Agonist	FST	Rat	32 mg/kg; iv; acute	Reduced immobility	Torregrossa et al. 2006
UFP-502	Agonist	FST	Rat	30-100 nmol; icv; acute	Reduced immobility	Torregrossa et al. 2006
UFP-512	Agonist	FST	Rat	0.3, 1 mg/kg; ip; acute	Reduced immobility	Vergura et al. 2008
		FST	Mouse	0.01 nmol; icv; 0.1 mg/kg; ip; acute	Reduced immobility	Vergura et al. 2008
NIH 11082	Agonist	TST	Mouse	16, 32 mg/kg; ip; acute	Reduced immobility	Naidu et al. 2007
AZD 2327	Agonist	LH	Rat	1, 10 mg/kg; oral; 3d	Reversal of escape deficit	Hudzik et al. 2011
ADL 5859	Agonist	FST	Mouse	3, 10 mg/kg; ip; acute	Reduced immobility	Huang et al. 2016
KNT-127	Agonist	FST	Mouse	1 mg/kg; ip; acute and 5d;	Reduced immobility	Nozaki et al. 2014
		OB	Rat	3 mg/kg; ip; 14d	Reversal of emotionality	Gotoh et al. 2017
		FST	Mouse	1 mg/kg; sc; acute	Reduced immobility	Saitoh et al. 2011
Indirectly Acting DOP Agonists						
RB101	Indirect agonist	FST	Rat	32 mg/kg; iv; acute 100 mg/kg; ip; acute	Reduced immobility	Jutkiewicz et al. 2006
		FST	Mouse	80 mg/kg; ip; acute	Reduced immobility	Nieto et al. 2005
		LH	Rat	5 mg/kg; iv; 3d	Reversal of escape deficit	Tejedor-Real et al. 1998
RB 38A and RB 38B	Indirect agonist	LH	Rat	R38A: 6 μg; icv; 3d R38B; 30 μg; icv; 3d	Reversal of escape deficit	Tejedor-Real et al. 1995
Opiorphin	Indirect agonist	FST	Rat	1, 2 mg/kg; iv; acute	Reduction in immobility	Javelot et al. 2010
		TST	Mouse	1-6 μg; icv; acute	Reduction in immobility	Yang et al. 2011

Table 1.10 Directly and indirectly acting DOP agonists and their effects on behaviour in rodent models and tests. D=days, FST=forced swim test, LH=learned helplessness; OB=olfactory bulbectomy, icv=intracerebroventricular, ip=intraperitoneal, iv=intravenous, NS=not specified, sc=subcutaneous, SDS=social defeat stress, TST=tail suspension test.

1.8.6.4 Opioid Combination Therapies

Modulation of the opioid system by agents that act on a single opioid receptor subtype may not be sufficient either for efficacy purposes or to counteract the adverse effects that occur with activation of certain opioid receptor subtypes. With this in mind, opioid drug combinations, that attempt to improve/maintain the efficacy of opioid modulation whilst at the same time reducing the adverse effect profile, have been examined. As such, it would be anticipated that these drugs could have the potential to be a more favourable prospect as antidepressants than the currently marketed opioid drugs. Table 1.11 exhibits the strategy and use of opioid combination therapy as an antidepressant target to address the behavioural deficits in rodent model of depression and tests of 'depressive-like' behaviour.

Buprenorphine is an opioid drug that has come under considerable preclinical investigation in recent years, due to a unique combination of features. Buprenorphine is a partial MOP agonist, which acts as a functional antagonist at the KOP (Lutfy and Cowan, 2004). Buprenorphine administration reduces immobility time in the FST in mice (Falcon et al., 2015; 2016), as well as in SPD and WKY rats (Burke et al., 2019a), with the effects in mice shown to last for over 24 hours (Falcon et al., 2015). WKY rats injected with a single injection of buprenorphine are shown to display a reduction in immobility for up to a week after administration (Smith et al., 2019). A role for the KOP in the mechanism of action of buprenorphine has been suggested as this reduction in immobility in the FST in mice can be prevented by administration of the KOP antagonist norBNI, but not by the MOP antagonist clocinnamox (Almatroudi et al., 2015). With this in mind, buprenorphine has been shown to increase locomotor functioning in rats (Smith et al., 2019). Moreover, buprenorphine reduces the latency to approach in the NIHY model in mice (Falcon et al., 2016). In animals of depression, chronic administration of buprenorphine reverses the behavioural deficits associated with the CMS model in mice (Falcon et al., 2016) and attenuates the hyperactivity associated with the OB model in rats (Burke et al., 2019b). An analogue of buprenorphine is BU10119, which possesses KOP and MOP antagonist properties. Acute administration of BU10119 has also been shown to reduce immobility time in the FST and reduce the latency to approach in the NIHY in mice (Almatroudi et al., 2018).

Another strategy with regards to buprenorphine, is to combine it with another opioid compound that is capable of reducing the MOP agonist properties of buprenorphine; that central locus for the association with addiction liability. Combination of buprenorphine and naltrexone produces a reduction in immobility time in the FST and reduced latency to approach in the NIHY test in mice (Almatroudi et al., 2015). Combination of buprenorphine with the selective MOP antagonist samidorphan, has been shown to reduce immobility time in the FST in SD and WKY rats (Burke et al., 2019a; 2019b, Smith et al., 2019), decrease burying behaviours in rats (Smith et al., 2019) and attenuate hyperactivity in the OB rat model (Burke et al., 2019b). This combination has also been shown to have the advantage of attenuating the locomotor sensitization effects of buprenorphine which are seen following chronic administration in rats (Burke et al., 2019b). Smith et al. (2019) also showed that samidorphan was able to reduce buprenorphine-induced extracellular 5-HT and DA in the prefrontal cortex and nucleus accumbens, suggesting that the actions and addition of samidorphan may attenuate the reinforcing reward properties of the MOP agonism in buprenorphine. Translated to the human scenario, this combination would have the benefit of antidepressant efficacy, whilst reducing addiction potential associated with the MOP.

Another combination strategy has been proposed that combines actions at the KOP and DOP targets, with the differential distribution of KOPs and DOPs suggesting that a combination of agents affecting these two receptor types might have synergistic actions (Huang et al., 2016). Combination of LY2444296, the short-acting KOP antagonist with ADL5859, a selective nonpeptide DOP agonist, which individually were shown to have antidepressant activity in their own right, have been shown to have a clear synergistic effect with a reduction in immobility in the FST, and at doses which do not produce any locomotor activation (Huang et al., 2016).

Besides using two drugs to produce a certain opioid receptor profile, there have been efforts to design single chemical entities that have the desired opioid receptor affinities. For example, *m*-CF_3-PhSe is an MOP and DOP agonist and a KOP antagonist, which reduces immobility time in mice in the FST (Brüning et al., 2011, Rosa et al., 2017) and TST (Rosa et al., 2017), and has also been shown to increase sociability (Rosa et al., 2018a). These effects of *m*-CF_3-PhSe were still apparent following repeated administration, an effect that is lost after repeated exposure to morphine; where tolerance is evident (Rosa et al., 2017). The mixed nature of *m*-CF_3-

PhSe was confirmed by the ability of naltrindole, a DOP antagonist, and naloxonazine, an MOP antagonist to block the FST effects whilst nor-BNI, a KOP antagonist enhanced its effects (Rosa et al., 2017). In addition, antagonists of the 5-HT_{1A}, 5-$HT_{2A/2C}$ and 5-HT_3 receptors can block the behavioural effects of *m*-CF_3-PhSe associated with repeated forced swim stress in mice, highlighting an interaction with the serotonergic system (Rosa et al., 2018b). 5'-AMN and 5'MABN are potent KOP and MOP antagonists which demonstrate a reduction in immobility time in the FST in mice after a single injection, and whose effects are still evident up to two weeks after this single injection (Casal-Dominguez et al., 2013)

An alternative single compound with multiple opioid receptor affinities is 3CS-nalmefene, an MOP antagonist, and KOP and DOP partial agonist. Chronic treatment with 3CS-nalmefene has been shown to reverse the FST and NOR deficits in the IFN-α rat, in conjunction with revering the inflammatory and endocrine alterations associated with this model (Callaghan et al., 2018). ATPM-ET is an experimental compound that has KOP agonist and MOP partial agonist properties that has been shown to reduce immobility time in mice in the FST and TST, with both effects being blocked by the KOP antagonist nor-BNI, but not by the selective MOP antagonist β-FNA; suggesting its effects are principally mediated as an agonist of the KOP (Wang et al., 2016).

In conclusion, combining two opioid compounds together presents beneficial antidepressant effects in many tests and animal model of depression. Perhaps this strategy should be more firmly utilised in future research as the addictive potential of certain selective opioid compounds that have been shown to have antidepressant function, can attempt to continue to be utilised for their antidepressant potential.

Compound	Mechanism	Test	Species	Dose(s); route; duration	Finding	Reference
Buprenorphine	MOP partial agonist, KOP antagonist	FST FST FST NIHY CMS OB FST	Mouse Mouse Rat Mouse Mouse Rat Rat (WKY)	0.065-2 mg/kg; ip; acute 0.25 mg/kg; ip; acute 0.1 mg/kg; sc; acute 0.25 g/kg; ip; acute 0.25 mg/kg; ip; 7d 0.1 mg/kg; sc; 21d 0.1, 1 mg/kg; sc; acute	Reduced immobility Reduced immobility Reduced immobility Reduced latency to approach Attenuation of prolonged immobility and sucrose preference deficit Attenuation of hyperactivity Reduced immobility	Falcon et al. 2015 Falcon et al. 2016 Burke et al. 2019a Falcon et al. 2015 Falcon et al. 2016; Robinson et al. 2017 Burke et al. 2019b Smith et al. 2019
Buprenorphine (BUP) and Naltrexone (NAL)	BUP - MOP partial agonist, KOP antagonist; NAL – KOP, DOP, MOP antagonist	FST NIHY	Mouse Mouse	BUP (1mg/kg), NAL (1 mg/kg); ip; acute BUP (1mg/kg), NAL (1 mg/kg); ip; acute	Reduced immobility Reduced latency to approach	Almatroudi et al. 2015 Almatroudi et al. 2015
BU10119	MOP antagonist, KOP antagonist	FST NIHY	Mouse Mouse	1 mg/kg; ip; acute 1 mg/kg; ip; acute	Reduced immobility Reduced latency to approach	Almatroudi et al. 2018 Almatroudi et al. 2018
m-CF_3-PhSe	MOP agonist, DOP agonist, KOP antagonist	FST FST TST FSS SI SA	Mouse Mouse Mouse Mouse Mouse Mouse	50, 100 mg/kg; oral; acute 50 mg/kg; oral; acute 5-50 mg/kg; oral; acute 1, 5 mg/kg; oral; prior to and after FSS 25 mg/kg; ig; 7d 5, 25 mg/kg; ig; 7d	Reduced immobility Reduced immobility Reduced immobility Reversal of behavioural deficits Increased sociability Reduced social avoidance	Bruning et al. 2011 Rosa et al. 2017 Rosa et al. 2017 Rosa et al. 2018b Rosa et al. 2018a Rosa et al. 2018a
ATPM-ET	KOP agonist, MOP partial agonist	FST TST	Mouse Mouse	0.5, 1 mg/kg; sc; acute 0.05-1 mg/kg; sc; acute	Reduced immobility Reduced immobility	Wang et al. 2016 Wang et al. 2016
LY2444296 (LY) and ADL5859 (ADL)	KOP antagonist DOP agonist	FST	Mouse	LY (3 mg/kg), ADL (0.84 mg/kg); ip; acute	Synergistically reduced immobility	Huang et al. 2016
3CS-nalmefene	MOP antagonist, DOP and KOP partial agonist	IFN-α	Rat	0.3 mg/kg; sc; 28d	Attenuation of immobility and novel object deficits	Callaghan et al. 2018
Buprenorphine (BUP) and Samidorphan (SAM)	BUP - MOP partial agonist, KOP antagonist; SAM – selective MOP antagonist	FST OB	Rat (SPD; WKY) Rat	BUP (0.1 mg/kg), SAM (0.3 mg/kg); sc; acute BUP (0.1 mg/kg), SAM (0.3 mg/kg); sc; 14d	Reduced immobility of BUP preserved with SAM Attenuation of hyperactivity by BUP, preserved with SAM administration	Burke et al. 2019a ; Smith et al. 2019 Burke et al. 2019b
5' AMN	MOP and KOP antagonist	FST	Mouse	1 mg/kg; NS; acute	Reduced immobility	Casal-Dominguez et al. 2013
5'MABN	MOP and KOP antagonist	FST	Mouse	1, 10 mg/kg; NS; acute	Reduced immobility	Casal-Dominguez et al. 2013

Table 1.11 Opioid combination and non-selective drug strategies and their effects on behaviour in rodent models and tests. CMS=chronic mild stress, d=days, FSS=forced swim stress, FST=forced swim test, icv=intracerebroventricular, IFN-α=interferon α, ip=intraperitoneal, iv=intravenous, MB=marble burying, NIHY=novelty-induced hypophagia, NS=not specified, OB=olfactory bulbectomy, SA=social avoidance, sc=subcutaneous, SI=social interaction, SPD=Sprague Dawley, TST= tail suspension test, WKY=Wistar-Kyoto.

1.8.7 Clinical evaluation of opioid modulating drugs in MDD

Despite the suggestions of antidepressant effect observed with currently marketed opioid analgesics there has been limited clinical evaluation due to the concerns about the safety profile of these opioid drugs. These concerns have been heightened by the recent rise in opioid-related deaths (Murphy et al., 2018). Perhaps tramadol, with its lower affinity for the MOP and consequently lower addictive potential than other marketed opioid analgesics may be the most promising of the existing drugs to be considered (Barber, 2011). However, to date the literature has only isolated case reports of antidepressant properties of tramadol in patients (Barber, 2011). With regards to buprenorphine, low doses have been shown to improve depressive symptoms in a small study involving treatment resistant patients (Karp et al., 2014). This is the only recent study to have been conducted that has examined buprenorphine in clinical depression.

Some of the experimental compounds described in this review have reached the clinical stage of their development. As discussed previously, the clinical development of KOP antagonists has been hindered by the original compounds having long durations of action (Carlezon and Krystal, 2016). However, the short-acting orally-bioavailable KOP antagonist LY2456302 (also known as Opra Kappa, and CERC-501) has just begun to be evaluated clinically in cocaine dependence, with early reports suggesting that it appears to be well-tolerated (Reed et al., 2018).

The selective DOP agonist AZD2327 has recently been investigated in patients that have comorbid depression and anxiety, as it has demonstrated efficacy not only in preclinical models of depression but also in anxiety (Hudzik et al. 2011, Richards et al., 2016). The dose of AZD2327 was one at which there would be minimal risk of eliciting convulsions, which as has been discussed is a common adverse effect associated with DOP agonist (Richards et al., 2016). Having said this, findings showed that although AZD2327 elicited a greater response than placebo, it did not reach statistical significance (Richards et al., 2016). Interestingly, there were greater effects seen in the anxious symptoms, suggesting that this compound, and potentially other selective DOP agonists might be more useful in the treatment of anxiety disorders rather than that of depression (Richards et al., 2016).

With regards the strategy of combination therapy, the combination of buprenorphine and the MOP antagonist samidorphan has been developed, and is known as ALKS 5461. This combination aims to lower the MOP activation seen with

buprenorphine, whilst enhancing the KOP antagonist properties, providing a balanced opioid effect that would have limited addiction liability (Ehrich et al., 2015). Initially, a small study involving treatment-resistant MDD patients demonstrated efficacy of ALKS 5461 (Ehrich et al., 2015). This was also followed up by a multicentre randomised double-blind phase II trial in treatment-resistant depression patients (Fava et al., 2016), and also in an open-label extensive study as an adjunctive therapy for MDD patients (Thase et al., 2019), both of which demonstrated the compound to have beneficial activity. These results suggest that it may have an augmentation role in treatment resistant depression, a sub-facet of depression that has yet to be therapeutically addressed (Ragguett et al., 2018).

In conclusion, it is clear that there is therapeutic and beneficial evidence for targeting the opioid system in MDD. Having said all this, more work needs to be done at the preclinical level to establish strategies that can address the topics of dose, chronic administration, selective opioid targeting, and additive potential.

1.9 Limitations of current research

It has been demonstrated that there is considerable research being currently conducted on evaluating the antidepressant potential of opioid-modulating drugs. The main principal single-site receptors that have emerged from these investigations suggest that KOP antagonists and DOR agonists may be plausible and novel targets in the treatment of depression. Although agonism of the MOP, in general, has also shown antidepressant activity, there are concerns around the development of selective MOP agonists for a number of safety reasons. Collectively, these compounds have been evaluated using a range of rodent models, but by far the most commonly encountered have been the acute FST and TST paradigms; two paradigms classically associated with detecting antidepressant-like activity in novel compounds for the treatment of MDD. In conjunction with this, these two tests can also be used to induce stress rather than just as antidepressant 'screens' *per se*, and as such are pertinent paradigms that have been utilised to examine the effects to the opioid system after acute and repeated stress exposure.

Certain opioid compounds have also been examined in animal models of depression, some of which include repeated dosing and more elaborate behavioural investigations that more appropriately represent the alterations seen in the clinical condition. However, there is no predefined protocol for preclinical antidepressant

evaluation, and in conjunction with this, the nature of the chronic models employed can often vary considerably. Indeed, having a consistent approach to conducting research using a particular model can vary between laboratories, and is an issue that should be addressed in order to validate an animal model and verify its reliability (Yin et al., 2016). Indeed, it is clear that any preclinical behavioural evaluations using animal models of depression, need to be able to not only establish efficacy of novel compounds and to compare this efficacy against established antidepressants, but also to be able to examine whether behavioural effects induced from the compound that suggest an adverse effect signal (such as increased locomotor functioning, sensitization, or a propensity to cause seizures) are also included. In conjunction with this, any compounds assessed should be able to facilitate promising behaviour and molecular effects via chronic rather than acute dosing regimens, as these more appropriately resemble the timecourse and treatment of antidepressants in the clinical condition.

The incorporation of central molecular endpoints is also an important aspect of such preclinical evaluations. Indeed, the growing awareness of the downstream events following GPCR activation and how these can be differentially affected by ligands, challenges the agonist/antagonist dichotomy (Wisler et al., 2014). As the opioid system and receptors are tightly co-localised throughout the CNS, knowledge of their interactions with each other, as well as with other GPCRs, is vital to the establishment of producing modulating targets in a favourable manner.

In conclusion, in order to address these gaps in the literature, a well-validated and reliable animal model of depression should be used to investigate the role of the opioid system in animal models of depression. The OB model is believed to be a well-established animal model of depression, that replicates the time it take for disease development, as well as having shown the fact that chronic rather than acute antidepressant treatment attenuates behavioural effects seen. In conjunction with this, removal of the olfactory bulbs has also been associated with alterations to the central opioid system, and behavioural features have been shown to be reduced by targeting the opioid system.

1.10 Hypothesis

It was hypothesised that olfactory bulbectomy (OB) in rats is associated with an extensive remodelling of key regions in the brain, resulting in biochemical change to

the opioid system, which cause changes in behaviour through cognitive and motivational processes, that symptomatically reflect depression. Pharmacological attenuation of such changes will be explored with conventional antidepressants, as well as with the novel use of opioid modulating drugs in the model.

1.11 Research Aims

The primary objective of this project was to assess the role of the opioid system in the OB rat model. This was undertaken with regards to three main aspects:

- Examination of social cognition
- Examination of the impact of stress
- Examination of pharmacological modulating compounds

A primary focus was placed on social cognition, a feature that has not been examined in OB rats and is an important feature of MDD. In addition to this, the opioid system has been shown to have a role in social cognitive functioning, and has been shown to increase sociability and cognitive functioning in rodents. Current antidepressant therapy has a poor history of specifically attenuating deficits in social function in depression and as such, it has been suggested that perhaps novel therapeutic strategies are needed. As such, we wanted to examine the effects of chronic administration with pharmacological drugs, with particular respect to opioid modulating drugs, on social cognition in OB rats.

The opioid system is closely linked to systems involved in the regulation of stress. Exposure to stress has been shown to cause adaptions in opioid levels in the brain and opioid modulators have been shown to inhibit stress-related responses in both animals and humans alike. As such, the effects of exposure to an acute and chronic stressor on central opioid functioning was examined in the rat, through use of the OB model as a ‘chronic’ stressor.

Lastly, in this introduction, an emphasis has been placed on the importance of developing reliable and reproducible animal models in psychiatric diseases, a subject that is of great concern in preclinical science. As such, the opportunity to validate the OB rat model and its behavioural responses was undertaken in order to authenticate this models place in preclinical literature. With all these factors in mind, the specific research aims of the project were as follows:

- To conduct a systematic review and meta-analysis of the olfactory bulbectomy (OB) rat model to validate its reliability as an animal model of depression
- To re-characterise the behavioural effects of olfactory bulbectomy (OB); with a particular focus on social cognition in the 3-chamber sociability test
- To investigate alterations to the central opioid system following acute (forced swim test) and chronic stressors (olfactory bulbectomy) in the rat
- To assess the effects of pharmacological intervention with opioid modulating drugs in the olfactory bulbectomised (OB) rat model of depression

Chapter 2:

A systematic review and meta-analysis of behavioural responses in the olfactory bulbectomised (OB) rat

2.1 Introduction

The utilisation of animals in biomedical research as models of disease are pivotal in being able to shed light on identifying disease mechanism and novel targets. However, against this backdrop, reproducibility and ethical considerations in pre-clinical experimentation is of growing concern in biomedical research (Hunter, 2017). Developing animal models of a disease or disorder can often incur many challenges, such as time, labour, cost, environment and workspace. Ethical considerations are vital in animal research and need to be taken into consideration when implementing animals as tools for scientific research, such that well-designed experiments that only use the amount of animals that are necessary are executed (Tannenbaum and Bennett, 2015). In addition to this, determining the validity of using animals to model a disease that will appropriately resemble the symptomatic and molecular changes seen in the clinical condition is of vital importance for furthering treatment and therapeutic strategies, and these models must be able to be appropriately replicated across laboratory settings. As experimental subjects, animals are very sensitive to external factors such as noise and environment, and heterogeneity across animal populations is frequently a defining feature when deeming significant effects. These factors are very important as they can cause difficulties in replicating animal models and can also cause variability in the results that are observed across different laboratories.

Recent endeavours have been made to address these concerns of ethics and reproducibility in preclinical research. The introduction of the ARRIVE (Kilkenny et al., 2010) and PREPARE (Smith et al., 2017) guidelines for the conduction of animal research, emphasise that experiments that use animals must be of ethical, accurate, transparent and reproducible disclosure, that endeavour to produce easy replication and report accurate and factual findings. All animal studies should be undertaken with the responsibility of embarking on an experiment that is in line with the 3 Rs; replacement, reduction and refinement (Russell and Burch, 1959). Initially coined by Russel and Burch (1959), the 3 Rs are important ethical principles that are now considered as ‘alternatives’ or ‘alternative methods’ for reducing any potential distress to animals in preclinical science (Tannenbaum and Bennett, 2015). Global organisations such as the National Centre for Replacement, Refinement and Reduction of Animals in Research (NC3Rs) in the United Kingdom, and the Institute for Laboratory Animal Research (ILAR) in the United States of America, are at the forefront of these advances and have been set up with the objective of making sure

these principles are actively initiated in animal research. These organisations endeavour to ethically minimise the unnecessary use of animals in experiments and to improve the overall welfare and well-being of animals that are used (ILAR, 2011, NC3Rs, 2019). These ethical changes have been actively accepted and welcomed by the scientific community, with the aim of improving the transparency of animal use in scientific research and with the aim of working with agencies that protect the welfare of animals rather than being in conflict with them. Indeed, Elliott Lilley, senior scientific officer to the Royal Society for the Protection of Cruelty to Animals (RSPCA), endorsed the creation of digital tours for the public of four animal laboratories in the United Kingdom in 2017, with the incentive that these initiatives allow the public a glimpse at the lives of research animals. In summary, it must be said that these foundations and guidelines for preclinical research have begun to address many of the ethical concerns associated with the use of animals in science, but much work is still needed with regards to the reproducibility element.

One major downfall of reproducibility across preclinical science, is the failure of published literature to fully disclose its experimental design and report coherent and transparent findings. Indeed, the methodological approaches and protocols used in animal studies are often poorly defined and lack consistency, leading to the increased risk of bias among findings and leading to ambiguity regarding the true effect of an intervention or model (Hooijmans et al., 2014, Kilkenny et al., 2009). In a survey by Kilkenny et al. (2009) examining 271 studies reporting animal research in the USA and UK by publically funded research establishments, they found that 41% of papers did not state the aim or hypothesis of the study, 41% did not state the number of animals used or their specific characteristics such as sex, strain, age or weight, 87% of papers did not mention the use of randomisation in allocating subjects to experimental groups, whilst 86% did not state that any form of blinding was used in making the measurements on the subjects, and finally 30% of papers did not describe their methods of statistical analysis or use a measure of error or variability. This paper highlights the importance of accuracy and transparency when reporting experimental design, methods, and results, and indicates that omitting such criteria in experimental design and publication has had a profoundly negative impact on producing ethically sound and scientifically robust research (Kilkenny et al., 2009). Species, sex, strain and the housing conditions of animals are all factors that must be considered when planning animal experimentation and when trying to reproduce results. Publication

bias can have detrimental results to animal research as a whole. The absence of negative findings in a given field can give an overall positive 'gloss' to a novel treatment or target, which may not necessarily be the case. The failure to report negative results or the act of omitting such results from publication can have many damaging outcomes, including contributing to the wastefulness of animals and in turn, life (Smith et al., 2017).

In clinical research, i.e. that uses human subjects, the degree of consensus between findings obtained from different experimental studies is often evaluated using tools such as systematic review and/or meta-analysis (Leucht et al., 2009) and are means to establish the extent of reproducibility (Gopalakrishnan and Ganeshkumar, 2013). A systematic review is conducted in order to identify, collect and review all relevant literature and data on a particular given topic with the over-arching objective of answering a specific scientific question (Vesterinen et al., 2014). A meta-analysis goes a step further by taking the information available in data format to determine the efficacy of a certain intervention, or established diagnostic marker, by evaluating its approximate effect size (Hooijmans et al., 2014). The Cochrane Library, formerly known as the Cochrane Collaboration, is well established as a network and collection of highly-standardised evidence-based systematic research reviews in clinical healthcare that inform and guide all healthcare and scientific research professionals on issues related to health. The first Cochrane review was published in 1995 and examined the administration of corticosteroids in premature babies in maturing their respiratory system. There were a number of small studies that were not statistically significant but when they were bundled together, it was demonstrated that this intervention was significantly effective (Crowley, 1995). As such, the Cochrane Library provides reliable results on healthcare issues that must meet strict criteria to be incorporated into the library, and that influence the decisions of healthcare professionals and policy-makers based upon solid scientific evidence. The Cochrane Library and its systematic reviews have proven very successful in providing evidence-based findings to all clinical–based research and its systematic reviews are highly recognised by both academic researchers and by healthcare bodies and institutions (Vesterinen et al., 2014).

Interestingly, such approaches have only recently started to spark interest pre-clinically (Hooijmans et al., 2014). Although it is much less explored in pre-clinical research, the utilisation of systematic review and meta-analysis should be more

frequently exploited in order to negate the necessity of unethical replication, as well as to properly assess the efficacy of a model, intervention or diagnostic marker. In a review by Hooijmans et al. (2014), he states that there are a number of advantages to conducting a meta-analysis on animal studies:

- Exploring a wider range of toxicities
- Exploring more mechanistic interventions
- Exploring more of the heterogeneity across diseases and disorders
- Accumulating all the data on a specific topic in one place
- Demonstrating how a given hypothesis or research question may have shifted its conclusions over time

By combining the results of a number of small animal studies together, we increase the power of the analysis and provide more of an insight into the significance of the effect (Hooijmans et al., 2014, Orset et al., 2016). For example, in a meta-analysis conducted by Sena et al. (2010), the efficacy of recombinant tissue plasminogen activator (rtPA) in the treatment of stroke which was induced experimentally in animals was examined. They concluded that sufficient evidence had been accumulated by 2001 to establish the efficacy of rtPA, with the use of around 1500 research animals (Hooijmans et al., 2014, Sena et al., 2010). However, by 2010 when the meta-analysis was conducted, an additional 1888 animals had been used (Hooijmans et al., 2014, Sena et al., 2010). This example demonstrates that by conducting a meta-analysis in a specific animal model, such as in this stroke model, it allows the scientific community to join together and avoid further needless replication, while also allowing us to fill in the gaps of a particular scientific question which may still yet remain unanswered (Vesterinen et al., 2014). In addition to this, the conduction of a meta-analysis on a model or diagnostic marker in preclinical experimentation has the addition of ascertaining if there indeed *is* an effect with an intervention, and the magnitude and reproducibility of such an intervention, before the researchers commit to utilising it. Having said all this, one limitation of a meta-analysis in both clinical and pre-clinical literature is the fact that we can only work with literature that has been published. The failure to report negative results in an intervention that is accustomed to having positive findings is unfortunately a tactic that is often implemented. Employed as a level of self-censorship, this approach can have a concerning impact to research in a

given field. Having said this, for preclinical work in particular, it can often be very difficult to get negative findings published, with many rejections seen during the peer-review process. With this in mind, the responsibility of publishing negative results is a two-pronged process. That being said, there are fundamental differences between conducting experiments in animals when compared to humans, such as in the experimental design, purpose and conduct of the experiments, and so many factors need to be taken into consideration when conducting a systematic review or meta-analysis pre-clinically (Vesterinen et al., 2014). Similar to humans, heterogeneity in animal studies is a major component in scientific research, such that sex, age, species, strain, dose and timing of intervention are all very important defining criteria that could influence the effect size that is measured (Hooijmans et al., 2014). Initiatives such as the Collaborative Approach to Meta-Analysis and Review of Animal Data from Experimental Studies (CAMARADES), established in 2004, and the SYstematic Review Centre for Laboratory Animal Experimentation (SYRCLE) have been established in order to instruct, assist, and guide in the conduction of systematic reviews and meta-analysis in preclinical research (Vesterinen et al., 2014). Although the Cochrane Library only publishes reviews on clinical data to-date, discussions between SYRCLE and the Cochrane Library of establishing a preclinical animal study methods group that will work in close co-operation with each other to create such a system for preclinical findings has begun (Ritskes-Hoitinga et al., 2014). By pooling a large set of data on an animal model or diagnostic marker, and systematically reviewing the findings and results, this can help to validate the intervention examined and quantify its authenticity as a reliable tool for investigation.

Bilateral OB is a commonly used animal model (Kelly et al., 1997). It is a well-established comprehensive model which has shown evidence for a number of the behavioural and biochemical abnormalities associated with depression (Song and Leonard, 2005). The most common behavioural alteration seen in the model is an increase in locomotion in the OF, and which in the OB model is attenuated with chronic but not acute antidepressant treatment. Using this behaviour as a primary endpoint, the model has reported predictive validity for detecting antidepressant-like efficacy (Kelly et al., 1997). In conjunction with this, the behavioural phenotypes of the OB model takes approximately two weeks to develop following surgery, which somewhat mirrors the protracted time-course of symptom development and progression in depression (Van Riezen and Leonard, 1990). Besides hyperactivity in

the OF, other OB-related alterations include anhedonia, maladaptive responses to stress, weight loss and cognitive impairment; features which indicate positive face validity. Having said this, results are not always repeatedly consistent in this model and variations in behavioural responses in the OB model have been shown (See Chapter 1, Section 1.7.4). As a result, and due to the fact that the OB model is being used as the paradigm of choice for the assessment of novel opioid modulating strategies in this project, it was decided that a systematic review and meta-analysis of the behavioural responses in the OB model would be conducted to assess the degree of reproducibility in findings across the various laboratories in the world that use the model. A retrospective pooled analysis of published papers and scientific data between the years 1999-2018 was undertaken from pre-clinical studies looking at depressive-like behavioural responses in OB rats. Only studies that examined the OB rat model with the aim of employing an intervention/treatment to modify the OB-related behavioural responses for evaluating antidepressant-like properties in the model were included. Only these studies were examined as they more accurately resembled our own studies where an intervention would also be undertaken to assess a modification in behaviour in the OB rat as a result of potential antidepressant compounds. Therefore, the specific aims of this chapter were as follows:

- To identify all of the primary literature studies that have used the OB rat model over the last twenty years (1999-2018) where an intervention/treatment was employed to modify behavioural responses in the model
- To evaluate the principal methodological differences between studies by conducting a systematic review of these aspects
- To conduct a meta-analysis and determine the effect size of the main behavioural endpoints that have been evaluated in the OB rat
- To further evaluate the OF test with regard to whether different test conditions are capable of producing greater effect sizes in the OB rat

2.2 Materials and Methods

2.2.1 Materials

Item	Source
GetData Graph Digitizer	www.getdata-graph-digitizer.com
Comprehensive Meta-Analysis®Software	www.meta-analysis.com
GraphPad Prism 8	Software provided by NUI Galway
Microsoft Excel	Software provided by NUI Galway

Table 2.1 Software packages used for the conduction of the systematic review and meta-analysis in Chapter 2.

2.2.2 Literature Search Strategy and Selection Criteria

The search strategy involved using the keywords 'olfactory bulbectomy' and 'rat' into three different search engines, namely PubMed, Embase and Web of Science. Only papers that included these search terms and that were published between the years 1999-2018 were included. In all, 1,092 papers across all three search engines were found. The OB procedure employed in each paper was carefully examined to ensure that it followed the accepted methodology (Kelly et al., 1997) for the generation of sham-operated and OB rats. Two reviewers examined and screened the abstracts and titles for all 1,092 papers for the eligibility criteria and to check that they were relevant. This included cross-referencing across search engines so that duplicates of papers were accounted for and eliminated (n=774). In all, 318 papers remained, and the titles and abstracts of all papers were checked and only papers that were in the English language were included (Figure 2.1). This further reduced the number to 306 papers. Further examination excluded papers that actually did not involve olfactory bulbectomy (n=10), that used unilateral bulbectomy rather than bilateral olfactory bulbectomy (n=12), were undertaken in mice (n=16), were undertaken *in vitro* (n=2), were conference abstracts only (n=8), or were literature review papers rather than original research papers (n=3).

The remaining 255 papers were divided up into categorises such that the specific aims, objectives and interventions used in each study were evaluated in order to classify the 'type of study' assessed (Figure 2.1). Only 'intervention' papers were chosen and used for further analysis. An intervention was defined as any treatment that has been employed to modify the OB-related behavioural responses for evaluating antidepressant-like properties in the model. All other 'types of studies' were excluded (n=122), with the aim of assessing the OB rat with regards its specific OB-related behavioural responses in depressive-like behaviours (Figure 2.1).

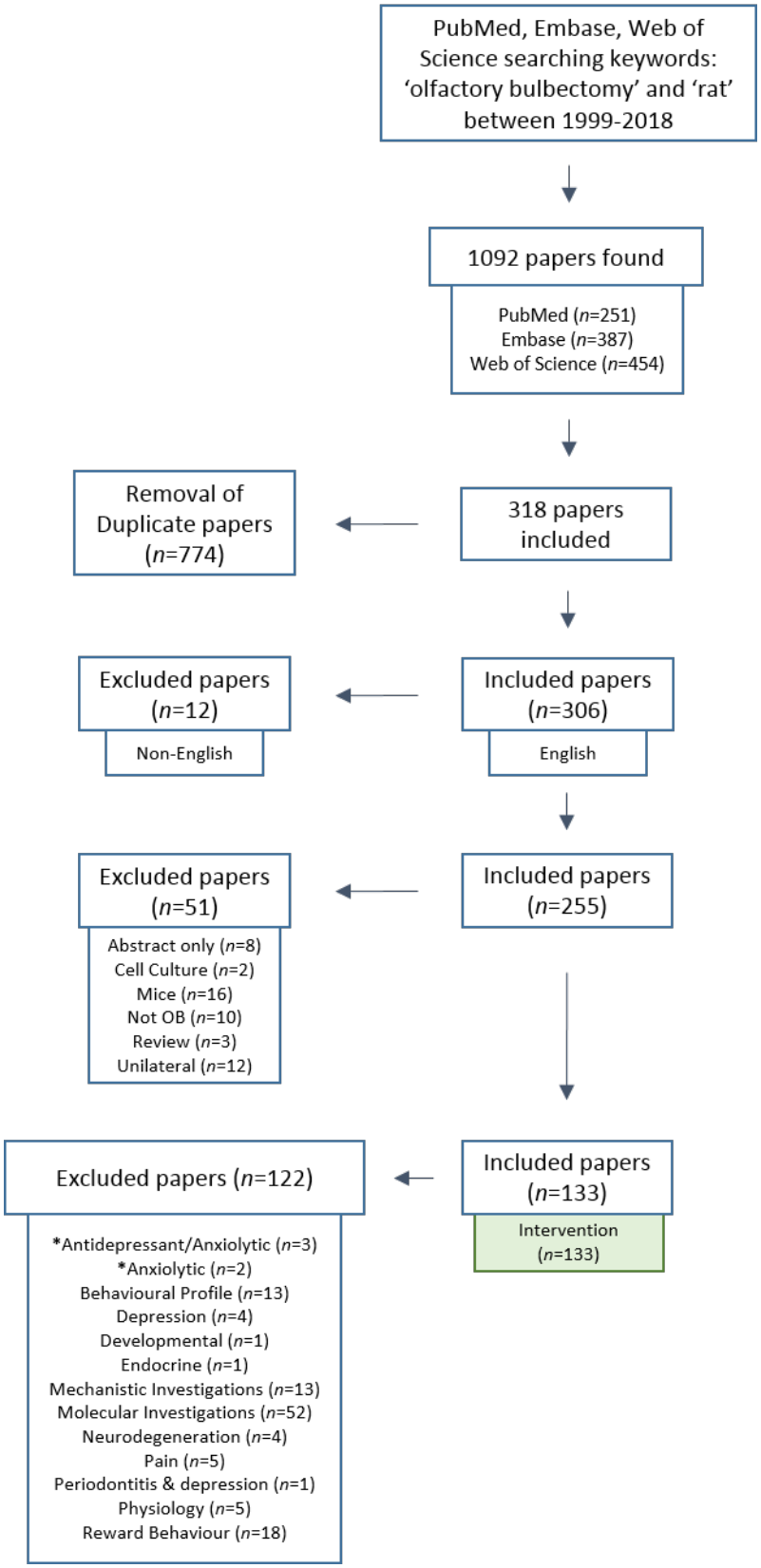


Figure 2.1 Summary of literature search strategy and selection criteria. Numbers in brackets equal the amount of papers included, excluded or per topic examined. *Antidepressant/anxiolytic papers (*n*=3) and anxiolytic papers (*n*=2) were excluded as they have no behavioural endpoints and examined only molecular measurements.

2.2.3 Data Collection and Outcome Measures

Of the 133 intervention papers remaining, data was extracted from all papers including the strain of rat used, the sex of rat that was used, the housing of the rats after surgery, the behavioural outputs/responses examined, and the frequency and number of papers published by the same university/laboratory groups, and lastly the nature of the intervention examined. The housing of the rats *after* surgery was examined and defined in this manner, as in some studies the housing of the rats before and after surgery altered and this option was chosen as it was the housing condition that the rats stayed in for the duration of the experiment. Nevertheless, housing pre-treatment or prior to surgery is also very important as early life experience can have huge potential to influence behaviour, especially when examining psychiatric illness such as depression where early life experience is often considered a risk factor of disease.

Of the behavioural outputs assessed, a number of classic behavioural tests for depressive-like and anxiety-like behaviour in the OB model were further analysed. This included examining:

- Distance moved in the OF test
- Immobility time in the FST
- Latency time, and number of trials in the PA test
- Open arm entries (number and percentage) and/or open arm duration (time and percentage) in the EPM
- Total hyperemotionality in the HE test
- Sucrose/Saccharin intake/preference in the SPT
- Social interaction time in the SI test

In the OF test, papers that specifically examined distanced moved in the OF test were included such that animals had to be placed into an unfamiliar environment. An unfamiliar environment was defined as a novel arena/apparatus that the animal had not been exposed to previously, that was brightly lit and was surrounded by tall aluminium/black or wooden walls. The data extracted included both the use of automated-tracking software and manual scoring techniques, where distance moved was defined as the number of sections crossed, the number of squares crossed, the distance moved in centimetres, ambulation scores, or the number of photobeams interrupted. Studies that examined locomotion in OB rats in any other form such as in

the homecage were not included, as the nature of the response in this environment would not be considered the same as that of in the OF. As the OF test was the highest examined behavioural test of all tests examined in the OB rat (*n*=107), further data were extracted with regards the specific set-up of this test, such that data on the shape of the OF arena and the duration that rats spent in the OF arena were extracted. Note that two papers were excluded from this part of the analysis as on closer inspection of the data these two papers published results that used exactly the same control responses for sham-operated and OB rats as had been published by the same author in a previous research paper.

In the FST, papers that specifically examined time spent immobile were included. Active behaviours such as swimming and climbing were not assessed. The data that was extracted included both the use of automated-tracking software and manual scoring techniques, where immobility was defined as the total duration spent immobile in seconds, or, by counts, i.e. both the continuous and count methods were used and included.

In the PA test, two parameters were assessed. Papers that specifically examined the latency of time it took for rats to enter the chamber, and that examined the number of trials it took for rats to learn to avoid the chamber were included. The time it took for rats to enter the chamber was defined as the latency, the latency time, the retention time or the time spent on the platform. The number of trials it took for rats to learn to avoid the chamber was defined as the number of trials or the trial number.

In the EPM, papers that specifically examined the number or percentage (%) of open arm entries, and the number or percentage (%) of time spent in the open arms by rats in the EPM were included; closed arm entries and closed arm time (number and percentage) was not assessed. Often, the data for the open arm entries would be expressed either as number or percentage of entries, or as time or percentage time and so the data was amalgamated such that there was one set of data for open arm entries, and one set for the open arm time. The open arm entries and time was defined as the total duration (s) that rats spent in the open arms, or, the total amount of entries (counts) that rats made into the open arms.

In the HE test, papers that specifically examined and scored the overall HE or total emotional score were included. The total HE score was equated as the sum of responses when rats were tested for attack, fight, startle, struggle and vocalisation

responses when primed in a manner to do so. These five responses were not included when examined singularly. The HE score was defined as the total emotional response made by rats.

In the SPT, papers that specifically examined the amount or intake of sucrose/saccharin consumed were included. The sucrose/saccharin intake or % preference was defined as sucrose/saccharin intake (g), or, % preference, i.e. *[sucrose intake/(sucrose intake + water intake)*100]*. In a similar fashion to the EPM parameters, the intake and % preference were amalgamated under a single parameter.

In the SI test, papers that specifically examined and scored the time that rats spent exploring the unfamiliar rat were included. Social interaction was described in the papers as crawling, sniffing, following, running, probing, grooming, climbing and mounting the unfamiliar rat, and these behaviours were summed to give a total social interaction time. These behaviours were not assessed independently. The pair of rats that were placed into the arena together were not cagemates and had had no prior interaction, but belonged to the same treatment group. The social interaction time was defined as the total time that the rats spent socially interacting with the other rat.

For all behavioural outputs assessed, if rats were tested in a behavioural response more than once, only the most recent (or last) time-point examined was included in the analysis. The reason for this was because this would typically be the timepoint when antidepressant effects would be evaluated, i.e. following repeated (typically 2-3 weeks) treatment. This would better match-up with the vast majority of other papers that measure only one time-point after antidepressant intervention, and also with our own project which will measure behavioural activity after pharmacological intervention in later chapters. The OF and HE test were the only two parameters to expose rats to these behavioural paradigms on more than one occasion. Theses mixed/repeated behavioural test experiences were acknoweledged and single verses repeated exposures to the test experience were noted. In the OF test, 16/105 papers exposed rats to the OF more than once. In the HE test, 9/14 papers exposed rats to the HE test on more than one occasion. Both of these parameters were checked to make sure that more than one exposure did not affect the overall mean outputs.

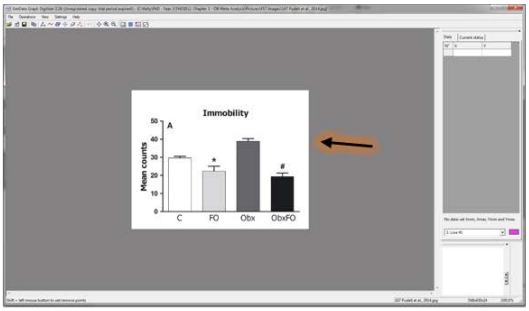
With regards the OB surgery itself, the length of time that rats were allowed to recover from surgery was not deemed a principle variable, i.e. if rats were left for longer than 14 day or shorter than fourteen days, the data was still included in analysis. Similarly, the time of testing after surgery was not deemed a principle variable, i.e. no matter the

time-point of testing after surgery (less or more than 14 days), all data were included. A behavioural response was excluded from the analysis if an author repeated the same findings in another research paper (*n*=3), if the paper did not include the *n* numbers (*n*=1), and if the data did not include any descriptive statistics (mean, median, standard deviation, interquartile range, etc.) (*n*=1).

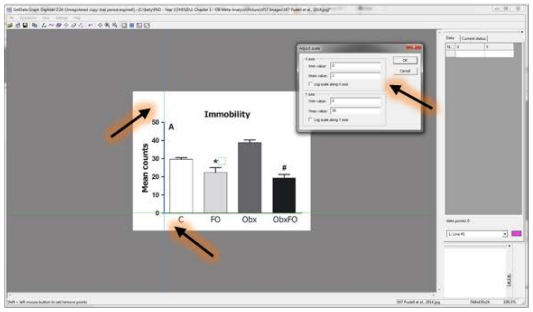
2.2.4 Data Extraction and Computation

Data was extracted on the control sham-operated and OB rats only. For sham-operated rats, this included rats that had undergone sham surgery and acted as 'controls'. Sham-operated rats were considered a 'control' if they had not been treated with any experimental drug (drug-naïve), and had received the vehicle, or the corresponding vehicle treatment. If a paper did not have a 'control' sham-operated rat that met these criteria, its behavioural response was not included (*n*=1). In a similar manner, data on OB rats was only included when an OB rat had undergone bilateral bulbectomy and was drug-naïve or had received vehicle-treatment. If a paper did not include a 'control' OB rat, this papers behavioural responses were not included (*n*=1).

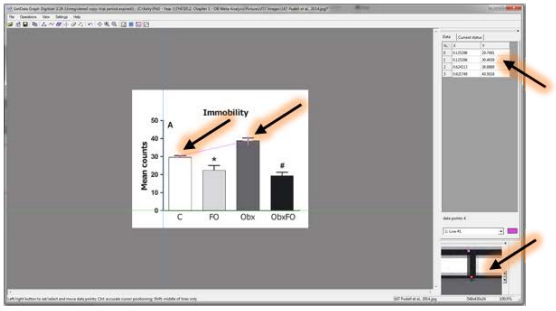
For all of the behavioural data, the mean and standard deviation was calculated in order to generate the data in a format for evaluation. In the vast majority of cases, data were expressed as mean and standard error of the mean (SEM). In such cases, the (SEM) was converted to the standard deviation using the following formula: *[standard error of the mean*square root(n number)]*. If a paper did not specifically state the *n* number of a group, giving instead a range for the *n* numbers of all groups, the average of this range was calculated, and this was used for the analysis e.g. *n*=8-12, the *n* used would be 10. On one occasion, data was expressed as non-parametric and was excluded due to difficulties in computing to mean and standard deviation (SD). Mean and SEM/SD values were taken directly from the papers when presented in tabular form or numerically in the text of the results sections. For data that was described through graphical depiction, the commercial software package GetData Graph Digitizer (www.getdata-graph-digitizer.com) was used to estimate values and calculate the mean and standard deviations (Figure 2.2).



Step 1: Import Image



Step 2: Set up scale for *x-axis* & *y-axis*



Step 3: Capture points on graph & calculate values for mean and SD of sham and OB rats

Figure 2.2 Estimated values for mean and standard deviations (SD) using the commercial software package GetData Graph Digitizer. The image was taken from the published paper and imported to GetData Graph Digitizer (1). The measurements for the *x-axis* and the *y-axis* were set using the 'set the scale' tool (2). Using the 'point capture mode' tool, the point for the mean and the SD was selected on the graph, and a value was generated for each of these in the panel on the top right-hand side (3). If data was depicted as SEM, the SD was calculated as described in the methods section above.

2.2.5 Statistical Analysis

All data for the characteristics of the studies were extracted and expressed as a percentage of the total findings, i.e. 133 papers, and this figure was represented in the pie chart or graph represented. For example, 71 papers used Sprague-Dawley rats out of the 133 papers, *[(71/133)*100]* meaning 53.38% of papers used Sprague-Dawley rats. The same approach was used for the OF characteristics, i.e. characteristics were expressed as a percentage of the total 105 OF papers. All data for the meta-analysis were mean and standard deviations, and converted where applicable as discussed in Section 2.2.4. Meta-analysis was performed using Comprehensive Meta-Analysis®Software (www.meta-analysis.com). The 95% confidence interval (95% CI) was calculated, which delineates the number of standard deviations in the difference between the means of sham-operated and OB rats. Outputs for meta-analysis were generated from Comprehensive Meta-Analysis®Software and exported to Microsoft Word. In meta-analysis outputs, the blue dots represent the effect size of each published result, with the lines representing the 95% CI; the thicker the blue dot, the more weighted the effect. The green dot on the bottom of the output represents the overall effect size. The effect sizes were shown to either 'Favour A' or 'Favour B'. Favours A indicates a reduction in the parameter, whilst favours B indicates an increase in the parameter when the OB group is compared to its sham-operated counterpart. The threshold for 'Favours A' verses 'Favours B' was set as 0.00 such that when an individual effect size and its 95% CI were both deemed to cross over the 0.00 line, the study was deemed as having a significant effect. However, if an individual effect size was seen to cross over the 0.00 but the CI passed through, rather than over, the 0.00 line, then a study was deemed to have a trend to 'Favour A' or 'Favour B' rather than to have a significant effect. All graphs were prepared using GraphPad Prism Version 8.

2.3 Results

2.3.1 Characteristics of the included studies

Of the 133 papers that were deemed eligible for analysis, the following characteristics of these papers were elucidated: the strain of rat that was used, the sex of rat that was used, the housing of the rats after surgery, the behavioural outputs/responses examined, the frequency and number of papers published by the same university/laboratory groups, and the nature of the intervention examined.

2.3.1.1 Strain of rats

The effects of strain are expressed in Figure 2.3. Of the 133 papers examined, 71 used the Sprague-Dawley strain, 57 used the Wistar strain, 2 used the Long Evans strain, 1 used the Lister-Hooded strain and 2 papers did not state the strain of rat that was used (Figure 2.3).

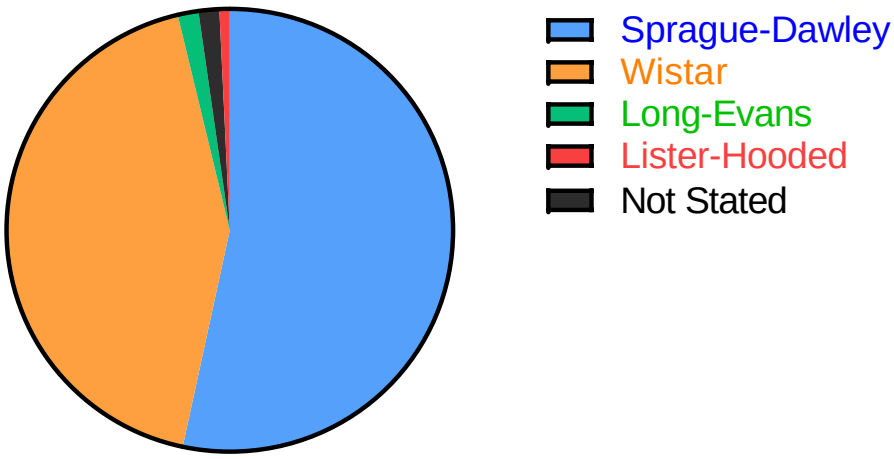


Figure 2.3 Strain of rats. Results are generated from the 133 OB intervention studies between 1999 and 2018. Data is expressed as a percentage of the total number of papers examined for this characteristic.

2.3.1.2 Sex of rat

The results for sex are expressed in Figure 2.4. Of the 133 papers examined, 126 studies used only male rats, 1 paper used only female rats and 3 papers used both male and female rats. Three papers did not specify which sex of rat was used. When the results are expressed in 5-year blocks over the 20 years, it can be seen that those 3 papers that used both males and females had taken place in the most recent time block (Figure 2.4B).

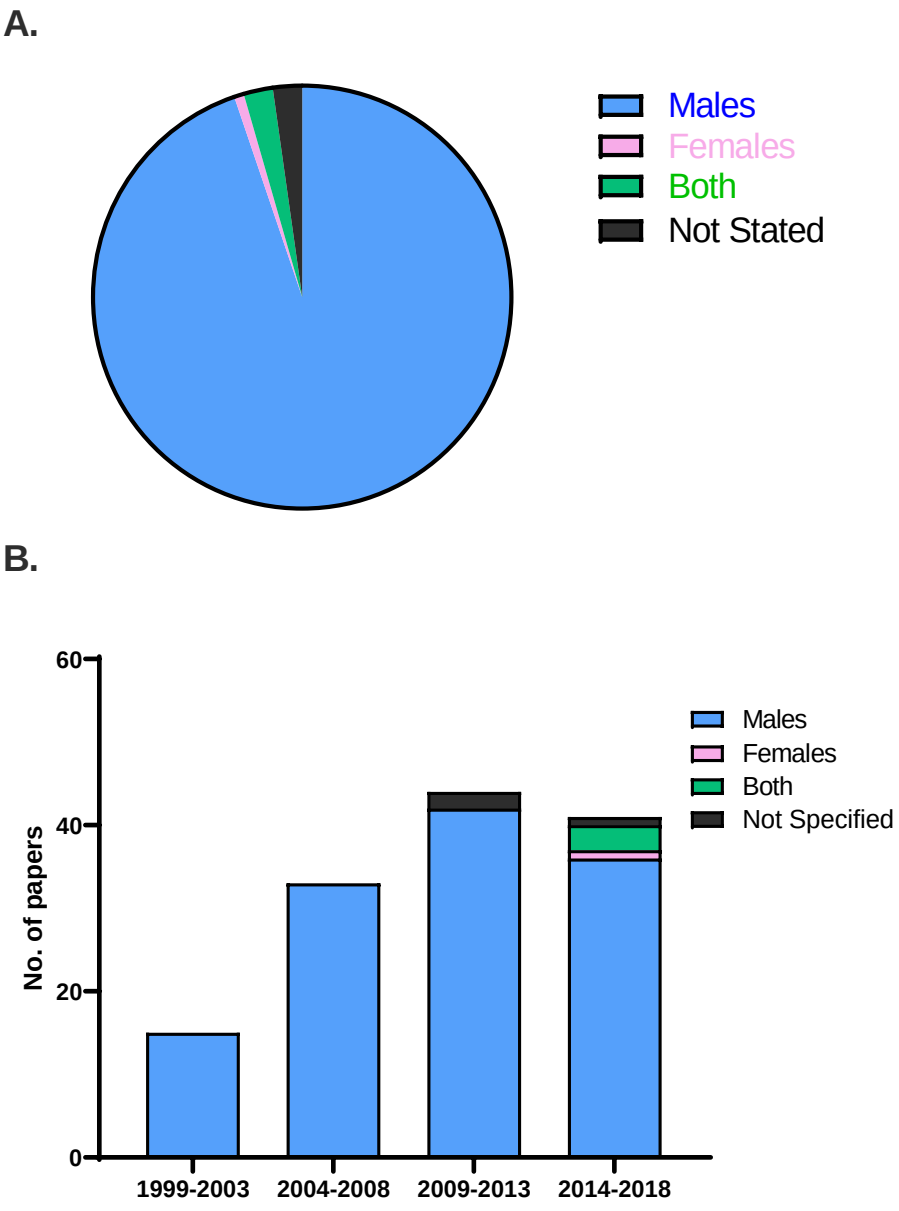


Figure 2.4 Sex of rats. Results are generated from the 133 OB intervention studies between 1999 and 2018. Sex of rat employed as represented as a piechart (A). The sex of rat employed as represented as a barchart with the number of years broken-down into 5-year blocks (B). The data in the piechart (A) is expressed as a percentage of the total number of papers examined for this characteristic.

2.3.1.3 Housing of rats after surgery

The effects of housing after surgery are expressed in Figure 2.5. Of the 133 papers examined, 46 papers did not state how the animals were housed after surgical operation, 41 papers singly-housed rats, 21 papers housed rats as 4 per cage and 13 papers used pair-housing. There were also 8 studies where 5 or more rats were housed per cage, and 4 studies that employed 3 rats per cage.

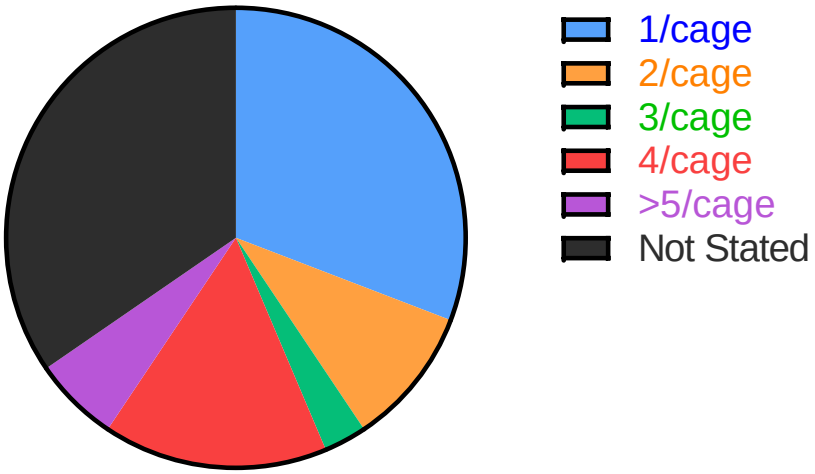


Figure 2.5 Housing of rats after surgery. Results are generated from the 133 OB intervention studies between 1999 and 2018. The data in the piechart is expressed as a percentage of the total number of papers examined for this characteristic.

2.3.1.4 Behavioural responses examined

Table 2.2 summarises the most commonly used behavioural responses evaluated in the papers, with the OF test in this model being by far the most utilised behavioural parameter used in 107 studies.

Behavioural Parameters	No. of Papers
Open Field Test	107*
Forced Swim Test	27
Passive Avoidance Test	20
Elevated Plus Maze	17
Hyperemotionality	16
Sucrose/Saccharin Consumption	15
Social Interaction Test	9
Morris Water Maze	3
Novelty-Suppressed Feeding	3
T-Maze	3

Table 2.2 Behavioural parameters examined and the frequency of times they are examined. Results are generated from the 133 OB intervention studies between 1999 and 2018. *subsequent analysis revealed that two of the papers used the same control groups.

2.3.1.5 Institution of corresponding author

Table 2.3 summarises the institutes where the corresponding author resided and which most likely represent the location of the experiments. Of the 133 papers examined, 57 different institutes examined the OB rat model of depression and published an intervention paper. The Birla Institute of Technology and Science, Pilani, Rajasthan, India published the most papers on the OB model with 19 publications, followed by Utrecht University in the Netherlands with 11 publications. There were 35 institutes that published just a single paper using the OB model.

Institute of Corresponding Author	No. of Papers
Birla Institute of Technology and Science, Pilani, Rajasthan, India	19
Utrecht University, Netherlands	11
McGill University, Montreal, Canada	8
National University of Ireland, Galway, Ireland	8
Polish Academy of Sciences, Krakow, Poland	8
Taisho Pharmaceutical Co. Ltd., Saitama, Japan	5
Maharashtra Institute of Pharmacy, Maharashtra, India	4
Universidad de Cantabria, Santander, Spain	4
University of Melbourne, Australia	4
National Center of Neurology and Psychiatry, Tokyo, Japan	3
Bharati Vidyapeeth University, Erandwane, Pune, India	2
Fudan University, Shanghai, China	2
Taisho Pharmaceutical Co. Ltd., Saitama, Japan	2
Maharashtra Institute of Pharmacy, Maharashtra, India	2
Universidad de Cantabria, Santander, Spain	2
Hoshi University, Tokyo, Japan	2
Jagiellonian University Medical College, Krakow, Poland	2
Medical University, Varna, Bulgaria	2
National Taiwan University, Taipei, Republic of China	2
Panjab University, Chandigarh, India	2
Peking University, Beijing, China	2
Universidad de Cordoba, Spain	2

Table 2.3 Institutes that published more than one paper on the OB rat model. Results are generated from the 133 OB intervention studies between 1999 and 2018.

2.3.1.4 Nature of the intervention

The nature of the intervention employed in the 133 papers was determined and results are summarised in Table 2.4–2.8. Interventions were classed under compounds that targeted the 'classic' neurotransmitter systems, the neuropeptide systems, other systems, interventions that used natural products, and finally, other interventions that did not meet the description of any of the above categories.

The most commonly employed drug interventions were focused on drugs targeting the central monoaminergic system, most particularly the serotonergic and noradrenergic systems. Within the serotonergic system, the SSRIs fluoxetine, citalopram, fluvoxamine, paroxetine and sertraline have been examined, whilst most subtypes of the 5-HT receptor have also featured, including the 5-HT_{1A} receptor full agonists (+)-S-20499 and 8-OH-DPAT, the 5-HT_{1A} receptor partial agonist buspirone, and the 5-HT_{1A} receptor antagonist WAY-100635. The 5-HT_{2A} receptor antagonists BIP-1 and ketanserin and the 5-HT_{2C} agonist WAY-163909 have also been examined. The 5-HT_3 receptor antagonist ondansetron has been examined alongside a range of experimental 5-HT_3 antagonists, whilst there have also been a 5-HT_4 receptor antagonist (RS 67333), a 5-HT_6 receptor partial agonist (EMD 386088) and a 5-HT_7 receptor antagonist (SB-269970) examined. Drugs with mixed serotonergic effects that have been evaluated include DSP-1053 (a SERT inhibitor and 5-HT_{1A} receptor partial agonist), SSA-426 (a SERT inhibitor and 5-HT_{1A} receptor antagonist), meta-Chlorophenylpiperazine (mCPP) (possessing mixed agonist/antagonist effects on various 5-HT receptors) and LSD (a 5-$HT_{1A/2A}$ receptor agonist). Melatonin has also been investigated, a hormone with a strong structural resemblance to 5-HT.

With regard to the noradrenergic and dopaminergic systems, the NET inhibitors desipramine and reboxetine and the DAT inhibitor bupropion have been evaluated, as well as the DA agonist's pramipexole and rotigotine. Drugs with multimodal actions, i.e. affecting more than one neurotransmitter system, have also been examined, with these including the dual 5-HT/NA inhibitors, imipramine, amitriptyline, lifepramine, milnacipran and F-98214-TA and the triple reuptake inhibitor DOV 216,303. Other multimodal drugs included [(-)-BPAP], WS-50030, lurasidone, MCL0042 and agomelatine.

Other neurotransmitter systems that have been examined include the glutamatergic, GABAergic and cholinergic systems. The glutamatergic system has also featured, with drugs acting on the NMDA receptor either as non-competitive

antagonists (ketamine, MK-801 and memantine), as well as the blockers magnesium and zinc. Metabotropic glutamate receptor agents have included MPEP (a metabotropic glutamate (mGlu) receptor 5 antagonist) and MTEP and MGS0039 (Group II mGlu receptor antagonists). In conjunction with this, riluzole and tianeptine, whose primary mechanism is affecting glutamate function, have also been examined. Targeting of the GABAergic system has included $GABA_B$ receptor antagonists (CGP 36742 and CGP 51176), positive allosteric modulators of $GABA_A$ receptors (diazepam and chlordiazepoxide), and the GABA enhancing agent, tiagabine. Finally, targeting of the cholinergic system has involved the acetylcholinesterase inhibitors tacrine and physostigmine, and nicotine.

With regards to neuropeptides, the neuropeptide Y (NPY) system has been most commonly explored with agents including NPY, [$Leu^{31}Pro^{34}$]PYY (a Y_1 receptor agonist), PYY^{3-36} (a Y_2 receptor agonist), JNJ-31020028 and BIIE0246 (Y_2 receptor antagonists), and [cPP^{1-7},NPY^{19-23},Ala^{31},Aib^{32},Gln^{34}]hPP (a Y_5 receptor agonist). Other neuropeptide drugs that have been evaluated in the OB model typically have focused on a single target such as the DOP agonists (KNT-127 and SNC80), CRF_1 receptor antagonist (R278995/CRA0450), neurokinin-2 (NK-2) receptor antagonist (saredutant), Vassopressin 1b (V1b) receptor antagonists (SSR149415, TASP02333278 and TASP0390325), melanin converting hormone (MCH) 1 receptor antagonists (TASP0382650 and TASP0489838), VIP and neuropeptide trefoil factor 3.

A number of other drugs have been examined, many of which have been marketed for other conditions, and which do not fall within the monoamine or neuropeptide categories. These include treatments that have anti-inflammatory properties, such as mincocycline, celecoxib, fish oil and ethyl-eicosapentaenoate (EPA), the sigma1 receptor agonists SA-4503 and igmesine, the phosphodiesterase 4 inhibitors etazolate and rolipram, the cannabinoid tetrahydrocannabinol (THC) and CB_1 receptor antagonist rimonabant, the adenosine A_{2A} receptor antagonist ZM 241385, the glucocorticoid synthesis inhibitor meyrapone, 17 beta-estradiol, the nitric oxide synthase (NOS) inhibitor TRIM and the cardiovascular drugs simvastatin (a HMG coA reductase inhibitor) and losartan (an angiotensin 1 receptor antagonist). Finally agents with more general actions have included vanillin, N-acetylcysteine and some derivatives of 1,2,4-triazino[5,6-b]indole-3-thione. There have also been a

number of studies where natural products, either as extracts or as a believed active principle have been examined.

A number of non-pharmacological interventions have also been explored in the OB rat, such as chronic exercise, wheel running, treadmill, environmental or dietary enrichment, REM sleep deprivation, or invasive interventions such as deep brain stimulation, transcranial magnetic stimulation and vagus nerve stimulation.

Target	Drug Name	Mechanism	Reference
Serotonin	Fluoxetine	SERT inhibitor	Mar et al. 2002; 2000; Mato et al. 2010; Riad et al. 2017; Rodríguez-Gaztelumendi et al. 2009; Roche et al. 2007; Wang et al. 2012
	Citalopram		Breuer et al. 2007; Pandey et al. 2014
	Fluvoxamine		Saitoh et al. 2007
	Paroxetine		Cryan et al. 1999; El Mansari et al. 2015
	Sertraline		Bissette 2001; Harkin et al. 1999
	(+)-S-20499	5-HT_{1A} receptor agonist	McGrath and Norman, 1999
	8-OH-DPAT		Jiang et al. 2014
	Buspirone	5-HT_{1A} receptor partial agonist	Mar et al. 2000; Sato et al. 2008
	WAY-100635	5-HT_{1A} receptor antagonist	Cryan et al. 1999
	BIP-1	5-HT_{2A} receptor antagonist	Pandey et al. 2010
	Ketanserin		Pandey et al. 2015
	WAY-163909	5-HT_{2C} receptor agonist	Rosenzweig-Lipson et al. 2007
	(4-phenylpiperazin-1-yl) (quinoxalin-2-yl) methanone (4a)	5-HT_3 receptor antagonist	Mahesh et al. 2012
	2-(4-methyl piperazin-1-yl)-1,8-naphthyridine-3-carbonitrile		Mahesh et al. 2007
	2-(4-phenylpiperazin-1-yl)-1, 8-naphthyridine-3-carboxylic acid (7a)		Gautam et al. 2013
	4i (N-(3-chloro-2-methylphenyl) quinoxalin-2-carboxamide)		Gupta et al. 2014
	N-(benzo[d] thiazol-2-yl)-3-ethoxyquinoxalin-2-carboxamide 6k		Kurhe et al. 2014

	N-(pyridin-3-yl) quinoxalin-2-carboxamide		Pandey et al. 2016
	N-n-butyl-3-ethoxyquinoxalin-2-carboxamide (6p)		Bhatt et al. 2013
	N-n-butylquinoxalin-2-carboxamide (4n)		Kumar et al. 2012
	QCF-3		Devadoss et al. 2010
	Ondansetron		Mahesh et al. 2010; Ramamoorthy et al. 2008;
	RS 67333	5-HT_4 receptor agonist	Lucas et al. 2007
	EMD 386088	5-HT_6 receptor partial agonist	Jastrzębska-Wiesek et al. 2017
	SB-269970	5-HT_7 receptor antagonist	Mnie-Filol et al. 2011
	DSP-1053	SERT inhibitor and 5-HT_{1A} partial agonist	Kato et al. 2015
	SSA-426	5-HT_{1A} antagonist and SERT inhibitor	Sukoff Rizzo et al. 2009
	mCPP	Mixed agonist/antagonist effects on 5-HT receptors	Rajkumar et al. 2009
	LSD	5-$HT_{1A/2A}$ agonist	Buchborn et al. 2014
Noradrenaline	Desipramine	NET inhibitor	Mar et al. 2000; Connor et al. 2000; Ho et al. 2004
	Reboxetine		Harkin et al. 1999
Dopamine	Pramipexole	D_2/D_3 receptor agonist	Breuer et al. 2009
	Rotigotine	$D_1/D_2/D_3$ receptor agonist	Bertaina-Anglade et al. 2006
	Buproprion	DAT inhibitor	El Mansari et al. 2015
	Imipramine	SERT and NET inhibitor	Breuer et al. 2007; Chambliss et al. 2004; Gigliucci et al. 2014; Keilhoff et al. 2006; Roche et al. 2008; Takahashi et al. 2008;
	Amitriptyline		Mar et al. 2000; Rafalo et al. 2017
	Lofepramine		Kelly and Leonard, 1999
	Milnacipran		Redmond et al. 1999; Saitoh et al. 2007
	F-98214-TA		Artaiz et al. 2005
	DOV 216,303	SERT, NET and DAT inhibitor	Breuer et al. 2008; Prins et al. 2011

	[(-)-BPAP]	increases monoamine release and reuptake	Tsunekawa et al. 2007
	WS-50030	D_2 receptor partial agonist/SERT inhibitor	Brennan et al. 2010
	Lurasidone	D_2 and 5-HT_2 receptor antagonist	Ishibashi et al. 2010
	MCL0042	MC_4 receptor antagonist and SERT inhibitor	Chaki et al. 2005
	Agomelatine	Melatonin agonist and 5-HT_{2C} antagonist	Norman et al. 2012
GABA	CGP 36742	$GABA_B$ receptor antagonist	Nowak et al. 2006
	CGP 51176		
	Diazepam	PAM of $GABA_A$ receptor	Wierońska et al. 2001
	Chlordiazepoxide		
	Tiagabine	GABA enhancement	Pistovcakova et al. 2008
Glutamate	Ketamine	Non-competitive NMDA antagonist	Holubova et al. 2016
	MK-801		Ho et al. 2004a; 2004b
	Memantine		Borre et al. 2011
	Magnesium	NMDA receptor	Pochwat et al. 2015
	Zinc		Nowak et al. 2003
	MPEP	mGlu receptor 5 antagonist	Pilc et al. 2002; Wierońska et al. 2002
	MTEP		Palucha et al. 2005
	MGS0039	Group II mGlu receptor antagonist	Palucha-Poniewiera et al. 2010
	Riluzole	Multiple effects on glutamate	Takahashi et al. 2011
	Tiantepine	Glutamate	Breivik et al. 2006
Acetylcholine	Tacrine	Acetylcholinesterase inhibitor	Hallam et al. 2004
	Physostigmine	Nicotinic receptor agonist	Vieyra-Reyes et al. 2008

Table 2.4 Drugs targeting the 'classical' neurotransmitter systems that have been examined in the OB rat model. Results are generated from the 133 OB intervention studies between 1999 and 2018. D=dopamine receptor, DAT=dopamine transporter, GABA=gamma aminobutyric acid, MC=melanocortin, mGlu=metrabotropic glutamate receptor, NET=norepinephrine transporter, NMDA=N-Methyl-D-aspartate receptor, PAM=positive allosteric modulator, SERT=serotonin transporter, 5HT=serotonin.

Target	Drug Name	Mechanism	Reference
Neuropeptide Y	NPY	Endogenous NPY receptor agonist	Goyal et al. 2009
	[Leu31Pro34]PYY	Y_1 receptor agonist	Morales-Medina et al. 2012c
	PYY3-36	Y_2 receptor agonist	Morales-Medina et al. 2012c
	JNJ-31020028	Y_2 receptor antagonist	Morales-Medina et al. 2012a
	BIIE0246		Morales-Medina et al. 2012c
	[cPP1-7,NPY19-23,Ala31, Aib32, Gln34]hPP	Y_5 receptor agonist	Morales-Medina et al. 2012b
Opioid	KNT-127	DOP agonist	Gotoh et al. 2017
	SNC80		Saitoh et al. 2008; Takahashi et al. 2008
CRF	R278995/CRA0450	CRF_1 receptor antagonist	Chaki et al. 2004
Neurokinin	Saredutant	NK-2 receptor antagonist	Louis et al. 2008
Vasopressin	SSR149415	V1b receptor antagonist	Breuer et al. 2009; Iijima and Chaki, 2007
	TASP0233278		Iijima et al. 2014
	TASP0390325		
Melatonin	Melatonin	Endogenous agonist	Tasset et al. 2010
	TASP0382650	Melanin-concentrating hormone (MCH)1 receptor antagonists	Chaki et al. 2015
	TASP0489838		
Vasoactive intestinal peptide	VIP	Endogenous VIP receptor agonist	Ivanova et al. 2014; 2012
Trefoil Factor	Neuropeptide Trefoil Factor 3	Agonist of EGF receptor	Li et al. 2015

Table 2.5 Drugs targeting the neuropepetide systems that have been examined in the OB rat model. Results are generated from the 133 OB intervention studies between 1999 and 2018. CRF=corticotropin releasing factor, DOP=delta opioid receptor, EGF=epidermal growth factor, NK=neurokinin, MCH=melanin-concentrating hormone, NPY=neuropeptide Y, Y=neuropeptide Y receptor, V=vasopressin, VIP=vasoactive intestinal peptide receptor.

Target	Drug Name	Mechanism	Reference
Anti-inflammatory	Minocycline	Microglia suppressant	Borre et al. 2012; Burke et al. 2014
	Celecoxib		Borre et al. 2012
	Fish oil	COX-2 inhibitor	Pudell et al. 2014
	Ethyl-eicosapentaenoate (EPA)		Song et al. 2009
Sigma	SA-4503	Sigma1 receptor agonist	Wang et al. 2007
	Igmesine		Bermack et al. 2002
Phosphodiesterase	Etazolate	Cyclic nucleotide phosphodiesterase 4 inhibitor	Jindal et al. 2015; 2012
	Rolipram		Jindal et al. 2015
Adenosine	ZM 241385	A_2A receptor antagonist	Padilla et al. 2018
Oestrogen	17 beta-estradiol	ER agonist	Tasset et al. 2008
Glucocorticoid	Metyrapone	Glucocorticoid synthesis inhibitor	Healy et al. 1999
Cannabinoid	THC	Cannabinoid receptor agonist	Elbatsh et al. 2012
	Rimonabant	CB1 receptor antagonist	Eisenstein et al. 2010; Elbatsh et al. 2012
Nitric Oxide	TRIM	NOS inhibitor	Gigliucci et al. 2014
HMG coA reductase	Simvastatin	HMG coA reductase inhibitor	Douma et al. 2011
Angiotensin	Losartan	Angiotensin 1 (AT1) receptor antagonist	Tashev and Ivanova, 2018
Other	N-acetylcysteine	Glutathione precursor, glutamate releaser	Smaga et al. 2012
Other	1,2,4-triazino[5,6-b]indole-3-thione derivatives	Range of biological activities	Aswar et al. 2012

Table 2.6 Drugs targeting the other systems that have been examined in the OB rat model. Results are generated from the 133 OB intervention studies between 1999 and 2018. AT1=angiotensin 1 receptor, A_{2A}=adenosine 2A receptor, CB=cannabinoid, COX=cyclooxegenase, EPA=Ethyl-eicosapentaenoate, ER=estrogen receptor, HMG CoA=3-hydroxy-3-methyl-glutaryl-coenzyme A, NOS=nitric oxidise synthase, THC=tetrahydrocanabinoil, TRIM= 1-(2-trifluoromethylphenyl) imidazole.

Compound	Source	Reference
20(S)-protopanaxadiol	Ginseng metabolite	Xu et al. 2010
4-hydroxyisoleucine	Constituent of fenugreek seeds (*Trigonella foenum-graecum*)	Kalshetti et al. 2015
Baicalein	Constituent of *Scutellaria baicalensis*	Chen et al. 2018; Yu et al. 2016
Curcumin	Constituent of turmeric	Chang et al. 2016; Xu et al. 2005
Ellagic acid	Constituent of *Punica granatum*	Kalshetti et al. 2015
Extract of Centella asiatica	Constituent of the small herbaceous plant family *Mackinlayaceae*	Kalshetty et al. 2012
Extract of Commiphora mukul	Constituent of the small tree family *Burseraceae*	Kalshetti et al. 2015
Extract of Mucuna pruriens	Constituent of the velvetbean plant family *Fabaceae*	Pati et al. 2010
Extract of Hedyotis corymbosa	Constituent of the flowering plant family *Rubiaceae*	Pawar et al. 2018
Extract of Panax quinquefolium	American ginseng	Rinwa and Kumar, 2014
Parthenolide	Constituent of *Tanacetum parthenium*	Pandey et al. 2008
Rhodioloside	Constituent of *Rhodiola rosea*	Zhang et al. 2016
Salidroside	Constituent of *Rhodiola rosea*	Yang et al. 2014
Sarsapogenin	Constituent of *Anemarrhena asphodeloides*	Feng et al. 2017
Silymarin	Polyphenolic flavanoid of Sylybum marianum	Thakare et al. 2017
Vanillin	Extracted from Vanilla Beans	Xu et al. 2015

Table 2.7 Natural products that have been examined in the OB rat model. Results are generated from the 133 OB intervention studies between 1999 and 2018.

Intervention	Reference
Chronic exercise	Van Hoomissen et al. 2011
Deep brain stimulation	Jiménez-Sánchez et al. 2016
Enriched diet	Borre et al. 2014
Environmental enrichment	Hendriksen et al. 2012
REM sleep deprivation	Maturana et al. 2015
Transcranial magnetic stimulation	Vieyra-Reyes et al. 2008
Treadmill exercise	Shin et al. 2017
Vagus nerve stimulation	Gebhardt et al. 2013
Wheel running	Chambliss et al. 2004

Table 2.8 Other forms of interventions that have been examined in the OB rat model. Results are generated from the 133 OB intervention studies between 1999 and 2018. REM=rapid eye movement.

2.3.2 Characteristics of the open field test

As the OF was the most examined behavioural parameter amongst all papers, it merited further investigation to determine the experimental features of the test that were utilised in each study.

2.3.2.1 Shape of the open field arena

The results for shape of the OF arena are expressed in Figure 2.6. Of the 105 paper that examined the OF, 58 papers used a circular or round-shaped OF, followed 42 papers that used a square-shaped OF. Three papers used a rectangular OF arena, and two papers did not state the shape of OF apparatus.

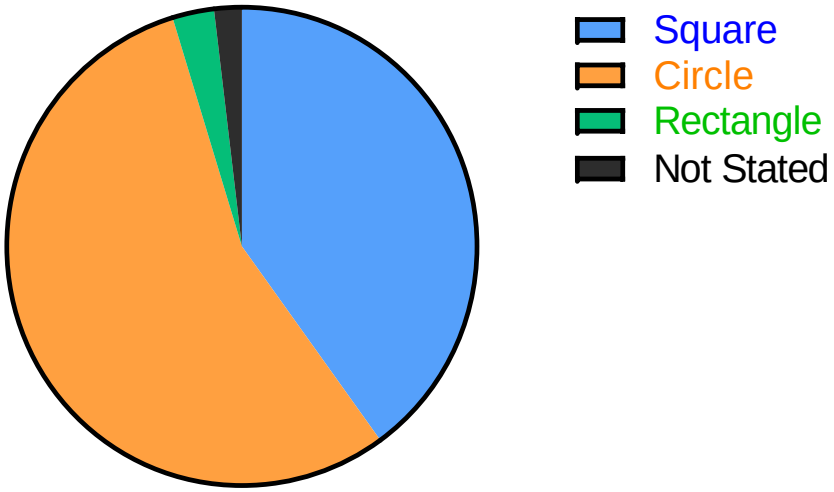


Figure 2.6 Shape of the open field arena. Results are generated from 133 studies that used the OB model as an intervention between 1999 and 2018. Data is expressed as a percentage of the total number of papers examined for this characteristic.

2.3.2.2 Duration of time tested in the open field arena

The results for duration of time tested in the OF arena are expressed in Figure 2.7. Of the 105 papers that examined the OF, 51 papers tested rats in the OF apparatus for 5 minutes, 37 papers tested rats for 3 minutes, 8 papers tested rats for 15 minutes and 5 papers tested rats for 10 minutes. For all other cases, only a single paper tested rats in the OF arena for the following times; 4 minutes, 6 minutes, 30 minutes and 40 minutes, and these were classified as 'other'.

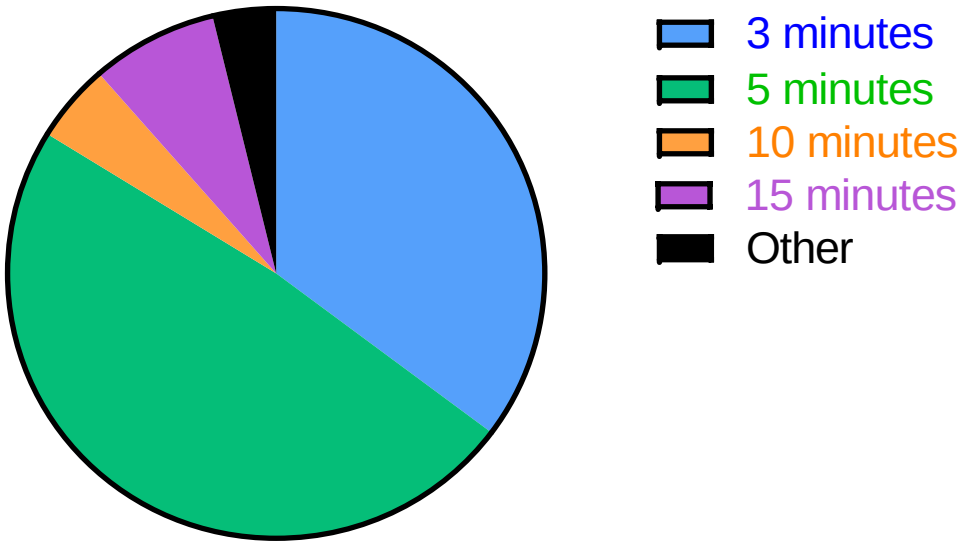


Figure 2.7 Duration rats were tested in the open field arena. Results are generated from 133 studies that used the OB model as an intervention between 1999 and 2018. Data is expressed as a percentage of the total number of papers examined for this characteristic.

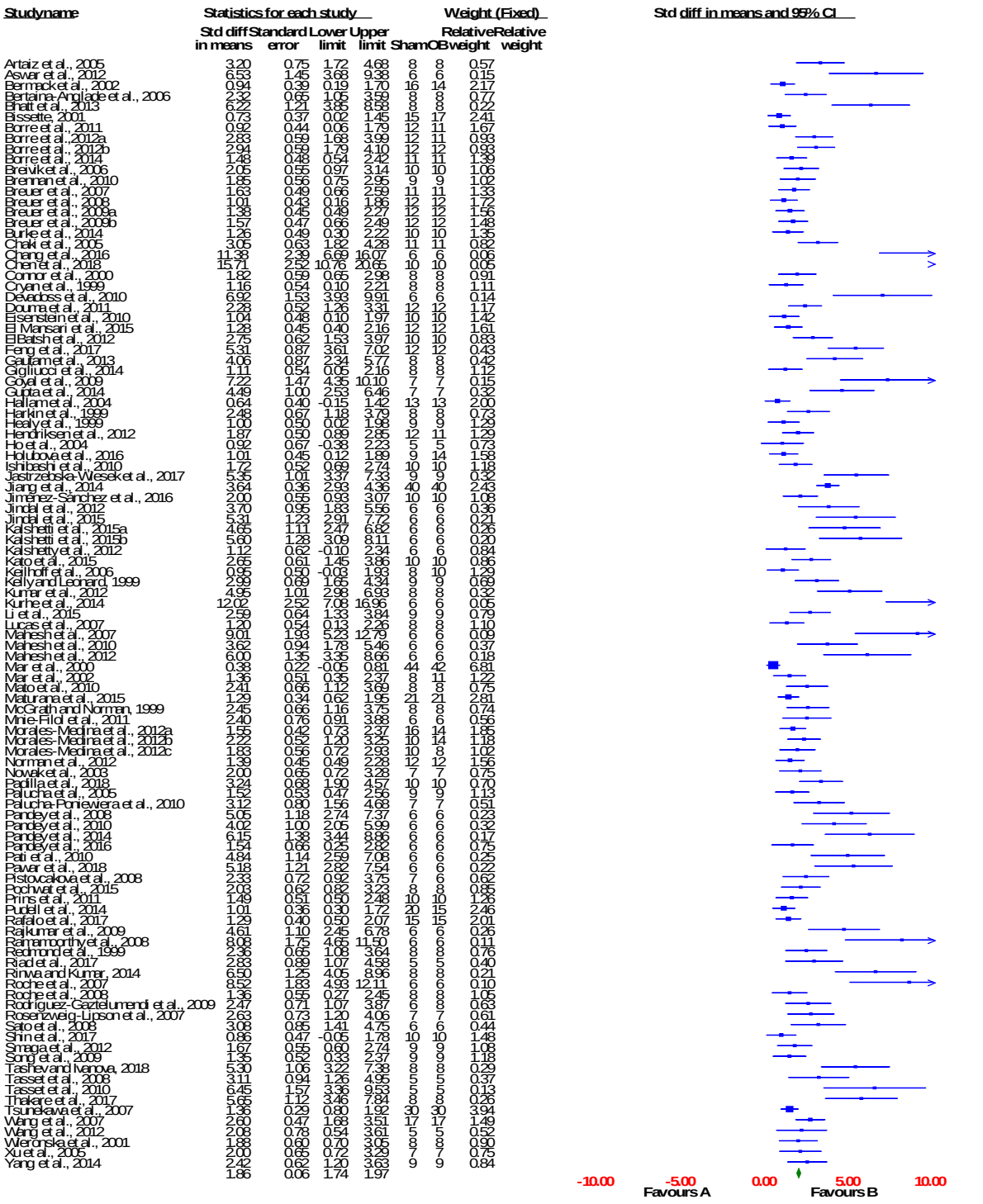
2.3.3 Meta-analysis of behavioural responses

A meta-analysis was undertaken for the seven most commonly used behavioural tests examined, where nine of the most commonly used behavioural responses were assessed. As such, the OF test, the FST, the EPM, the SPT, the PA, the SI and the HE were examined in the OB rat model of depression amongst the 133 papers examined.

2.3.3.1 Distance moved in the open field

A total of 107 papers examined the distance moved in the OF test of the 133 papers assessed, and 105 of these were eligible for examination. Two papers published results that appeared to exactly mimic results that had been published by the same author in a previous research paper.

The overall effect size recorded for distance moved in OB rats in the OF test was 1.86 (95% CI: 1.74 to 1.97) (Figure 2.8).



Meta Analysis

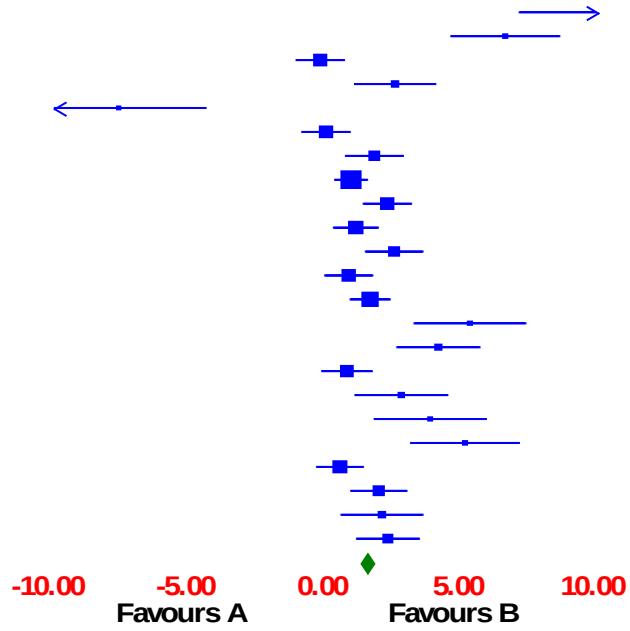
Figure 2.8 Meta-Analysis of distanced moved in the open field test in OB rat intervention papers published between the years 1999-2018. The overall effect size for distanced moved in OB rats in the OF test was 1.86 (*n*=105 papers). Favours A indicates a reduction in the parameter, whilst favours B indicates an increase in the parameter when the OB group is compared to its sham-operated counterpart. CI=confidence interval.

2.3.3.2 Immobility in the forced swim test

A total of 25 papers examined the FST of the 133 papers assessed, and 22 of these were eligible for examination. Three papers were excluded as one paper had no OB 'control' group, and two papers published results that appeared to exactly mimic results that had been published by the same author in a previous research paper. Of the 22 papers eligible, one paper examined the OB rat in two different strains; Long-Evans and Wistar rats, so results for each of these strains were included as two separate sets of data.

The overall effect size recorded for immobility in OB rats in the FST was 1.52 (95% CI: 1.29 to 1.75) (Figure 2.9).

Study name	Std diff in means	Standard error	Lower limit	Upper limit	Sham	OB	Relative weight	Relative weight
Chang et al., 2016	12.06	2.53	7.11	17.02	6	6	0.22	
Feng et al., 2017	6.62	1.04	4.59	8.66	12	12	1.31	
Healy et al., 1999	-0.18	0.47	-1.11	0.74	9	9	6.33	
Jindal et al., 2015	2.57	0.78	1.04	4.10	6	6	2.32	
Kalshetti et al., 2015	-7.60	1.66	-10.84	-4.36	6	6	0.52	
Kelly & Leonard, 1999	0.03	0.47	-0.89	0.95	9	9	6.35	
Li et al., 2015	1.81	0.56	0.71	2.90	9	9	4.51	
Maturana et al., 2015	0.95	0.33	0.31	1.59	21	21	13.32	
Morales-Medina et al., 2012a	2.28	0.47	1.36	3.21	16	14	6.39	
Morales-Medina et al., 2012b	1.12	0.44	0.26	1.98	12	12	7.32	
Morales-Medina et al., 2012c	2.53	0.56	1.44	3.63	15	9	4.53	
Padilla et al., 2018	0.86	0.47	-0.05	1.78	10	10	6.46	
Pudell et al., 2014	1.65	0.39	0.88	2.43	20	15	9.07	
Rinwa & Kumar, 2014	5.32	1.07	3.23	7.41	8	8	1.24	
Shin et al., 2017	4.16	0.80	2.60	5.72	10	10	2.23	
Smaga et al., 2012	0.80	0.49	-0.16	1.76	9	9	5.89	
Tasset et al., 2008	2.80	0.89	1.06	4.54	5	5	1.78	
Tasset et al., 2010	3.86	1.07	1.76	5.96	5	5	1.23	
Thakare et al., 2017	5.14	1.04	3.11	7.17	8	8	1.31	
Vieyra-Reyes et al., 2008a	0.54	0.46	-0.35	1.43	10	10	6.81	
Vieyra-Reyes et al., 2008b	1.97	0.54	0.90	3.04	10	10	4.76	
Wang et al., 2012	2.09	0.79	0.55	3.63	5	5	2.29	
Yang et al., 2014	2.31	0.61	1.11	3.50	9	9	3.82	
	1.52	0.12	1.29	1.75				



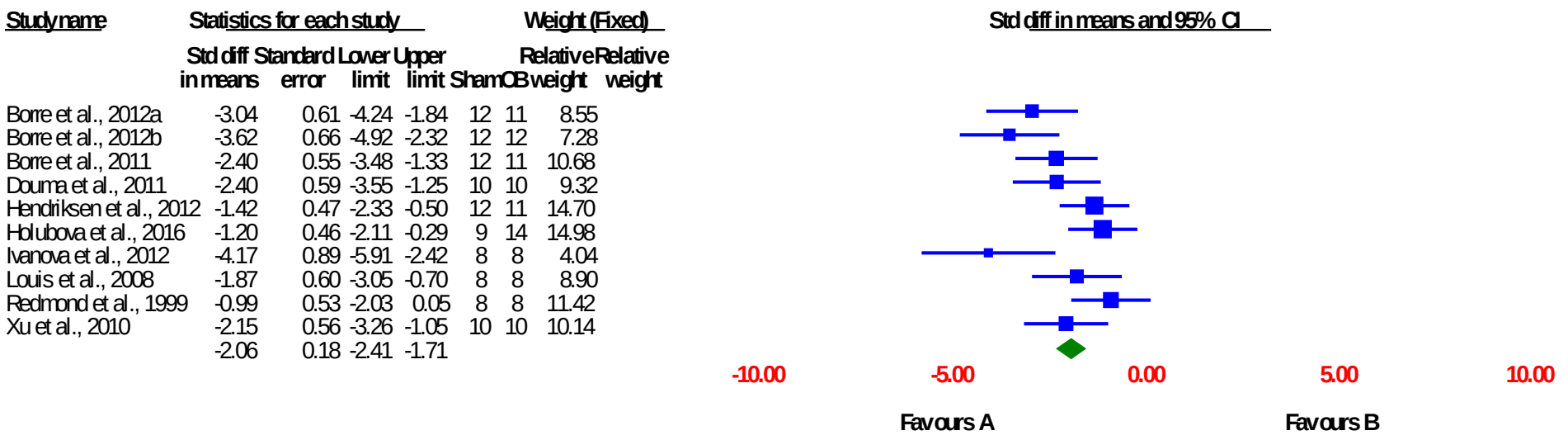
Meta Analysis

Figure 2.9 Meta-Analysis of immobility in the forced swim test in OB rat intervention papers published between the years 1999-2018. The overall effect size for immobility in OB rats in the FST was 1.52 (*n*=22 papers). Favours A indicates a reduction in the parameter, whilst favours B indicates an increase in the parameter when the OB group is compared to its sham-operated counterpart. CI=confidence interval.

2.3.3.3 Latency time to enter the chamber in the passive avoidance test

A total of 10 papers examined latency time to enter the chamber in the PA test of the 133 papers assessed, and 10 of these were eligible for examination.

The overall effect size recorded for latency time to enter the chamber in OB rats in the PA test was -2.06 (95% CI: -2.41 to -1.71) (Figure 2.10).



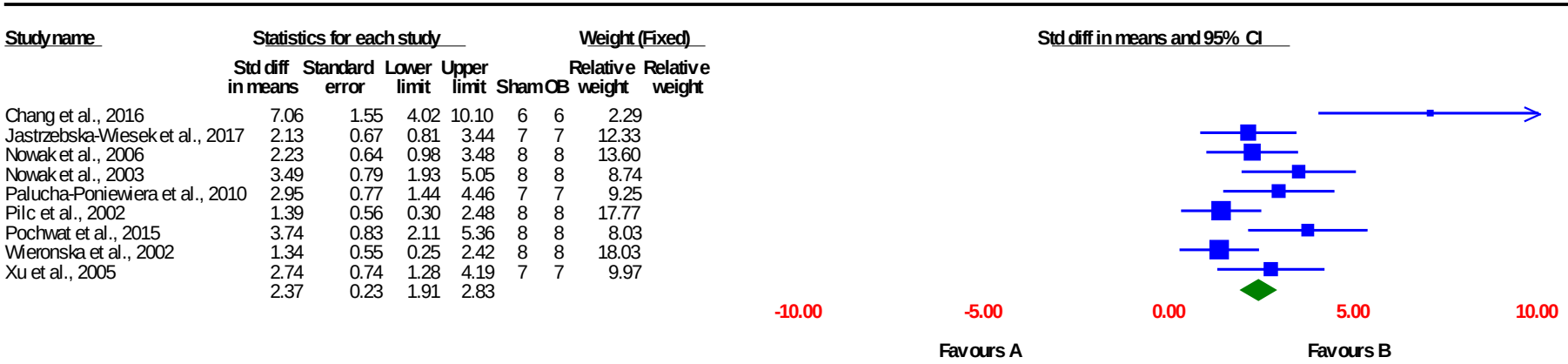
Meta Analysis

Figure 2.10 Meta-Analysis of latency time to enter the chamber in the passive avoidance test in OB rat intervention papers published between the years 1999-2018. The overall effect size for latency time to enter the chamber in the PA test in OB rats was -2.06 (*n*=10 papers). Favours A indicates a reduction in the parameter, whilst favours B indicates an increase in the parameter when the OB group is compared to its sham-operated counterpart. CI=confidence interval.

2.3.3.4 Number of trials taken in the passive avoidance test

A total of 10 papers examined the number of trials taken in the PA test of the 133 papers assessed, and 9 of these were eligible for examination. One paper was excluded as the data was non-parametric and could not be converted to mean and standard.

The overall effect size recorded for the number of trials taken in OB rats in the PA test was 2.37 (95% CI: 1.91 to 2.83) (Figure 2.11).



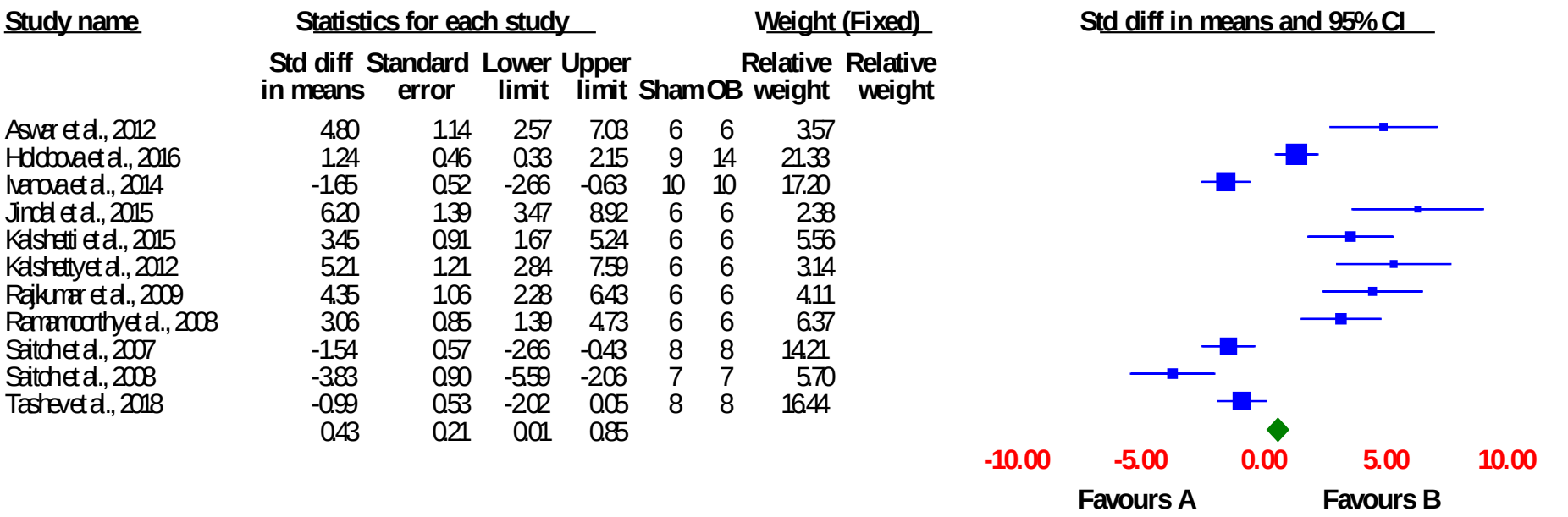
Meta Analysis

Figure 2.11 Meta-Analysis of the number of trials taken in the passive avoidance test in OB rat intervention papers published between the years 1999-2018. The overall effect size for the number of trials taken in the PA test in OB rats was 2.37 (*n*=12 papers). Favours A indicates a reduction in the parameter, whilst favours B indicates an increase in the parameter when the OB group is compared to its sham-operated counterpart. CI=confidence interval.

2.3.3.5 Open arm entries in the elevated plus maze

A total of 13 papers examined open arm entries in the EPM of the 133 papers assessed, and 11 of these were eligible for examination. Two papers were excluded as one paper used non-parametric data and was difficult to compute to mean and standard deviation, and one paper published results that appeared to exactly mimic results that had been published by the same author in a previous research paper.

The overall effect size recorded for open arm entries in OB rats in the EPM was 0.43 (95% CI: 0.01 to 0.85) (Figure 2.12).



Meta Analysis

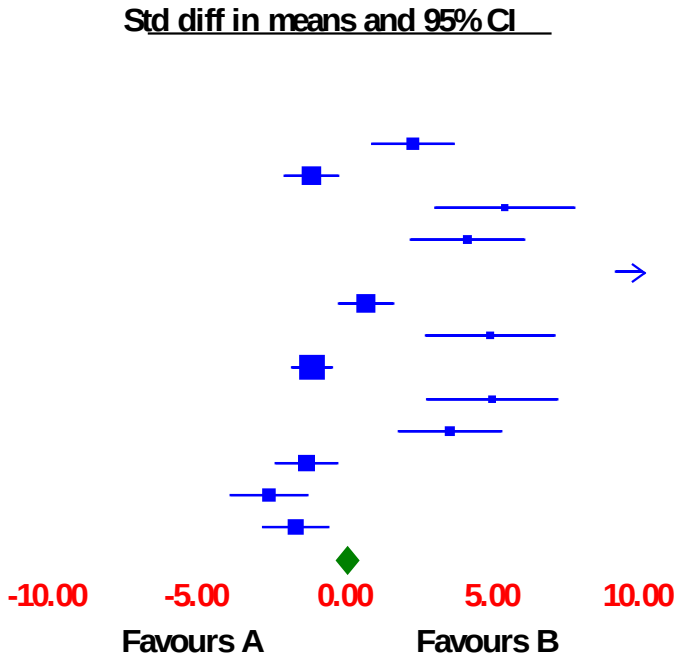
Figure 2.12 Meta-Analysis of open arm entries in the elevated plus maze in OB rat intervention papers published between the years 1999-2018. The overall effect size for open arm entries in OB rats in the EPM was 0.43 (*n*=11 papers). Favours A indicates a reduction in the parameter, whilst favours B indicates an increase in the parameter when the OB group is compared to its sham-operated counterpart. CI=confidence interval.

2.3.3.6 Open arm duration in the elevated plus maze

A total of 16 papers examined open arm duration in the EPM of the 133 papers assessed, and 13 of these were eligible for examination. Three papers were excluded as one paper failed to state any descriptive statistics (SEM or *n* numbers), one paper used non-parametric data that was difficult to compute to mean and standard deviation, and one paper published results that appeared to exactly mimic results that had been published by the same author in a previous research paper.

The overall effect size recorded for open arm duration in OB rats in the EPM was -0.07 (95% CI: -0.43 to 0.28) (Figure 2.13).

Study name	Std diff in means	Standard error	Lower limit	Upper limit	Sham	OB	Relative weight (Fixed)	Relative weight
Aswar et al., 2012	2.20	0.73	0.76	3.63	6	6	6.23	
Ivanova et al., 2014	-1.24	0.49	-2.20	-0.29	10	10	13.97	
Jindal et al., 2015	5.31	1.23	2.91	7.72	6	6	2.21	
Kalshetti et al., 2015	4.05	1.01	2.07	6.02	6	6	3.28	
Kalshetty et al., 2012	15.22	3.16	9.03	21.41	6	6	0.33	
Mar et al., 2002	0.61	0.50	-0.37	1.58	8	9	13.50	
Pandey et al., 2008	4.82	1.14	2.58	7.05	6	6	2.56	
Pudell et al., 2014	-1.22	0.37	-1.95	-0.49	20	15	24.15	
Rajkumar et al., 2009	4.89	1.15	2.63	7.14	6	6	2.51	
Ramamoorthy et al., 2008	3.45	0.91	1.67	5.24	6	6	4.02	
Saitch et al., 2007	-1.41	0.56	-2.51	-0.32	8	8	10.68	
Saitch et al., 2008	-2.69	0.69	-4.04	-1.33	8	8	7.01	
Tashev et al., 2018	-1.78	0.59	-2.93	-0.62	8	8	9.56	
	-0.07	0.18	-0.43	0.28				



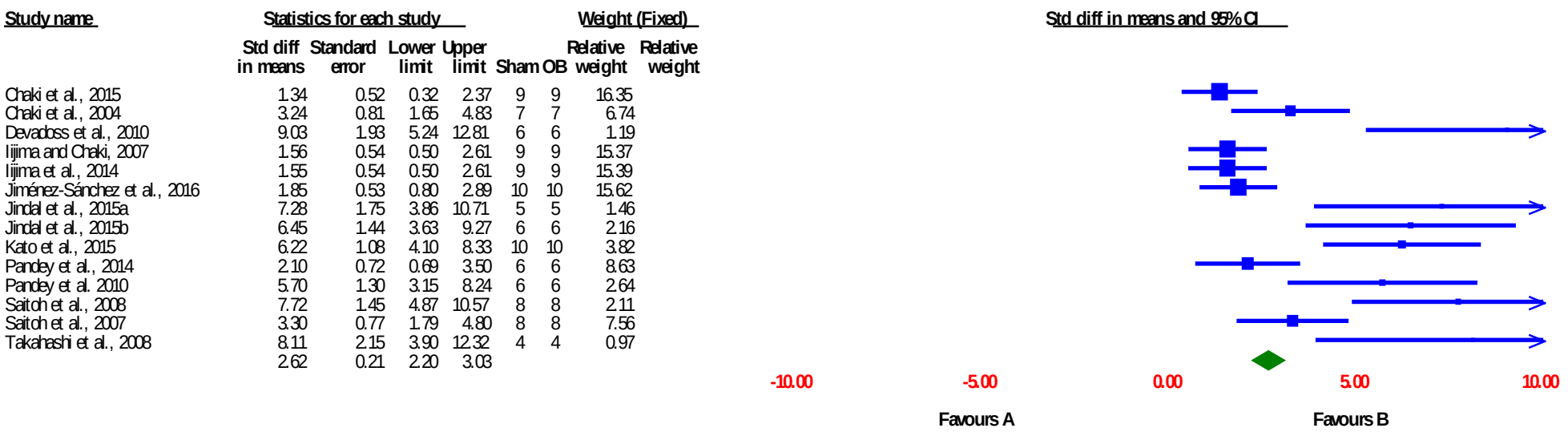
Meta Analysis

Figure 2.13 Meta-Analysis of open arm duration in the elevated plus maze in OB rat intervention papers published between the years 1999-2018. The overall effect size for open arm duration in OB rats in the EPM was -0.07 (*n*=13 papers).Favours A indicates a reduction in the parameter, whilst favours B indicates an increase in the parameter when the OB group is compared to its sham-operated counterpart. CI=confidence interval.

2.3.3.7 Hyperemotionality

A total of 16 papers examined HE in the HE test of the 133 papers assessed, and 14 of these were eligible for examination. Two papers were excluded as there was no sham-operated control group.

The overall effect size recorded for the overall HE score in OB rats in the HE test was 2.62 (95% CI: -2.20 to 3.03) (Figure 2.14).



Study name	Std diff in means	Standard error	Lower limit	Upper limit	Sham	OB	Relative weight
Chaki et al., 2015	1.34	0.52	0.32	2.37	9	9	16.35
Chaki et al., 2004	3.24	0.81	1.65	4.83	7	7	6.74
Devadoss et al., 2010	9.03	1.93	5.24	12.81	6	6	1.19
Iijima and Chaki, 2007	1.56	0.54	0.50	2.61	9	9	15.37
Iijima et al., 2014	1.55	0.54	0.50	2.61	9	9	15.39
Jiménez-Sánchez et al., 2016	1.85	0.53	0.80	2.89	10	10	15.62
Jindal et al., 2015a	7.28	1.75	3.86	10.71	5	5	1.46
Jindal et al., 2015b	6.45	1.44	3.63	9.27	6	6	2.16
Kato et al., 2015	6.22	1.08	4.10	8.33	10	10	3.82
Pandey et al., 2014	2.10	0.72	0.69	3.50	6	6	8.63
Pandey et al. 2010	5.70	1.30	3.15	8.24	6	6	2.64
Saitoh et al., 2008	7.72	1.45	4.87	10.57	8	8	2.11
Saitoh et al., 2007	3.30	0.77	1.79	4.80	8	8	7.56
Takahashi et al., 2008	8.11	2.15	3.90	12.32	4	4	0.97
	2.62	0.21	2.20	3.03			

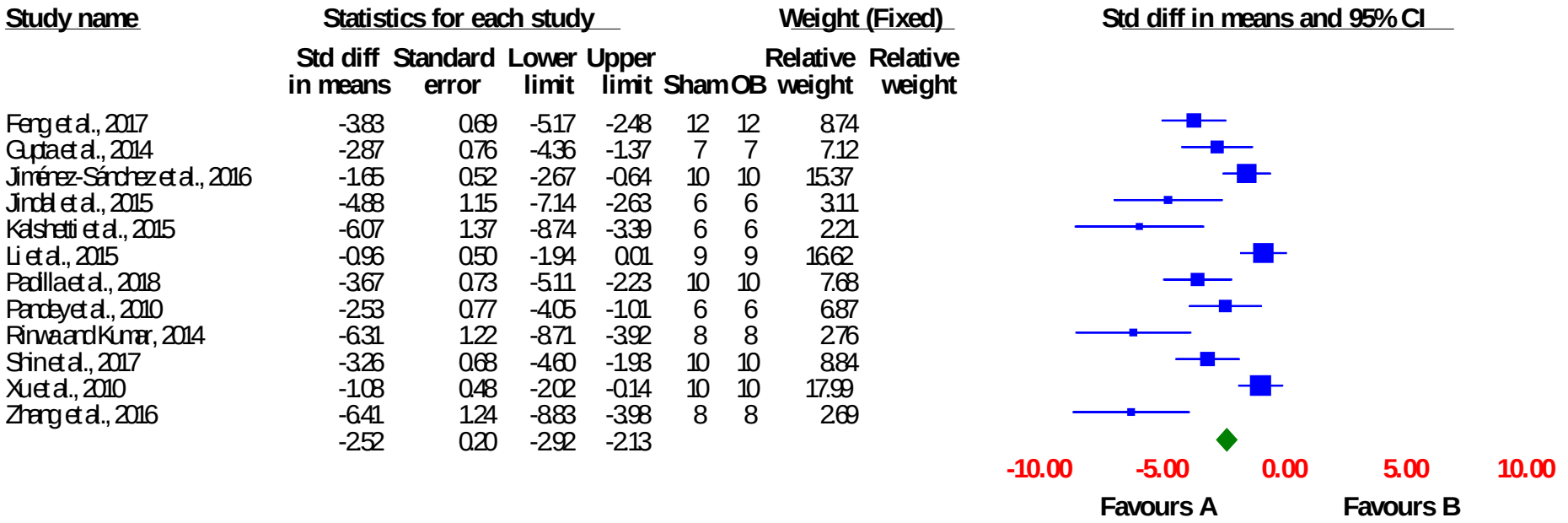
Meta Analysis

Figure 2.14 Meta-Analysis of overall hyperemotionality score in the hyperemotionality test in OB rat intervention papers published between the years 1999-2018. The overall effect size for overall HE score in OB rats in the HE test was 2.62 (*n*=14 papers). Favours A indicates a reduction in the parameter, whilst favours B indicates an increase in the parameter when the OB group is compared to its sham-operated counterpart. CI=confidence interval.

2.3.3.8 Sucrose/Saccharin consumption

A total of 15 papers examined sucrose/saccharin consumption in the SPT of the 133 papers assessed, and 12 of these were eligible for examination. Three papers were excluded as one paper failed to state any descriptive statistics (SEM or SD), and two papers published results that appeared to exactly mimic results that had been published by the same author in a previous research paper.

The overall effect size recorded for sucrose/saccharin consumption in OB rats in the SPT was -2.52 (95% CI: -2.92 to -2.13) (Figure 2.15).

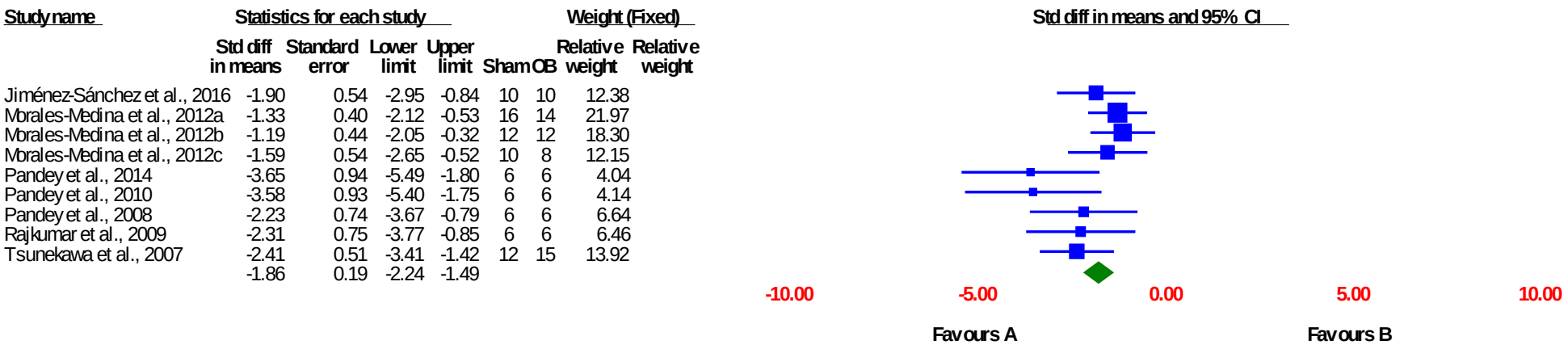


Meta Analysis

Figure 2.15 Meta-Analysis of sucrose/saccharin consumption in the sucrose preference test in OB rat intervention papers published between the years 1999-2018. The overall effect size for sucrose/saccharin consumption in the SPT in OB rats was -2.52 (*n*=12 papers). Favours A indicates a reduction in the parameter, whilst favours B indicates an increase in the parameter when the OB group is compared to its sham-operated counterpart. CI=confidence interval.

2.3.3.9 Social interaction time

A total of 9 papers examined social interaction time in the SI test of the 133 papers assessed, and 9 of these were eligible for examination. The overall effect size recorded for social interaction time in OB rats in the SI test was -1.86 (95% CI: -1.49 to -2.24) (Figure 2.16).



Meta Analysis

Figure 2.16 Meta-Analysis of social interaction time in the social interaction test in OB rat intervention papers published between the years 1999-2018. The overall effect size for social interaction time in the SI test in OB rats was -1.86 (*n*=9 papers). Favours A indicates a reduction in the parameter, whilst favours B indicates an increase in the parameter when the OB group is compared to its sham-operated counterpart. CI=confidence interval.

2.4 Discussion

Systematic reviews and meta-analysis have become important instruments in the assessment and appraisal of therapeutic interventions (Leucht et al., 2009). Utilisation of these analytical and systematic techniques aid in the validation of therapeutic treatments and help to validate models of differing scientific methodology. These techniques are particularly useful for evaluating preclinical data that is generated from different laboratories that often use different or distinct methodological approaches. The aim of this chapter was to conduct a systematic review and meta-analysis on behavioural responses in the OB rat model between the years 1999-2018, where interventions have been employed to assess their impact on these behavioural responses. From an extensive literature search across the three databases, there were 133 published papers that fully met all criteria. The principle methodological differences between studies were assessed and a system review of the following aspects were examined; strain of rat, sex of rat, housing of rat after surgery, institute of corresponding author, nature of intervention, and the most popular behavioural response explored. These results will now be discussed in detail.

Firstly, it was immediately evident that the Albino strains (Sprague Dawley and Wistar) have been overwhelmingly used in the OB rat model. Together, these two strains account for over 96% of the published papers on this model between the years 1999-2018. These findings are in concordance with those found across all areas of research involving rats, particularly with regards to behaviour, where the Sprague-Dawley followed by the Wistar strain are the most popular strains of rat used (Ellenbroek and Youn, 2016). The Sprague-Dawley strain is actually derived from the Wistar strain (Parker et al., 2014). The Sprague-Dawley strain has a high fertility rate, is calm in temperament, easily handled, has a long lifespan, grows quickly, and is strongly disease resistance (Parker et al., 2014). All of these features combined make it a promising candidate for animal research. Coinciding with this is the concept of consistency of use, and the fact that researchers tend to use the same strains as others in the field in order for findings to be more comparable. In conjunction with this, Albino strains are easily accessible and available from commercial suppliers, whereas other strains are often considered more ‘exotic’ in nature and so can be more difficult to purchase and breed. Taking all of this into account, the selection of strain employed is very much dependent on the type of intervention or disease being investigated,

especially with regards any behavioural examinations, and with previous experience and availability of strains.

Secondly, there was a clear preference for the use of male rats in the OB experiments that were examined. Strikingly, only four papers published between the years 1999-2018 examined female rats in the OB rat model, three of which actually used both sexes (Gupta et al., 2014, Pudell et al., 2014, Shin et al., 2017, Thakare et al., 2017). In the past, the use of female rats in scientific investigation was less prevalent, as application of the female sex was associated with the idea that dependant on which stage the females were at in the oestrous cycle, this would generate different and varying findings (Beery and Zucker, 2011, Prendergast et al., 2014). As a result, female rats were only used in scientific research in the past to study gender-specific female conditions and disorders. Having said this, this justification that females show more variability and are more troublesome in research, is without proper foundation, and as such needs serious reappraisal (Beery and Zucker, 2011, Mogil and Chanda, 2005). The importance of using female rats and animals in scientific research has been a topic of discussion in the last few decades, with emphasis being placed on the fact that many disorders and diseases that exist are not gender-specific and so both sexes should be taken into consideration and utilised to cover the heterogeneity in diseases and disorders (Beery and Zucker, 2011, Hughes, 2007, Prendergast et al., 2014). The National Institute of Health (NIH) now recommends that both sexes be included in any scientific research proposals in order to fully represent the effects to both sexes in the clinical scenario, and that a plausible justification for the use of only one sex must be explained in any grants or applications prior to the stage of successful funding (Shansky and Woolley, 2016, ILAR, 2011). Indeed, Shansky and Woolley (2016) state that this new policy implemented by the NIH will be of great value to neuroscience, and that addressing this disproportion of research using one gender or sex must be overcome in order to further our scientific knowledge of the body and the brain. Indeed, it can be seen that in publications in more recent years, more scientists are trying to accommodate the use of both sexes into their research, though this still appears to be with very slow movement (Hughes, 2007). This is also beginning to be the case with the OB rat model, with investigation of this particular paradigm attempting to encompass a more active approach to assessing behavioural features, with all the papers that implement female rats in this systematic review having been published in the last five years (Figure 2.3).

The housing of rats after surgery also produced fruitful information on this model and the methodological approaches to it as a whole. Remarkably, over 35% of papers did not report how the rats were housed after surgery. The lack of full disclosure in the experimental design on these papers, is adding to the scientific struggle when it comes to reproducibility of results and scientific transparency. The ARRIVE guidelines clearly state in the methods section of an experiment that details of 'the experimental unit' should be clearly stated, such that 'a single animal, group or cage of animals' is clearly described in the study design (Kilkenny et al., 2010). Housing conditions and parameters have been shown to have significant effects on animals, in particular rodents tend to associate in social groups, with paired-housing and group-housing shown to promote well-being in rats and reduce stress (Turner et al., 2014, Wallace et al., 2009). In contrast to this fact, the highest stated housing condition in OB rat papers published during this timeframe was that of singly-housing. This is not surprising however, as the OB syndrome is known to develop an irritable state, and so regular animal handling is often recommended in order to combat this attribute (Leonard and Tuite, 1981, van Riezen et al., 1977). Nevertheless, singly-housing animals is not always a good feature to exploit as singly-housing animals has been shown to induce stress in animals, with increased HPA-axis activity and anxiety-like behaviour being seen in socially-isolated animals, when compared to group and pair-housed counterparts (Linge et al., 2013, Pinelli et al., 2017, Wallace 2009). In general and if possible, paired or group-housing should try to be employed in all animal studies, in order to negate the necessity for singly-housing, and any negative effects to the well-being of the animal that may be associated with it.

With regards the institution of the corresponding author, a number of academic institutes worldwide have been shown to utilise the OB model. The importance of publishing research so that it can be shared with the scientific community is a crucial and essential part of the scientific research itself, as it shares and updates information on the subject and helps to prevent unnecessary replication. In order to further disease prevention and treatment, industrial laboratories also need to contribute by sharing their findings so that these results can be amalgamated and related to pre-existing knowledge on the subject. Nevertheless, the amount of published papers on the OB rat model covering both the eastern and western hemispheres of the world, indicates the diversity of the OB rat model, and validates it as a universal model used worldwide.

The nature of the intervention in each paper was also examined. The interventions that have been evaluated to assess whether the behavioural changes in the OB rat could be reversed have been extensive, and have universally demonstrated efficacy. Most of the marketed antidepressants have been evaluated in the OB model over the 20-year period of this investigation, often as positive controls to compare novel treatments against. Interestingly, there is a vast range of papers that have examined natural products and other forms of strategies, indicating that the nature of the intervention employed is not always strictly pharmacological in basis.

A number of behavioural parameters have been assessed in the OB rat in papers between the years 1999-2018. In this systematic review we choose to report only the top ten behaviours assessed. Unsurprisingly, the OF test was the most frequently used test to analyse the behavioural effects in OB vs. sham-operated rats. As mentioned above, hyperactivity is classically associated with the OB syndrome and this feature is seen to be reversed with chronic but not acute antidepressant treatment (Kelly et al., 1997). The fact that the OF is a locomotor and anxiety-like behavioural test, and was the most commonly explored behavioural parameter in this model, with 107/133 papers examining it, is in line with what we would have expected. The FST was the second most commonly explored test with 20% of OB rat papers investigating stress responses using this paradigm. Again, this is unsurprising as the FST is most commonly used to assess antidepressant efficacy (Lucki, 1997, Slattery and Cryan, 2012), and all of these papers are intervention studies and so many would examine antidepressant potential and it would be expected that the FST would be used to assess and screen such intervention compounds. A number of other tests were utilised to examine behaviour in the OB rat, relating to cognitive, anxiety-like, anhendonic and emotional responses, all of which are prevalent features of MDD. As the OF test was unanimously the most common test explored, it was decided that further investigations would be undertaken to examine the shape of the OF arena and the amount of time animals were placed in the arena for. A round or circular-shaped arena was the most popular form used, followed very closely by a square arena. Irrespective of the nature of the OF, a robust effect was observed in the meta-analysis in this particular parameter, which will be discussed in the next paragraph. With regards time tested in the OF, the two most popular timings were 5 minutes of testing, followed very closely by 3 minutes of testing. The reason for this may be on account of a habituation effect, whereby OB rats have been shown to habituate to the OF arena over time and

following subsequent exposure (Gigliucci et al., 2014, Holubova et al., 2016). As such, short and acute exposures may better assess their locomotor and anxiety-like behaviour in this test.

Finally, we will discuss the results of the nine behaviour parameters where meta-analysis was performed. A summary of the overall effect sizes of the meta-analysis for each of the nine behavioural parameters is given in Table 2.9 Summary of the overall effects sizes and 95% CI for each of the meta-analysis untaken in the olfactory bulbectomised rat model.. A meta-analysis calculates the effect size of a study by examining the standardised mean difference between two groups in an experiment (Coe, 2002). Coe (2002), describes that the effect size is a tool that allows scientists "to move beyond the simplistic 'Does it work or not?' to the far more sophisticated, 'How well does it work in a range of contexts?'", that places emphasis on the efficacy of an intervention or diagnostic tool in comparison to other relative experiments, rather than on the statistically significant result per experiment (Coe, 2002).

Behavioural Response	Effect size (95% CI)
Distance moved in the OF	1.86 (1.74 to 1.97)
Immobility in the FST	1.52 (1.29 to 1.75)
Latency time to enter the chamber in the PA test	-2.06 (-2.41 to -1.71)
Number of trials taken in the PA test	2.37 (1.91 to 2.83)
Open arm entries in the EPM	0.43 (0.01 to 0.85)
Open arm duration in the EPM	-0.07 (-0.43 to 0.28)
Hyperemotionality in the HE test	2.62 (-2.20 to 3.03)
Sucrose/Saccharin consumption in the SPT	-2.52 (-2.92 to -2.13)
Social interaction time in the SI test	1.86 (-1.49 to -2.24)

Table 2.9 Summary of the overall effects sizes and 95% CI for each of the meta-analysis untaken in the olfactory bulbectomised rat model. Results are generated from 133 studies that used the OB model as an intervention between 1999 and 2018. CI=confidence interval, EPM=elevated plus maze, FST=forced swim test, HE=hyperemotionality, OF=open field, PA=passive avoidance, SI=social interaction, SPT=sucrose/saccharin preference test.

With regard the distanced moved in the OF, from the meta-analysis we can conclude that there is a high degree of reproducibility in the OF test using OB rats as a model for depression, as all papers that examined this parameter in the OB rat found a significant effect of increased distanced moved in the OF in OB rats when compared to sham-operated rats. Once again, it is very important to note that all of this was

irrespective of the shape of the apparatus or time tested, delineating a robust effect between this model and this behavioural parameter.

From the meta-analysis performed on immobility in the FST, there was again a significant degree of reproducibility in this parameter. Overall, the pooled data on immobility in the FST displayed a behavioural response in the OB rat model, in that OB rats spend significantly more time immobile than sham-operated rats, a behaviour which is thought to be indicative of behavioural despair and learned hopelessness, resembling this feature in MDD. Therefore it can be stated that in general a reproducible behavioural response of increased immobility is seen in OB rats when examined in the FST.

With regards the meta-analysis conducted on the latency time and the number of trials in the PA test, there was a significant degree of reproducibility in both of these parameters. The overall the effect sizes indicated that all OB rats in these studies took less time to enter the chamber than sham-operated rats, and took longer to learn to avoid the negative compartment or chamber. Overall, the meta-analysis conducted in the PA test in the OB rat model, highlighted a deficit in cognitive functioning in the OB rat model; one that appears to be reliably replicated across studies.

Open arm entries and duration in the EPM were also examined in the OB rat model via a meta-analysis. With regards the open arm entries an overall effect was again seen in the model in this behavioural response, but to a much lower degree and magnitude of effect than was found in the OF and FST. The overall effect size was 0.43, indicating that OB rats tended to make more entries into the open arms than their sham-operated counterparts. With regards open arm duration, the effect was so low that it cannot be considered that there is a difference between OB and sham-operated rats. Taking this into account, the data from open arm time in the EPM in OB rats is much less consistent between studies. Overall, the meta-analysis on open arm entries in the EPM in the OB rat demonstrated a weak deficit and response in this model. Open arm duration in the EPM in OB rats is much less reliable in its output and results when replicated across laboratories.

With regards the meta-analysis performed on HE scores in the HE test, a deficit in OB rats when compared to sham-operated rats in all studies observed, with OB rats displaying significantly higher HE scores when compared to sham-operated rats, relevantly relatable to the demeanour of irritability and aggitation that is often

associated with this model, and can be seen in the case of MDD (Leonard and Tuite, 1981, van Riezen et al., 1977).

A meta-analysis was conducted on sucrose/saccharin consumption in the SPT in the OB rat model, and results revealed that OB rats have a lower intake of these two food sources than sham-operated rats, therefore supporting the anhedonic profile in this model.

Lastly, the final meta-analysis was conducted on social interaction time in the SI test. The results demonstrate a robust effect of social deficit in OB rats, in that they spend less time interacting in social play and exploration when faced with social stimulus/stimuli. Social cognitive deficits have been shown in patients with MDD (Hirschfield et al., 2000), so this feature of the OB rat model is another characteristic behavioural trait that may represent the impaired cognitive processing which is seen in the clinical condition of the disorder.

The aim of this chapter was to assess the OB rat model as a model of depression in papers published between the years 1999-2018 that had the aim of employing an intervention to modify the OB behavioural response. Over the past twenty years, the OB rat model has primarily been undertaken in male albino rats (Sprague-Dawley and Wistar), with rats being traditionally singly-housed and tested in a number of behavioural parameters. This model has been utilised as a model of depression in numerous laboratories worldwide for assessing both antidepressant treatment and mechanistic action. The OF is by far the most commonly used behavioural parameter for assessing the OB model as a model of depression, with the FST, PA test, and EPM, also being widely explored, and the HE, SPT and SI test also having been investigated. Overall, the papers examined exhibited strong experimental design, conduct and reporting of results indicating a good scientific rigor in the laboratories that use this model. Some papers failed to report particular methodological approaches, especially the housing of rats after surgery, and so it is recommended that particular care be taken when disclosing experimental approaches in order to further support clear scientific documentation. The meta-analysis conducted in this chapter revealed that there is a high degree of reproducibility in the OF test, FST, PA test, HE test, SPT, and SI test using the OB rat as a model of depression. The EPM revealed less consistent results, with open arm entries displaying a much weaker but positive OB behavioural response in this test, but with open arm duration revealing less reliable replication across the studies observed. In conclusion, the systematic review and the meta-analysis

performed on the OB rat papers published between the years 1999-2018, reveal that the OB model is a well-incorporated and utilised animal model with consistent findings in many behavioural responses that may relate to the symptoms of depression.

In order to improve future studies exploring the OB rat model, scientists should employ paired-/group-housing techniques, rather than singly-housing OB animals in order to be in line with the 3Rs, particularly the principle of refinement. The principle of refinement places emphasis upon reducing any stress or suffering to the animal and overall improving animal welfare and wellbeing (Russel and Burch, 1959). Housing is a vital component of this principle, with welfare bodies promoting the use of paired-/group-housing to ethically improve the use of animals in science (ILAR, 2011). Future studies should also attempt to utilise female rats as well as male rats to encourage hetergeniety across scientific research. The use of both sexes in preclinical research is a more appropriate representation of disease pathology in a population, and in the case of drug treatments or therapies that reach the stage of clinical trials, it is important that these compounds be assessed in female organisms preclinically and before entering human trials, in order to better understand the effect of a drug (Hughes, 2007). Lastly, future studies should endeavour to report all findings accurately and coherently so that findings and experiments can be easily repeated and reproduced amoung the scientific community. This has been the basis for the aim of this Chapter and it is this researcher's belief that by conducting a systematic review and more importantly a meta-analysis on a given subject, it reinforces and reveals the models/interventions/therapeutic use in scientific research. This in turn supports the priniciples of the 3Rs, in that animals will be used minimally, replication is easy, and finally that ethically the animal welfare and use of animals in science is performed correctly.

Chapter 3:

Characterisation of the behavioural effects of olfactory bulbectomy; focus on social cognition

3.1 Introduction

Psychiatric disorders such as depression are associated with a number of social cognitive deficits. Social cognition is the ability to detect, perceive, interpret and adapt to social stimuli. Social cognitive dysfunction has been shown in patients with MDD, as well as being a risk factor for MDD (Godard et al., 2011; 2012). Firstly, and as stated previously in DSM-5, individuals with depression have been shown to have increased social withdrawal and separation from society (DSM-5). In a recent review, social dysfunction in depression can be classified under an umbrella of categorical symptoms including increased sensitivity to social rejection, social anhedonia, impaired social recognition and reduced empathy (Kupferberg et al., 2016). Theory of mind (ToM) is known as 'the ability to attribute mental states (feelings, beliefs, intentions, and desires) to others and understand and predict others' behaviour based on their mental states' (Bora et al., 2016). Bora et al. (2016) conducted a meta-analysis of all ToM studies conducted in MDD patients and found significant impairments in ToM measurements, with the degree of impairment correlating with symptom severity. Hirschfield et al. (2000) conducted a MEDLINE search of all clinical papers between the years 1988-1999 that discussed social functioning in depression. Hirschfield et al. (2000) found that depressive disorders, such as MDD, are associated with severe social impairments, when compared to healthy individuals and other medical conditions, stating that the 'enormous personal, social, and economic impact of depression, due in no small part to the associated impairments in social functioning, is often underappreciated'. Neuroimaging studies in patients with MDD have also revealed that when tested in social cognitive tasks, patients with depression have difficulties with recognition and processing of emotional facial expressions, and with verbal cues such as prosody; which emphasises language tone, inflection, pitch and/rhythm (Kan et al., 2004, Knight and Baune, 2019, Lee et al., 2005, Weightman et al., 2014). As such, it is clear that adapting to our complex social environment is indeed a critical ability that is damaged or inhibited in individuals with depression (Bora et al., 2016).

As discussed in the introduction, stressful life-events can contribute to the pathophysiology and development of MDD. Social environmental stressors in the form of social rejection, loss of rank or social status, and loss of situational control, are experienced as traumatic life-events and are associated with depression susceptibility (Brown et al., 1993, Czeh et al., 2016, Pryce et al., 2017). Animal models

that use social stressors produce 'depressive-like' behaviours, and these stressors that are utilised attempt to recreate the everyday-life stressful scenarios experienced by humans. Social defeat stress is an animal model that utilises loss of social rank or control as its basis. In this test, an animal is placed into the homecage of another animal, whereby the intruder is attacked and becomes the submissive entity in the encounter, resulting in social defeat and psychosocial impairment (Grimwood et al., 2011, Rygula et al., 2005; 2006). Rats exposed to 5 weeks of social defeat stress display impaired responding to stressors and anhedonia-like behaviour, including increased immobility in the FST, reduced exploratory behaviour, reduced locomotor activity and reduced sucrose preference (Rygula et al., 2005; 2006); all of these behavioural deficits were reversed by chronic administration of the SSRI antidepressant citalopram (Rygula et al., 2006). Such animal models highlight the impact and status that social functioning has and that social stress and social cognition are vital aspects in this mental disorder (Rygula et al., 2006).

The OB model is a well-established animal model which has measurable face, construct and predictive validity (Harkin et al., 2003, Kelly et al., 1997, Song and Leonard, 2005). As such it is a model used extensively that has shown its efficacy for encompassing a number of pathophysiological theories that are indicated in depression, as well as a number of behavioural qualities that are seen as depressive-like in nature. In conjunction with this, it has reported predictive validity for detecting antidepressant efficacy, in addition to testing novel antidepressant therapies, with 'depressive-like' behavioural endpoints being reduced in the model after antidepressant treatments. However, aspects of MDD that have not been examined as strongly in this OB model is that of sociability, social motivation and social cognitive functioning. As pointed out in section Section 1.7.7, although cognition and cognitive deficits have been examined in the OB model (Hendrickson et al., 2012, Mucignat-Caretta et al., 2004, Primeaux and Holmes, 1999), none of these cognitive tests have incorporated any *social* cognitive elements. Similarly, and as mentioned in the General Introduction in Chapter 1, OB animals have been examined in the SI test, which is a behavioural test that examines an animal's social interplay and exploration of another animal, which has relevance for human social behaviour (Pandey et al., 2014). SI is shown to be decreased in OB animals in comparison to sham-operated counterparts (Jiménez-Sánchez et al., 2016, Morales-Medina et al., 2012a; 2012b; 2012c, Pandey et al., 2009; 2010; 2014, Rajkumar et al., 2009, Tsunekawa et al., 2008). These

alterations have been reversed with chronic paroxetine (Pandey et al., 2009), imipramine (Tsunekawa et al., 2008), amitriptyline (Pandey et al., 2010), and escitalopram (Pandey et al., 2014, Rajkumar et al., 2009) treatment. Other than the SI test, the only behaviour that has measured sociability of sorts in OB animals is that of sexual behaviour, where once again this behavioural aspect is seen to be decreased in OB animals, highlighting social dysfunction after OB surgery (Edwards et al., 1990). In conjunction with this, most SI paradigms are usually confined to one short acute novel experience or examination, such that animals are exposed to an unfamiliar or novel animal for a concise period of time, and this novel experience is not repeated. In the meta-analysis that was conducted in the previous chapter, that examined the SI test in the OB model between the years 1999-2018, the SI test or any other SI paradigm was only examined on a single occasion, or just once in OB animals, with no paper examining repeated exposure to SI paradigms (Morales-Medina et al., 2012a; 2012b; 2012c, Rajkumar et al., 2009, Tsunekawa et al., 2007). Pandey et al. (2010; 2014) exposed two separate cohorts of OB animals to the SI test at one week and two weeks post-dosing with antidepressants (three and four weeks post-surgery), and found that OB animals have diminished SI at both time-points, with a trend for an even greater degree of impairment at the second or latter time-point. Having said this, SI and sexual behavioural paradigms do not take into account the cognitive aspect of social functioning, and as such, social memory processing and motivation.

The 3-chamber sociability test is a test of social recognition and memory (Crawley, 2004, Moy et al., 2004). In this regard it assesses an animal social processing and motivational behaviour, in addition to examining the animal's memory functioning. Primarily used to assess social impairment in autism models, the 3-chamber sociability test incorporates three important aspects in social cognitive function; that of social approach, social novelty and social recognition (Crawley, 2004, Moy et al., 2004). The 3-chamber sociability test consists of an arena that is split into three chambers and the test itself involves three consecutive 10-minute time-trials (Figure 3.1). In the first trial, an animal is placed into an empty arena with three chambers and allowed to explore all three chambers. This is known as the *habituation trial*. In the second trial, a novel conspecific animal is introduced and placed into a restricted cage in either the left or right chamber, with an adjacent empty cage in the far chamber. The test animal can now explore the novel conspecific animal or the empty cage; this is known as the *sociability trial*. In the third trial, the novel

conspecific animal becomes the familiar animal and a second novel conspecific animal is placed into what once was the empty cage. Again, the test animal can explore the 3 chambers for ten minutes; this trial is known as the *social preference/social novelty trial.*

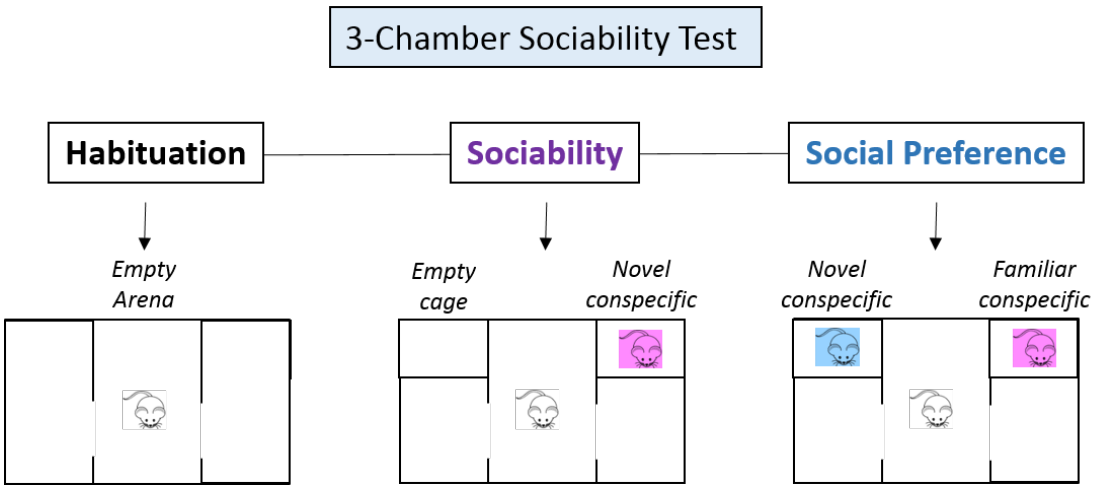


Figure 3.1 Diagram of the 3-chamber sociability test.

Many parameters can be assessed, including distance moved, % preference, time spent interacting and number of contacts made. This social cognitive test also has the added strength that no direct contact is made with the conspecific, limiting aggressive and sexual behaviour from either animal (Moy et al., 2004). As such, pure auditory, olfactory and visual communication allows for social cognitive functioning to be distinctly assessed (Moy et al., 2004). In conjunction with this, this test is distinctly unique from the novel object recognition, as animals are allowed to interact and discriminate between an animate rather than inanimate objects. Similar to behavioural parameters examined in the meta-analysis in Chapter 3, variations exist in the 3-chamber sociability test with regards exploration times and how these are described, and also with the duration of time that animals are tested for (Table 3.1). The 3-chamber sociability test also tends to be performed in mice rather than in rats and regardless of species, animals tend to be group-housed prior to testing (Table 3.1). In this lab, and as shown in the results of the meta-analysis and systematic review in Chapter 2, there is a past history of singly-housing OB rats in order to assess their homecage-locomotor activity, and as such our lab has a history of working with singly-housed OB rats and has characterised the OB model under this specificity.

Sociability Trial							
Exploration Time (s)	Description of Exploration	Duration of Test	Housing	Species	Strain	Sex	Reference
367 ± 36	Time spent in Social Chamber	10 mins	NS	Mice	C57BL/6J	M	Dogra et al. 2016*
144 ± 16	Time spent investigating Social Stimulus Animal	10 mins	Group	Rats	SPD	M	Reilly et al. 2015
133 ± 12						F	
371 ± 54	Social Chamber Time	10 mins	Paired	Rats	Wistar	M	Smith et al. 2015
169 ± 44	Time spent in Chamber	5 mins	Group	Rats	SPD	M	Eagle et al. 2013
153 ± 40	Sniffing Time	10 mins	Group	Mice	C57BL/6J	M	Moy et al. 2013
105 ± 46	Sniffing Time	10 mins	Group	Mice	C57BL/6J	M	Chadman, 2011
87 ± 30	Time spent in Chamber	10 mins	Group	Mice	C57BL6J	M	Kaidanovich-Beilin et al. 2011
217 ± 58	Time spent around Stranger 1 Cage	10 mins	Group	Mice	KO	NS	Tanda et al. 2009
292 ± 33	Time spent in Chamber	10 mins	Group	Mice	C57BL/6J	M	Moy et al. 2004
Social Preference Trial							
Exploration Time (s)	Description of Exploration	Duration of Test	Housing	Species	Strain	Sex	Reference
174 ± 43	Novel Investigation	10 mins	Paired	Rats	Wistar	M	Smith et al. 2018*
165 ± 47						F	
95 ± 10	Time spent investigating Novel Stimulus Animal	10 mins	Group	Rats	SPD	M	Reilly et al. 2015
74 ± 9						F	
408 ± 63	Novel Chamber Time	10 mins	Paired	Rats	Wistar	M	Smith et al. 2015
157 ± 59	Time spent in Chamber	5 mins	Group	Rats	SPD	M	Eagle et al. 2013
122 ± 33	Sniffing Time	10 mins	Group	Mice	C57BL/6J	M	Moy et al. 2013
77 ± 51	Sniffing Time	10 mins	Group	Mice	C57BL/6J	M	Chadman, 2011
75 ± 27	Time spent in Chamber	10 mins	Group	Mice	C57BL6J	M	Kaidanovich-Beilin et al. 2011
194 ± 90	Time spent around Stranger 2 Cage	10 mins	Group	Mice	KO	M	Tanda et al. 2009
278 ± 41	Time spent in Chamber	10 mins	Group	Mice	C57BL/6J	M	Moy et al. 2004

Table 3.1 Exploration times of the novel animal in the sociability and social preference trials, and the housing of rats and mice tested in to 3-chamber sociability test. The table shows time spent exploring the novel animal in the sociability and social preference trials of control rats, the descriptions used for time interacting/exploring, the duration of time that animals were tested, and the housing, species, strain, and sex used in studies. Data are expressed as mean ± standard deviation. F=female, KO=knockout, M=male, mins=minutes, NS=not stated, SPD=Sprague Dawley. *Dogra et al. (2016) only examined sociability, Smith et al. (2018) only examined social novelty preference.

In conjunction with this, OB rats have a well-established history of irritability and as such are often found to be aggressive in disposition (van Riezen et al., 1977). Regular handling and the pairing of two OB animals with two sham-operated animals has been shown to reduce this aggravation and irritable behaviour in the syndrome (Leonard and Tuite, 1981, van Riezen et al., 1977); but overall this is another reason OB rats in this lab have been traditionally singly-housed. Having said this, social isolation in itself is an important factor when assessing depression, as social isolation in humans has been shown to be correlated with loneliness and depression (Matthews et al., 2016), and social isolation in animals, such as maternal deprivation and social isolation stress, cause many depressive-like behaviours, in addition to neurochemical effects that resemble the effects seen in MDD (Krishnan and Nestler, 2011). For example, Isovich et al. (2001) showed that the striatal DAT, a factor of the dopaminergic system seen to be decreased in depression, and important in motivation and the reward pathway, is decreased after social defeat stress, but only in animals that are isolated after testing (Pryce et al., 2017). Wallace et al. (2009) showed that socially-isolated rats have increased latency to ejaculate, spend less time in the open arms of the EPM and have reduced % sucrose preference in comparison to pair-housed rats (Wallace et al., 2009). In a study by Westenbroek et al. (2005), examining gender-specific behavioural effects of social isolation in rats, pair-housing was shown to have a stress-reducing effect on both genders in comparison to isolated counterparts, with pair-housing a male rat with a female rat seen to prevent several of the behavioural effects of a CMS model. Pinelli et al. (2017) examined the effects of single, paired and group-housing on male and female rats, and found that all group-housed rats were shown to have decreased movement in the OF, group-housed males were shown to have decreased CRH mRNA in the hypothalamus, and all single-housed rats were shown to be significantly more inactive in the homecage than paired and group-housed counterparts. Isolation in OB mice has also been shown to enhance hyperactivity and exploratory behaviour in the OF and increase latency to novelty-suppressed feeding (Linge et al., 2013). Indeed, in the same study, sham-operated mice were shown to have increased anxiety-like behaviour in the OF after social isolation stress, when compared to their group-housed counterparts (Linge et al., 2013). Rodents in particular are pack animals and so prefer to be in groups than alone. Therefore by socially isolating a rat, regardless of surgical procedure, it is possible that we may be inducing a depressed state in the rat by enhancing stress through simply their housing condition

alone. Indeed, if patients with MDD exhibit social dysfunction, and social deficits are seen in animal models of depression, the housing of the animals could well be an important factor, with singly-housing an animal presenting a confound, when examining social cognition and function in models of depression. In conjunction with this, animal ethics committees and animal protective welfare bodies, are moving away from the procedure of singly-housing parameters, with paired- or group-housing being seen as better for the overall health and well-being of research animals (ILAR, 2011).

Lastly, *SI deficits* that exist in the OB model are attenuated with chronic antidepressant treatment (Pandey et al., 2009; 2010; 2014, Rajkumar et al., 2009, Tsunekawa et al., 2008), and in turn, *cognitive deficits* that exist in the OB model have also been shown to be attenuated with chronic antidepressant treatment (Jesberger and Richardson, 1986, van Riezen et al., 1977). Taking this into account, it would be hypothesised that chronic antidepressant treatment would attenuate any social cognitive deficits, if any, that are examined in the OB model. As a result, it was decided that two experiments would be undertaken to re-characterise the OB rat model of depression in this lab in terms of:

1) Social cognition; a factor that to the best of our knowledge has not been examined in this model before.
2) The impact of housing; a factor that is important when examining SI, and also with regards animal welfare moving forward in animal research.
3) Social cognitive dysfunction with antidepressant therapy; such that if any social cognitive deficits were seen, the impact that chronic conventional antidepressant treatment would be examined using two different classes of antidepressant drugs; a classic TCA and a classic SSRI.
4) Opioid receptor expression; to assess if alterations in the opioid system in limbic regions of the brain exist following OB surgery.

Therefore, the specific aims of the experiments in this chapter were as follows:

Experiment 1:

- To assess the effects of single versus paired-housing on naïve, sham-operated and OB rats

- To assess the effects of single versus paired-housing on the characteristic hyperactivity in OB rats in the OF test
- To assess the effects of single verses paired-housing on anxiety-like behaviour in the EPM in OB rats
- To characterise social cognition in the 3-chamber sociability test in the OB rat model of depression
- To analyse opioid receptor mRNA expression in the hippocampus, amygdala and nucleus accumbens after OB removal

Experiment 2:

- To characterise social cognition in the 3-chamber sociability test in the OB rat model of depression using singly-housed animals
- To assess the effects of chronic dosing of two conventional antidepressants drugs; fluoxetine (FLX) and desipramine (DMI), on social cognition in the OB rat model of depression

Note: The opportunity to examine the effects of chronic antidepressant treatment in the 3-chamber sociability test in OB rats arose as a part of another study that was already underway in our lab, and so the 3-chamber sociability test was added in as an additional testing parameter within the study design of that experiment. As a result, all animals were already singly-housed in this experiment and hence this is why there is a difference in housing between our two experiments in this chapter.

3.2 Materials and Methods

3.2.1 Animals

Experiments were carried out on a total of 92 (*experiment 1*) and 72 (*experiment 2*) male Sprague Dawley rats (200-250 g on arrival, obtained from Charles River, UK). In *experiment 1*, 64 rats were used as test subjects and 28 rats served as conspecifics for the 3-chamber sociability test. In *experiment 2*, 48 rats were used as test subjects and 24 rats served as conspecifics for the 3-chamber sociability test. On arrival, rats were housed in groups of 4 per cage, in plastic cage bottoms (42 cm x 25.5 cm x 13 cm) with a metal grated cage top with plastic water bottles (North Kent Plastics, Coalville, Leicestershire, UK). Cages contained 3Rs paper bedding (Fibrecycle Ltd., Scunthorpe, Lincolnshire, UK). Prior to surgery, cages also contained sizzle-nesting material for environmental enrichment (LBS Biotechnology, Horley, UK), and rats were given nutritional enrichment once a week (CocoPops, Tesco PLC, Ireland). This protocol was introduced and has had no impact on the behavioural responses (Castro et al., 2016). Test rats did not receive enrichment after surgery. All test rats were housed prior to surgery (based on bodyweights) and were randomly allotted to their surgery groups. These allotted groups were checked for statistical significance using IBM SPSS Statistics Version 24 software to make sure that there was no significant difference between groups prior to the commencement of the study. The conspecific rats were randomly housed in pairs based on bodyweights at the same time as the test rats, and conspecific rats continued to receive sizzle-nesting material and environmental enrichment throughout the duration of the study. All rats were housed in an environment with controlled temperature (20-24°C) and humidity (45-65%) (Monitor, Radionics Ltd, Dublin, Ireland), in a 12:12 h light-dark cycle, lights on from 08:00 h. Animals had access to food (*Exp. 1* 20% protein rodent diet Advanced Protocol® Verified 75 IF Irradiated (5V75), LabDiet®, Brentwood, MO, USA; *Exp. 2* 14% protein diet, Harlan-Teklad-2014 Maintenance Diet, Harlan Laboratories, Belton, Loughborough, UK) and water *ad libitum*. Bodyweight, food, and water consumption were monitored daily (weighing scales, Mason Technology, Dublin, Ireland) from one week pre-surgery until sacrifice.

All animal procedures were carried out under the approval of the Animal Care and Research Ethics Committee (ACREC), National University of Ireland, Galway (NUIG) (12/NOV/07). All procedures for this project were approved for authorisation

from the Health Products Regulatory Authority (HPRA) and in compliance with EU Directive 86/609 (HPRA Authorisation ID: AE19125/P006).

3.2.2 Experimental Design

Male Sprague Dawley rats (200-250 g) underwent surgery (sham or OB) under isoflurane anaesthesia (*Experiment 1 and 2*), or did not undergo surgery (naïve) (*Experiment 1*) four days after initial arrival. In *experiment 1*, rats were singly-housed or pair-housed (destined to become either paired shams, paired OB, or a sham-operated rat paired with an OB) prior to surgery (*n*=8/group), see Figure 3.2. This housing condition would persist for the duration of the study. Rats received either a sham or OB surgical procedure, and following recovery from surgery were kept in the assigned housing condition. In the case of mortality, particularly in pair-housed rats, a designated substitute was included. In *experiment 2*, rats were singly-housed prior to surgery, and two weeks post-surgery rats received daily subcutaneous injections of vehicle (saline), desipramine or fluoxetine (both 10 mg/kg s.c in a dose volume of 4 ml/kg) for three weeks (*n*=8/group). In *experiment 1*, rats were tested in the EPM and OF test (two weeks post-surgery), followed by the 3-chamber sociability test (four weeks post-surgery) and were re-tested in the OF test (five weeks post-surgery). Immediately after re-exposure to the OF at five weeks post-surgery, rats were euthanized via decapitation (Figure 3.2). In *experiment 2*, rats were tested in the 3-chamber sociability test (four weeks post-surgery) and were euthanized at five weeks post-surgery via decapitation (Figure 3.2).

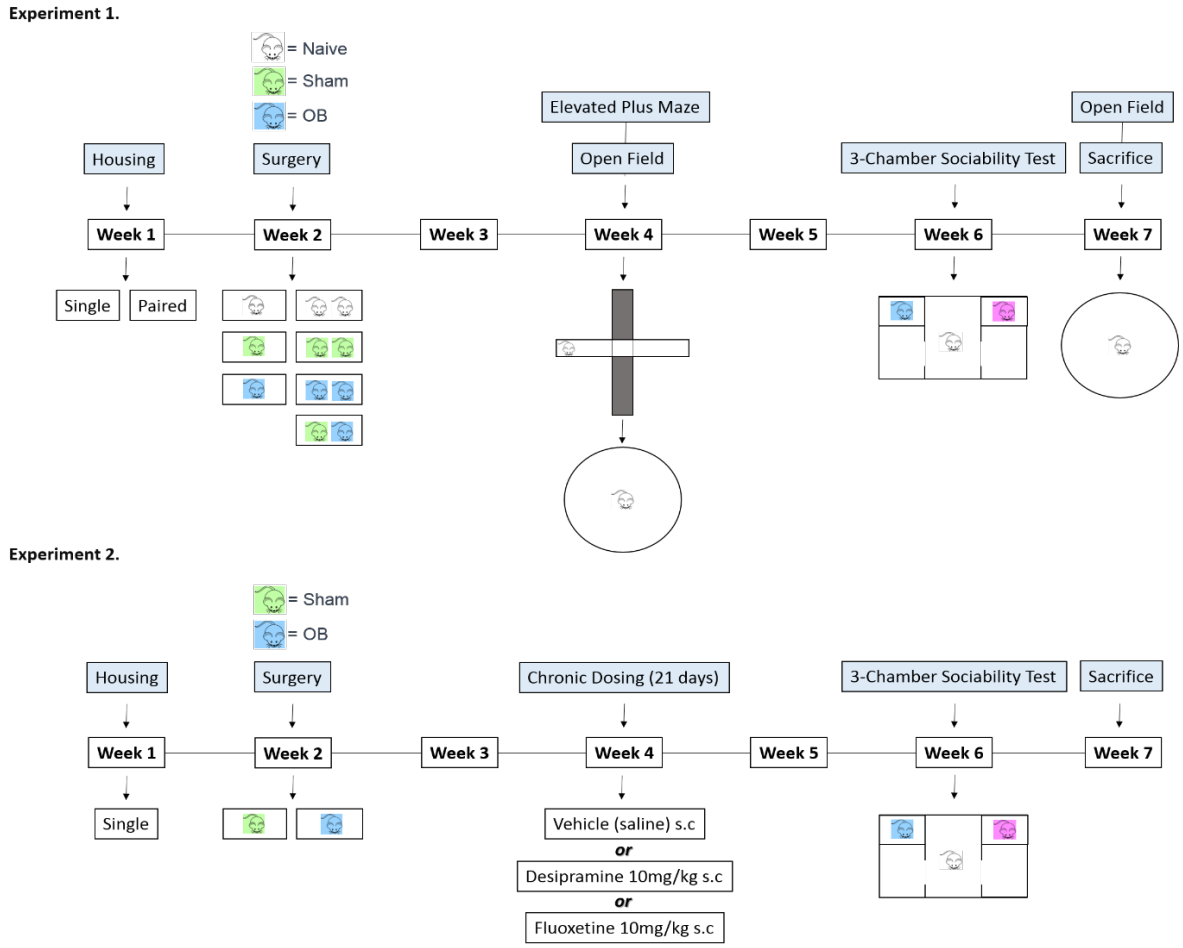


Figure 3.2 Experimental Design for experiment 1 and experiment 2.

3.2.3 OB Surgery

3.2.3.1 Aseptic Surgery Technique

Prior to surgery all equipment and surgical tools were autoclaved and sterilised. A surgery tool pack was made up consisting of a scalpel, large scissors, small scissors, large forceps, small forceps and a bulldog clips clipper. Packs were wrapped in tinfoil, placed into an autoclave bag and autoclaved prior to surgery. A new pack was opened after every four rats, and the previous pack was removed until that evening for cleaning. Similarly, a new scalpel blade was used after every 4-5 rats. Gauze, blue tissue paper, tinfoil, cotton buds, bulldog clips, the blunted needle, drill-bits and suture clips were also all placed into autoclave bags and sterilised the evening prior to surgery.

The surgeon scrubbed-in aseptically prior to surgery (See Appendix A) and aseptic procedures were conducted throughout the surgical procedure. An assistant brought the animal into the room and shaved the animals head. The surgeon placed

the animal into the stereotaxic frame using the earbars, directly after which they changed into a fresh pair of sterile surgical gloves. Care was taken not to touch the animal directly from this moment on, particularly the fur, and autoclaved pieces of tinfoil were placed on the ends of items such as tubes of cream used in surgery, or on the knobs of the anaesthetic machine, in order to remain sterile throughout surgery. A fresh pair of sterile surgical gloves was used per surgery. A drape was put on the surface of the table and the first tinfoil pack was opened, with the surgical tools placed on this sterile surface. After each item of equipment was finished being used it was handed to a second assistant who cleaned it with warm water and Rapidex® (Cat# D3657, Williams Medical Supplies Ltd., Wales), placed it in a hot bead steriliser and returned it to the draped area for use for the next surgery. One assistant acted as a direct sterile surgical assistant; thus handed the surgeon the tools, cleaned the tools and watched the animals that were in recovery in the room. The second surgical assistant's role was to watch over the animals; thus this assistant brought the animal from its homecage, anaesthetised the animal, shaved the animals head, monitored it after surgery in the recovery stage, gave it the post-operative injections, returned it to its homecage and observed the animal back in its homecage in between subsequent surgeries. This assistant also monitored respiration throughout the surgical procedure, counting respiration every 5-minutes.

Each day after surgery, all tools were thoroughly cleaned in Rapidex® (which disintegrates blood), were dried thoroughly and were sterilised in a hot bead steriliser. The new packs were then made up each evening and placed into the autoclave overnight for surgery the next morning.

3.2.3.2 Olfactory Bulbectomy Surgery Procedure

Rats were weighed each day from one week prior to surgery and their bodyweights were noted. Rats were randomly allocated to surgery groups. The night before surgery rats were given a ramekin of semisolid food (food diet mixed and softened with warm water) to encourage eating and strength pre-surgery. Surgeries were carried out as previously described in this lab by Burke et al. (2010) and Roche et al. (2007). The morning of surgery, rats were weighed in their holding room and brought into the surgery room where they were anaesthetised using isoflurane (Cat# CP0009, Chanelle, Ireland, 5.0% for induction and 2.0% for maintenance in 0.5 L/min O_2) and the hair

on their head was shaved. The rat was then manually positioned into the stereotaxic frame (Harvard Apparatus, MA, USA) using the earbars, with the frame being connected to an isoflurane pump to maintain anaesthesia throughout the duration of surgery. Reflexes were assessed after which eye drops (Blink®, Boots, Galway) were placed onto the eyes of the rat to prevent the eyes from drying out. The shaved part of the head was swabbed in a circular motion with iodine/betadine (7.5% w/v, iodinated povidone, Videne®, Ecolab Ltd., Leeds, UK) to prevent bacterial infection. The initial incision into the skull was made in a vertical line along the midline with a sterilised scalpel blade (Swan Morton). The skin on each side was pulled back and secured from the site of surgery using two bulldog clips (Fine Science Tools, Heidelberg, Germany). Bupivacaine (0.2 ml) (Bupivacaine Hydrochloride, AstraZeneca, Dublin, Ireland) and Norocaine® (Lidocaine Hydrochloride, Cat# NB0041, Chanelle Veterinary, Ireland) were instilled onto the dura of the skull to allow for pain relief and reduce inflammation. The periosteum was removed and bregma was located by eye, and the positions for drilling two parallel burr holes were marked with the tip of the scalpel blade; ~5 mm rostral to bregma and 2 mm lateral to the midline (Figure 3.3).

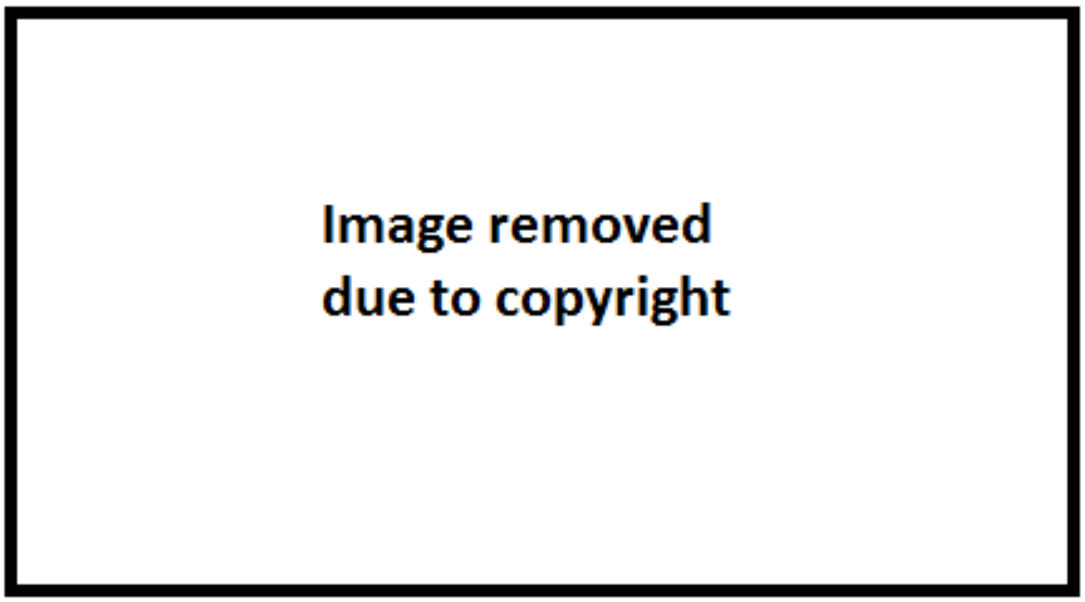


Figure 3.3 Illustrated image of the rat brain in the skull showing the positioning of the olfactory bulbs, bregma, and burr holes in relation to olfactory bulbectomy surgery. The illustration shows the brain regions that olfactory bulbs project to. Image taken from (Kelly et al., 1997).

A drill was then used to puncture two burr holes (2 mm in diameter) into the skull. Immediately after, a blunt hypodermic needle (16G – 1.6 x 40 mm, BD Microlance,

Oxford, UK) was placed into the holes which was attached to a vacuum pump (Super Vega Suction Machine, Medguard, Meath) and was used to aspirate the olfactory bulbs. Extreme care is taken with the positioning of the needle so as not to damage the cortex of the brain during insertion and aspiration. An empty 'suction' sound can be heard when all of the bulbs have been removed, immediately after which the holes are plugged with haemostatic sponge (Septodont, France) to halt the bleeding. The bulldog clips were then removed and the skin was sutured using sterile Michel suture clips (7.5 x 1.75 mm, Fine Science Tools, UK). Sham-operated rats underwent the same procedure but the head of the drillbit (size 8, Transmore Ltd., Dublin) is manually twisted to mark the two holes in the skull until the dura is pierced, taking care that the drill does not enter the skull cavity. After suturing, rats were placed on oxygen to promote oxygenation and waking, and before waking rats were injected with 1 ml of sterile saline s.c (Sodium Chloride Braun Melsungen AG, University Late Night Pharmacy, Galway) to promote hydration. Rats were placed into a recovery cage which had a heating pad (Peco Services Ltd., Cumbria, UK) underneath it and were monitored until they were fully mobile and awake. Once fully awake, rats were injected with Rimadyl® (Carpofen, Cat# BE0067 Chanelle Veterinary, Ireland, 5 mg/kg at a dose volume of 1 ml/kg s.c) as pain relief, and were returned to their homecage. Animals were given a subsequent dose of Rimadyl® (5 mg/kg at a dose volume of 1 ml/kg s.c) 24-hours post-operation and were closely monitored over the next 48 hour period, with bodyweight, food and water consumption all being measured. Rats were also given a fresh ramekin of mushy food the night of surgery. Each OB surgery lasted about 10-15 minutes. If a rat was unwell, it was singly-housed for a short period of time and given a ramekin of warm porridge to promote recovery. If a rat continued to deteriorate, and lost up to 20% of pre-surgical bodyweight, the veterinary doctor was called and euthanasia was performed. Successful OB lesioning was verified upon sacrifice of the animal (Figure 3.4). If any bulb remained, or if the frontal cortex had been damaged, rats were removed from further behavioural and molecular analysis.

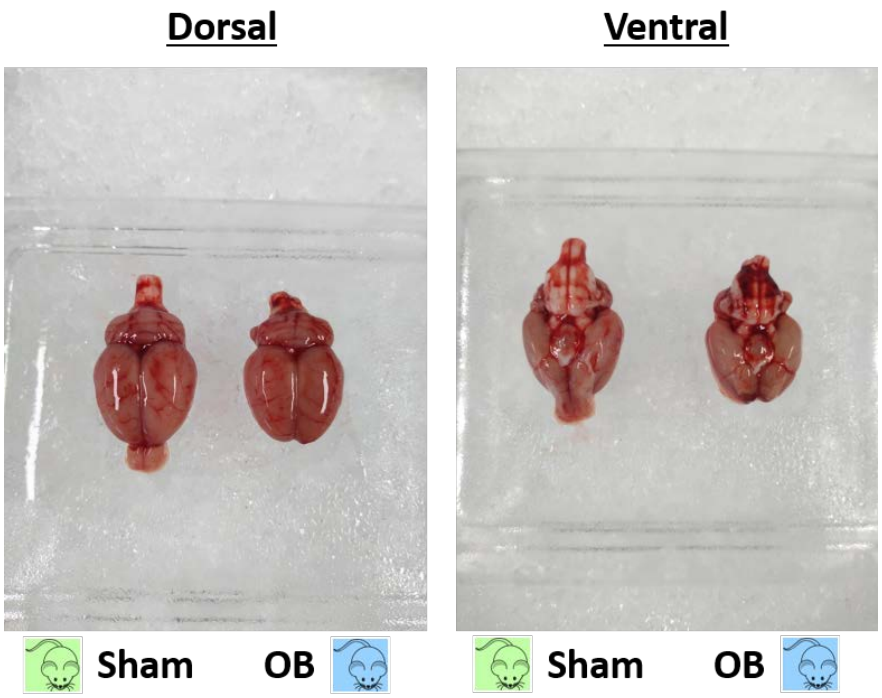


Figure 3.4 Verification of sham-operated and OB lesioning upon rat sacrifice and brain dissection. Successful OB surgery; all bulbs removed and no cortical damage.

3.2.4 Drugs

The selection of antidepressant doses was based upon literature review, and previous findings from this laboratory (Simpson et al., 2012a; 2012b). For *experiment 2*, desipramine hydrochloride (Cat# D3900, Sigma-Aldrich, Dublin) and fluoxetine hydrochloride (Cat# PHR1394, Sigma-Aldrich, Dublin) were both dissolved in saline. For each drug rats were given a dose of 10 mg/kg drug, at a dose volume of 4 ml/kg. Saline was used as the vehicle. All drugs were injected via subcutaneous (s.c) administration (syringes and needles, Lohans Pharmacy, Galway). All drugs were injected at the same time each day, in the afternoon between 14:00-18:00 h. If a rat was to be behaviourally tested that day, that animal was injected after testing. All drugs were made up in batch quantities and aliquoted with the amount of millilitres needed per day calculated previously. Aliquots were frozen at -20°C, and were thawed at room temperature on the morning of the day they were needed. Most of the OB studies examined report chronic dosing with antidepressants for 21 days, with behavioural testing commencing after at least two weeks of drug administration. Thus, the 21 day dosing regime was selected for the study.

3.2.5 Behavioural Testing

3.2.5.1 Open Field Test

As mentioned earlier, the OF is a test that is used to look at anxiety-like behaviour in a novel aversive environment. A rat is placed into a brightly lit novel aversive environment from which they cannot escape. The OF is separated into an inner zone and an outer zone, with the inner zone being more brightly lit and as such more aversive for the rat than the peripheral zone. The time spent in each zone can be measured. The longer amount of time that the animal spends in the centre of the arena is indictative of 'anxiolytic-like' behaviour. Locomotor activity is a key feature that is measured using this apparatus and as such, a rat's distance moved can be calculated. As discussed in chapter two, there is huge variation in the dimensions of the OF, and the duration of testing. The OF apparatus in this lab consists of a white circular base (75 cm in diameter), with aluminium mirrored walls (41 cm high) (apparatus designed and built by Mr. Ambrose O'Halloran, NUI Galway). The floor of the OF is divided up into 10 cm squares by think black lines. Positioned in a square shape overhead are four 60 watt bulbs that are attached to dimmer switches. Light intensity was manipulated using the dimmer switches so that the arena had a light intensity of between 180-220 lux, as determined using a lux meter (Iso-Tech ILM350, Radionics Ltd, Dublin Ireland) at the base of the arena. Each rat was removed from their homecage and brought to a separate unfamiliar room for testing. The rat was placed into the centre of the OF, facing away from the researcher and the door of the room. The experimenter left the room, closed the door and started the timer. Rats were allowed to explore the arena for 5 minutes. Immediately after the test ended, the rat was removed from the OF and brought back to its homecage. The apparatus was cleaned between each test with warm soapy water and a dishcloth to remove any olfactory smells, with the apparatus dried thoroughly before the next subject entered. Testing for the OF always took place between 08:10-11:30 h. All video footage was recorded on a DVR recorder which was connected to camera located 92 cm above the centre of the OF. Video-tracking software Ethovision®XT (version 11.5) (www.noldus.com) was used to later analyse and track the recorded footage. Using Ethovision®XT software, the inner and outer zones were illustrated and allowed for automated distance moved (cm), and time spent in inner or outer zone (sec) to be measured (Figure 3.5).

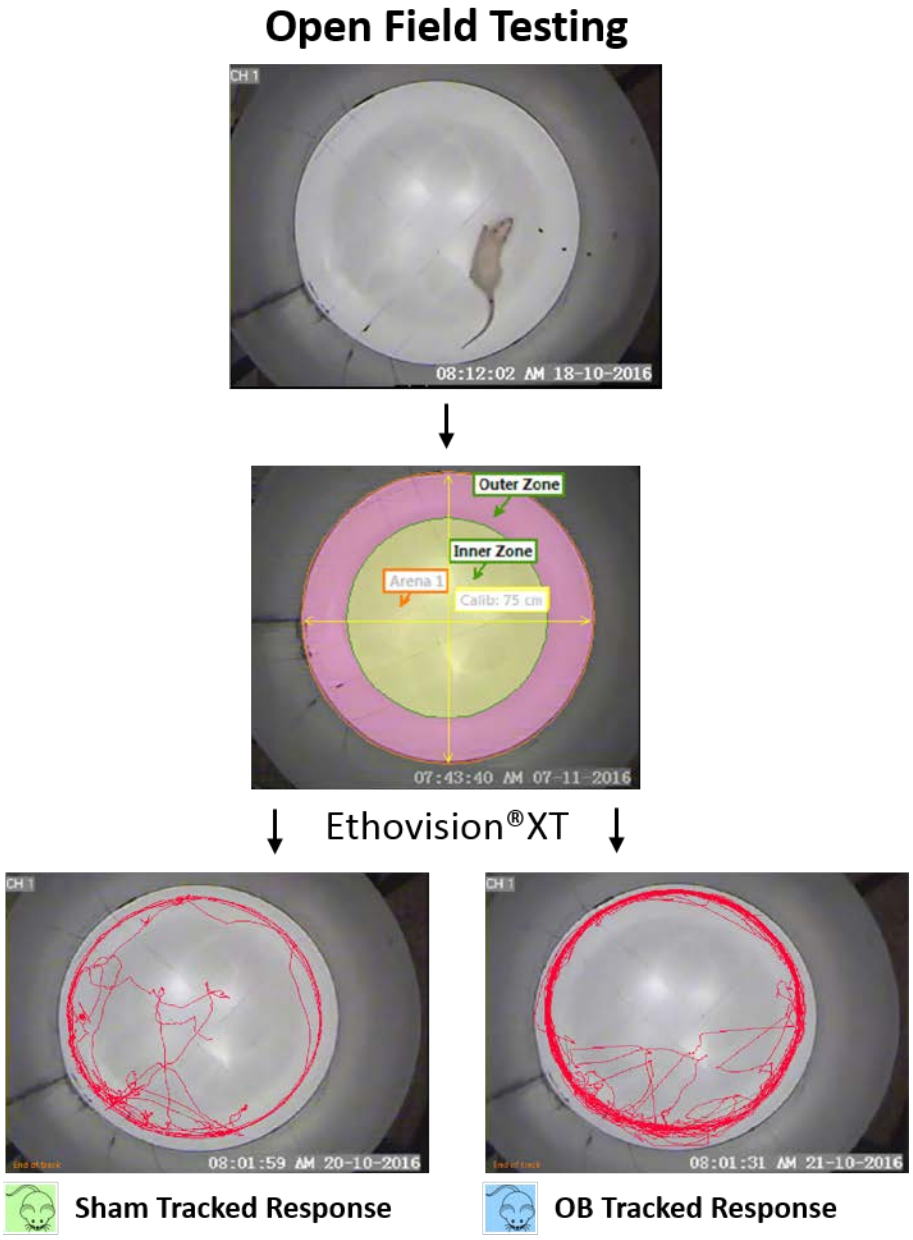


Figure 3.5 Open field apparatus and distanced tracked using Ethovision®XT Software. Rats were tested in the OF apparatus for 5 minutes and video footage was recorded. Footage was analysed using Ethovision®XT software and the inner and outer zones were illustrated and defined. Distanced moved per rat was tracked, with the above image illustrating the increased movement in the OB rats (thicker red lines representing more distance travelled of the arena) in comparison to the sham-operated rats.

3.2.5.2 Elevated Plus Maze

The EPM is a test of anxiety-like behaviour. It consists of a maze in the shape of a cross with two open or exposed arms, and two walled or enclosed arms (Figure 3.6). It is based upon the principle of thigmotaxis whereby a rat has a natural instinct to avoid open spaces and prefers to remain adjacent to vertical surfaces. The amount of time and the number of entries animals make into the open and closed arms measures 'anxiety-like' behaviour with the more time the animal spends in the open arms being seen as anxiolytic-like behaviour. The EPM apparatus is raised off of the ground (55 cm), with each arm measuring 50 cm x 13 cm, and the central platform being smaller at 13 cm x 10 cm (apparatus designed and built by Mr. Ambrose O'Halloran, NUI Galway). The enclosed walls measure 30 cm in height. A single 60 watt bulb is suspended over each arm (1.2 m), with each bulb being attached to dimmer switches allowing for different light intensities to be used. The light intensity over the end of each closed arm was between 50-60 lux, and the light intensity of each of the open arms was between 100-110 lux. Each rat was removed from their homecage and brought to a separate unfamiliar room for testing. The rat was placed with their head and front two paws in the centre of the EPM, facing the open arm and facing away from the researcher and the door of the room. The experimenter left the room, closed the door and started the timer. Rats were allowed to explore for 5 minutes. Immediately after the test ended, the rat was removed from the EPM and brought into a separate room where they were tested in the OF apparatus. The EPM was cleaned between each test with warm soapy water and a dishcloth to remove any olfactory smells, with the apparatus dried thoroughly before the next subject entered. Testing for the EPM always took place between 08:10-11:30 h. All video footage was recorded on a DVR recorder which was connected to a camera located 1.3 metres above the centre of the EPM. Video-tracking software Ethovision®XT (version 11.5) was used to later analyse and track the recorded videos. Using Ethovision®XT software, the open arms, closed arms and centre zone was illustrated, and allowed for automated distance moved in the arena to be measured (cm) (Figure 3.6). Manual scoring of the time spent, and number of entries into the open arms, closed arms and centre zone (sec) could also be measured via the 'mutually exclusive' setting in Ethovision®XT and was done with an experimenter who was blind to all experimental groups. Open arm time and arm open arm entries were reported. Total arm time and total arm entries

were also calculated (open arm + closed arm), in order to determine % open arm time (open arm time/total arm time*100) and % open arm duration (open arm duration/total arm duration*100).

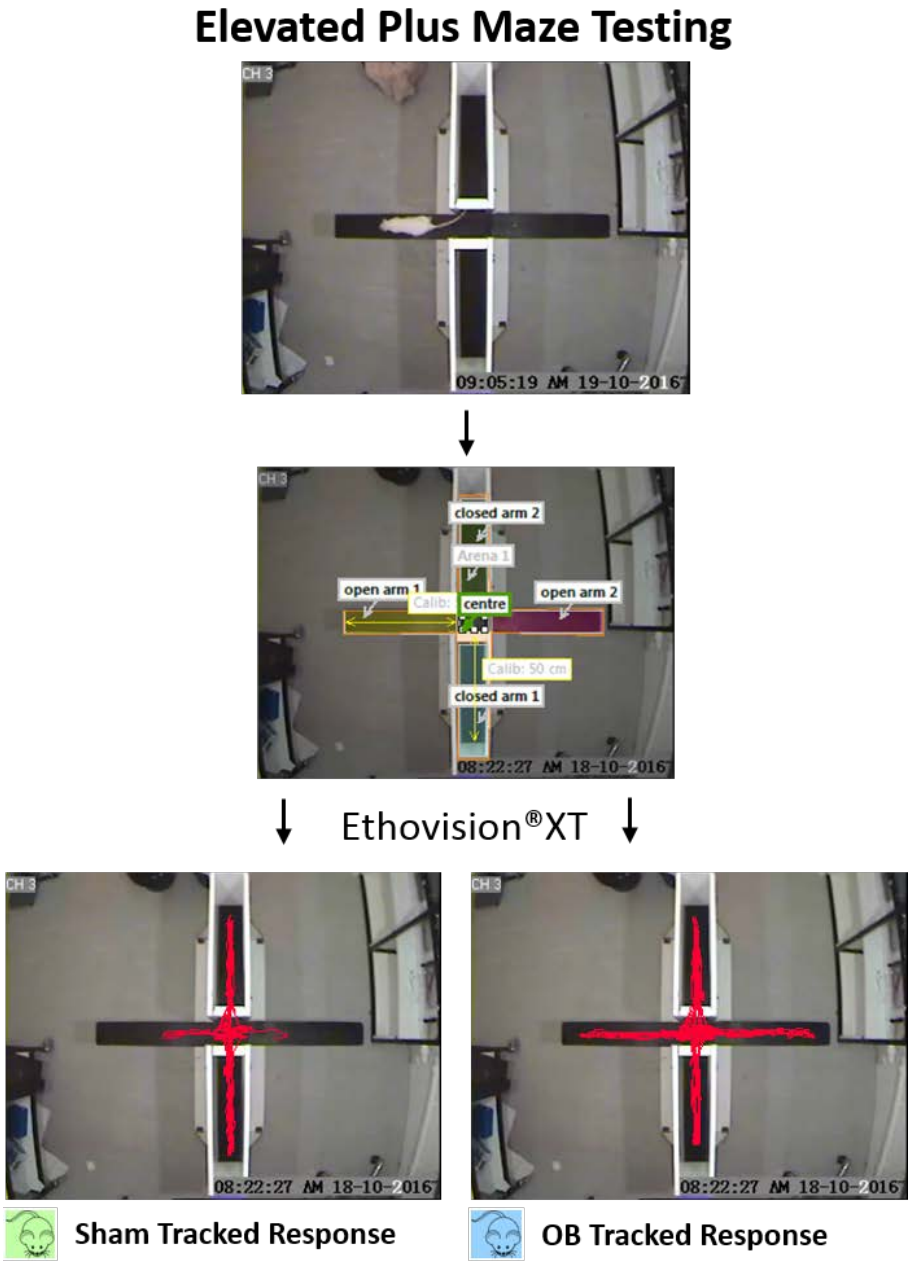


Figure 3.6 Elevated plus maze apparatus and distanced tracked using Ethovision®XT Software. Rats were tested in the EPM apparatus for 5 minutes and video footage was recorded. Footage was analysed using Ethovision®XT software and the two open and two closed arms were illustrated and defined, along with the centre zone. Distanced moved per rat was tracked, with the above image illustrating the movement in the OB rats (thicker red lines representing more distance travelled of the arena) in comparison to the sham-operated rats.

3.2.5.3 3-Chamber Sociability Test

The 3-Chamber Sociability test is a test for measuring social recognition and memory. Based upon Crawley's (2004) paradigm, it measures an animal's social approach to another unfamiliar organism on two separate occasions, testing sociability, and testing social novelty or preference. The total 3-chamber sociability apparatus measures 90.5 x 46 x 40 cm in dimensions, and is separated into three separate chambers; the centre chamber, the left chamber and the right chamber (apparatus designed and built by Mr. Ambrose O'Halloran, NUI Galway) (Figure 3.7). The left and right chambers mirror each other each, and are located on either side of the far ends of the arena (Figure 3.7). Each chamber measures 46 x 30.16 cm. Three bulbs (100W) were located above the arena (99 cm), one over the centre of each chamber and each bulb was attached to a master dimmer switch to control light intensity. Light intensity used was 45-48 lux over the centre chamber and 35-38 lux over the left and right chambers. The front panel of the arena is Perspex glass, with the other 3 panels made of opaque grey plastic sheeting. Similarly, the two panels or walls that separate the three chambers are also made of Perspex glass, such that the animal can see through the two walls the separate each chamber. In the left and right chambers, a metal grate can be slotted in 10 cm from the back wall of the chamber to create a restricted area (30 x 10 cm) where a novel rat, known as a 'conspecific' rat, can be placed into this restricted zone. In this way, the test rat can interact with the novel conspecific rat by pure olfactory, visual and auditory cues. The 3-chamber is separated into three distinct consecutive 10-minute time-trials, the *habituation trial* (exploration of the empty arena), the *sociability trial* (exploration of a novel conspecific rat verses a parallel empty cage) and finally, the *social preference trial* (exploration of the now familiar conspecific rat, verses a second novel conspecific rat, which is placed into what once was the empty cage). The rat was taken out from its homecage and tested in a separate unfamiliar room and always begins each trial in the centre chamber. After the *habituation trial*, the test rat is encouraged into the centre chamber and is restricted within this chamber by placing a water bottle at the entrance to the left and right chambers. While the test rat is here, the metal grate is added to each of the left and right chambers and a novel conspecific is placed into one side, with the opposite restricted area left empty (*sociability trial*). A lid is placed onto each restricted cage to stop the conspecific from escaping. The side of the chamber (left or right) that the first novel conspecific was placed into is rotated on each day of testing to avoid a side of preference in test rats.

The water bottles are removed and placed onto the lids of the restricted cages and the experimenter leaves the room, closes the door and starts the timer. After the *sociability trial*, the test rat is again trapped into the centre chamber with a water bottle blocking each entrance, and a second novel conspecific is placed into what was originally the empty cage (*social preference trial*). The lid is placed on top of the new novel conspecifics cage and the two water bottles are removed from each entrance and placed onto the restricted cages. The experiment leaves the room, closes the door and starts the timer. After the social preference trial, the test animal is returned to his homecage, as are the two novel conspecifics. The lids, metal grates and water bottles are removed from the arena and are thoroughly washed down with warm soapy water and a dish cloth, as is the entire arena. Similarly the arena, metal grates, lids and water bottles are all dried thoroughly. The conspecifics are housed in cages of two and each pair was used together per rat tested, i.e. each pair from a cage consisted of the novel rat and the familiar rat. The test rat had never come into contact with the conspecifics they were tested with previous to 3-chamber sociability test exposure. A conspecific rat could be tested once a day for four days consecutively before be given a break from testing for one day in accordance with the guidelines approved in this project by the HPRA. 3-Chamber sociability test testing always took place between 09:00-17:30 h. All video footage was recorded on a DVR recorder which was connected to a camera located (84 cm) above the centre of the centre chamber. Using Ethovision®XT software the right chamber, right interaction zone, left chamber, left interaction zone, and the centre chamber were all illustrated, and allowed for automated distance moved in the arena (and per zone) to be measured (cm) (Figure 3.7). The degree of habituation to the arena was calculated as the *social preference* (3^{rd}) trial as a percentage of the habituation (1^{st}) trial. A lower % habituation score indicated that a rat habituated to the arena over time. Manual scoring of the time spent interacting with the empty cage, novel animal, familiar animal and second novel animal (sec) could be measured via the 'mutually exclusive' setting in Ethovision®XT, and scoring was done by an experimenter who was blind to the experimental groups.

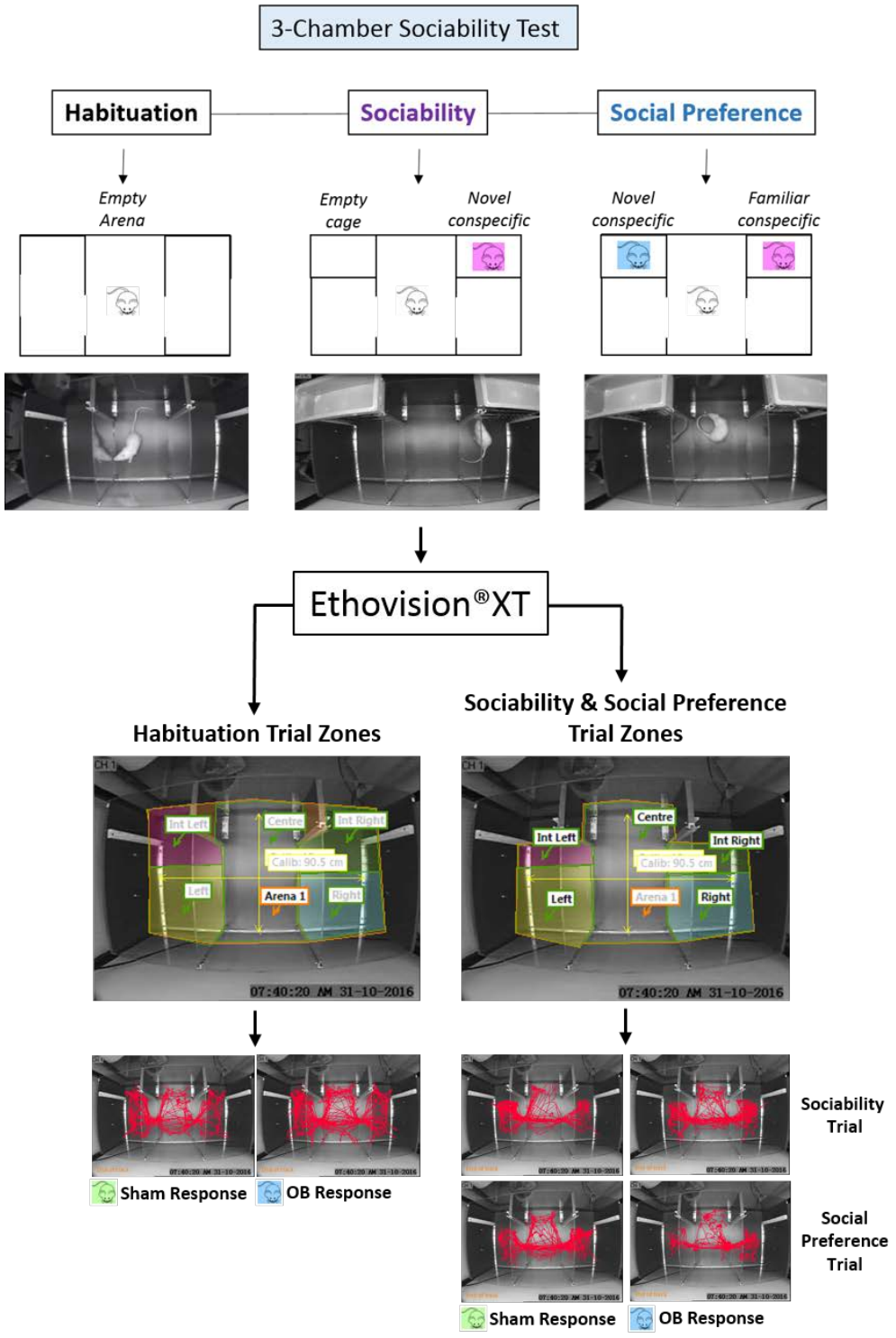


Figure 3.7 3-Chamber sociability test apparatus and distanced tracked using Ethovision®XT Software. Rats were tested in the 3-chamber sociability test apparatus for 30 minutes and video footage was recorded. Footage was analysed using Ethovision®XT software and the left, right, centre, right interaction and left interaction zones were illustrated and defined. A separate arena setting had to be drawn for the habituation trial as compared to the sociability and social preference trial, as the rat had access to the whole arena in this first trial. Distanced moved per rat was tracked, with the above image illustrating the movement in the OB rats in comparison to the sham-operated rats (thicker red lines representing more distance travelled of the arena and per zone).

3.2.6 Animal Sacrifice and Brain Removal

For post-mortem analysis, animals were sacrificed via live decapitation using a guillotine. Immediately after decapitation, the skin on the top of the head was lifted and cut off using a scissors. The optic ridge between the eyes was then broken using a rongeur. A small scissors was used to cut a small shallow line along the base of the skull and the bone was pulled back. A larger scissors was then used to cut along the midline of the skull along the saggital plane and the parietal and frontal bones were peeled to the side. Using a spatula, the optic nerve was severed and the brains were carefully lifted out from the skull. At this point, successful OB removal could be confirmed and care was taken when removing the brain from the skull so that any bulb that may still be present, or even attached to the prefrontal cortex, could be noted (Figure 3.4). Once removed, the brain was immediately placed on tinfoil in dry ice. The brains were snap-frozen on tinfoil on an upside-down Petri dish, in a bed of solid CO_2 pellets (to maintain form and structure) and stored at -80°C until molecular analysis.

3.2.7 Brain Dissection and Tissue Collection

Brains were removed from -80°C storage and placed onto a bed of CO_2 pellets in a Styrofoam box. The brain was then moved, per dissection, onto wet filter-paper on an upside-down glass petri dish that lay in a box of ice. The brain was then allowed to thaw slightly so that deeper regions could be teased apart and accessed. The hippocampus, amygdala and nucleus accumbens were dissected from both sides of the brain and weighed. The left side of each region was used for PCR analysis. The hippocampus and amygdala were halved on the sagittal plane due to their large size. Each PCR sample weighed about 30 mg per tissue.

3.2.8 Detection of MOP, DOP, KOP gene expression using real-time quantitative polymerase chain reaction (qRT-PCR)

3.2.8.1 RNA Isolation

Total RNA was extracted from homogenised samples using the NucleoSpin RNA II total RNA isolation kit and manual (Macherey-Nagel, Fischer Scientific, Ireland). 354 µl of RA1 buffer containing 1% β-mercaptoethanol (Cat# M6250, Sigma Aldrich, Ireland) was added to 30 mg tissue and homogenised with an Ultra-Turrax Polytron

tissue disrupter (Fischer Scientific, Ireland), to lyse the tissue. The lysate was then transferred to a NucleoSpin® filter (violet ring) which was fitted in a collection tube, and the tube was centrifuged (Hettich Zentrifugen MIKRO 185, Davidson and Hardy Ltd, Ireland) at 11,000 g for 1 minute. Once finished, RNA binding conditions were adjusted by removing the NucleoSpin® filter (violet ring), and adding 350 µl of 70% molecular grade ethanol (Cat# E7023, Sigma-Aldrich, Ireland) to the lysate and mixing it by pipetting up and down seven times. The lysate was then added onto the NucleoSpin® filter (blue ring), which was fitted into a collection tube, and the tube was centrifuged for 30 seconds at 11,000 g. This step allowed for RNA to bind to the blue ringed NucleoSpin® filter. From this step on the blue ringed NucleoSpin® filter was kept and contained the RNA. The blue ringed NucleoSpin® filter was placed into a new collection tube and 350 µl of membrane desalting buffer (MDB) was added to the filter and the tube centrifuged at 11,000 g for 1 minute. The flow-through was discarded and the tube was centrifuged again at 11,000 g for a further 1 minute to make sure the membrane was fully dried. Salt removal allows for the next step involving the rDNase reaction mixture to digest the DNA more thoroughly. A 10% v/v rDNase reaction mixture was prepared using 10 µl of reconstituted rDNase and adding 90µl of reaction buffer for rDNase (both supplied in the kit). This mixture was made up in a separate Eppendorf and 95 µl of this rDNase reaction mixture was added directly to the filter membrane of the blue ringed NucleoSpin® filter. The blue ringed NucleoSpin® filter tubes were taken off of ice and left to incubate at room temperature for 15 minutes to digest the DNA. 200 µl of buffer RAW2 was added to the filter and centrifuged at 11,000 g for 30 seconds. Buffer RAW2 deactivates the rDNase reaction mixture. The flow-through was discarded and 600 µl of buffer RA3 was added to the filter and centrifuged at 11,000 g for 30 seconds. Again, the flow-through was discarded and 250 µl of buffer RA3 was added to the filter and centrifuged at 11,000 g for 2 minutes. The blue ringed NucleoSpin® filter was placed into a new RNA-free Eppendorf tube and 50 µl of RNase-free water (supplied in the kit) was added directly to the centre of the filter and centrifuged at 11,000 g for 1 minute to elute the RNA. The eluted RNA Eppendorf tubes were stored on ice until RNA quantification was completed and then RNA samples were stored at -80°C until equalisation.

3.2.8.2 RNA Quantification and Equalisation

The concentration, quality and purity of the isolated RNA was assessed with a Maestro NanoDrop Spectrophotometer (Medical Supply, Dublin). RNA concentration was determined by calculating optical density (OD) at 260nm. RNA quality was determined by calculating the OD_{260}/OD_{280} ratio, and the purity was determined using the OD_{260}/OD_{230} ratio, where a value in between 1.3-2.0 was regarded as good quality and pure RNA. The same RNase-free water was used throughout quantification when the 'blank' was determined before each reading, and only samples with a ratio value >1.3 were used. After quantification, all samples were equalised to the same RNA concentration for each region (Table 3.2) by the addition of RNase-free water (supplied in the kit). Samples were then vortexed to ensure mixing and were frozen at -80°C until cDNA synthesis.

Region	Mean RNA concentration (µg/µl)	Quality (260:280)	Purity (260:230)	Equalised RNA concentration (µg/µl)
Hippocampus	245 ± 69	2.0-2.3	1.6-2.1	150
Amygdala	321 ± 74	1.9-2.3	1.9-2.1	175
Nucleus Accumbens	123 ± 49	2.1-2.5	1.9-2.1	45

Table 3.2 The RNA concentration, quality, purity, and equalised RNA concentration, per brain region after RNA isolation.

3.2.8.3 Complimentary DNA (cDNA) Synthesis

Equal amounts of RNA from each sample per brain region were synthesised into RNA. All reagents used were purchased from BioSciences Ltd., Dublin, Ireland. Two master mixes (MM1 and MM2) were prepared in advance of cDNA synthesis (Table 3.3). 10 µl of equalised RNA from each sample was placed into a new Eppendorf tube and 2 µl of MM1 was placed on the lid of each tube. The tube was centrifuged at 10,000 g for 10 seconds so the MM1 would be mixed with the equalised RNA. Tubes were then placed into a thermocycler (MJ Research, Bio-Rad, Fannin, Dublin) to be heated at 65°C for 5 minutes. Samples were quickly chilled on ice and then the tubes were centrifuged at 10,000 g for 10 seconds. Samples were placed back on ice and 7 µl of MM2 was added to the lid of each tube. The tube was centrifuged at 10,000 g for 10 seconds so the MM2 would be mixed with the equalised RNA mixture. Tubes were then placed into a thermocycler (MJ Research, Bio-Rad, Fannin, Dublin) to be heated at 37°C for 2 minutes. The tubes were again centrifuged at 10,000 g for 10 seconds. 1

µl of superscript III reverse transcriptase was carefully pipetted into the bottom of the tube and mixed in by stirring the pipette tip. The tubes were incubated at room temperature for 10 minutes, and loaded onto the thermocycler for incubation at 50°C for 50 minutes, followed directly after by heating of the mixture to 70°C for 15 minutes. The high temperature process deactivated the reaction. The tubes were removed from the thermocycler and diluted 1:4 (20 µl: 60 µl) with RNase-free water (supplied in the kit). Samples were vortexed and stored at -80°C until qRT-PCR.

Master Mix 1 (MM1)	
Random Primers (250 ng/µl)	1 µl
10Mm dNTP mix	1 µl
Total per sample	**2 µl**
Master Mix 2 (MM2)	
5X First Strand Buffer	4 µl
0.1M DTT	2 µl
RNase OUT	1 µl
Total volume per sample	**7 µl**

Table 3.3 Reagents and corresponding volumes used to make-up master mix 1 (MM1) and master mix 2 (MM2) for cDNA synthesis.

3.2.8.4 Quantitative Real-Time PCR (qRT-PCR) Analysis of Gene Expression

Quantitative Real-Time PCR (qRT-PCR) Analysis of Gene Expression with the synthesised cDNA using the Applied Biosciences StepOne Plus™ Real-Time PCR System (BioSciences, Dublin). Gene expression of target proteins were determined using commercially available Taqman gene expression assays (BioSciences Ltd., Dublin) containing forward and reverse target primers and FAM-labelled Taqman MGB probes (Target genes and assay ID's are listed in Table 3.4). FAM-labelled β-actin was used as the endogenous control gene. All samples were run in singleplex assays. All cDNA samples were removed from the -80°C freezer and placed on ice to thaw out. While the samples thawed, a Taqman master mix (Table 3.5) was prepared and kept on ice prior to pipetting into MicroAmp® optical 96-well plates (Applied BioSciences, Dublin). Each cDNA sample was vortexed and 2.5 µl of each cDNA sample was pipetted into a MicroAmp® optical 96-well plate. All samples were pipetted in duplicate. 7.5 µl of the Taqman master mix was then pipetted into the wells so that the total volume per well equalled 10 µl. A non-template control, containing

no cDNA sample but with the 7.5 µl of the Taqman master mix and 2.5 µl of RNase-free water were added in duplicate to each plate. The plate was sealed with adhesive sealing film (Cat# 4311971, Applied Biosystems, BioSciences, Dublin), ensuring that no air-bubbles were in any wells. The plate was centrifuged at 10,000 for 10 seconds to ensure mixing and wrapped in tinfoil. Plates were then transferred to the Applied Biosystems StepOnePlus™ Real Time machine (BioSciences, Dublin) and ran under the following preset cycle; 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of (95°C for 15 seconds followed by 60°C for 1 minute).

Target Gene	**Assay ID**	**Fluorescent Label**
MOP *(Oprm1)*	Rn01430371_m1	FAM
KOP *(Oprk1)*	Rn00567737_m1	FAM
DOP *(Oprd1)*	Rn00561699_m1	FAM
Endogenous Control Gene	**Assay ID**	**Fluorescent Label**
β-Actin *(Actb)*	Rn00667869_m1	FAM

Table 3.4 Assay ID's and fluorescent labels of target genes and the endogenous control gene.

Taqman Master Mix	
Taqman Reagent	5 µl
Taqman Primer *(ex. Oprm1 for MOP)*	0.5 µl
RNase-free water	2 µl
Total volume per sample	**7.5 µl**

Table 3.5 Reagents and corresponding volumes used to make-up Taqman master mix.

3.2.8.5 Analysis of qRT-PCR Data

Amplification plots and copy thresholds (Ct) values were examined using Applied Biosystems 7500 system SDS Software 1.3.1 (Figure 3.8) and were exported to Microsoft Excel for further analysis. The 2- $^{\Delta\Delta Ct}$ method was used to determine gene expression (Livak and Schmittgen, 2001). This method is used to assess relative gene expression by comparing gene expression of experimental samples to that of control samples, thus allowing determination of the fold change in mRNA expression between groups. This involved 3 steps:

1) Ct values are normalised to the endogenous control gene for each sample, i.e. Ct target gene – Ct endogenous control gene = ΔCt
2) Ct values are normalisation to the control sample, i.e. ΔCt sample – mean ΔCt of control group = ΔΔCt
3) The fold difference is determined and given as $2^{-\Delta\Delta Ct}$. The $2^{-\Delta\Delta Ct}$ values were expressed as a percentage of the mean of the $2^{-\Delta\Delta Ct}$ values of the control group, i.e. singly-housed sham-operated group

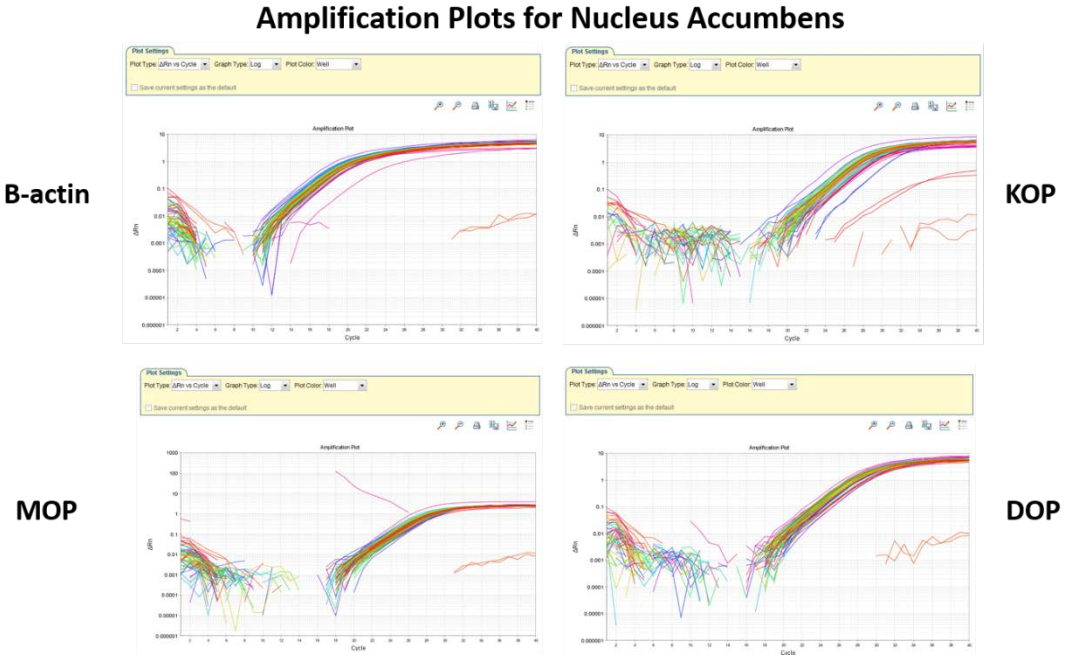


Figure 3.8 Sample amplification plots for β-Actin, the MOP, the KOP and the DOP in the nucleus accumbens. Images taken from Applied Biosystems 7500 system SDS Software 1.3.1. DOP=delta opioid receptor, KOP=kappa opioid receptor, MOP=mu opioid receptor.

3.2.9 Statistical Analysis

All statistical analysis was performed using IBM SPSS Statistics Version 24 software package. In all datasets, the presence of possible outliers was checked by assessing the distribution of data. In case a data point fell out of the range of (mean-2*standard deviation) to (mean+2*standard deviation), it was considered an outlier and excluded from subsequent analysis. Data were expressed as mean ± standard deviation, unless they are deemed non-parametric, in which case the data were expressed as median ± interquartile range. All data was tested for normality using Shapiro-Wilks test for normality, and all data was also tested for Levene's test for homogeneity of variance.

If data were deemed parametric, they were analysed using two-way ANOVA, followed by *post-hoc* Student Newman-Keuls test; $p<0.05$ was deemed statistically significant. If data was non-parametric, Kruskal-Wallis, followed where appropriate by *post-hoc* multiple Mann-Whitney U tests, with Bonferroni corrections were used. Note that a two-way ANOVA repeated measures could have been used to examine the distance moved in the 3-chamber sociality test but this method of analysis was not chosen. This was based upon the evidence that during our literature search examining the papers that employ the use of the 3-chamber sociability test, the majority of papers examine the results by a two-way ANOVA, considering each trial as a separate entity as each trial is considered contextual different. As a result, we undertook our analysis in the same manner. All graphs were prepared using GraphPad Prism Version 8.

3.3 Results

3.3.1 *Experiment 1:* Effects of social cognition, and single verses paired housing, in the OB rat model of depression

3.3.1.1 Mortality rates and verification after OB surgery

Firstly, it must be noted that of the sixty rats that had undergone surgery, eleven rats died in the post-operative and/or recovery period. Of the eleven rats that died, all deaths occurred as a result of olfactory bulbectomy surgery, with deaths occurring in the recovery cage, or within 4 hours after being removed to their homecage. Autopsy revealed that four rats had damage to the PFC (left or right hemisphere), and that the seven remaining rats had no damage to the cortex and as such cause of death was unconfirmed. Olfactory bulbectomy surgery is often associated with relative numbers of death post-surgery, as is sometimes, but not always, reported in the literature. However, the higher than expected number of deaths at this time was thought to be associated with an issue the isoflurane anaesthetic rigs, which had been recently been serviced prior to the commencement of surgery, in addition to other research groups also encountering higher unexplained surgical mortality rates at that current time. Upon completion and verification of OB removal at the end of the study, there were no animals excluded from further analysis for incomplete or excessive bulb removal.

3.3.1.2 Bodyweight gain (two and five weeks post-surgery)

With regards bodyweight gain at two weeks post-surgery, two-way ANOVA revealed that there was a significant effect of surgery [$F_{(2,58)}$=7.280, p=0.002], and a significant effect of housing [$F_{(1,58)}$=6.393, p=0.014], but no surgery x housing interaction effect [$F_{(2,58)}$=0.766, p=0.470] (Figure 3.9A). *Post-hoc* analysis revealed that OB rats, when pair-housed with another OB rat, had a significant reduction in bodyweight gain at two weeks post-surgery, when compared to paired sham-operated counterparts (p<0.05).

With regards bodyweight gain at five weeks post-surgery, two-way ANOVA revealed that there was a significant effect of surgery [$F_{(2,56)}$=6.200, p=0.004], and a significant effect of housing [$F_{(1,56)}$=3.394, p=0.071], but no surgery x housing interaction effect [$F_{(2,56)}$=0.087, p=0.917] (Figure 3.9B). *Post-hoc* analysis revealed that OB rats, when pair-housed with another OB rat, had a significant reduction in bodyweight gain at five weeks post-surgery, when compared to paired sham-operated counterparts (p<0.05).

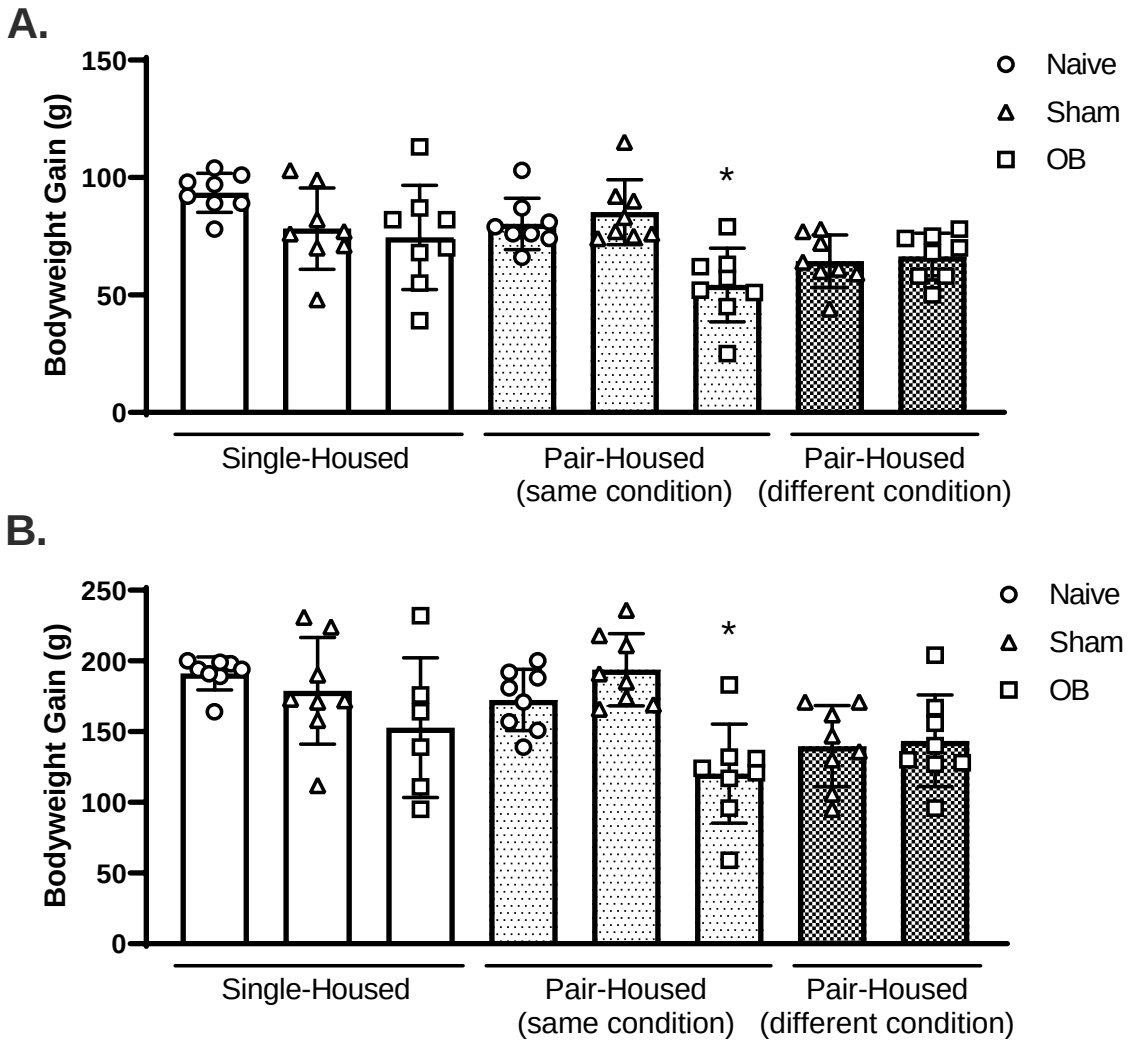


Figure 3.9 Bodyweight gain in singly- and pair-housed naïve, sham-operated and OB rats two weeks post-surgery. The bars of singly-housed rats are shaded in white, pair-housed rats (of the same condition) are shaded in spotted grey pattern, and pair-housed rats (of different conditions) are shaded in darker black pattern. OB rats who were pair-housed with another OB rat, had a significant reduction in bodyweight gain at two weeks (A) and five weeks (B) post-surgery, when compared to pair-housed sham-operated counterparts. Data are expressed as mean ± standard deviation (n=6-8 per group). *p<0.05 vs. appropriate sham-operated counterpart.

3.3.1.3 Elevated Plus Maze Results

3.3.1.3.1 Distance moved in the elevated plus maze (two weeks post-surgery)

Distance moved in the EPM was found to be non-parametric as it failed Levene's test for homogeneity of variance and Shapiro-Wilks test of normality, as such Kruskal-Wallis followed by *post-hoc* with Mann-Whitney U tests were performed. A significant difference in distance moved in the EPM was found between groups [K(7)=25.008, p=0.001] (Figure 3.10). *Post-hoc* analysis revealed that OB rats, when singly-housed or when paired together with another OB rat, moved significantly more in the EPM than when compared to appropriate sham-operated counterparts (p<0.05 with Bonferroni correction (p<0.016)).

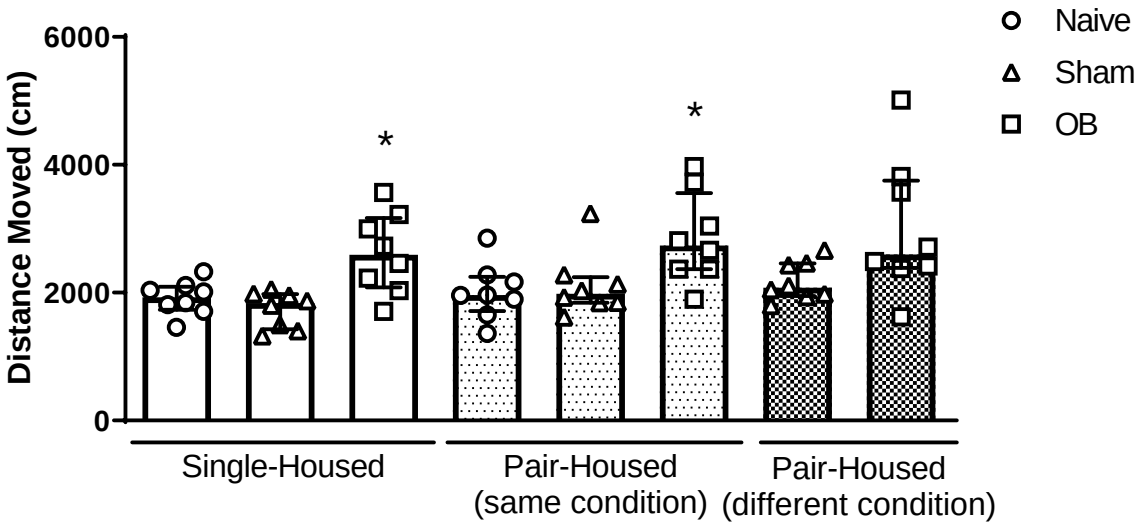


Figure 3.10 Distance moved in the elevated plus maze by naïve, sham-operated and OB rats two weeks post-surgery. The bars of singly-housed rats are shaded in white, pair-housed rats (of the same condition) are shaded in spotted grey pattern, and pair-housed rats (of different conditions) are shaded in darker black pattern. Singly-housed OB rats, and OB rats who were pair-housed with OB rat, moved significantly more in the EPM, than their appropriate sham-operated counterparts. Data are expressed as median ± interquartile range (n=8 per group). *p<0.05 vs. appropriate sham-operated counterpart.

3.3.1.3.2 % Open arm entries and % open arm duration in the elevated plus maze (two weeks post-surgery)

With regards % open arm entries in the EPM at two weeks post-surgery, two-way ANOVA revealed that there was a significant effect of surgery [$F_{(2,58)}$=5.614, p=0.006], but no effect of housing [$F_{(1,58)}$=0.163, p=0.688], or effect of surgery x housing interaction [$F_{(2,58)}$=1.259, p=0.292] (Figure 3.11A). *Post-hoc* analysis revealed that there was no significant difference between experimental conditions.

With regards % open arm duration in the EPM at two weeks post-surgery, two-way ANOVA revealed that there was a significant effect of surgery [$F_{(2,58)}$=4.374, p=0.017], but no effect of housing [$F_{(1,58)}$=0.107, p=0.745], or effect of surgery x housing interaction [$F_{(2,58)}$=0.904, p=0.411] (Figure 3.11B). *Post-hoc* analysis revealed that there was no significant difference between experimental conditions.

3.3.1.3.3 Open arm entries and open arm duration in the elevated plus maze (two weeks post-surgery)

With regards the number of open arm entries in the EPM at two weeks post-surgery, two-way ANOVA revealed that there was a significant effect of surgery [$F_{(2,57)}$=14.705, p<0.001], but no effect of housing [$F_{(1,57)}$=0.045, p=0.833], or effect of surgery x housing interaction [$F_{(2,57)}$=1.019, p=0.368] (Figure 3.11C). *Post-hoc* analysis revealed that singly-housed OB rats, and OB rats that were paired together, made significantly more open arm entries than their appropriate sham-operated counterparts (p<0.05).

With regards the duration spent in the open arms in the EPM at two weeks post-surgery, two-way ANOVA revealed that there was a significant effect of surgery [$F_{(2,58)}$=4.025, p=0.023], but no effect of housing [$F_{(1,58)}$=0.503, p=0.481], or effect of surgery x housing interaction [$F_{(2,58)}$=0.824, p=0.444] (Figure 3.11D). *Post-hoc* analysis revealed that there was no significant difference between experimental conditions.

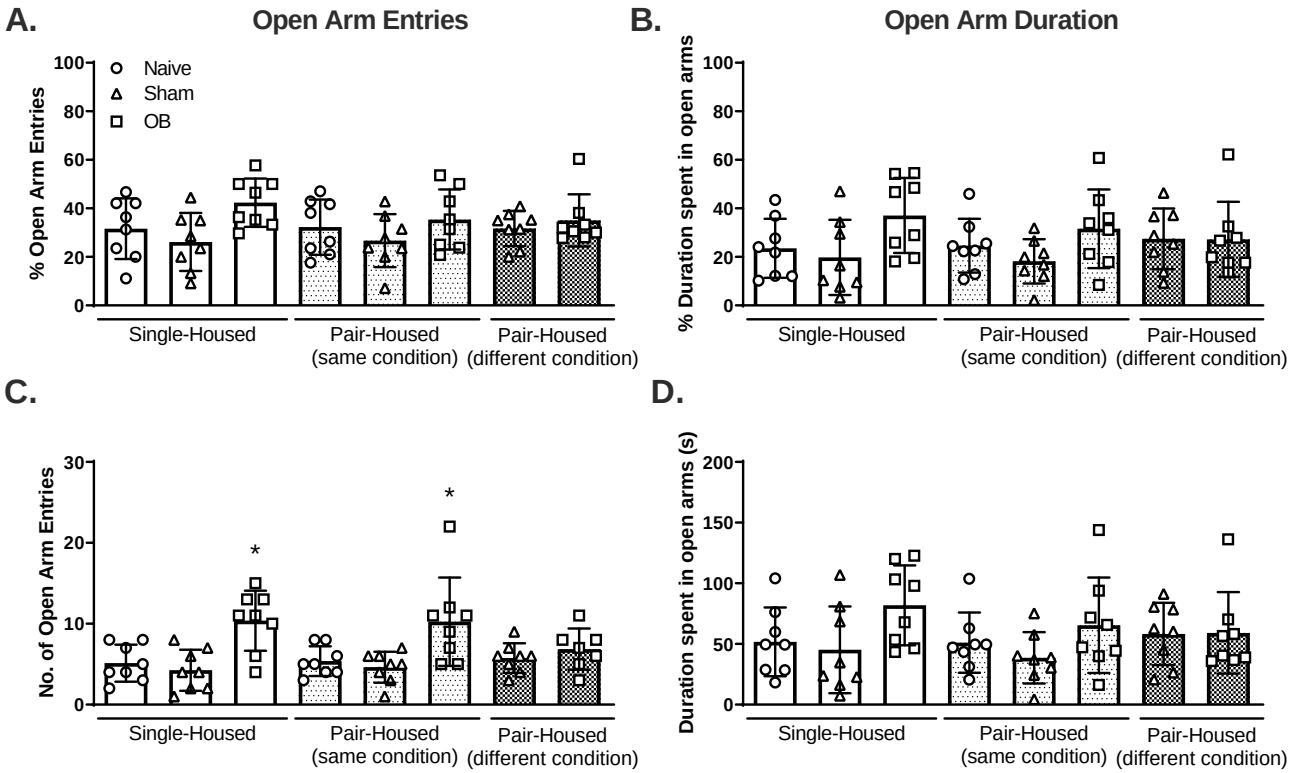


Figure 3.11 Open arm entries and duration spent in the open arm (percentage and raw values) in the elevated plus maze made by naïve, sham-operated and OB rats two weeks post-surgery. The bars of singly-housed rats are shaded in white, pair-housed rats (of the same condition) are shaded in spotted grey pattern, and pair-housed rats (of different conditions) are shaded in darker black pattern. Singly-housed OB rats, and OB rats that were pair-housed with one another OB, had a significant increase in the number of open arm entries made, in comparison to appropriate sham-operated counterparts (C). There was no significant difference in % open arm entries (A), % duration spent in the open arms (B) or the duration spent in the open arms (D). Data are expressed as mean ± standard deviation (A, B, C, D) (n=7-8 per group). *p<0.05 vs. appropriate sham-operated counterpart.

3.3.1.4 Open Field Results

3.3.1.4.1 Distance moved in the open field (two weeks post-surgery)

Distance moved in the OF at two weeks post-surgery was found to be non-parametric as it failed Levene's test for homogeneity of variance and Shapiro-Wilks test of normality, as such Kruskal-Wallis followed by *post-hoc* with Mann-Whitney U tests were performed. A significant difference in distance moved was found between groups [$K(7)=22.140$, $p=0.002$] (Figure 3.12A). *Post-hoc* analysis with Mann-Whitney U test followed by Bonferroni correction revealed a significant difference in that singly-housed OB rats had increased distance moved in comparison to singly-housed sham-operated rats, and similarly, in that OB rats that were pair-housed with sham-operated rats had significantly increased distance moved when compared to sham-operated rats who had been pair-housed with OB rats ($p<0.05$ with Bonferroni correction ($p<0.016$)). There was no significant difference between OB rats that had been paired with one another when compared to sham-operated rats that had been pair-housed together. Similarly, no significant differences were seen in sham and naïve rats, regardless of housing condition.

3.3.1.4.2 Time spent in the inner circle of the open field (two weeks post-surgery)

With regards time spent in the inner circle of the OF at two weeks post-surgery, two-way ANOVA revealed that there was no significant effect of surgery [$F_{(2,58)}=2.545$, $p=0.087$], housing [$F_{(1,58)}=0.081$, $p=0.777$], or any surgery x housing interaction [$F_{(2,58)}=0.231$, $p=0.795$] (Figure 3.12B).

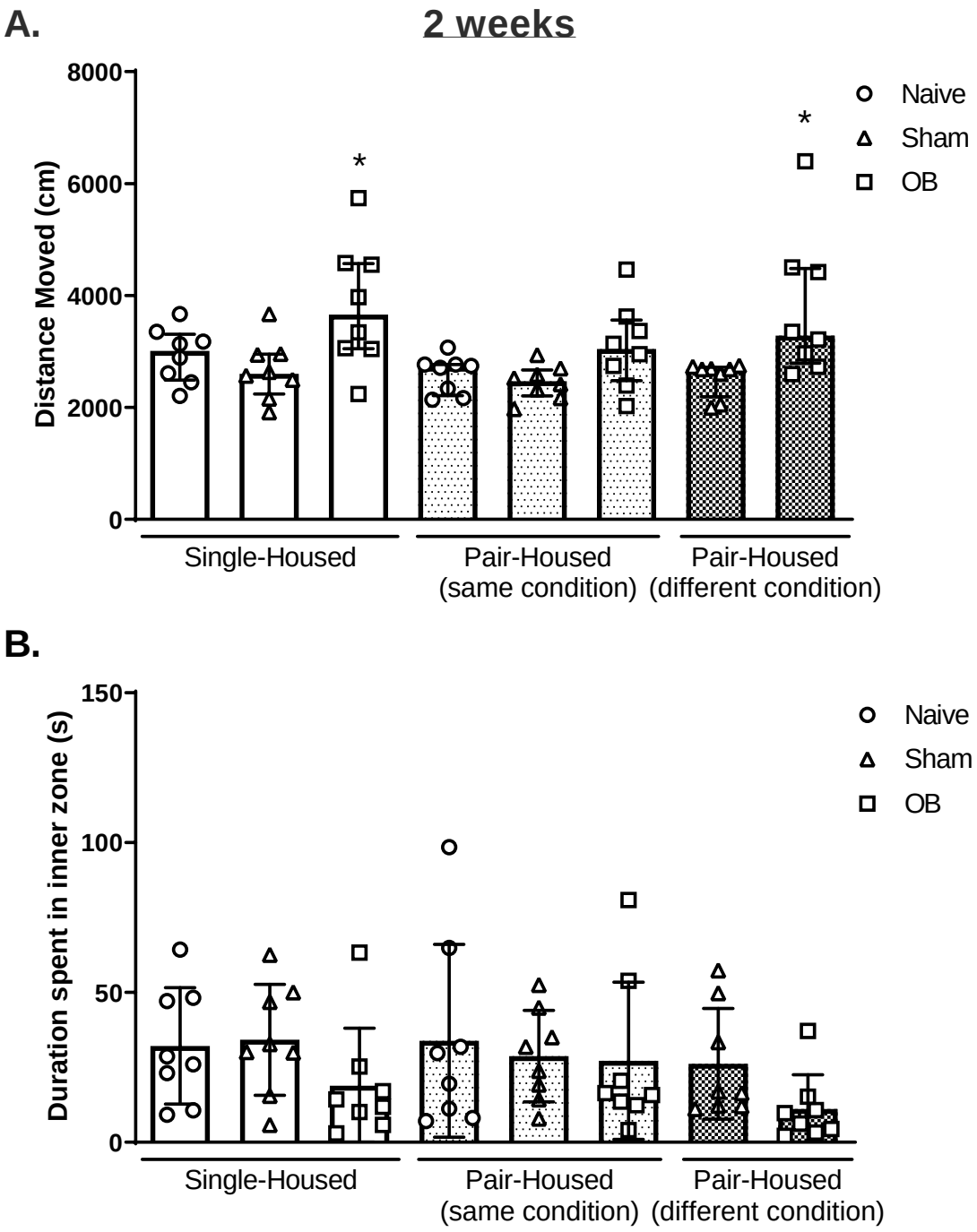


Figure 3.12 Distance moved in the open field (A) and duration spent in the inner zone (B) of the open field by naïve, sham-operated and OB rats two weeks post-surgery. The bars of singly-housed rats are shaded in white, pair-housed rats (of the same condition) are shaded in spotted grey pattern, and pair-housed rats (of different conditions) are shaded in darker black pattern. (A) Singly-housed OB rats, and OB rats who were paired with a sham-operated rat, moved significantly more than their appropriate sham-operated counterparts. (B) There was no significant difference between groups with regards time spent in the inner zone of the OF. Data are expressed as median ± interquartile range (A) and as mean ± standard deviation (B) (n=8 per group). *p<0.05 vs. appropriate sham-operated counterpart.

3.3.1.4.3 Distance moved in the open field (five weeks post-surgery)

With regards distance moved in the OF five weeks post-surgery, two-way ANOVA revealed that there was a significant effect of housing [$F_{(1,58)}$=9.043, p=0.004], but no effect of surgery [$F_{(2,58)}$=1.565, p=0.218], or effect of surgery x housing interaction [$F_{(2,58)}$=0.161, p=0.852] (Figure 3.13A). *Post-hoc* analysis revealed no significant difference between experimental conditions.

3.3.1.4.4 Time spent in the inner circle of the open field (five weeks post-surgery)

Time spent in the inner zone was found to be non-parametric as it failed Levene's test for homogeneity of variance and Shapiro-Wilks test of normality, as such Kruskal-Wallis followed by *post-hoc* with Mann-Whitney U tests were performed. A significant difference in time spent in the inner zone of the OF was found between groups [K(7)=15.577, p=0.029] (Figure 3.13B). *Post-hoc* analysis revealed that OB rats, when paired together with another OB rat, spent significantly less time in the inner zone of the OF when compared to pair-housed sham-operated rats (p<0.05 with Bonferroni correction (p<0.016)).

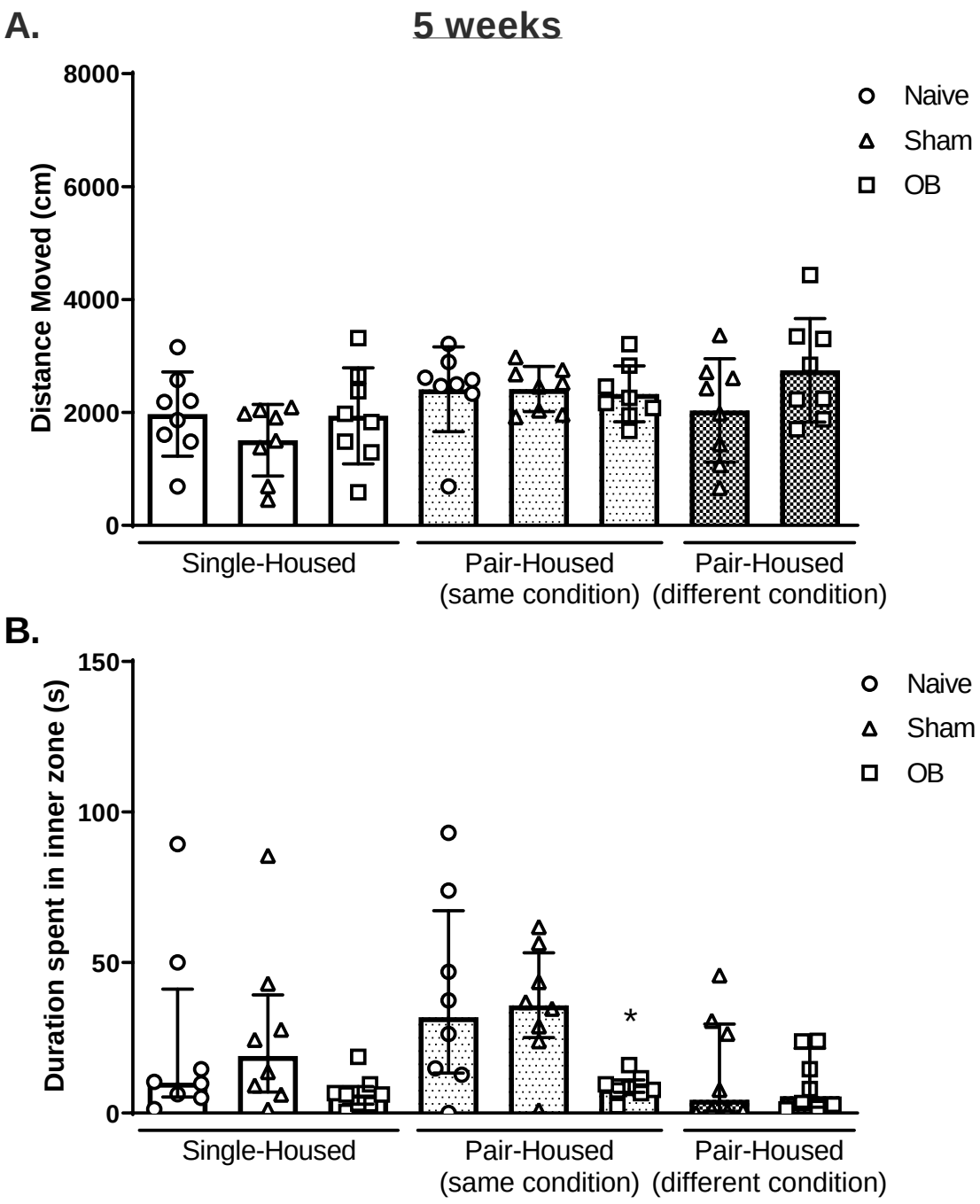


Figure 3.13 Distance moved in the open field (A) and duration spent in the inner zone (B) of the open field by naïve, sham-operated and OB rats five weeks post-surgery. The bars of singly-housed rats are shaded in white, pair-housed rats (of the same condition) are shaded in spotted grey pattern, and pair-housed rats (of different conditions) are shaded in darker black pattern. (A) The was no significant difference between groups with regards distance moved in the OF (B) OB rats, when paired together with another OB rat, spent significant less time in the inner zone of the OF when compared to pair-housed sham-operated rats. Data are expressed as mean ± standard deviation (A) and median ± interquartile range (B) (n=8 per group). *p<0.05 vs. appropriate sham-operated counterpart.

3.3.1.5 3-Chamber Sociability Test Results

3.3.1.5.1 Distance moved in the 3-chamber sociability test

Distance moved was analysed per trial using two-way ANOVA. With regards distance moved in the habituation trial, two-way ANOVA revealed that there was a significant effect of surgery [$F_{(2,58)}$=3.290, p=0.044] and a significant effect of housing [$F_{(1,58)}$=14.566, p<0.001], but that there was no surgery x housing interaction effect [$F_{(2,58)}$=0.802, p=0.453] (Figure 3.14). *Post-hoc* analysis revealed that there was no significant difference between experimental groups.

With regards distance moved in the sociability trial, two-way ANOVA revealed that there was no significant effect of surgery [$F_{(2,58)}$=2.131, p=0.128], housing [$F_{(1,58)}$=3.827, p=0.055], or any surgery x housing interaction effect [$F_{(2,58)}$=0.684, p=0.509] (Figure 3.14).

Lastly, with regards distance moved in the social preference trial, two-way ANOVA revealed that there was a significant effect of surgery [$F_{(2,58)}$=12.020, p<0.001], but no effect of housing [$F_{(1,58)}$=1.343, p=0.251], and no surgery x housing interaction [$F_{(2,58)}$=2.307, p=0.109] (Figure 3.14). *Post-hoc* analysis revealed that OB rats, when pair-housed, regardless of who they are paired with, move significantly less than their appropriate pair-housed sham-operated counterparts (p<0.05).

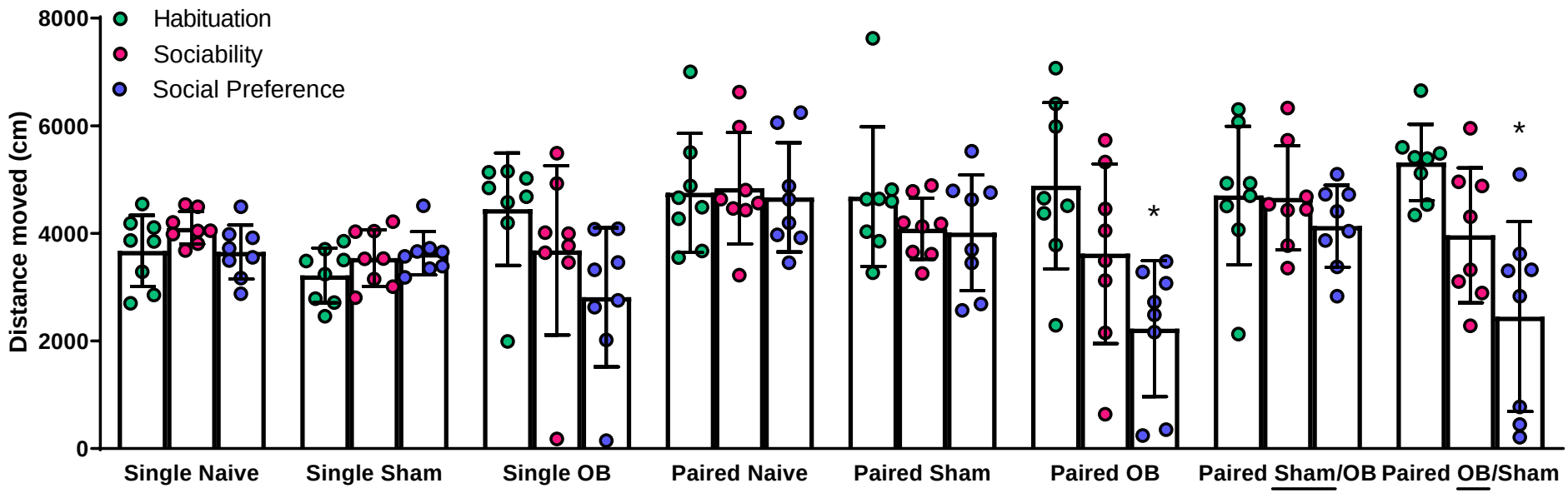


Figure 3.14 Distance moved in the habituation, sociability and social preference trial of the 3-chamber sociability test. The three white bars for each experimental group represent the distance moved per group per trial. The distance moved in the habituation trial is shaded in green, the sociability trial in pink, and the social preference trial is in blue. There was no significant difference between groups with regards distance moved in the habituation or sociability trial. However in the social preference trial, OB rats who were pair-housed, regardless of cagemate, moved significantly less than pair-housed sham-operated counterparts. Data are expressed as mean ± standard deviation (n=8 per group). *p<0.05 vs. appropriate sham-operated counterpart.

3.3.1.5.2 % Habituation in the 3-chamber sociability test

As a result of the pattern for OB rats to display a decreased distance moved across the 30 minute period of testing in the 3-chamber sociability test, it was decided to examine and express the data in terms of % habituation to the arena, i.e. to see if there was a difference in % habituation to the arena across surgery groups and housing conditions, but particularly with regards to OB rats. The % habituation score was calculated as described in the methods section 3.2.9.

With regards % habituation, two-way ANOVA revealed that there was a significant effect of surgery [$F_{(2,58)}$=17.731, p<0.001] and a significant effect of housing [$F_{(1,58)}$=5.691, p=0.020], but that there was no surgery x housing interaction effect [$F_{(2,58)}$=0.802, p=0.453] (Figure 3.15). *Post-hoc* analysis revealed that all OB rats, regardless of housing condition, scored significantly lower in % habituation, indicating that OB rats habituate to the arena over time in comparison to sham-operated and naïve rats (p<0.05).

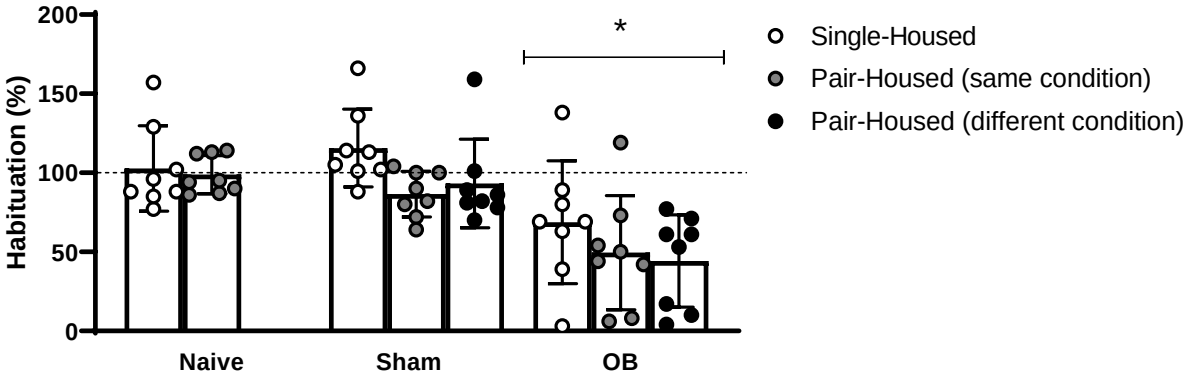


Figure 3.15 % Habituation of naïve, sham-operated and OB rats under singly- or pair-housing conditions in the 3-chamber sociability test. Singly-housed rats are shaded in white circles, pair-housed rats (of the same condition) are shaded in grey circles, and pair-housed rats (of different conditions) are shaded in black circles. OB rats, regardless of housing condition, had significantly lower % habituation scores in comparison to sham-operated and naïve rats. Data are expressed as mean ± standard deviation (n=8 per group). *p<0.05 vs. appropriate sham-operated counterpart.

3.3.1.5.3 *Sociability Trial:* Time spent interacting with the empty cage and novel animal

With regards time spent interacting with the empty cage in the sociability trial, two-way ANOVA revealed that there was a significant effect of surgery [$F_{(2,56)}$=3.908, p=0.026], but no effect of housing [$F_{(1,56)}$=0.545, p=0.463], or a surgery x housing interaction effect [$F_{(2,56)}$=0.311, p=0.734] (Figure 3.16). *Post-hoc* analysis revealed that there was no significant difference between the experimental groups.

With regards time spent interacting with the novel animal in the sociability trial, two-way ANOVA revealed that there was a significant effect of surgery [$F_{(2,56)}$=14.562, p<0.001], but no effect of housing [$F_{(1,56)}$=0.363, p=0.549], or a surgery x housing interaction effect [$F_{(2,56)}$=0.062, p=0.940] (Figure 3.16). *Post-hoc* analysis revealed that OB rats, when singly-housed and paired with one another, spent significantly less time interacting with the novel animal in comparison to their appropriate sham-housed counterparts, in the sociability trial (p<0.05).

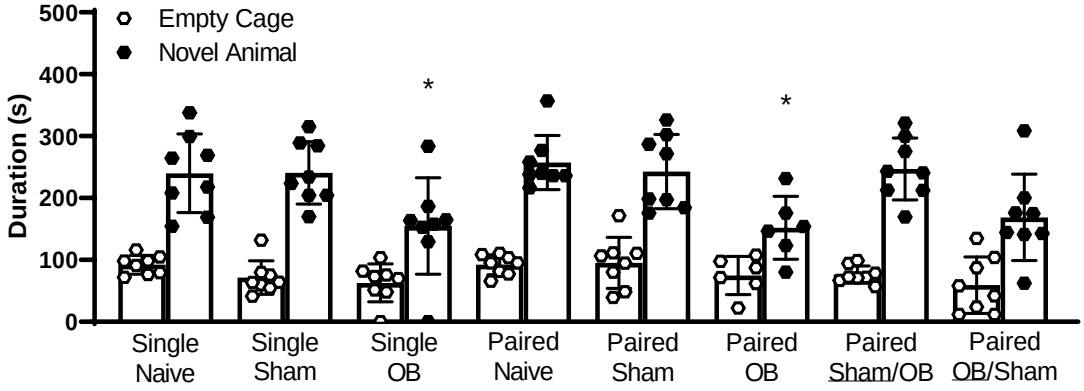


Figure 3.16 Duration spent interacting with the empty cage and the novel animal in the sociability trial. The circles that are shaded in white represent time spent interacting with the empty cage and the circles that are shaded in black represent time spent interacting with the novel animal. OB rats, when singly-housed or pair-housed with one another, spent significantly less time interacting with the novel animal in comparison to their appropriate sham-housed counterparts. There was no significant difference between experimental groups with regards time spent interacting with the empty cage. Data are expressed as mean ± standard deviation (n=8 per group). *p<0.05 vs. appropriate sham-operated counterpart.

3.3.1.5.4 *Social Preference Trial:* Time spent interacting with the familiar animal and novel animal

With regards time spent interacting with the familiar animal in the social preference trial, two-way ANOVA revealed that there was no significant effect of surgery [$F_{(2,56)}$=1.100, p=0.340], housing [$F_{(1,56)}$=0.350, p=0.557], or a surgery x housing interaction [$F_{(2,56)}$=1.321, p=0.275] (Figure 3.17).

With regards time spent interacting with the novel animal in the social preference trial, two-way ANOVA revealed that there was a significant effect of surgery [$F_{(2,56)}$=22.995, p<0.001], but no effect of housing [$F_{(1,56)}$=0.133, p=0.717], or a surgery x housing interaction effect [$F_{(2,56)}$=1.010, p=0.371] (Figure 3.17). *Post-hoc* analysis revealed that all OB rats, regardless of housing conditions, spent significantly less time interacting with the novel animal in comparison to their appropriate sham-operated counterparts, in the social preference trial (p<0.05).

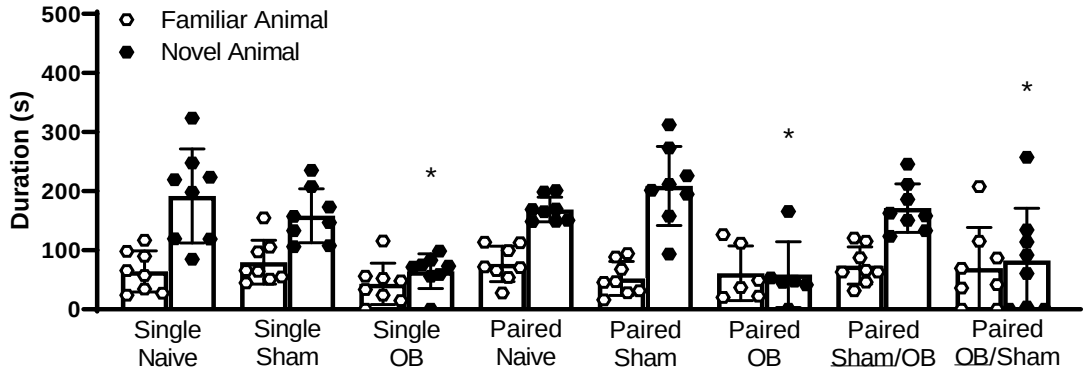


Figure 3.17 Duration spent interacting with the familiar animal and the novel animal in the social preference trial. The circles that are shaded in white represent time spent interacting with the familiar animal and the circles that are shaded in black represent time spent interacting with the novel animal. OB rats, regardless of housing condition, spent significantly less time interacting with the novel animal in comparison to their sham-operated counterparts. There was no significant difference between experimental groups with regards time spent interacting with the familiar animal. Data are expressed as mean ± standard deviation (n=8 per group). *p<0.05 vs. appropriate sham-operated counterpart.

3.3.1.6 Opioid receptor mRNA expression in the hippocampus, amygdala and nucleus accumbens

Opioid receptor mRNA expression was only looked at in four of the eight experimental groups; singly-housed sham-operated, singly-housed OB, OB rats who were paired with sham-operated animals, and likewise sham-operated rats who had been pair-housed with OB rats. There were several reasons for this. Firstly, the opioid system profile for singly-housed sham-operated and OB animals was investigated as we wanted to see if there was any difference under the current housing conditions that are used for OB rats in our laboratory. Second, going forward it was decided that if the OB profile was maintained, a pair-housed scenario would be utilised in future studies rather than singly-housing the animals. Thirdly, all behavioural data was scored first, and as a result of the blunted hyperactivity in OB rats who had been paired together, it was decided that going forward all experimental studies would use OB rats who had been paired with sham-operated rats to negate the necessity of singly-housing animals, and as such in the interest of time, only the OB rats who were paired with sham-operated rats, and likewise sham-operated rats who had been pair-housed with OB rats were assessed for their opioid receptor mRNA expression profile.

3.3.1.6.1 MOP (*Oprm1*) mRNA expression in discrete brain regions

With regards *Oprm1* mRNA expression in the amygdala, two-way ANOVA revealed that there was a significant effect of housing and a significant surgery x housing interaction effect (Table 3.6). *Post-hoc* analysis revealed that singly-housed OB rats had increased *Oprm1* mRNA expression in the amygdala when compared to singly-housed sham-operated counterpart, and to paired OB rats ($p<0.05$).

Two-way ANOVA revealed that there was no significant difference (surgery, housing, surgery x housing interaction effect) in *Oprm1* mRNA expression between sham-operated and OB rats in the hippocampus or nucleus accumbens (Table 3.6).

Group	Hippocampus	Amygdala	Nucleus Accumbens
Single Sham	1 ± 0.25	1 ± 0.30	1 ± 0.4
Single OB	0.91 ± 0.17	**1.62 ± 0.53***	1.01 ± 0.35
Paired Sham/OB	1.06 ± 0.38	0.95 ± 0.32	1.07 ± 0.22
Paired OB/Sham	0.91 ± 0.27	**0.90 ± 0.15+**	0.97 ± 0.30
Effect			
Surgery	$F_{(1,26)}$=1.113, p=0.301	$F_{(1,23)}$=3.472, p=0.075	$F_{(1,26)}$=0.121, p=0.730
Housing	$F_{(1,26)}$=0.068, p=0.797	**$F_{(1,23)}$=6.150, p=0.021**	$F_{(1,26)}$=0.015, p=0.903
Surgery x Housing	$F_{(1,26)}$=0.060, p=0.808	**$F_{(1,23)}$=4.634, p=0.042**	$F_{(1,26)}$=0.184, p=0.672

Table 3.6 MOP (Oprm1) mRNA expression in discrete brain regions. *Oprm1* mRNA expression is expressed as a percentage of the single-housed sham-operated group. *Oprm1* mRNA expression was significantly increased in the amygdala of singly-housed OB rats, when compared to singly-housed sham-operated rats, and pair-housed OB rats. There was no significant difference in *Oprm1* mRNA expression between sham-operated and OB rats in the hippocampus and nucleus accumbens. Data are expressed as mean ± standard deviation (n=6-8 per group). *$p<0.05$ vs. appropriate sham-operated counterpart, +$p<0.05$ vs. single OB.

3.3.1.6.2 KOP (*Oprk1*) mRNA expression in discrete brain regions

With regards *Oprk1* mRNA expression in the amygdala, two-way ANOVA revealed that there was a significant effect of surgery, but no effect of housing, or a surgery x housing interaction, (Table 3.7). *Post-hoc* analysis revealed that singly-housed OB rats had increased *Oprk1* mRNA expression in the amygdala when compared to singly-housed sham-operated counterpart ($p<0.05$).

Two-way ANOVA revealed that there was no significant difference (surgery, housing, surgery x housing interaction effect) in *Oprk1* mRNA expression between sham-operated and OB rats in the hippocampus, or nucleus accumbens (Table 3.7).

Group	Hippocampus	Amygdala	Nucleus Accumbens
Single Sham	1 ± 0.53	1 ± 0.50	1 ± 0.21
Single OB	0.84 ± 0.33	**2.33 ± 0.87***	0.79 ± 0.33
Paired Sham/OB	0.96 ± 0.35	1.10 ± 0.73	0.92 ± 0.18
Paired OB/Sham	1.03 ± 0.54	1.59 ± 0.92	0.95 ± 0.31
Effect			
Surgery	$F_{(1,25)}=0.063$, $p=0.803$	**$F_{(1,24)}=8.177$, $p=0.009$**	$F_{(1,26)}=0.728$, $p=0.401$
Housing	$F_{(1,25)}=0.199$, $p=0.660$	$F_{(1,24)}=1.006$, $p=0.326$	$F_{(1,26)}=0.147$, $p=0.705$
Surgery x Housing	$F_{(1,25)}=0.435$, $p=0.516$	$F_{(1,24)}=1.708$, $p=0.204$	$F_{(1,26)}=1.345$, $p=0.257$

Table 3.7 KOP (*Oprk1*) mRNA expression in discrete brain regions. *Oprk1* mRNA expression is expressed as a percentage of the single-housed sham-operated group. *Oprk1* mRNA expression was significantly increased in the amygdala of singly-housed OB rats, when compared to singly-housed sham-operated rats. There was no significant difference in *Oprk1* mRNA expression between sham-operated and OB rats in the hippocampus and nucleus accumbens. Data are expressed as mean ± standard deviation (n=6-8 per group). *$p<0.05$ vs. appropriate sham-operated counterpart.

3.3.1.6.3 DOP (*Oprd1*) mRNA expression in discrete brain regions

With regards *Oprd1* mRNA expression in the amygdala, two-way ANOVA revealed that there was a significant effect of surgery, but no effect of housing or a surgery x housing interaction, (Table 3.8). *Post-hoc* analysis revealed that there was no significant difference between the groups.

Two-way ANOVA revealed that there was no significant difference (surgery, housing, surgery x housing interaction effect) in *Oprd1* mRNA expression between sham-operated and OB rats in the hippocampus or nucleus accumbens (Table 3.8).

Group	Hippocampus	Amygdala	Nucleus Accumbens
Single Sham	1 ± 0.21	1 ± 0.37	1 ± 0.23
Single OB	1.01 ± 0.33	1.43 ± 0.27	0.85 ± 0.11
Paired Sham/OB	1.06 ± 0.33	1.03 ± 0.15	0.98 ± 0.27
Paired OB/Sham	1.04 ± 0.35	1.22 ± 0.22	0.97 ± 0.29
Effect			
Surgery	$F_{(1,25)}$=0.004, p=0.950	**$F_{(1,22)}$=7.307, p=0.013**	$F_{(1,26)}$=0.817, p=0.374
Housing	$F_{(1,25)}$=0.122, p=0.730	$F_{(1,22)}$=0.627, p=0.437	$F_{(1,26)}$=0.273, p=0.605
Surgery x Housing	$F_{(1,25)}$=0.014, p=0.905	$F_{(1,22)}$=1.096, p=0.306	$F_{(1,26)}$=0.555, p=0.463

Table 3.8 DOP (*Oprd1*) mRNA expression in discrete brain regions. *Oprd1* mRNA expression is expressed as a percentage of the single-housed sham-operated group. There was no significant difference in *Oprd1* mRNA expression between sham-operated rats and OB rats in the hippocampus, amygdala and nucleus accumbens. Data are expressed as mean ± standard deviation (n=6-8 per group).

3.3.2 *Experiment 2:* Effects of chronic dosing with fluoxetine (FLX) and desipramine (DMI), on social cognition in the OB rat model of depression

3.3.2.1 Distance moved in the 3-chamber sociability test

Distance moved was analysed per trial using two-way ANOVA, unless data was non-parametric in which a Kruskal-Wallis test was used. With regards distance moved in the habituation trial, two-way ANOVA revealed that there was no significant effect of surgery [$F_{(1,42)}$=2.339, p=0.134], drug [$F_{(2,42)}$=0.977, p=0.385], or a surgery x drug interaction effect [$F_{(2,42)}$=0.675, p=0.515] (Figure 3.18).

With regards distance moved in the sociability trial, two-way ANOVA revealed that there was a significant effect of surgery [$F_{(1,42)}$=24.445, p<0.001], but no effect of drug [$F_{(2,42)}$=2.475, p=0.096], or any surgery x drug interaction effect [$F_{(2,42)}$=1.659, p=0.203] (Figure 3.18). *Post-hoc* analysis revealed that fluoxetine caused a significant decrease in distance moved in OB rats when compared to OB vehicle-treated rats in the sociability trial (p<0.05). It was also shown that OB rats treated with fluoxetine and desipramine moved significantly less when compared to their sham-treated counterparts in the sociability trial (p<0.05).

Lastly, distance moved in the social preference trial was found to be non-parametric as it failed Levene's test for homogeneity of variance and Shapiro-Wilks test of normality, as such Kruskal-Wallis was used. There was a significant difference between groups with regards distance moved in the social preference trial [K(5)=17.494, p=0.104] (Figure 3.18). *Post-hoc* analysis revealed that desipramine-treated OB rats moved significantly less than sham-operated rats treated with desipramine (p<0.05 with Bonferroni correction (p<0.016)).

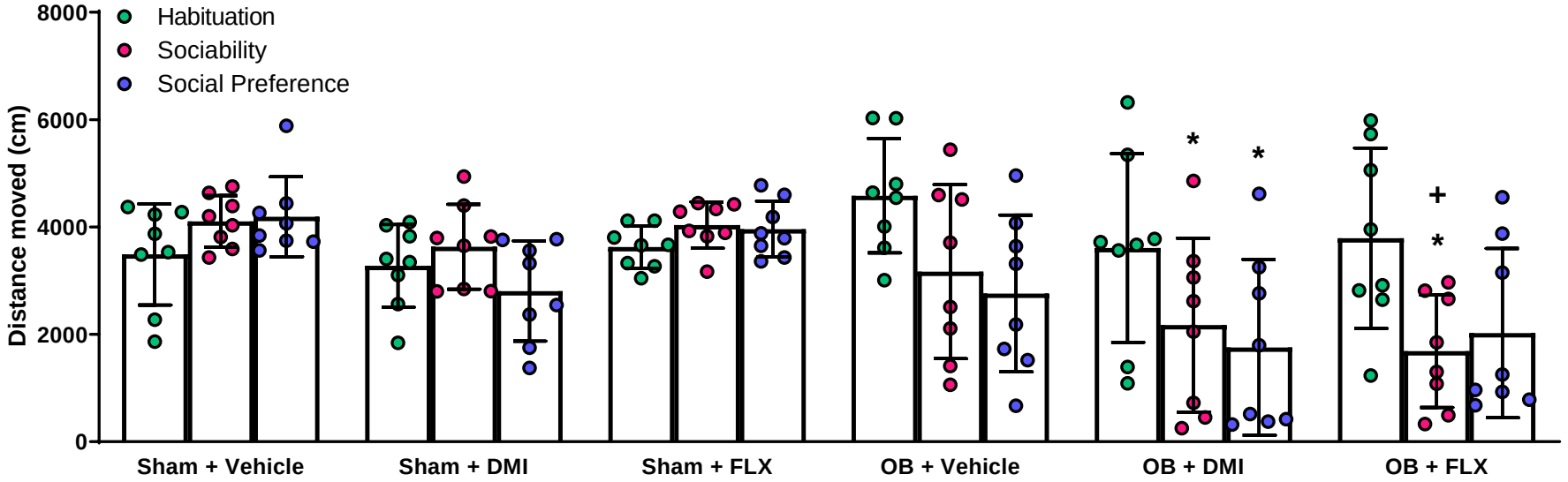


Figure 3.18 Distance moved in the habituation, sociability and social preference trial of the 3-chamber sociability test. The three white bars for each experimental group represent the distance moved per group per trial. The distance moved in the habituation trial is shaded in green, the sociability trial in pink, and the social preference trial is in blue. There was no significant difference between groups with regards distance moved in the habituation trial. In the sociability trial, fluoxetine and desipramine caused a significant decrease in distance moved in OB rats when compared to sham-treated counterparts. Fluoxetine also caused a significant decrease in OB rats when compared to vehicle-treated OB rats. In the social preference trial, desipramine also caused a decrease in distance moved in OB rats when compared to sham-treated counterparts. Data are expressed as mean ± standard deviation (n=8 per group). *p<0.05 vs. appropriate sham-treated counterpart; +p<0.05 vs. OB + Vehicle group.

3.3.2.2 % Habituation in the 3-chamber sociability test

Similar to *experiment one*, a trend was displayed for OB rats to have a reduction in distance moved across the 30 minute period of testing in the 3-chamber sociability test. As a result, it was again decided to examine and express the data in terms of % habituation to the arena, i.e. to see if there was a difference in % habituation to the arena with regards to OB rats, and to see if this pattern significantly matched the pattern in *experiment one*. The % habituation score was calculated as described in the methods section 3.2.9.

With regards % habituation in the 3-chamber sociability test, two-way ANOVA revealed that there was a significant effect of surgery [$F_{(1,42)}$=42.319, p<0.001] and a significant effect of drug [$F_{(2,42)}$=4.071, p=0.024], but that there was no surgery x drug interaction effect [$F_{(2,42)}$=0.895, p=0.416] (Figure 3.19). *Post-hoc* analysis revealed that all OB rats, regardless of drug treatment, scored significantly lower in % habituation, indicating that OB rats habituate to the arena over time in comparison to sham-operated rats (p<0.05). Desipramine also caused a significantly lower % habituation score in sham-operated rats when compared to vehicle-treated counterparts (p<0.05).

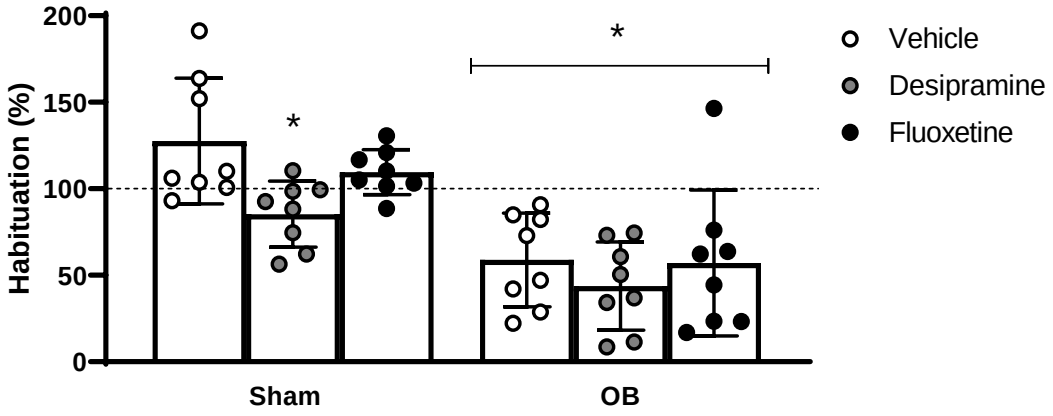


Figure 3.19 % Habituation of sham-operated and OB rats in the 3-chamber sociability test after chronic dosing with vehicle, desipramine or fluoxetine. Vehicle-treated rats are shaded in white circles, desipramine-treated rats are shaded in grey circles, and fluoxetine-treated rats are shaded in black circles. OB rats, regardless of drug treatment, had significantly lower % habituation scores in comparison to sham-operated rats. Desipramine also lowered % habituation in sham-operated rats when compared to sham-operated vehicle-treated animals. Data are expressed as mean ± standard deviation (n=8 per group). *p<0.05 vs. appropriate sham-treated counterpart.

3.3.2.3 *Sociability Trial:* Time spent interacting with the empty cage and novel animal

With regards time spent interacting with the empty cage in the sociability trial, two-way ANOVA revealed that there was a significant effect of surgery [$F_{(1,42)}$=15.901, p<0.001], but no significant effect of drug [$F_{(2,42)}$=2.184, p=0.125], or any surgery x drug interaction effect [$F_{(2,42)}$=2.034, p=0.143] (Figure 3.20). *Post-hoc* analysis revealed that fluoxetine caused a reduction in time spent interacting with the empty cage in OB rats in comparison to sham-operated animals (p<0.05).

With regards time spent interacting with the novel animal in the sociability trial, two-way ANOVA revealed that there was a significant effect of surgery [$F_{(1,42)}$=65.820, p<0.001], drug [$F_{(2,42)}$=3.801, p=0.030], and a surgery x drug interaction [$F_{(2,42)}$=4.048, p=0.025] (Figure 3.20). *Post-hoc* analysis revealed that in the sociability trail all OB rats, regardless of drug treatment, spent significantly less time interacting with the novel animal in comparison to their sham-operated counterparts (p<0.05). Fluoxetine also caused a significant decrease in time spent interacting with the novel animal in sham-operated rats when compared to vehicle-treated rats (p<0.05).

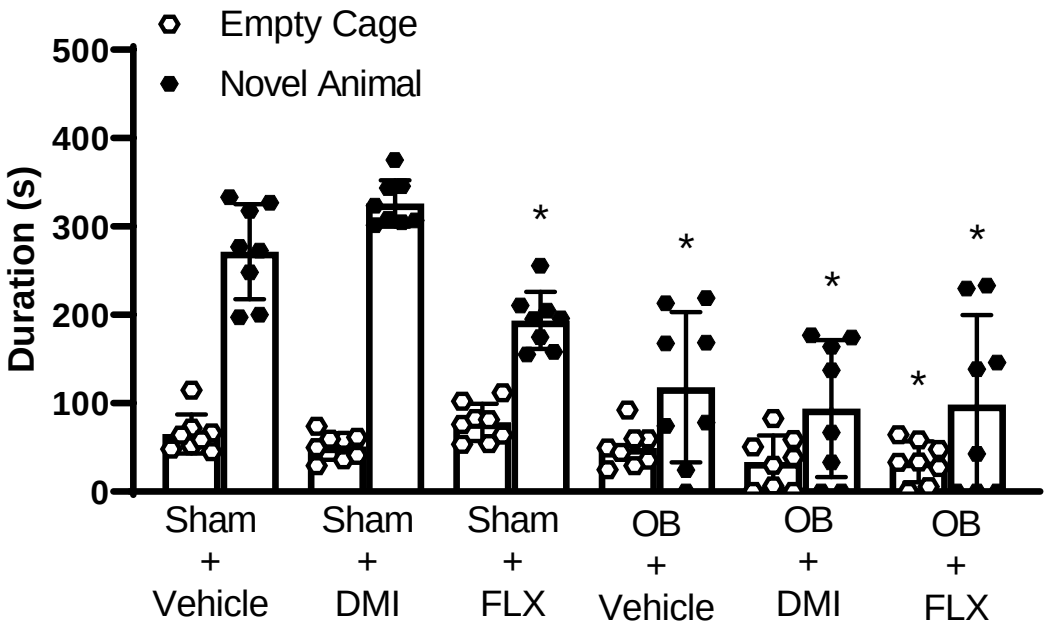


Figure 3.20 Duration spent interacting with the empty cage and the novel animal in the sociability trial. The circles that are shaded in white represent time spent interacting with the empty cage and the circles that are shaded in black represent time spent interacting with the novel animal. Fluoxetine caused a significant decrease in the time spent interacting with the empty cage in OB rats when compared to sham-operated animals. All OB rats, regardless of drug treatment, spent significantly less time interacting with novel animal in comparison to sham-operated counterparts. Fluoxetine also caused a significant decrease in time spent interacting with the novel animal in sham-operated animals when compared to vehicle-treated sham-operated rats. Data are expressed as mean ± standard deviation (n=8 per group). *p<0.05 vs. appropriate sham-treated counterpart.

3.3.2.4 *Social Preference Trial:* Time spent interacting with the familiar animal and novel animal

Time spent interacting with the familiar animal was found to be non-parametric as it failed Levene's test for homogeneity of variance and Shapiro-Wilks test of normality, as such Kruskal-Wallis followed by *post-hoc* with Mann-Whitney U tests were performed. A significant difference in time spent interacting with the familiar animal was found between groups [$K(5)=22.537$, $p<0.001$] (Figure 3.21). *Post-hoc* analysis revealed that desipramine caused a significant decrease in time spent interacting with the familiar animal in OB rats when compared to sham-treated rats ($p<0.05$ with Bonferroni correction ($p<0.016$)).

Time spent interacting with the novel animal was also found to be non-parametric as it failed Levene's test for homogeneity of variance and Shapiro-Wilks test of normality, as such Kruskal-Wallis followed by *post-hoc* with Mann-Whitney U tests were performed. A significant difference in time spent interacting with the novel animal was found between groups [$K(5)=20.808$, $p=0.001$] (Figure 3.21). *Post-hoc* analysis revealed that desipramine caused a significant decrease in time spent interacting with the novel animal in OB rats when compared to sham-treated rats ($p<0.05$ with Bonferroni correction ($p<0.016$)).

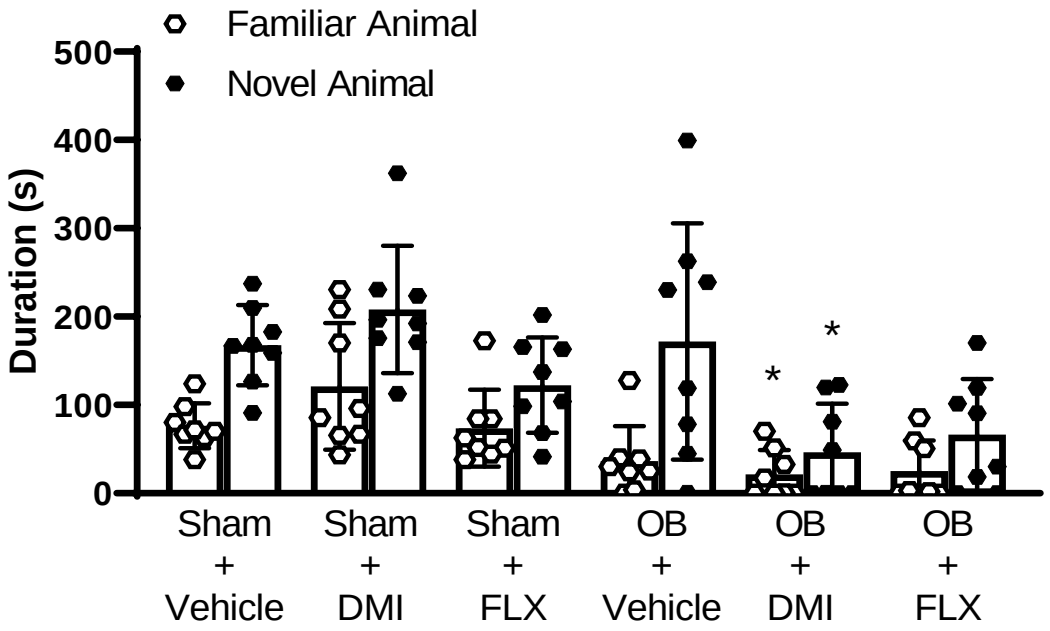


Figure 3.21 Duration spent interacting with the familiar animal and the novel animal in the social preference trial. The circles that are shaded in white represent time spent interacting with the familiar animal and the circles that are shaded in black represent time spent interacting with the novel animal. Desipramine caused a significant decrease in the time spent interacting with the familiar and novel animals in OB rats when compared to sham-treated rats. Data are expressed as median ± interquartile range (n=8 per group). *p<0.05 vs. appropriate sham-treated counterpart.

3.4 Discussion

The aim of this project is to assess the social cognitive and motivational deficits in the OB model with a view to use this paradigm to detect the antidepressant potential of opioid modulating drugs. In order to do so, the first step was to characterise the OB model in our laboratory and to assess social cognition, an attribute of depression which, to the best of our knowledge, has not been examined in the OB model to date. At the same time, it was decided to look at the effects of housing within the model, a feature that is detrimental in social functioning as isolation verses group-setting scenarios can effect consequential SI and processing. As mentioned in the introduction, this lab has a history of working with singly-housed animals and as such the effects of differential housing had never been assessed. By singly-housing an animal, isolation is introduced, which in itself could be regarding as inducing depression, and hypothetically might exacerbate or heighten any depressive- and anxiety-like behaviours. In animal research, there has been a recent movement towards minimising singly-housing animals in response to animal well-being and animal welfare authorities (ILAR, 2011, NC3Rs, 2019). As such, the purpose of re-characterising the OB model under housing condition, and ascertaining any differential effects on behaviours as a result of housing parameters was undertaken. In addition to these two main aims, we also wanted to assess whether; the hyperactivity in the OF in OB animals would be affected by housing conditions, whether there would be an effect of housing conditions on anxiety-like behaviour via examination in the EPM, and lastly, the effect of OB surgery and differential housing on the opioid system.

Therefore, the focus of our first experiment was to assess the effects of singly- verses pair-housing in naïve, sham-operated and OB animals, with regards social cognition, anxiety-like behaviour, locomotion in the OF and effects to the opioid system. The aim of using naïve rats was to assess whether sham operation itself, i.e. undergoing the surgical operation, has an effect on the behaviour and biochemical disposition of the animal. Hence, a naïve or non-operated group was added, and singly-housed permutations for both naïve rats and sham-operated rats were also included. Overall, it was evident that naïve rats and sham-operated rats behaved similarly to each other in all behaviours examined, irrespective of housing condition. A similar finding was observed by Qu et al. (2015), who found no differences in behaviour of sham-operated and non-operated rats after exposure to the OF, SPT and FST. This

indicates that sham-operated animals can fully be accepted as negative controls in animal research, and that exposure to sham surgery and anaesthetic procedure does not alter the behavioural profile of rats when compared to animals who have not undergone such surgical manipulations. Care has been taken to improve the surgery procedure, aseptic technique and the post-operative care of the rats after OB to reduce any adverse effects upon recovery. As this reduction in bodyweight is not seen in the other OB rats housed under different conditions, it's logical to think that this particular housing condition of placing two OB rats together, is the underlying factor effecting the health and well-being of the animal.

With regards to bodyweight gain, the only change of note was a significant reduction in bodyweight gain in OB rats who had been pair-housed with another OB rat. OB lesioning has been shown to have no effect on bodyweight (Morales-Medina et al., 2012a; 2012b), or has been shown to cause a decrease in bodyweight (Saitoh et al., 2008). As such bodyweight data in the OB model can be variable, as can be seen with the three different OB groups in this study. As to why OB rats when paired with another OB animal showed a significant loss in weight gain, as is seen in the clinical scenario of depression, could be due to an aggravation in the syndrome, or even increased stress effect, when housed with a likened 'depressed' cagemate.

With regards to the EPM results, we found that singly-housed OB rats and OB rats which had been pair-housed with an OB cagemate, displayed an increase in distance moved in the EPM at two weeks post-surgery, but that this effect was blunted when OB rats were pair-housed with a sham-operated rat. The EPM uses a higher lux lighting in its two open arms and as such could be paralleled to the brightly lit anxiogenic environment that is created in the OF. Indeed, previous research in our lab has shown that that singly-housed OB rats, both male and female, display an increase in locomotion in the EPM (Doherty, unpublished), so perhaps the hyperlocomotion seen in these OB rats could be seen as similar to the classic hyperactivity that is associated with OB rats in the OF test. Holubova et al. (2016) also found an increase in distance moved in OB rats when compared to sham-operated rats in the EPM, however, they did not comment on how the animals were housed. Interestingly, in our study this wasn't the case with OB rats when pair-housed with a sham-operated rat. As mentioned in the introduction, OB animals have a history of irritability and aggression (Mucignat-Caretta et al., 2004, van Riezen et al., 1977) and perhaps by pairing two OB animals together we are exacerbating this effect, but this may not be

the case when OB rats are pair-housed with a sham-operated animal. It was noted by the researcher in this study, that although blind to the experimental groups, OB rats that were pair-housed with one another play-fought more and often had a higher tendency towards aggression when being handled. With regards open arm time and entries, there was also no significant difference between sham-operated and OB rats in the duration spent in the open arms (seconds and percentages) or in the % open arm entries in the EPM. There was a significant difference in number of open arm entries though, with singly-housed OB rats and OB rats that were pair-housed with each other displaying a significant increase in open arm entries in comparison to sham-operated counterparts indicating anxiolytic-like behaviour in the EPM, and as has been found in many previous OB studies (Kalshetti et al., 2012; 2015a; 2015b, Saitoh et al., 2008). Indeed even OB rats who had been paired with sham-operated rats displayed a trend for an increase in open arm entries in comparison to sham-operated rats, however, in our experiment, it was not significant. In a study by Stepanichev et al. (2016), they also found no difference in the number of open arm entries made by OB rats when compared to sham-operated rats, as did McGrath and Norman (1998; 1999), so this grouping in this study isn't a standalone case, although again it didn't specify whether animals continued to be group-housed post-surgery.

With regards to OF testing, rats were tested in this apparatus at two time points; two weeks post-surgery and five week post-surgery. At two weeks post-surgery, a significant increase in locomotor activity was found in singly-housed OB rats and in OB rats that were pair-housed with sham-operated rats, as is well established in a number of OB animals in OB literature (Burke et al., 2013; 2015, Kalshetty et al., 2012, Roche et al., 2007; 2008). However, hyperactivity was blunted in OB rats who were paired with one another. It is interesting to note that this time-point of two weeks post-surgery, is also when the EPM was tested, and that these results in the OF at two weeks post-surgery could be taken in tandem with the EPM results, with the idea that the increase in distance moved in the EPM in the singly-housed OB group may be very similar to that of the increase in locomotion in this group in the OF. When pairing two OB rats together, we lost the hyperactivity in the OF. Few studies comment on housing OB animals in pairs alone, and if they are housed as two OB animals together, it is usually always with two sham-operated cagemates in order to reduce the antagonistic nature of the phenotype (Kelly et al., 1997). At five weeks post-surgery, there was no difference in naïve, sham-operated and OB rats with regards distance travelled in the

OF, i.e. there was a loss in hyperactivity in all of our OB groups, regardless of housing. Reasons for this may include the fact that this is a second exposure to the OF and as such it is no longer a completely novel environment and may no longer be as aversive to the animals as they are now familiar with the apparatus. In conjunction with this, animals had also underwent two other different behavioural tests in the meantime and prior to this second exposure to the OF, and so exposure to these tests could also have an effect. Re-exposure to the OF in our laboratory, has been shown to attenuate the hyperactive effect in OB animals, with the idea that OB rats perhaps habituate to the environment on repeated exposure. A loss of hyperactivity in OB animals over-time in the OF has also been shown in other studies, with a loss in novelty and a pronounced habituation effect being thought to be the cause (Holubova et al., 2016, Zueger et al., 2005). Holubova et al. (2016) has also shown that on a ten minute exposure in the OF test, OB rats display hyperactivity in the first two minutes of exposure but that by the last two minutes this hyperactivity is lost or normalised to sham-operated rats, describing this as OB rats becoming habituated to the environment. Gigliucci et al. (2014) also showed that rats repeatedly tested in the OF at 14, 21 and 28 days post-surgery, had a reduction in hyperactivity over the 3 time-points, and a complete loss of significant difference from sham-operated animals by 28 days post-surgery. In fact, in this experiment, it was noted that even some sham-operated and naïve groups appeared to move slightly less in the OF on this second exposure. With regards time spent in the inner zone of the OF, there was no difference between naïve, sham-operated and OB rats at two week post-surgery, but at five weeks post-surgery, OB rats who were pair-housed with other OB rats, spent significantly less time in the centre of the OF, indicating an anxiogenic effect in this model and an anxiety-like phenotype when compared to pair-housed sham-operated rats. This has been found previously in our lab (Burke et al., 2015), and is also reported in other OB animal studies (Zueger et al., 2005). Interestingly, when an OB rat was singly-housed or was pair-housed with a sham-operated rat, a trend for reduction in the inner zone was also seen, but this was not statistically significant. A lack of difference between sham-operated and OB animals in inner zone time has also been shown in OB studies (Kalshetti et al., 2015b, Burke et al., 2013). A previous study in our lab had a similar finding, with a trend for a reduction in time spent in the inner zone by singly-housed OB rats, but that this trend did not reach statistical significance (Burke et al., 2013). In another study by Burke et al. (2010), there was no significant difference between

sham-operated and OB rats in the time spent in the inner zone of the OF. Having said this, in comparison to exposure at week two, the trend for reduction in time spent in the inner zone by OB animals is interesting and although not significant, perhaps this is again in line with a habituation effect; in that OB animals recognise this apparatus and have learnt not to spend as much time in the centre zone due to its aversive nature. This lack of significance in the singly- and pair-housed (different conditions) OB groups may also be as a result of the movement made by their specific sham-operated counterparts groups rather than as a result of the OB movement *per se*. For example, sham-operated rats who were paired with OB animals appear to display a prominent reduction in inner zone time in comparison to other sham-operated groups. As to why this may be is unknown, but it is likely that these animals have also habituated to the environment.

Finally, we move on to the 3-chamber sociability test results, and as such social cognition in the OB model of depression. It was hypothesised that as a well-established model of depression, the OB animal model may have social cognitive deficits, and as a result, a number of parameters within the test were used to assess this (Gigliucci et al., 2014). Firstly, we looked at distance moved over the three trials, hypothesising that the distance moved in the habituation trial would be of interest as it could be compared to the novel OF arena, minus the bright lux lighting and the aversive reflective walls. However, no significant difference was found in this trial or the sociability trial, but a decrease in distance moved in the social preference trial in paired OB rats, regardless of who they were paired with, was found. This effect was not seen in singly-housed OB animals, although a similar trend in reduced distance travelled was evident. Interestingly, this was also the case with OB vehicle-treated rats in the antidepressant study (also singly-housed), which could be said to be comparable to the singly-housed OB rats in the housing study. The lack of significant reduction in the singly-housed OB rats in both studies, when compared to the paired OB groups, could be due to the idea that to the singly-housed OB animal all exposures to another animal are more novel than to that of an OB who is use to interacting with his other cagemate. With this in mind, a singly-housed OB rat may be more motivated to travel a distance to visit a novel conspecific than that of an OB rat who is not already unaccustomed to SI.

A pattern in OB rats in both studies was noticed upon analysis of the locomotion data, in that all OB rats, regardless of housing and drug treatment,

appeared to have an overall decrease in distance travelled in the arena over time. In contrast, sham-operated rats, again in both studies, tended to explore the arena to a similar degree within each phase or trial tested. As a result, % habituation for naïve, sham-operated and OB animals was examined, and in both studies, regardless of housing or drug treatment, OB rats displayed a significant habituation to the arena over time in comparison to their appropriate sham-operated counterparts. As discussed above, OB animals have been proven to habituate to the OF over time, with a reduction in distance moved in the latter minutes of testing, along with an abolition of hyperactivity on repeated exposure (Gigliucci et al., 2014, Holubova et al., 2016, Zueger et al., 2005). Perhaps this habituation effect is a feature of the model that is not only restricted to the OF apparatus. As to what this reduction in distance moved over time might mean, is still open for debate, but it shows a lack in motivation to move, bearing resemblance to the fatigue, lack of motivation and lack of interest in clinical depression. Although as discussed above we examined % habituation overtime and found an effect in all our OB animals regardless of housing and treatment in both experiments, a two-way repeated measures could have also been valuable to run in addition to this, to address if there is a difference across the distanced moved behaviour in the three trials in these animals by the means of a different statistical test.

With regards to the sociability trial and time spent interacting with the novel animal, in the housing study there was a distinct trend for all OB animals to spend less time interacting with the novel animal in comparison to naïve and sham-operated animals, but only the OB rats who had been singly-housed, or paired with another OB rat were shown to be significantly different. In the antidepressant study, in which all OB animals were singly-housed, all OB rats, regardless of treatment spent significantly less time with the novel animal when compared to sham-operated counterparts. Firstly, this reduction in time spent with the novel animal in OB animals when compared to sham-operated rats could be analogous to the social dysfunction and loss in social responding in patients with MDD. Patients with MDD display social withdrawal and diminished social interaction, so the reduction in time with the novel animal shown by OB animals in comparison to sham-operated rats could mirror this socially impaired symptom in everyday-life in depressed individuals when compared to healthy individuals. In conjunction with this, this sociability deficit in the OB model was not attenuated with chronic dosing with conventional antidepressants. The lack of effect in these two different classes of antidepressants in attenuating the sociability

deficit in OB animals could also be compared to the clinical situation where a lack of effect of antidepressants in attenuating social dysfunction in MDD is also seen. With this in mind, an additional feature of depression can be included as a novel facet in the OB model, with a unique social cognitive characteristic in the model which is not attenuated by chronic antidepressant treatment having been established. We found no differences in either study with regards time spent interacting with the empty cage, but this was expected as the magnitude of preference between an animate-being verses an inanimate object is much greater (Moy et al., 2004).

With regards to the social novelty/preference trial and time spent interacting with the now familiar animal verses the novel animal, in the housing study all OB animals, both singly- and pair-housed, spent significantly less time with the novel animal when compared to sham-operated counterparts. In the antidepressant study, we didn't find any difference between sham-operated and OB vehicle-treated animals with regards time spent interacting with the novel animal. As to why our singly-housed OB animals in each study display two different results is hard to say. The singly-housed OB rats in our antidepressant study would have been exposed to chronic injection of vehicle for 21 days and as such, had twice as much handling as the OB animals in the housing study. Moy et al. (2013) also comments that 'a drawback to the social novelty task is that the magnitude of preference between two stranger mice is typically less than the magnitude of preference between social and non-social stimuli in the sociability phase' so perhaps this is why our OB rats in this study (which displayed a significant deficit in sociability in the sociability trial), do not exhibit a deficit in social novelty in the social preference trial. On top of this, singly-housed vehicle-treated OB rats also display quite a bit more variability in their time spent exploring the novel animal in the social preference trial, whereas in the housing study the data is very tight in the singly-housed OB group. The deficit in social preference in the housing study across all OB animals, regardless of housing, displays a lack of social preference and deficit in social novelty and interaction in OB rats. Another explanation for this reduction in time spent with the second novel animal in comparison to sham-operated rats could be social avoidance, in that OB animals want to spend more time alone than time spent interacting. Another reason could also be memory deficit, in that OB animals spend less time with the novel animal in comparison to sham-operated rats as there differentiation between the novel conspecific and familiar conspecific is not as strong as in sham-operated and naïve

rats. It must restated that the 3-chamber sociability test can also be viewed as a memory test, with the third trial of the test looking at memory and recognition (Crawley, 2004).

In *experiment 2*, chronic antidepressant treatment with conventional antidepressants fluoxetine and desipramine had varying effects on social cognition in sham-operated and OB rats, but overall these two classes of antidepressants did not attenuate any of the SI deficits that were seen in OB rats. If anything, rather than attenuate the changes in social function and cognition in OB rats, both fluoxetine and desipramine, often had the opposite effect and intensified them. Desipramine in particular, appeared to have a negative effect on OB rats, and in some cases in sham-operated rats as well. In OB animals, desipramine caused a significant decrease in distance moved in the sociability and social preference trial, and a significant decrease in time spent interacting with the familiar and novel animal in the social preference trial, all when compared to sham-operated rats. It did not attenuate the % habituation in OB animals, or increase sociability in OB animals. In sham-operated animals, desipramine reduced % habituation in this group. Overall, desipramine could have had sedative effect with regards locomotion in the sociability and social preference trials. With regards cognitive function, chronic desipramine treatment has been shown to impair the performance of rats in the T-maze when compared to controls with a reduction in correct responses and an increase delay in time to choose the arm (Clinton et al., 2006). Acute dosing of 10 mg/kg of desipramine in rats has also been shown to impair performance in novel maze and familiar maze reconsolidation, with increased errors in both mazes when compared to controls and a negative effect on spatial memory (Watts et al., 2012). In another study, the effects of tetrabenazine, a monoamine transporter-type 2 inhibitor that induces depressive symptoms and reduces choice effort, was examined on effort-related and motivational behaviour in rats in an operant conditioning paradigm, with results showing that desipramine failed to reverse the effects of tetrabenazine, and actually worsened motivational behaviour by significantly suppressing lever presses to a more higher magnitude when given in combination, and even alone (Yohn et al., 2016). As a result, desipramine may be a poor choice to reverse the cognitive dysfunction seen in OB model due to its sedative effect and evidence of impairment of cognitive function.

The SSRI fluoxetine significantly reduced distance moved in the sociability trial in OB rats and also caused them to spend significantly less time interacting with

the empty cage. Fluoxetine did not attenuate % habituation scores, or time spent interacting with either of the novel conspecifics in the sociability or social preference trial. In the sociability trial fluoxetine actually reduced the time than sham-operated rats spent with the novel animal in comparison to vehicle, and reduced the time that OB animals spent interacting with the novel animal to a further degree in comparison to sham-operated rats. Interestingly, Moy et al. (2013) found similar results, in that acute dosing with 10 mg/kg of fluoxetine in mice decreased sniffing time towards the novel animal in the sociability test in comparison to vehicle-treated mice, indicating a loss in social approach and producing social avoidance or sedation. Fluoxetine has also been shown to decrease SI time in rats in a dose-dependent manner with 5-10 mg/kg of the SSRI causing a significant social avoidance and anxiogenic effect (Bagdy et al., 2001). Low dose of citalopram, another SSRI, has been shown to reduce SI in rats, indicating an enhancement of anxiety-like behaviour, anxiogenic action and encouragement of social avoidance (Dekeyne et al., 2000). With this in mind, perhaps SSRI's, such as fluoxetine, are not the correct therapeutic choice of drug for attenuating the social cognitive decline in this particular model. Indeed, in a study conducted in patients with MDD, Kasper et al. (1999) commented that reboxetine, a drug that targets the noradrenergic system, was shown to be much more effective in improving social functioning than fluoxetine, a serotonergic drug.

Lastly, with regards the discussion of behaviour in both experiments and as discussed previously, a number of behavioural endpoints were not consistently shown in both experiments and it must be noted that a limitation of the lack of change or effect in OB animals in both experiments, may be due to the extra handling and injection regimen. In *experiment 1*, animals were handled everyday but were not exposed to any injection procedure, or in this manner any added stress. In contrast in *experiment 2*, animals were also exposed to handling everyday, but had the addition of injection stress in the afternoon and in this manner also extra handling. In this regard, a limitation of comparing these two studies is the fact that the animals in one experiment received twice as much handling as the other, and were also exposed to added stress in the form of injection exposure. In turn this may have effected their behavioural responses in the tests examined. In hindsight, future studies could encompass injection stress with saline, and planned handling, so that both cohorts could be exactly comparable.

Lastly, the MOP, DOP and KOP mRNA expression was examined in three discrete limbic brain regions in sham-operated and OB rats in *experiment 1*. Overall, no significant differences in MOP, KOP or DOP mRNA expression between sham-operated and OB rats was found in the hippocampus or nucleus accumbens. In the amygdala, a surgery effect was found with regards the KOP and DOP receptors in that singly-housed OB rats displayed a significant elevated level of KOP mRNA in the amygdala in comparison to sham-operated counterparts, and a similar trend was seen in this group with DOP mRNA in this region, although this was not statistically significant. To the best of our knowledge, a significant elevated level of KOR mRNA has not been published in any OB literature to date. The examination of opioid expression in the OB model is a novel field and so very little evidence of consequential effects to the opioid system after OB exists thus far. In a study by Hirsch et al. (1980), a reduction in KOP and DOP opioid binding was seen at one week post-surgery in OB mice that was seen to return to baseline by four weeks post-surgery. There is a number of differences between our studies, not including the fact that the study by Hirsch et al. (1980) was performed in mice as opposed to rats, post-mortem changes were normalised by 4 weeks post-surgery, whereas ours occur at five weeks post-surgery, and lastly the fact that Hirsch reported on opioid receptor binding while this study examined mRNA expression. PET binding potential in patients with MDD has also revealed a decrease in KOP binding potential, indicating lower *in vivo* KOP availability, in the amygdala-anterior cingulate cortex-ventral striatal neural circuit (Pietrzak et al., 2014). The reason the elevation in KOP mRNA expression was only seen in singly-housed OB rats and not OB rats housed with sham-operated rats is unknown. One potential explanation could be that by singly-housing these animals for a prolonged period of time they were exposed to added stress, contributing to the elevated levels of KOP mRNA expression in the amygdala, a region associated with fear, survival instinct and emotion.

In conclusion, differential housing in rats had a number of effects, but overall sham-operated and naïve animals behaved in a similar manner. It was established that housing OB animals in pairs resulted in differential behavioural outputs that were often dependent upon the condition of their said cagemate. OB animals when paired together actually appeared to cause a heightening or intensification of their syndrome, producing increased anxiety-like behaviour in the OF, causing an increase in irritability (as noted by the handlers) and caused a loss in characteristic hyperactivity

in the OF. Housing two OB animals together also had a negative effect on bodyweight with a significant reduction in bodyweight in this group from two weeks post-surgery to sacrifice at five-weeks. There was very little negative effect on OB animals that were paired with sham-operated rats, and overall paired OB rats, regardless of housing with sham-operated rats or OB animals, showed similar social cognitive changes when tested in social cognitive parameters. Importantly, OB rats that were paired with sham-operated rats also maintained the classic hyperactivity in the OF. As a result, and in keeping with animal welfare authorities that wish to negate the necessity for singly-housing, this group and housing condition will be used in future experiments. The most prominent result from both experiments is that OB caused altered social cognition, with pronounced reduction in sociability and social preference, and a remarkable habituation to the test arena. These results add an additional behavioural alteration to the model, and one that was not seen to be altered by chronic antidepressant treatment. Other classes of drugs, whether antidepressant or not, may be needed in order to reverse or normalise these novel and unique social cognitive alterations seen in the OB model.

Chapter 4:

Alterations in the central opioid system following exposure to acute (forced swim) and chronic (olfactory bulbectomy) stressors in the rat

4.1 Introduction

Stress is a major component in psychiatric illness, such as depression. Stress can be defined as an interaction with a noxious or unpleasant stimulus that results in a number of behavioural and physiological responses that can cause possible detrimental effects to the individual's health if experienced repeatedly (Drolet et al., 2001). Having said this, not everyone exposed to stress succumbs to its effects in the same manner, with a sub-domain of individuals being resilient to the detrimental effects of repeated exposure to stress, and with a further sub-domain being more susceptible to the negative effects of stressful events, and in turn to the development of psychiatric illness such as depression (Southwick et al., 2005). As stated in the introduction, stressful or traumatic life events, and early life stress, are considered major risk factors in the onset of depression (DSM-V). Stress causes an increase in ACTH and cortisol, two biomarkers that are shown to be elevated in individuals with MDD, and markers that represent activation of the HPA-axis, an area of interest associated with the pathophysiology of depression. Repeated exposure to stressful stimuli is believed to cause over-activation and dysfunctionality of the HPA-axis system that is seen in certain individuals who suffer from depression. In tandem with this, BDNF, a growth factor that is involved in the response to stress, has been shown to be decreased in the brains of patients with MDD when examined in post-mortem tissue (Dwivedi et al., 2003), and in the plasma of MDD patients *in vivo* (Karege et al., 2005a, Karege et al., 2005b, Shimizu et al., 2003).

Animal models of depression often use stress as a component in which to facilitate or initiate the model itself, such that the symptoms of behavioural despair and pathophysiological traits that are seen in the clinical case of depression are exhibited. Models such as CMS, social isolation, social defeat stress, LH, OB, sleep deprivation and maternal deprivation all explore and induce an element of stress in the animal, whether it be invoked by acute or chronic means, that symptomatically and neurochemically resemble facets of the clinical condition. For example, BDNF has been shown to be reduced in both acute and chronic stress-induced paradigms in animals, such as the social isolation model and CMS model (Barrientos et al., 2003, Nibuya et al., 1995, Smith et al., 1995, Zhang et al., 2019). Corticosterone, the equivalent to cortisol in humans and an animal biomarker of stress and HPA-axis activation, is increased in animals after exposure to stress such as OB (Rinwa and Kumar, 2013, Thakare et al., 2017, Yang et al., 2014), CMS (Fracchia et al., 1992,

Réus et al., 2012, Song et al., 2006, Wang et al., 2018) and LH (Song et al., 2006, Vollmayr et al., 2001). Indeed, tests of behavioural anxiety and despair such as the FST, the TST, the OF test, the SI test and the EPM, all involve placing the animal into an unknown environment in which their HPA-axis system is activated due to unpleasant or stressful stimuli.

The FST assesses behavioural responses to a physical stressor that was initially established to detect and examine the efficacy of antidepressant drugs in rodents (Porsolt et al., 1977, Slattery and Cryan, 2012). Also known as the Porsolt test, as it was first described by Porsolt et al. (1977), the FST involves the animal, usually a rat or mouse, being placed into a cylinder of water for a period of time in which the animal initially struggles to escape from the cylinder (exhibited with intense swimming and climbing behaviours), followed by a cessation of this struggling behaviour, with the animal instead adapting a position of immobility; indicative of passive behaviour or helplessness (Slattery and Cryan, 2012). This immobility in the FST is considered to reflect a failure in stress-coping mechanisms, which is indicated by the abolition of persistence and perseverance to escape (Slattery and Cryan, 2012). When antidepressant effects are being evaluated in rats, the FST usually involves two exposures separated by 24 hours, namely the pre-swim and the test swim with drug administration's taking place between these two phases. The pre-swim lasts 15-minutes and is performed so that animals are exposed to the stressful environment and acclimatise to the arena, and learn to adapt immobility-like behaviour due to the inescapable nature of the test (behavioural despair). Exactly 24 hours after the pre-swim, the swim test is undertaken, whereby animals are again placed into the cylinder for a period of 5 minutes. Antidepressant treatment reduces the time that animals spend immobile and as such increases escape behaviours, indicative of promoting perseverance and wilfulness in the animal. Drug treatments are either given as three injections at 1, 5 and 23.5 hours before the subsequent swim test, or as two injections with one injection 1 hour before the swim test and the other injection immediately after the pre-swim; both dosing regimens have been shown to be successful in detecting known antidepressant efficacy (Slattery and Cryan, 2012). The FST has revealed consistent and reliable finding across numerous laboratories and animal strains due to its ease of use and reproducibility (Detke et al., 1995). Traditionally, it was considered as a tool in which to 'screen' novel compounds for their antidepressant-like efficacy rather than as a stress test, albeit this thinking has been

challenged, in recent years. However, few studies have examined the effects of administration of drugs chronically rather than acutely in this test, which would more appropriately resemble the action of antidepressants in the clinical scenario. This would aid in the abolition of false positive results with acute administration of novel compounds, in conjunction with highlighting effects of drugs that may require more chronic administration to cause an effect (Detke et al., 1997). One major pitfall of the Porsolt et al. (1977) FST is its lack of consistency and sensitivity in detecting the antidepressant-like effects of SSRI antidepressants (Lucki et al., 1997). As a result, refinements were made to the original FST, with adaptations increasing the throughput and reliability of SSRI efficacy. Increasing the volume of water, identifying the 'active' behaviours of swimming and climbing, and most interestingly, changing the method of scoring to a time-sampling (count) method, rather than using a cumulative (continuous) time method, have all added further strength to the FST (Detke et al., 1995, Lucki, 1997).

Firmly regarded as a test with strong predictive validity, the FST uses the appearance of a stressful event and environment, via the exposure to an inescapable swim stress, to create a traumatic experience in animals, in order to mirror stressful events. In recent years the FST has received much criticism as to how appropriately this test may relevantly relate to the clinical case of depression. Debate over whether the rodents in the test cease swimming as a result of helplessness, or as a result of the animals learning that they will be rescued sooner upon a cessation of movement, are just two of the questions being asked (Reardon, 2019). In conjunction with this, animal rights groups have been actively campaigning to end its use, with large deliberation being placed over its overuse as a method and test for examining depression (Reardon, 2019). Indeed, it is argued that 'while no single animal test can capture the full complexity of a human disorder, these tests in particular are recognized by many scientists as lacking sufficient mechanistic specificity to be of general use in clarifying the neurobiological mechanisms underlying human depression' (Reardon, 2019). Nevertheless, the FST is one of the most commonly used assays to examine 'depressive-like' behaviour in rodents, implementing exposure to a stressful stimulus in order to illicit both behavioural and molecular alterations that reflect alterations seen in the clinical condition. Exposure to this test is associated with increased HPA-activity and decreased BDNF expression, all symptoms and markers that are shown to be altered in MDD (Badowska-Szalewska et al., 2010, Brown et al., 2014, Connor et

al., 1997). As such, its potential and its use so far for furthering depression research should not be ignored.

With this in mind, the FST has two functions *per se*, and can be considered as a screen for testing antidepressant efficacy (with repeated exposures), or solely as a test for examining 'depressive-like' behaviour in animals and as such behavioural despair (only singular exposure necessary). Having said this, regardless of acting as a screen for antidepressant compounds, or examining 'depressive-like' behaviour, by its properties alone, exposure to this forced swim paradigm is exposure to an acute stress, and as such, this apparatus can be regarded as having a third function; that of exposure to a stressor via the means of an involuntary or forced swim. It is this category of pure 'stressor' behaviour that is examined in this Chapter. Bearing this in mind, the FST is often used to induce behavioural deficits (e.g. LH or CMS), or in tandem with other animal models (e.g. social defeat stress (SDS), chemical-induction or genetic strain). As discussed in previous chapters, the OB model is regarded as an animal model with strong face, construct and predictive validity. Unlike the FST which is considered an acute stressful experience, the OB model evokes stress on a more long-term basis by surgical manipulation, resulting in changes such as chronic irritability, sleep disturbances, psychomotor agitation, increases in corticosterone and CRF, decreases in BDNF in the hippocampus and loss of synaptic density, but to name a few, and all of which are indicators of stress arousal (Morales-Medina et al., 2013, Thakare et al., 2017, Wang et al., 2012, Yang et al., 2014, Yang et al., 2015). The FST has been examined in the OB model, with the majority of studies looking at two exposures and indicating an increase in immobility that is reversed by acute antidepressant treatment (see Chapter 2). Nevertheless, some researchers have also found no difference in immobility time in OB rats when compared to sham-operated rats (Kelly and Leonard, 1999, Stepanichev et al., 2016). Vieyra-Reyes et al. (2008) found that this immobility in OB animals was strain dependent; with OB Wistar rats showing immobility in the forced swim but OB Long-Evans rats showing no difference when compared to sham-operated counterparts. Few studies have reported the effects to swimming and climbing behaviours after OB surgery, but OB mice have been reported to spend significantly more time swimming (Kalshetti et al., 2015b, Linge et al., 2013) and less time climbing than their sham-operated counterparts (Linge et al., 2013). In contrast, Morales-Medina et al. (2013) found that OB rats spent significantly less time

swimming, with no differences in climbing behaviour when compared to sham-operated rats.

In conjunction with this, the majority of OB model studies that examine the FST tend to use the original continuous method of scoring, rather than the count method or indeed an automated tracking measure. The potential for an automated tracking technique to be used could assist in reducing the variability and inconsistency in scores associated with manual scoring techniques. Manual scoring techniques are often quite subjective in nature, and so have the potential for human bias and human error. In addition, an automated version of tracking activity would provide a tool that could be easily reproduced across laboratory settings with the potential for more consistent, accurate and precise results. Indeed, many of the studies examining the FST in the OB model do not fully clarify what scoring method is used. The findings are summarised in Table 4.1. As a result, using the forced swim in combination with the OB model melds the action of acute and chronic stressors together and allows for further implications of stress in preclinical depression research.

Housing	Strain	Sex	Sham	OB	Method of Scoring	Pre-swim	Immobility Effect	Duration of Test	Reference
Rats									
Single	SPD	M	162 ± 2	124 ± 2	Automated	No	↓	5 mins	Kalshetti et al. 2015b
Single	SPD	M	72 ± 20	142 ± 38	Continuous	No	↑	4 mins*	Yang et al. 2014
Paired	SPD	M	188 ± 22	309 ± 27	Continuous	No	↑	10 mins	Morales-Medina et al. 2013
Paired	SPD	M	184 ± 16	301 ± 24	Continuous	No	↑	10 mins	Morales-Medina et al. 2012a
Paired	SPD	M	300 ± 42	392 ± 108	Continuous	No	↑	10 mins	Morales-Medina et al. 2012b
Paired	SPD	M	147 ± 82	312 ± 59	Continuous	No	↑	10 mins	Morales-Medina et al. 2012c
Single	Wistar	M and F	58 ± 14	159 ± 24	NS	Yes	↑	5 mins	Thakare et al. 2017
NS	Wistar	M	108 ± 4	141 ± 8	Continuous	Yes	↑	5 mins	Smaga et al. 2017
Group	Wistar	F	196 ± 28	204 ± 32	NS	Yes	–	5 mins	Stepanichev et al. 2016
Single	SPD	M	102 ± 14	142 ± 28	NS	Yes	↑	5 mins	Li et al. 2015
Single	Wistar	M	73 ± 24	193 ± 31	Continuous	Yes	↑	5 mins	Rinwa and Kumar, 2013
NS	Wistar	M	122 ± 45	168 ± 68	Continuous	Yes	↑	5 mins	Smaga et al. 2012
NS	SPD	M	118 ± 55	215 ± 36	NS	Yes	↑	5 mins	Wang et al. 2012
Group	Wistar	M	91 ± 17	160 ± 11	Continuous	Yes	↑	5 mins	Tasset et al. 2008
Single	Wistar	M	73 ± 2	95 ± 5	NS	Yes	↑	5 mins	Vieyra-Reyes et al. 2008
Single	LE	M	78 ± 3	97 ± 16	NS	Yes	–	5 mins	Vieyra-Reyes et al. 2008
Group	SPD	M	195 ± 45	196 ± 31	NS	Yes	–	5 mins	Kelly and Leonard, 1999
Mice									
Single	C57BL6	M	159 ± 18	108 ± 6	Automated	No	↓	5 mins	Linge et al. 2013
Group	C57BL6	M	162 ± 22	96 ± 10	Automated	No	↓	5 mins	Linge et al. 2013
Group	DDY	M	104 ± 13	164 ± 10	Continuous	Yes	↑	5 mins	Han et al. 2009

Table 4.1 Examination of the forced swim test in the OB model of depression. The table show immobility scores for sham-operated and OB animals in the FST, the length of time animals were tested, the method of scoring that was used, as well as the housing conditions, sex and strain of the animals. Data are expressed as mean ± standard deviation. F=female, LE=Long Evans, M=male, NS=not stated, SPD=Sprague Dawley, -=no significant difference, ↓=decreasing, ↑=increasing, *=tested for 6 minutes but only the last 4 minutes scored for immobility.

The central opioid system has been implicated in regulating the stress response in rodents (McCubbin, 1993) as it is densely populated in regions of the brain that are involved in the regulation of stress, such as the hypothalamus, prefrontal cortex, amygdala and hippocampus (McCubbin, 1993). Rodents exposed to stress paradigms have been shown to have altered central opioid expression. Shirayama et al. (2004), found that dynorphin A immunoreactivity was increased in the hippocampus of rats exposed to acute immobilisation, the FST and a LH paradigm. DYN mRNA is increased in the nucleus accumbens and striatum of rats exposed to a social defeat model (Bérubé et al., 2013), and PDYN mRNA is increased in the nucleus accumbens of rats exposed to the FST (Chartoff et al., 2009). ENK mRNA has been shown to be decreased in the amygdala of rats after social defeat stress and chronic unpredictable stress (Bérubé et al., 2013; 2014). MOP KO mice have been shown to have increased open arm entries in the EPM and decreased immobility time in the FST and TST, indicating that the blockage of the MOP receptor may play a role in anxiolytic and antidepressant-like behaviour (Ide et al., 2010).

In turn, opioid modulating drugs have been shown to alleviate stress-induced anxiety- and depressive-like behaviour in rodents. KOP antagonists have been shown to decrease immobility time in the FST (DIPPA), increase open arm entries and percentage duration (JDTic), and reduce symptoms in the LH model (norBNI) (Knoll et al., 2011, McLaughlin et al., 2003, Shirayama et al., 2004). MOP agonists have been shown to decrease immobility time in the FST and TST (Endomorphin 1 and 2) (Fichna et al., 2007), and DOP agonists have been shown to increase open arm duration (SNC80), decrease emotionality scores (SNC80, KNT-127), and decrease immobility time in the FST (SNC80, KNT-127, (+)BW373U86, JOM-13, deltorphin II, DPDPE) (Broom et al., 2002, Gotoh et al., 2017, Saitoh et al., 2004; 2008; 2011, Torregrossa et al., 2005; 2006). Pharmacological and genetic manipulation of the opioid system has also been shown to reverse the neurochemical effects that are caused by stress including HPA-axis activation and reductions in BDNF, but these effects are dependent upon the receptor that is targeted. Acute administration of nonpeptidic DOP agonists have been shown to increase BDNF mRNA expression in the hippocampus and the frontal cortex of rats (Torregrossa et al., 2004; 2005; 2006). Increases in corticosterone after exposure to restraint stress, the TST and repeated FST were reduced in MOP KO mice when compared to the wild-type controls (Ide et al., 2010).

Taking all of this into consideration, it is clear to see that the opioid system plays a crucial role in stress regulation and the response to stress in animals. The opioid system can both activate and reduce stress-induced neuroendocrine and behavioural responses depending on which receptor system is targeted, while at the same time having the ability to stimulate these systems and their components in a resting or non-stressed state (Drolet et al., 2001). The role of the opioid system in the OB model is less explored, and basal opioid levels in this chronic model have yet to be fully characterised. The effect of an acute and well-validated stressor, such as the forced swim, alone and in combination with the more longer-lasting stress adaptations of OB, on the opioid system in discrete brain regions would determine if acute and/or chronic stress has an overarching cumulative effect on the opioid system, which could in turn have implications in these models and within the field of research as a whole. As such, the objective of this study was to examine the effects of acute swim stress and OB, alone or in combination, on the mRNA expression of opioid receptors and the precursors for their endogenous ligands, in a number of brain regions implicated in stress and depression. It was hypothesised that exposure to the acute and chronic stressors, alone and in combination, would cause differential behavioural effects in sham-operated and OB rats, and that an increase in mRNA opioid expression would be seen in a number of brain regions.

Therefore, the specific aims of the experiment in this chapter were as follows:

- To assess the behavioural effects of immobility, swimming and climbing after an acute 15-minute force swim stress exposure on rats, alone or in combination with OB surgery
- To compare the techniques used for scoring immobility, swimming and climbing behaviours by examining manual scoring methods (continuous method and count method), and automated tracking methods (activity analysis methods via tracking software)
- To analyse c-fos mRNA expression in sham-operated and OB rats as a marker for the induction of stress after swim stress exposure
- To analyse opioid receptor and pre-propeptide mRNA expression in the hippocampus, amygdala, prefrontal cortex and hypothalamus after swim stress and/or olfactory bulbectomy

4.2 Materials and Methods

4.2.1 Materials

This was carried out as described in Chapter 3, Section 3.2.1. All materials for animal sourcing, husbandry, monitoring and surgery were kept consistent with *experiment 1* in Chapter 3. All materials for behavioural recording, and analyses were kept the same as in Chapter 3. All qRT-PCR kits and reagents used were kept consistent with those used in Chapter 3.

4.2.2 Animals

Experiments were carried out on a total of 36 male Sprague Dawley rats (175-200 g on arrival, obtained from Charles River, UK). On arrival, rats were housed in groups of 4 per cage, in plastic cage bottoms (42 cm x 25.5 cm x 13 cm) with a metal grated cage top with plastic water bottles (North Kent Plastics, Coalville, Leicestershire, UK). Cages contained 3Rs paper bedding (Fibrecycle Ltd., Scunthorpe, Lincolnshire, UK). Prior to surgery, cages also contained sizzle-nesting material for environmental enrichment (LBS Biotechnology, Horley, UK), and rats were given nutritional enrichment once a week (CocoPops, Tesco PLC, Ireland) which was stopped after surgery. All rats were housed in an environment with controlled temperature (20-24°C) and humidity (45-65%) (Monitor, Radionics Ltd, Dublin, Ireland), in a 12:12 h light-dark cycle, lights on from 08:00 h. Animals had access to food (20% protein rodent diet Advanced Protocol® Verified 75 IF Irradiated (5V75), LabDiet®, Brentwood, MO, USA) and water *ad libitum*. Bodyweight, food, and water consumption were monitored daily (weighing scales, Mason Technology, Dublin, Ireland) from one week pre-surgery until sacrifice. All rats were pair-housed one week prior to surgery (based on body weights) and were randomly allotted to their surgery groups. These allotted groups were checked for statistical significance using IBM SPSS Statistics Version 24 software to make sure that there was no significant difference between groups prior to the commencement of the study.

All animal procedures were carried out under the approval of the Animal Care and Research Ethics Committee (ACREC), National University of Ireland, Galway (NUIG) (12/NOV/07). All procedures for this project were approved for authorisation from the Health Products Regulatory Authority (HPRA) and in compliance with EU Directive 86/609 (HPRA Authorisation ID: AE19125/P006).

4.2.3 Experimental Design

Male Sprague Dawley rats (200g-250 g) underwent surgery (sham or OB) under isoflurane anaesthesia ten days after initial arrival. Rats were pair-housed (a sham-operated rat paired with an OB) a week prior to surgery (*n*=9/group), see Figure 4.1. Five weeks following surgery, rats were placed in the FST apparatus for 15 minutes, or left undisturbed in their homecage. Rats who were exposed to an acute forced swim were returned to their homecage immediately after and 45 minutes later, rats were euthanized via decapitation and brains removed and dissected. Five weeks post-surgery was chosen as the testing timepoint so that the timepoint of post-mortem analysis would be the same as in Chapter 3. This 45-minute timepoint was chosen in order to examine the effects of the acute force swim stress exposure to the central opioidergic system (Alkermes Inc., Internal data, Loguinov et al., 2001, Smith et al., 2019). Connor et al. (1997) has shown that activation of the HPA-axis is seen at 15 minutes post-forced swim exposure, with return to baseline levels within 120 minutes of exposure, with the caveat of two exposures rather than one exposure. In conjunction with this, Browne et al. (2014) has shown that 30 minutes after a single 15 minute acute FST exposure, plasma corticosterone levels are increased in mice.

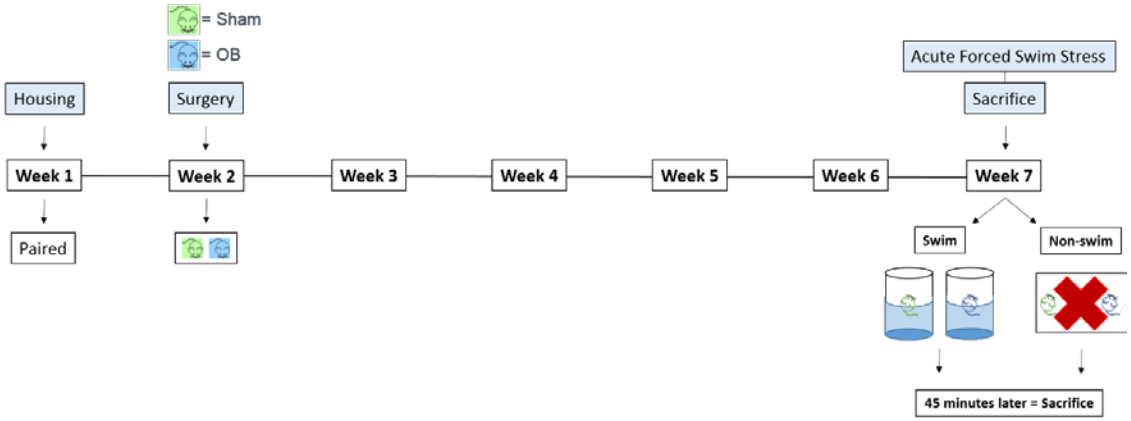


Figure 4.1 Experimental design for forced swim stress experiment.

4.2.4 OB Surgery

This was carried out as described in Chapter 3, with some modifications. Thirty minutes prior to surgery, all rats undergoing surgery that day were given a dose of buprenorphine (Cat# RP0003, Chanelle Veterinary, Ireland, 0.03 mg/kg at a dose volume of 1 mg/kg s.c) to apply pain relief throughout the surgical procedure. Once a rat was placed in the earbars, a subcutaneous injection of dexamethasone (Depo-

Medrone, Pfizer Healthcare, Ireland, 5 mg/kg at a dose volume of 1 ml/kg s.c) was given to the animal to reduce potential swelling in the brain following the post-operative period.

4.2.5 Behavioural Testing

4.2.5.1 Forced Swim Stress

As stated in the introduction of this chapter, the FST, originally established by Porsolt et al (1977) and with later modifications (Detke et al., 1995, Lucki, 1997), is a test of behavioural despair in which an animal is placed into a cylinder of water for a period of time and either continuously struggles to escape or adapts a position of immobility indicative of 'learned helplessness'. In this study, we chose to use the forced swim apparatus and the 15 minute pre-swim as our acute stressor, as this apparatus and test is a well-validated and reliably reproducible test that has been proven to induce acute stress in a number of animal models and strains tested. On the day of testing rats were removed from their homecage in pairs (with their cagemate) and brought into an unfamiliar room where the FST was set-up. The cylinders (45 x 20 cm) were filled to 30 cm with water (23-25°C) which was regularly checked for temperature (apparatus designed and built by Mr. Ambrose O'Halloran, NUI Galway). Two researchers placed the rats into the filled cylinders, with two animals (i.e cagemates) tested in adjacent cylinders for 15 minutes. After each swim rats were removed from the cylinder and were dried thoroughly with a towel and returned to their homecage in pairs until sacrifice. The cylinder was emptied after each swim, and cleaned thoroughly. The cylinder was then refilled with fresh water for the following trial. Exposure to the forced swim stress took place between 09:00-13:00 h. All video footage was recorded on a DVR recorder which was connected to a camera located directly across from the forced swim cylinders. Video-tracking software Ethovision®XT (version 11.5) was used to later analyse and track the recorded footage. The files were scored by an observer blind to all experimental conditions.

The FST was scored both manually and through automated-tracking using Ethovision®XT software (Figure 4.2). For manual scoring, the continuous and count method were both used. Four behaviours were scored, swimming, climbing, immobility-strict and immobility-lenient. Immobility was split into strict and lenient in order to fully class true immobility. Immobility consists of a rat floating in the water

without struggling, with the rat making movements necessary to keep its head above the water. Immobility-strict consists of strict static behaviour and firmly no movements at all. Immobility-lenient involves the animal staying afloat with more gentle and subtle movements that are not quite as rigorous as swimming and do not involve moving into another quadrant of the cylinder. Swimming consists of the rat making horizontal movements throughout the swim cylinder which includes crossing into another quadrant of the cylinder, as well as diving up and down in the cylinder. Climbing consisted of upward movements of the forepaws, and breaking the surface of the water, most frequently against the side of the cylinder. For the continuous method, behaviours were scored continuously for the full 15-minute duration of the trial. For the count method, at each 5 s interval during the test the behaviour that was present at that moment was scored, so that by the end of the 15 minutes there was a maximum 180 counts in total. Manual scoring was done using the 'mutually exclusive' setting on Ethovision®XT.

For the automated-tracking method, the activity analysis option in Ethovision®XT software (version 11.5) was ticked and behaviours were automatically calculated once activity threshold parameters had been set. The activity thresholds were set-up as follows; highly active (>10%), active (8-10%), moderately active (4-8%), and inactive (<4%). The activity thresholds were considered as behaviours in the following way; highly active (climbing), active (swimming), moderately active (immobility-lenient), and inactive (immobility-strict) (Figure 4.3; Figure 4.4).

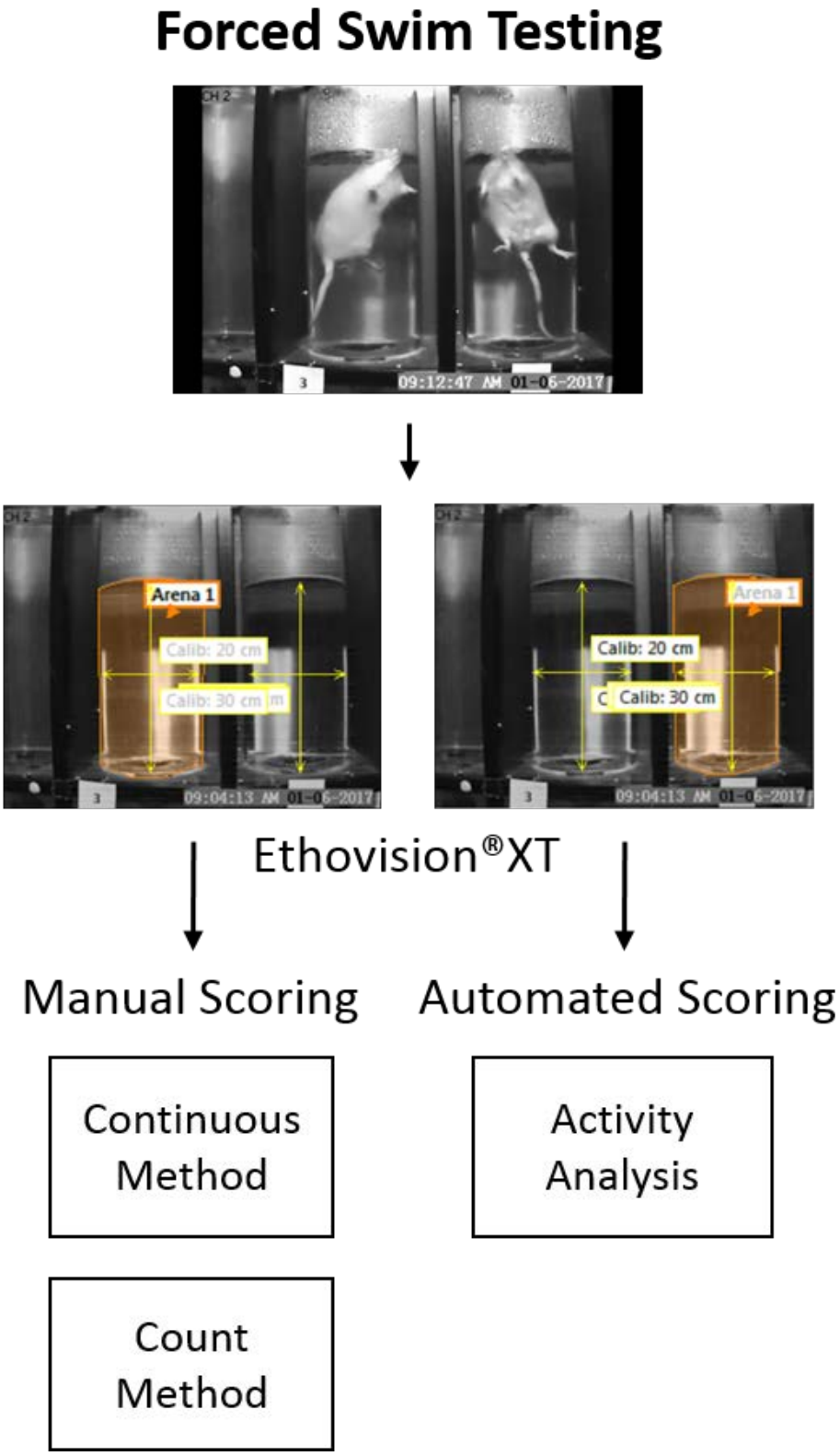


Figure 4.2 Forced swim test apparatus and scoring techniques using Ethovision®XT Software. Rats were tested in the forced swim apparatus for 15 minutes and video footage was recorded. Footage was analysed using Ethovision®XT software, and videos were scored manually (continuous and count method) and automatically (activity analysis).

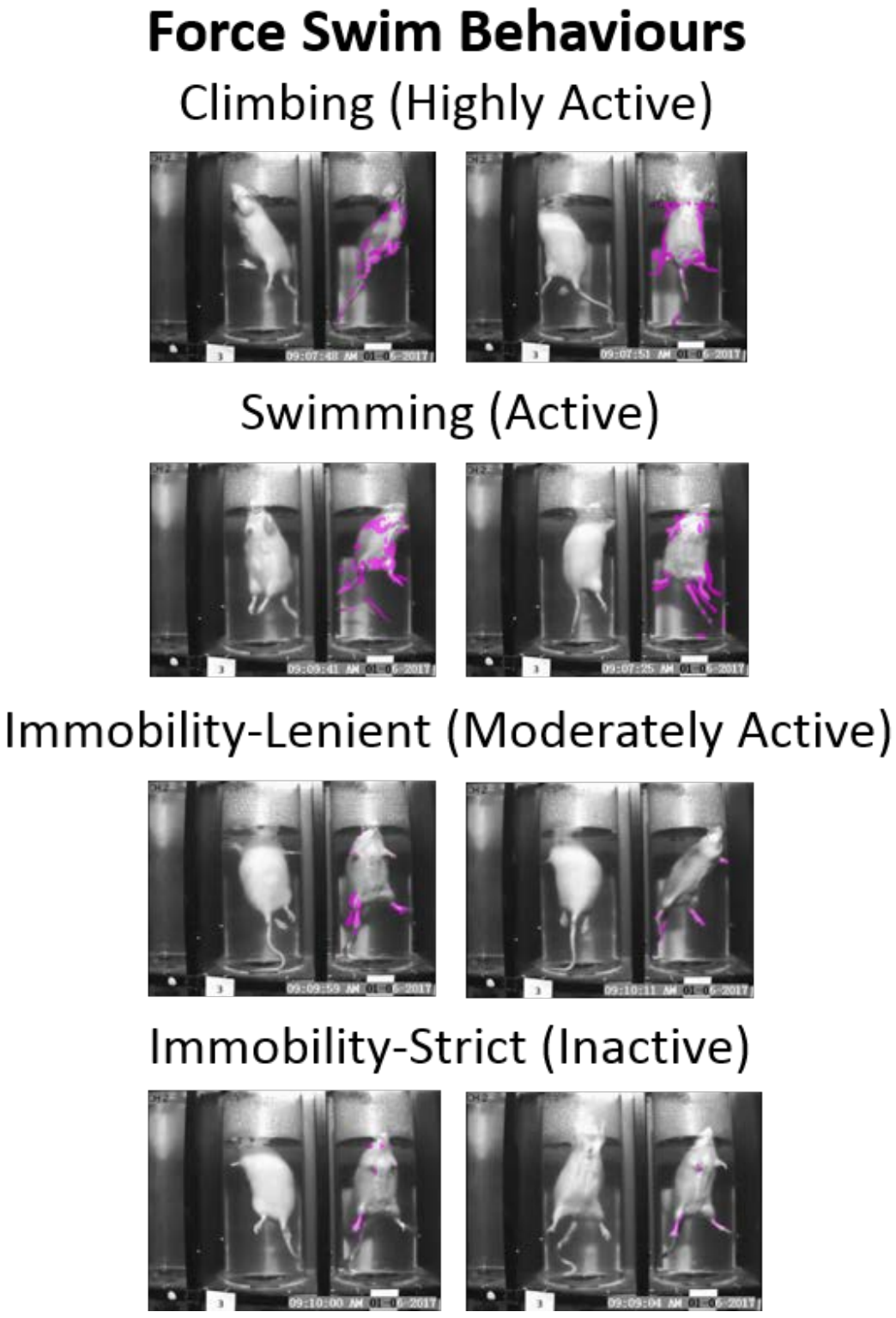


Figure 4.3 Forced swim test behaviours and activities scored using Ethovision®XT Software. Rats were tested in the forced swim apparatus for 15 minutes and footage was analysed using Ethovision®XT software. The activity thresholds were considered as behaviours in the following way; highly active (climbing), active (swimming), moderately active (immobility-lenient), and inactive (immobility-strict). The more purple the colouring on the rat, the more activity and movement being made by the rat.

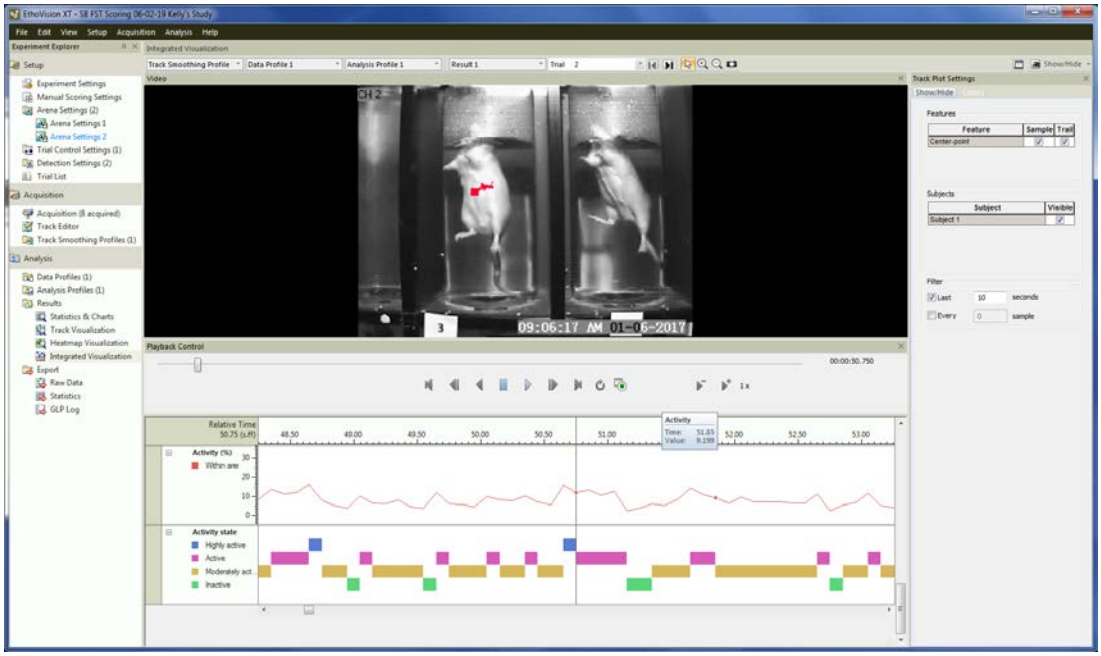


Figure 4.4 Activity analysis setting on ethovision®XT software used to score forced swim test behaviours. The activity thresholds/states were tracked over the 15-minute forced swim stress with the activity states set as highly active (purple), active (pink), moderately active (orange), and inactive (green). The sequence of activity states tracked per second can be seen in the bottom panel of the image above, with the longer the length of the coloured line indicating the longer length of duration of an activity state.

4.2.6 Animal Sacrifice and Brain Removal

This was carried out as described in Chapter 3, section 3.2.7. All rats exposed to the forced swim stress were killed in pairs exactly 45 minutes after removal from the apparatus. A timer was set after each set of swims to keep timing of sacrifice accurate. Between the 45-minute intervals, non-swim rats were removed from their homecages in pairs (a sham and an OB) and euthanized so as to have all rats and post-mortem tissue harvested at the same time-point. The brain was dissected immediately after removal from the skull rather than frozen for later dissection (See section 4.2.7).

4.2.7 Brain Dissection and Tissue Collection

Following removal the brain was placed onto wet filter-paper on an upside-down glass petri dish that lay in a box of ice. The hippocampus, amygdala, prefrontal cortex and hypothalamus were dissected from both sides of the brain and weighed. Regions were placed into eppendorfs and were snap frozen in a bed of solid CO_2 pellets (to maintain form and structure) and stored at -80°C until molecular analysis. The left and right side of the brain were randomised for each region so that equal amounts of left and

right sides were used for PCR analysis per group. 708 µl of RA1 buffer containing 1% β-mercaptoethanol was added to each tube and homogenised. This larger quantity was added to fully homogenise whole left/right larger brain regions, rather than to cut them in half. 354 µl of the homogenised sample was then taken for PCR analysis. The other half of the homogenised sample was frozen at -80°C in case it would be needed for later use.

4.2.8 Detection of opioid receptor and precursor peptides gene expression using real-time quantitative polymerase chain reaction (qRT-PCR)

4.2.8.1 RNA Isolation

This was carried out as described in Chapter 3, section 3.2.9.1

4.2.8.2 RNA Quantification and Equalisation

This was carried out as described in Chapter 3, section 3.2.9.2. After quantification, all samples were equalised to the same RNA concentration for each region (Table 4.2) by the addition of RNase-free water (supplied in the kit).

Region	Mean RNA concentration (µg/µl)	Quality (260:280)	Purity (260:230)	Equalised RNA concentration (µg/µl)
Hippocampus	199 ± 41	2.2-2.3	1.9-2.1	120
Amygdala	216 ± 45	2.1-2.4	1.6-2.1	115
Pre-frontal Cortex	176 ± 58	2.1-2.4	1.3-2.1	85
Hypothalamus	313 ± 82	2.2-2.8	2.0-2.1	170

Table 4.2 The RNA concentration, quality, purity, and equalised RNA concentration, per brain region after RNA isolation.

4.2.8.3 Complimentary DNA (cDNA) Synthesis

This was carried out as described in Chapter 3, section 3.2.9.3.

4.2.8.4 Quantitative Real-Time PCR (qRT-PCR) Analysis of Gene Expression

This was carried out as described in Chapter 3, section 3.2.9.4. Target genes and assay ID's are listed in Table 4.3. VIC-labelled β-actin was used as the endogenous control gene. All samples were run in multiplex assays. Preparation for Taqman master mix is seen in Table 4.4.

Target Gene	Assay ID	Fluorescent Label
MOP *(Oprm1)*	Rn01430371_m1	FAM
KOP *(Oprk1)*	Rn00567737_m1	FAM
DOP *(Oprd1)*	Rn00561699_m1	FAM
POMC *(Pomc)*	Rn00595020_m1	FAM
PDYN *(Pdyn)*	Rn00571351_m1	FAM
PENK *(Penk)*	Rn00567566_m1	FAM
c-Fos *(cfos)*	Rn02396759_m1	FAM
Endogenous Control Gene	**Assay ID**	**Fluorescent Label**
β-Actin *(Actb)*	Rn00667869_m1	VIC

Table 4.3 Assay ID's and fluorescent labels of target genes and the endogenous control gene.

Taqman Master Mix	
Taqman Reagent	5 µl
Taqman Primer *(ex. Oprm1 for MOP)*	0.5 µl
Endogenous Control Gene *(ex. β-actin)*	0.5 µl
RNase-free water	1 µl
Total volume per sample	**7.5 µl**

Table 4.4 Reagents and corresponding volumes used to make-up Taqman master mix.

4.2.8.5 Analysis of qRT-PCR Data

This was carried out as described in Chapter 3, section 3.2.9.5. Figure 4.4Figure 4.5 shows amplification plots for all opioid peptides and receptors in the amygdala. The control group in this case was the non-swim sham-operated group.

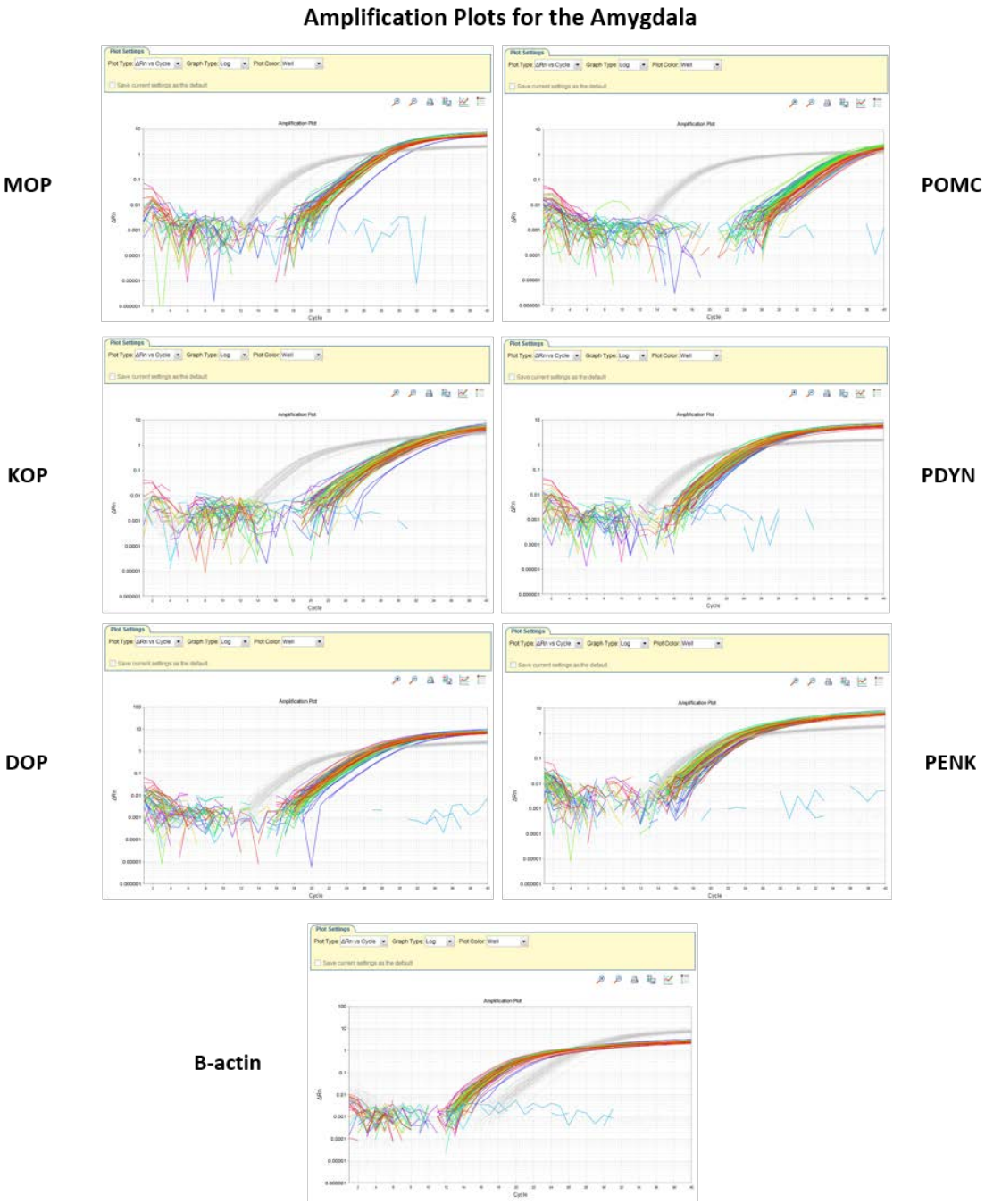


Figure 4.5 Sample amplification plots for β-Actin, MOP, KOP, DOP, PENK, PDYN and POMC in the amygdala. Images taken from Applied Biosystems 7500 system SDS Software 1.3.1. DOP=delta opioid receptor, KOP=kappa opioid receptor, MOP=mu opioid receptor, PDYN=prodynorphin, PENK=preproenkephalin, POMC=proopiomelanocortin.

4.2.9 Statistical Analysis

All statistical analysis was performed using IBM SPSS Statistics Version 24 software package. In all datasets, the presence of possible outliers was checked by assessing the distribution of data. In case a data point fell out of the range of (mean-2*standard deviation) to (mean+2*standard deviation), it was considered an outlier and excluded from subsequent analysis. Data were expressed as mean ± standard deviation, unless they are deemed non-parametric, in which case the data were expressed as median ± interquartile range. All data was tested for normality using Shapiro-Wilks test for normality, and all data was also tested for Levene's test for homogeneity of variance. All bodyweight and opioid receptor and peptide data was analysed using two-way ANOVA, and followed by *post-hoc* Student Newman-Keuls test; $p<0.05$ was deemed statistically significant. For behavioural data, total duration data was analysed with independent-samples *t*-test. Timebins data was analysed by one-way repeated measures ANOVA, followed by *post-hoc* with independent-samples *t*-test with a Bonferroni correction for multiple comparisons, where $p<0.003$ was deemed statistically significant. For the repeated measures, Mauchly's test of Sphericity was assumed at $p>0.05$, and if this was violated ($p<0.05$), then the Greenhouse Geisser correction was used. All graphs were prepared using GraphPad Prism Version 8.

4.3 Results

4.3.1 Mortality rates and verification after OB surgery

Of the 40 rats that had undergone surgery, 4 rats died in the post-operative and/or recovery period. Of the 4 rats that died, all deaths occurred as a result of olfactory bulbectomy surgery, with deaths occurring in the recovery cage, or within 4 hours after being removed to their homecage. Autopsy revealed that two rats had slight damage to the PFC (left hemisphere), and that the two remaining rats had no damage to the cortex, with complete bilateral bulb removal and as such cause of death was unconfirmed. Upon completion and verification of OB removal at the end of the study, there were no animals excluded from further analysis for incomplete or excessive bulb removal.

4.3.2 Bodyweight gain (five weeks post-surgery)

With regards bodyweight gain at five weeks post-surgery, two-way ANOVA revealed that there was no significant effect of surgery [$F_{(1,32)}$=3.743, p=0.062], swim [$F_{(1,32)}$=0.620, p=0.437], or any surgery x swim interaction effect [$F_{(1,32)}$=0.106, p=0.747] (Figure 4.6).

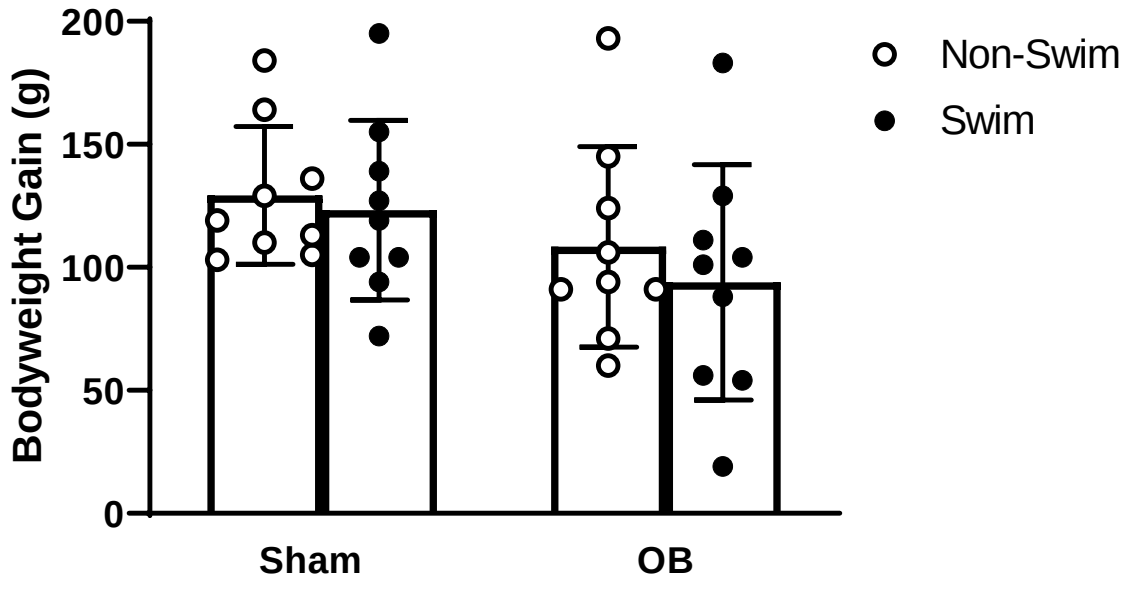


Figure 4.6 Bodyweight gain in sham-operated and OB rats at five weeks post-surgery. There was no significant difference between bodyweight gain in sham-operated and OB rats at five weeks. All bodyweights are prior to forced swim exposure. Data are expressed as mean ± standard deviation (n=9 per group).

4.3.3 Forced Swim Exposure – Behavioural Analysis

As mentioned in the methods, behavioural analysis for the forced swim stress was analysed via three different scoring methods. Behaviour was manually scored via the continuous method and the count method, and behaviour was also automatically scored via the activity analysis option in Ethovision®XT (11.5). Four behaviours were scored, as opposed to the regular three behaviours, in order to classify immobility under two types. For the continuous and count method, swimming, climbing, immobility-strict and immobility-lenient, were manually scored. For the automated analysis method, highly active, active, moderately active and inactive were automatically scored.

4.3.3.1 Continuous Method

There was no significant difference in total duration spent swimming [$t(16)=0.638$, $p=0.532$], climbing [$t(16)=0.887$, $p=0.388$], immobility-strict [$t(16)=0.409$, $p=0.688$], or immobility-lenient [$t(16)=0.639$, $p=0.532$] between sham-operated and OB rats (Figure 4.7)

With regards swimming, a repeated measures ANOVA revealed an effect of time [$F_{(4.840,77.434)}=2.643$, $p=0.031$] but no time x surgery interaction effect [$F_{(4.840,77.434)}=0.744$, $p=0.589$], in that swimming increased over time (Figure 4.8A). Between subjects effect revealed no effect of surgery [$F_{(1,16)}=0.408$, $p=0.532$]. *Post-hoc* analysis revealed no significant difference between sham-operated and OB rats on swimming at any particular minute timepoint ($p<0.05$ with Bonferroni correction ($p<0.003$)).

With regards climbing, a repeated measures ANOVA revealed an effect of time [$F_{(3.165,50.637)}=27.079$, $p<0.001$] but no time x surgery interaction effect [$F_{(3.165,50.637)}=1.167$, $p=0.333$], in that climbing decreased over time (Figure 4.8B). Between subjects effect revealed no effect of surgery [$F_{(1,16)}=0.786$, $p=0.388$]. *Post-hoc* analysis revealed no significant difference between sham-operated and OB rats on climbing at any particular minute timepoint ($p<0.05$ with Bonferroni correction ($p<0.003$)).

With regards immobility-strict, a repeated measures ANOVA revealed an effect of time [$F_{(4.212,67.393)}=5.510$, $p=0.001$] but no time x surgery interaction effect [$F_{(4.212,67.393)}=0.770$, $p=0.555$] (Figure 4.8C). Between subjects effect revealed no

effect of surgery [$F_{(1,16)}$=0.167, p=0.688]. *Post-hoc* analysis revealed no significant difference between sham-operated and OB rats on immobility-strict at any particular minute timepoint (p<0.05 with Bonferroni correction (p<0.003)).

With regards immobility-lenient, a repeated measures ANOVA revealed an effect of time [$F_{(4.746,75.944)}$=4.419, p=0.002] but no time x surgery interaction effect [$F_{(4.746,75.944)}$=0.663, p=0.645] (Figure 4.8D). Between subjects effect revealed no effect of surgery [$F_{(1,16)}$=0.408, p=0.532]. *Post-hoc* analysis revealed no significant difference between sham-operated and OB rats on immobility-lenient at any particular minute timepoint (p<0.05 with Bonferroni correction (p<0.003)).

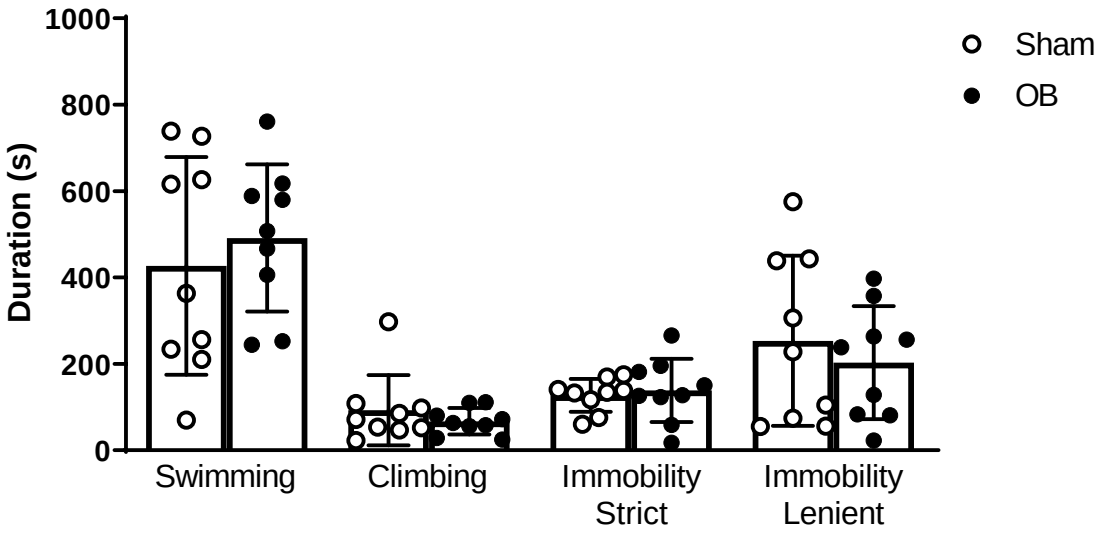


Figure 4.7 Continuous method scoring of total duration spent swimming, climbing and immobile by sham-operated and OB rats in the forced swim. There was no significant difference in the total duration of time spent swimming, climbing, immobility-strict or immobility-lenient between sham-operated and OB rats. Data are expressed as mean ± standard deviation (n=9 per group).

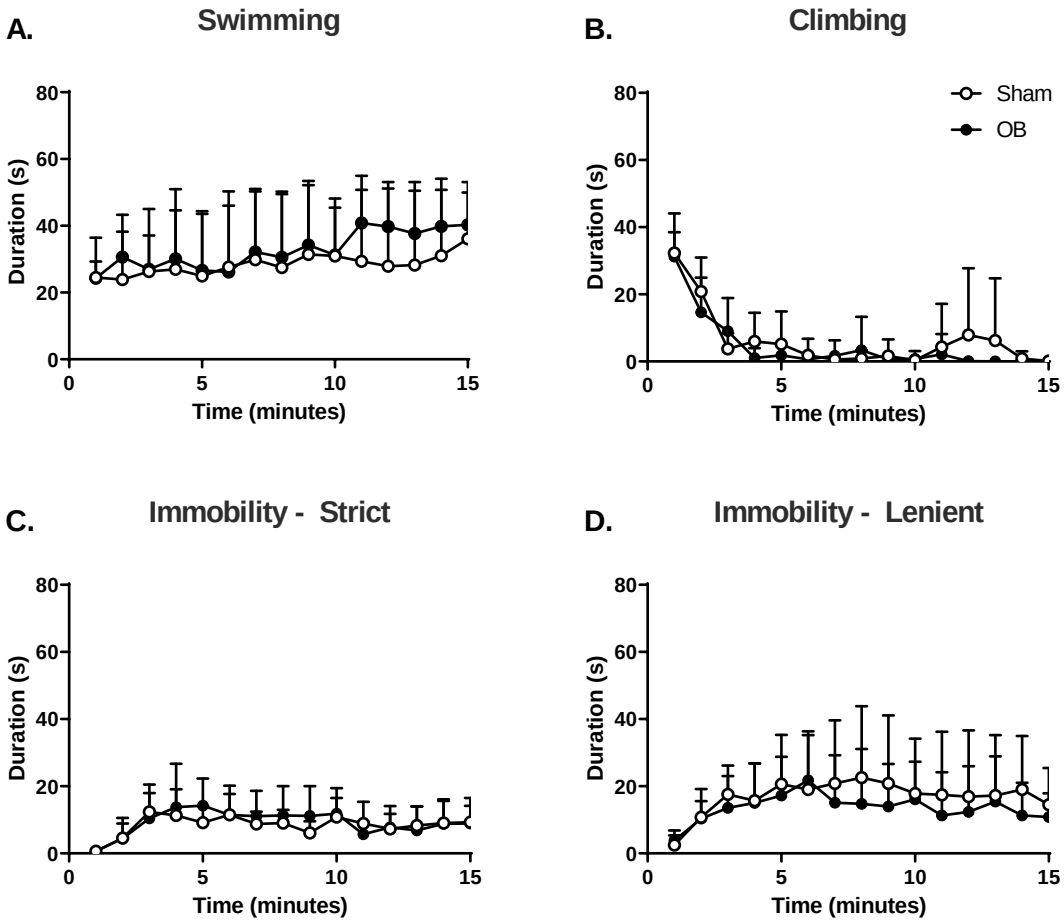


Figure 4.8 Continuous method scoring of time spent swimming, climbing and immobile by sham-operated and OB rats in the forced swim when broken down into 1-minute timebins. There was no significant difference between sham-operated and OB rats in the time spent swimming (A), climbing (B), immobility-strict (C) or immobility-lenient (D) over time when broken down into 1-minute time bins. Data are expressed as mean ± standard deviation (*n*=9 per group).

4.3.3.2 Count Method

There was no significant difference in total counts for swimming [$t(16)=0.923$, $p=0.370$], climbing [$t(16)=0.326$, $p=0.749$], immobility-strict [$t(16)=0.041$, $p=0.968$], or immobility-lenient [$t(16)=1.464$, $p=0.163$] between sham-operated and OB rats (Figure 4.9).

With regards swimming, a repeated measures ANOVA revealed an effect of time [$F_{(5.588,89.414)}=2.235$, $p=0.051$] but no time x surgery interaction effect [$F_{(5.588,89.414)}=0.648$, $p=0.681$] (Figure 4.10A). Between subjects effect revealed no effect of surgery [$F_{(1,16)}=0.685$, $p=0.420$]. *Post-hoc* analysis revealed no significant difference between sham-operated and OB rats on swimming counts at any particular minute timepoint ($p<0.05$ with Bonferroni correction ($p<0.003$)).

With regards climbing, a repeated measures ANOVA revealed an effect of time [$F_{(5.349,85.581)}=24.599$, $p<0.001$] but no time x surgery interaction effect [$F_{(5.349,85.581)}=2.435$, $p=0.038$], in that climbing counts decreased over time (Figure 4.10B). Between subjects effect revealed no effect of surgery [$F_{(1,16)}=0.426$, $p=0.523$]. *Post-hoc* analysis revealed no significant difference between sham-operated and OB rats on climbing counts at any particular minute timepoint ($p<0.05$ with Bonferroni correction ($p<0.003$)).

With regards immobility-strict, a repeated measures ANOVA revealed an effect of time [$F_{(14,224)}=4.354$, $p<0.001$] but no time x surgery interaction effect [$F_{(14,224)}=0.513$, $p=0.924$], in that immobility–strict counts increased over time (Figure 4.10C). Between subjects effect revealed no effect of surgery [$F_{(1,16)}=0.018$, $p=0.895$]. *Post-hoc* analysis revealed no significant difference between sham-operated and OB rats on immobility-strict counts at any particular minute timepoint ($p<0.05$ with Bonferroni correction ($p<0.003$)).

With regards immobility-lenient, a repeated measures ANOVA revealed an effect of time [$F_{(14,224)}=10.367$, $p<0.001$] but no time x surgery interaction effect [$F_{(14,224)}=0.670$, $p=0.802$] (Figure 4.10D). Between subjects effect revealed no effect of surgery [$F_{(1,16)}=1.080$, $p=0.314$]. *Post-hoc* analysis revealed no significant difference between sham-operated and OB rats on immobility-lenient counts at any particular minute timepoint. ($p<0.05$ with Bonferroni correction ($p<0.003$)).

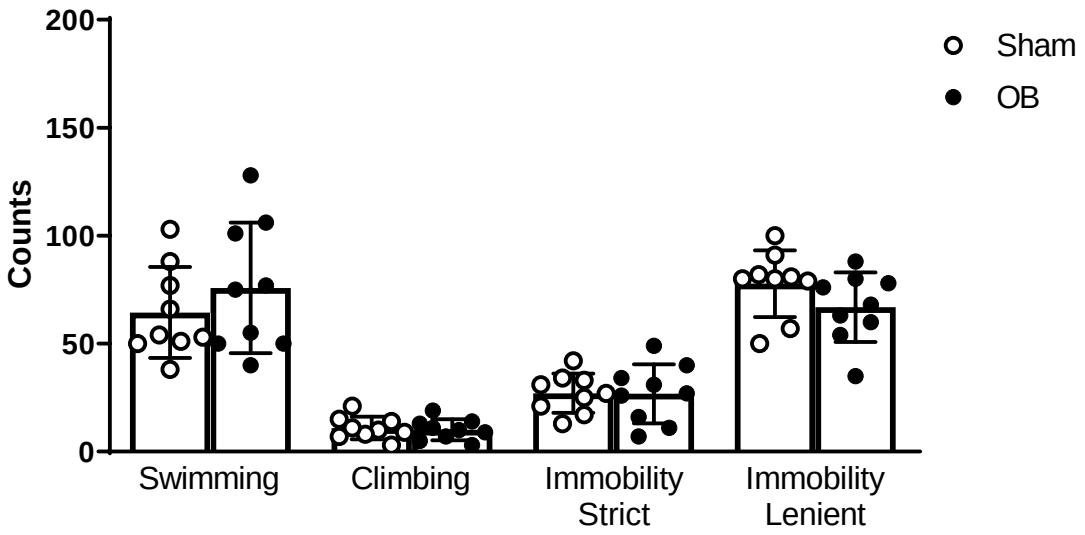


Figure 4.9 Counts method scoring of total duration spent swimming, climbing, immobility-strict and immobility-lenient by sham-operated and OB rats in the forced swim. There was no significant difference in the total counts for swimming, climbing immobility-strict or immobility-lenient between sham-operated and OB rats. Data are expressed as mean ± standard deviation (*n*=9 per group).

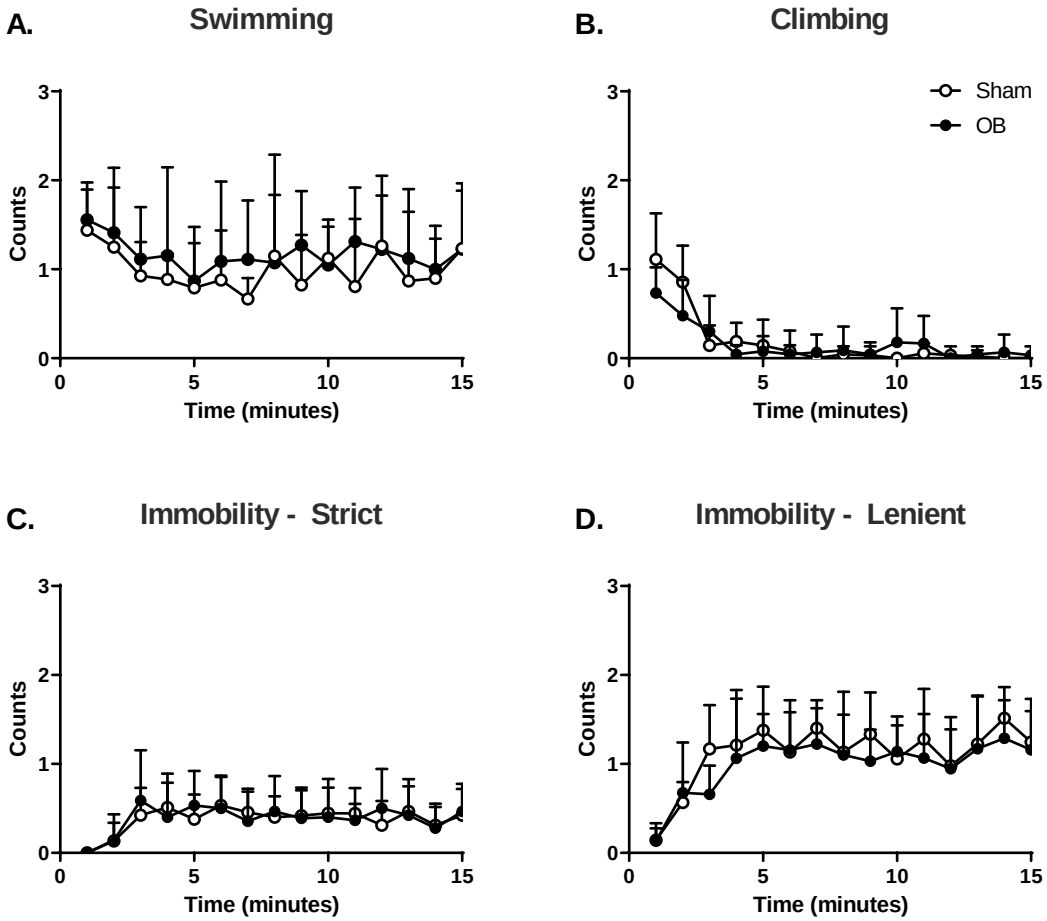


Figure 4.10 Counts method scoring of time spent swimming, climbing, immobility-strict and immobility-lenient by sham-operated and OB rats in the forced swim when broken down into 1-minute timebins. There was no significant difference between sham-operated and OB rats in the total counts for swimming (A), climbing (B), immobility-strict (C) or immobility-lenient (D) over time when broken down into 1-minute time bins. Data are expressed as mean ± standard deviation (*n*=9 per group).

4.3.3.3 Automated Activity Analysis

There was no significant difference in total duration spent highly active [$t(16)=2.595$, $p=0.020$], active [$t(16)=1.191$, $p=0.251$], moderately active [$t(16)=1.694$, $p=0.110$], or inactive [$t(16)=0.798$, $p=0.437$] between sham-operated and OB rats (Figure 4.11).

With regards duration spent highly active, a repeated measures ANOVA revealed an effect of time [$F_{(2.114,33.824)}=165.001$, $p<0.001$] but no time x surgery interaction effect [$F_{(2.114,33.824)}=1.274$, $p=0.294$], in that duration spent highly active decreased over time (Figure 4.12A). Between subjects effect revealed a significant effect of surgery [$F_{(1,16)}=5.145$, $p=0.038$]. *Post-hoc* analysis revealed no significant difference between sham-operated and OB rats on duration spent highly active at any particular minute timepoint ($p<0.05$ with Bonferroni correction ($p<0.003$)).

With regards duration spent active, a repeated measures ANOVA revealed an effect of time [$F_{(2.818,45.091)}=85.210$, $p<0.001$] but no time x surgery interaction effect [$F_{(2.818,\ 45.091)}=0.751$, $p=0.520$], in that duration spent active decreased over time (Figure 4.12B). Between subjects effect revealed no effect of surgery [$F_{(1,16)}=1.361$, $p=0.260$]. *Post-hoc* analysis revealed no significant difference between sham-operated and OB rats on duration spent active at any particular minute timepoint ($p<0.05$ with Bonferroni correction ($p<0.003$)).

With regards duration spent moderately active, a repeated measures ANOVA revealed an effect of time [$F_{(3.769,60.297)}=3.124$, $p=0.023$] but no time x surgery interaction effect [$F_{(3.769,60.297)}=0.440$, $p=0.768$] (Figure 4.12C). Between subjects effect revealed no effect of surgery [$F_{(1,16)}=0.600$, $p=0.450$]. *Post-hoc* analysis revealed no significant difference between sham-operated and OB rats on duration spent moderately active at any particular minute timepoint ($p<0.05$ with Bonferroni correction ($p<0.003$)).

With regards duration spent inactive, a repeated measures ANOVA revealed an effect of time [$F_{(3.620,57.921)}=20.672$, $p<0.001$] but no time x surgery interaction effect [$F_{(3.620,57.921)}=0.391$, $p=0.796$], in that duration spent inactive increased over time (Figure 4.12D). Between subjects effect revealed no effect of surgery [$F_{(1,16)}=0.020$, $p=0.889$]. *Post-hoc* analysis revealed no significant difference between sham-operated and OB rats on duration spent inactive at any particular minute timepoint ($p<0.05$ with Bonferroni correction ($p<0.003$)).

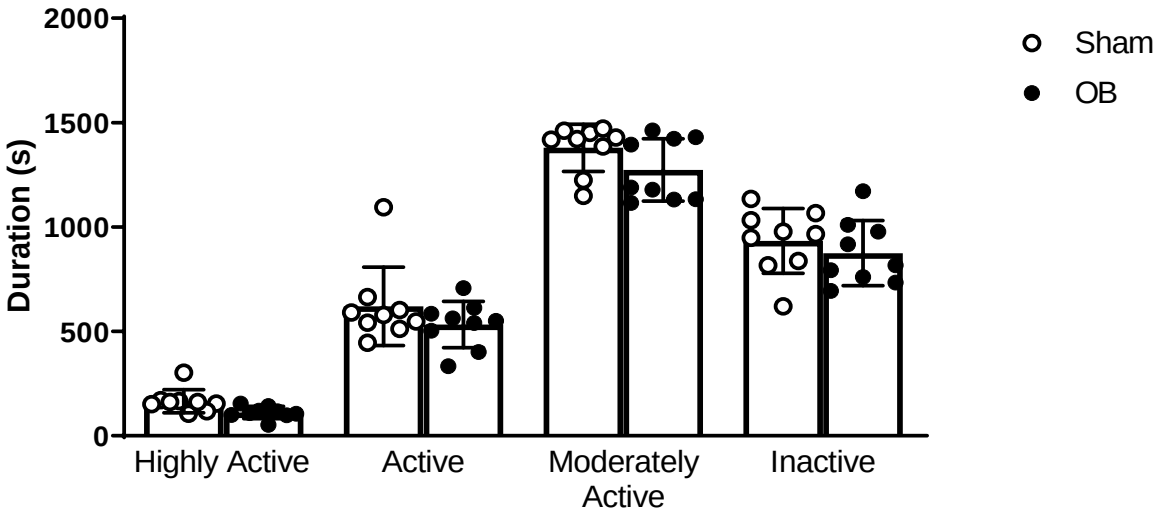


Figure 4.11 Automated activity analysis of total duration spent highly active, active, moderately active and inactive by sham-operated and OB rats in the forced swim. There was no significant difference in the total duration of time spent highly active, active, moderately active or inactive between sham-operated and OB rats. Data are expressed as mean ± standard deviation (*n*=9 per group).

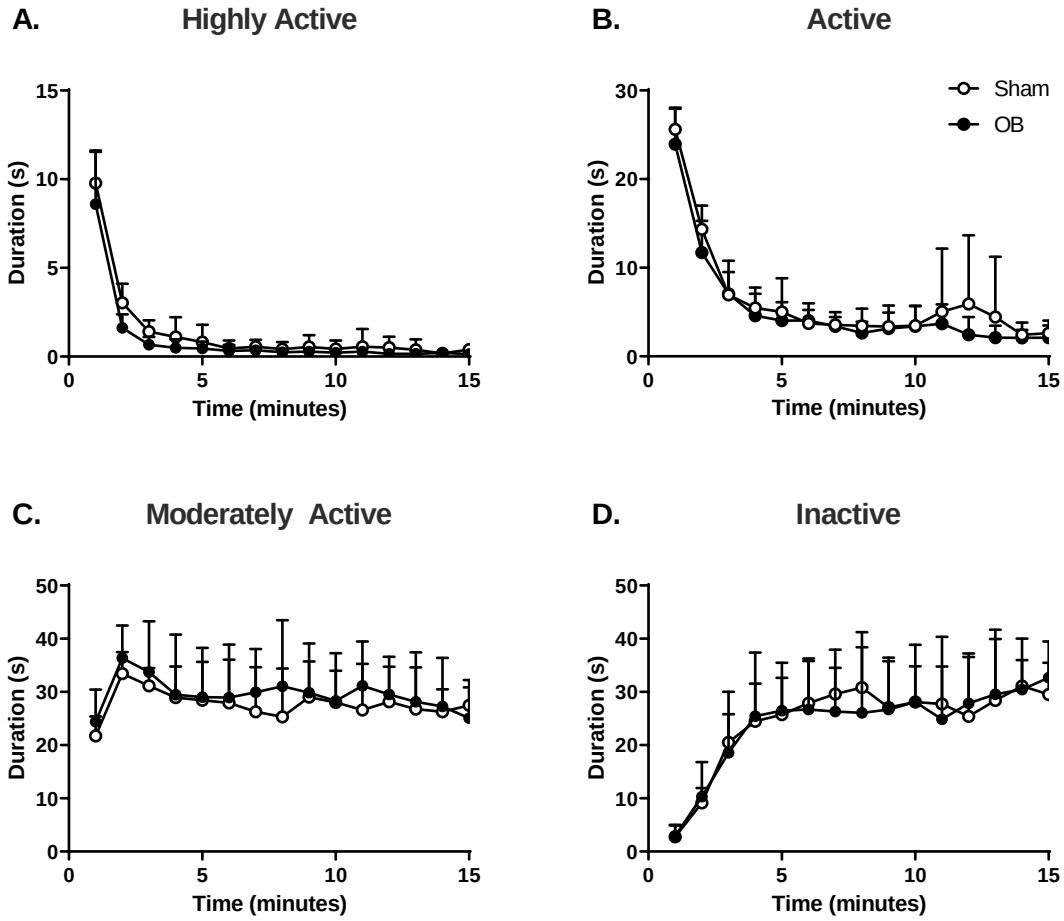


Figure 4.12 Automated activity analysis of time spent highly active, active, moderately active and inactive by sham-operated and OB rats in the forced swim when broken down into 1-minute timebins. There was no significant difference between sham-operated and OB rats in the time spent highly active (A), active (B), moderately active (C), or inactive (D) in the forced swim when broken down into 1-minute time bins. Data are expressed as mean ± standard deviation (*n*=9 per group).

4.3.4 Opioid receptor and pre-propeptide mRNA expression in the hippocampus, amygdala, hypothalamus and prefrontal cortex

4.3.4.1 MOP system

4.3.4.1.1 MOP (*Oprm1*) mRNA expression in discrete brain regions after swim stress and olfactory bulbectomy exposure

With regards *Oprm1* mRNA expression in the hypothalamus, two-way ANOVA revealed that there was a significant surgery x swim interaction effect, but no surgery or swim effect (Table 4.5). However, *post-hoc* analysis revealed that there was no significant differences between the groups.

With regards *Oprm1* mRNA expression in the hippocampus, amygdala or prefrontal cortex, two-way ANOVA revealed no significant difference (surgery, swim, or surgery x swim interaction effect) in *Oprm1* mRNA expression between sham-operated and OB rats, regardless of swim exposure (Table 4.5).

Group	Hippocampus	Amygdala	Hypothalamus	Prefrontal Cortex
Sham Non-Swim	1 ± 0.17	1 ± 0.21	1 ± 0.35	1 ± 0.42
Sham Swim	0.79 ± 0.23	1.03 ± 0.09	1.30 ± 0.22	0.98 ± 0.43
OB Non-Swim	0.82 ± 0.14	0.92 ± 0.19	1.16 ± 0.25	1 ± 0.41
OB Swim	0.91 ± 0.18	0.91 ± 0.19	0.93 ± 0.33	1.04 ± 0.38
Effect				
Surgery	$F_{(1,24)}$=0.120, *p*=0.732	$F_{(1,31)}$=2.289, *p*=0.140	$F_{(1,32)}$=1.006, *p*=0.323	$F_{(1,29)}$=0.305, *p*=0.585
Swim	$F_{(1,24)}$=0.595, *p*=0.448	$F_{(1,31)}$=0.015, *p*=0.903	$F_{(1,32)}$=0.096, *p*=0.758	$F_{(1,29)}$=1.528, *p*=0.226
Surgery x Swim	$F_{(1,24)}$=3.712, *p*=0.066	$F_{(1,31)}$=0.070, *p*=0.793	**$F_{(1,32)}$=6.447, *p*=0.016**	$F_{(1,29)}$=1.554, *p*=0.223

Table 4.5 MOP (*Oprm1*) mRNA expression in discrete brain regions. *Oprm1* mRNA expression is expressed as a percentage of the sham-operated non-swim group. There was no significant difference in *Oprm1* mRNA expression between sham-operated and OB rats in the hippocampus, amygdala, hypothalamus or prefrontal cortex. Data are expressed as mean ± standard deviation (*n*=7-9 per group).

4.3.4.1.2 POMC (*Pomc*) mRNA expression in discrete brain regions after swim stress and olfactory bulbectomy exposure

With regards *Pomc* mRNA expression in the prefrontal cortex, two-way ANOVA revealed that there was a significant surgery effect and a surgery x swim interaction effect, but no swim effect (Table 4.6). *Post-hoc* analysis revealed that OB non-swim rats had increased *Pomc* mRNA expression in the prefrontal cortex when compared to sham-operated non-swim rats ($p<0.05$).

With regards *Pomc* mRNA expression in the hippocampus, amygdala or hypothalamus, two-way ANOVA revealed no significant difference (surgery, swim, or surgery x swim interaction effect) in *Pomc* mRNA expression between sham-operated and OB rats, regardless of swim exposure (Table 4.6).

Group	Hippocampus	Amygdala	Hypothalamus	Prefrontal Cortex
Sham Non-Swim	1 ± 0.77	1 ± 0.68	1 ± 0.31	1 ± 0.21
Sham Swim	0.71 ± 0.44	0.85 ± 0.33	0.71 ± 0.26	1.72 ± 0.59
OB Non-Swim	0.86 ± 0.44	1.05 ± 0.63	0.70 ± 0.16	**1.97 ± 0.71***
OB Swim	0.79 ± 0.42	0.81 ± 0.37	0.74 ± 0.31	1.72 ± 0.70
Effect				
Surgery	$F_{(1,31)}$=0.022, p=0.882	$F_{(1,31)}$=0.000, p=0.985	$F_{(1,32)}$=1.913, p=0.176	**$F_{(1,29)}$=4.816, p=0.036**
Swim	$F_{(1,31)}$=0.871, p=0.358	$F_{(1,31)}$=1.076, p=0.308	$F_{(1,32)}$=1.851, p=0.183	$F_{(1,29)}$=1.138, p=0.295
Surgery x Swim	$F_{(1,31)}$=0.316, p=0.578	$F_{(1,31)}$=0.057, p=0.813	$F_{(1,32)}$=3.168, p=0.085	**$F_{(1,29)}$=4.964, p=0.034**

Table 4.6 POMC (*Pomc*) peptide mRNA expression in discrete brain regions. *Pomc* mRNA expression is expressed as a percentage of the sham-operated non-swim group. *Pomc* mRNA expression was increased in the prefrontal cortex of OB non-swim rats when compared to sham-operated non-swim controls. There was no significant difference in *Pomc* mRNA expression between sham-operated and OB rats in the hippocampus, amygdala or hypothalamus. Data are expressed as mean ± standard deviation (n=8-9 per group). *$p<0.05$ vs. sham-operated non-swim rats.

4.3.4.2 KOP system

4.3.4.2.1 KOP (*Oprk1*) mRNA expression in discrete brain regions after swim stress and olfactory bulbectomy exposure

With regards *Oprk1* mRNA expression in the hippocampus, two-way ANOVA revealed that there was a significant swim effect and a surgery x swim interaction effect, but no surgery effect (Table 4.7). *Post-hoc* analysis revealed that there was a swim effect, in that sham-operated swim rats had decreased *Oprk1* mRNA expression in the hippocampus in comparison to sham-operated non-swim rats ($p<0.05$). There was also a surgery effect in that OB non-swim rats had decreased *Oprk1* mRNA expression in the same region in comparison to sham-operated non-swim rats ($p<0.05$). OB swim rats, in contrast had increased *Oprk1* mRNA expression in the hippocampus in comparison to sham-operated swim rats ($p<0.05$).

With regards *Oprk1* mRNA expression in the prefrontal cortex, two-way ANOVA revealed that there was a significant surgery effect, swim effect, and a surgery x swim interaction effect (Table 4.7). *Post-hoc* analysis revealed that there was a swim effect, in that sham-operated swim rats had decreased *Oprk1* mRNA expression in the prefrontal cortex in comparison to sham-operated non-swim rats ($p<0.05$). There was also a surgery effect in that OB non-swim rats had decreased *Oprk1* mRNA expression in the same region in comparison to sham-operated non-swim rats ($p<0.05$).

With regards *Oprk1* mRNA expression in the amygdala and hypothalamus, two-way ANOVA revealed no significant difference (surgery, swim, or surgery x swim interaction effect) in *Oprk1* mRNA expression between sham-operated and OB rats, regardless of swim exposure (Table 4.7).

Group	Hippocampus	Amygdala	Hypothalamus	Prefrontal Cortex
Sham Non-Swim	1 ± 0.27	1 ± 0.30	1 ± 0.26	1 ± 0.20
Sham Swim	**0.46 ± 0.11***	0.80 ± 0.14	1.12 ± 0.12	**0.69 ± 0.14***
OB Non-Swim	**0.61 ± 0.13***	0.96 ± 0.21	1.12 ± 0.18	**0.55 ± 0.19***
OB Swim	**0.77 ± 0.22^{+}**	1.03 ± 0.29	0.96 ± 0.20	0.54 ± 0.11
Effect				
Surgery	$F_{(1,31)}$=0.339, p=0.564	$F_{(1,30)}$=1.170, p=0.288	$F_{(1,32)}$=0.061, p=0.807	**$F_{(1,29)}$=24.361, p<0.001**
Swim	**$F_{(1,31)}$=7.017, p=0.013**	$F_{(1,30)}$=0.478, p=0.495	$F_{(1,32)}$=0.097, p=0.757	**$F_{(1,29)}$=7.267, p=0.012**
Surgery x Swim	**$F_{(1,31)}$=25.424, p<0.001**	$F_{(1,30)}$=2.330, p=0.137	$F_{(1,32)}$=3.871, p=0.058	**$F_{(1,29)}$=5.993, p=0.021**

Table 4.7 KOP (*Oprk1*) mRNA expression in discrete brain regions. *Oprk1* mRNA expression is expressed as a percentage of the sham-operated non-swim group. *Oprk1* mRNA expression was significantly decreased in the hippocampus and prefrontal cortex of sham-operated swim and OB non-swim rats in comparison to sham-operated non-swim rats. OB swim rats were also found to have significantly decreased *Oprk1* mRNA expression in the hippocampus in comparison to sham-operated swim rats. There was no significant difference in *Oprk1* mRNA expression between sham-operated and OB rats in the hypothalamus and amygdala. Data are expressed as mean ± standard deviation (n=8-9 per group). *p<0.05 vs. sham-operated non-swim rat, ^{+}p<0.05 vs. sham-operated swim rat.

4.3.4.2.2 PDYN (*Pdyn*) mRNA expression in discrete brain regions after swim stress and olfactory bulbectomy exposure

With regards *Pdyn* mRNA expression in the hippocampus, two-way ANOVA revealed that there was a significant swim effect, but no surgery effect, or swim x surgery interaction effect (Table 4.8). However, *post-hoc* analysis revealed that there was no significant difference in *Pdyn* mRNA expression in the hippocampus between sham-operated and OB animals, regardless of swim stress exposure.

With regards *Pdyn* mRNA expression in the prefrontal cortex, two-way ANOVA revealed that there was a significant swim effect, and swim x surgery interaction, but no surgery effect (Table 4.8). *Post-hoc* analysis revealed that OB swim rats had significantly increased *Pdyn* mRNA expression in the prefrontal cortex in comparison to OB non-swim rats ($p<0.05$).

With regards *Pdyn* mRNA expression in the amygdala or hypothalamus, two-way ANOVA revealed no significant difference (surgery, swim, or surgery x swim interaction effect) in *Pdyn* mRNA expression between sham-operated and OB rats, regardless of swim exposure in the amygdala or hypothalamus (Table 4.8).

Group	Hippocampus	Amygdala	Hypothalamus	Prefrontal Cortex
Sham Non-Swim	1 ± 0.15	1 ± 0.37	1 ± 0.09	1 ± 0.37
Sham Swim	1.14 ± 0.32	0.92 ± 0.20	1.10 ± 0.15	0.89 ± 0.42
OB Non-Swim	0.80 ± 0.22	0.76 ± 0.18	1.02 ± 0.15	0.34 ± 0.22
OB Swim	1.04 ± 0.26	0.83 ± 0.32	0.98 ± 0.15	**1.34 ± 0.82**$^{+}$
Effect				
Surgery	$F_{(1,30)}$=2.754, p=0.107	$F_{(1,30)}$=2.543, p=0.121	$F_{(1,31)}$=1.082, p=0.306	$F_{(1,27)}$=0.271, p=0.607
Swim	**$F_{(1,30)}$=4.206, p=0.049**	$F_{(1,30)}$=0.001, p=0.973	$F_{(1,31)}$=0.399, p=0.532	**$F_{(1,27)}$=5.022, p=0.033**
Surgery x Swim	$F_{(1,30)}$=0.248, p=0.622	$F_{(1,30)}$=0.543, p=0.467	$F_{(1,31)}$=1.892, p=0.179	**$F_{(1,27)}$=7.931, p=0.009**

Table 4.8 Peptide PDYN (*Pdyn*) mRNA expression in discrete brain regions. *Pdyn* mRNA expression is expressed as a percentage of the sham-operated non-swim group. *Pdyn* mRNA expression was significantly increased in the prefrontal cortex of OB swim rats in comparison to OB non-swim rats. There was no significant difference in *Pdyn* mRNA expression between sham-operated and OB rats in the hippocampus, hypothalamus and amygdala. Data are expressed as mean ± standard deviation (n=7-9 per group). $^{+}p<0.05$ vs. OB non-swim rat.

4.3.4.3 DOP system

4.3.4.3.1 DOP (*Oprd1*) mRNA expression in the discrete brain regions after swim stress and olfactory bulbectomy exposure

With regards *Oprd1* mRNA expression in the hypothalamus, two-way ANOVA revealed that there was a significant surgery x swim interaction effect, but no effect of surgery or of swim alone (Table 4.9). However, *post-hoc* analysis revealed that there was no significant difference between the groups.

With regards *Oprd1* mRNA expression in the prefrontal cortex, two-way ANOVA revealed that there was a significant surgery effect, but no swim effect, or swim x surgery interaction effect (Table 4.9). *Post-hoc* analysis revealed that OB rats, regardless of swim exposure or not, had significantly decreased *Oprd1* mRNA expression in the prefrontal cortex in comparison to their appropriate sham-operated rat counterparts ($p<0.05$).

With regards *Oprd1* mRNA expression in the hippocampus and the amygdala, two-way ANOVA revealed that there was no significant difference (surgery, swim, surgery x swim interaction effect) in *Oprd1* mRNA expression between sham-operated and OB rats, regardless of swim. (Table 4.9).

Group	Hippocampus	Amygdala	Hypothalamus	Prefrontal Cortex
Sham Non-Swim	1 ± 0.39	1 ± 0.77	1 ± 0.22	1 ± 0.20
Sham Swim	0.89 ± 0.20	0.66 ± 0.22	1.11 ± 0.14	1.07 ± 0.26
OB Non-Swim	0.92 ± 0.34	0.69 ± 0.18	1.17 ± 0.24	**0.70 ± 0.22***
OB Swim	0.89 ± 0.18	0.80 ± 0.38	0.99 ± 0.08	**0.70 ± 0.19^{+}**
Effect				
Surgery	$F_{(1,32)}$=0.127, p=0.724	$F_{(1,32)}$=0.273, p=0.605	$F_{(1,31)}$=0.078, p=0.783	**$F_{(1,31)}$=17.776, $p<0.001$**
Swim	$F_{(1,32)}$=0.428, p=0.518	$F_{(1,32)}$=0.521, p=0.475	$F_{(1,31)}$=0.246, p=0.623	$F_{(1,31)}$=0.212, p=0.649
Surgery x Swim	$F_{(1,32)}$=0.190, p=0.666	$F_{(1,32)}$=1.935, p=0.174	**$F_{(1,31)}$=4.945, p=0.034**	$F_{(1,31)}$=0.184, p=0.671

Table 4.9 DOP (*Oprd1*) mRNA expression in discrete brain regions. *Oprd1* mRNA expression is expressed as a percentage of the sham-operated non-swim group. *Oprd1* mRNA expression was significantly decreased in the prefrontal cortex of OB rats, when compared to appropriate sham-operated control counterparts. There was no significant difference in *Oprd1* mRNA expression between sham-operated and OB rats in the hippocampus, hypothalamus and amygdala. Data are expressed as mean ± standard deviation (n=8-9 per group). *$p<0.05$ vs. sham-operated non-swim rat, $^{+}p<0.05$ vs. sham-operated swim rat.

4.3.4.3.2 PENK (*Penk*) mRNA expression in discrete brain regions after swim stress and olfactory bulbectomy exposure

With regards *Penk* mRNA expression in the hippocampus, two-way ANOVA revealed that there was a significant surgery x swim interaction effect, but no swim or surgery effect alone (Table 4.10). *Post-hoc* analysis revealed that the sham-operated swim rats had decreased *Penk* mRNA expression in the hippocampus in comparison to sham-operated non-swim rats ($p<0.05$). It was also shown that OB non-swim rats had decreased *Penk* mRNA expression in the hippocampus in comparison to sham-operated non-swim rats ($p<0.05$).

With regards *Penk* mRNA expression in the prefrontal cortex, two-way ANOVA revealed that there was a significant surgery effect, but no swim effect, or swim x surgery interaction effect (Table 4.10). *Post-hoc* analysis revealed that OB rats, regardless of swim exposure or not, had significantly decreased *Penk* mRNA expression in the prefrontal cortex in comparison to their appropriate sham-operated rat counterparts ($p<0.05$).

With regards *Penk* mRNA expression in the amygdala and hypothalamus, two-way ANOVA revealed that there was no significant difference (surgery, swim, surgery x swim interaction effect) in *Penk* mRNA expression between sham-operated and OB rats, regardless of swim. (Table 4.10).

Group	Hippocampus	Amygdala	Hypothalamus	Prefrontal Cortex
Sham Non-Swim	1 ± 0.12	1 ± 0.48	1 ± 0.11	1 ± 0.20
Sham Swim	**0.74 ± 0.13***	1.26 ± 0.33	0.97 ± 0.10	0.99 ± 0.17
OB Non-Swim	**0.75 ± 0.18***	0.94 ± 0.27	0.98 ± 0.13	**0.65 ± 0.15***
OB Swim	0.91 ± 0.21	0.95 ± 0.60	1.02 ± 0.16	**0.54 ± 0.14^{+}**
Effect				
Surgery	$F_{(1,29)}$=0.384, p=0.540	$F_{(1,31)}$=1.276, p=0.267	$F_{(1,32)}$=0.107, p=0.746	**$F_{(1,32)}$=46.906, p<0.001**
Swim	$F_{(1,29)}$=0.650, p=0.427	$F_{(1,31)}$=0.713, p=0.405	$F_{(1,32)}$=0.026, p=0.874	$F_{(1,31)}$=0.986, p=0.328
Surgery x Swim	**$F_{(1,29)}$=12.777, p=0.001**	$F_{(1,31)}$=0.614, p=0.439	$F_{(1,32)}$=0.635, p=0.431	$F_{(1,31)}$=0.793, p=0.380

Table 4.10 PENK (*Penk*) mRNA expression in discrete brain regions. *Penk* mRNA expression is expressed as a percentage of the sham-operated non-swim group. In the hippocampus, sham-operated swim rats and OB non-swim rats had significantly decreased *Penk* mRNA in comparison to sham-operated non-swim rats. *Penk* mRNA expression was significantly decreased in the prefrontal cortex of OB rats when compared to appropriate sham-operated control counterparts. There was no significant difference in *Penk* mRNA expression between sham-operated and OB rats in the hypothalamus and amygdala. Data are expressed as mean ± standard deviation (n=8-9 per group). *p<0.05 vs. sham-operated non-swim rat, ^{+}p<0.05 vs. sham-operated swim rat.

4.3.5 *c-fos* mRNA expression in the hypothalamus after swim stress and olfactory bulbectomy exposure

With regards *c-fos* mRNA expression in the hypothalamus, two-way ANOVA revealed that there was a significant swim effect, but no surgery effect, or swim x surgery interaction effect (Table 4.11). *Post-hoc* analysis revealed that swim rats, regardless of surgery group, had significantly increased levels of *c-fos* mRNA in the hypothalamus in comparison to appropriate non-swim counterparts ($p<0.05$).

Group	Hypothalamus
Sham Non-Swim	1 ± 0.48
Sham Swim	**8.82 ± 1.85***
OB Non-Swim	0.78 ± 0.35
OB Swim	**7.51 ± 2.30***
Effect	
Surgery	$F_{(1,30)}=1.821$, $p=0.187$
Swim	**$F_{(1,30)}=165.440$, $p<0.001$**
Surgery x Swim	$F_{(1,30)}=0.912$, $p=0.347$

Table 4.11 *c-fos* mRNA expression in the hypothalamus. *c-fos* mRNA expression is expressed as a percentage of the sham-operated non-swim group. Swim rats, both sham-operated and OB, had significantly increased expression of *c-fos* mRNA in the hypothalamus, in comparison to their non-swim counterparts. Data are expressed as mean ± standard deviation (n=8-9 per group). *$p<0.05$ vs. non-swim counterpart.

4.4 Discussion

In this study, the overall aim was to investigate the effects of acute and chronic stressors, alone and in combination, on the mRNA expression of opioidergic receptors and pre-propeptides. Swim stress was used as the acute stressor, while the procedure and adaptions as a result of OB surgery were considered as exposure to the chronic stressor. Behaviour in the forced swim stress was examined by a number of methods, c-fos mRNA expression was examined as a marker of stress induction after exposure to the acute swim stress and opioid receptor and precursor peptide mRNA expression was examined in a number of regions related to stress and depression. Sham-operated rats exposed to swim stress exhibited a characteristic initial increase in escape behaviour followed by immobility, an effect which did not differ in OB rats. An increase in c-fos expression was observed in all swim rats, confirming that swim exposure induced neuronal activation in the hypothalamus. Analysis of opioid receptor expression revealed a number of different findings with effects strictly attributed to the KOP and DOP systems and specifically in the hippocampus and prefrontal cortex.

Firstly, with regards bodyweight gain, and as seen with our sham-operated and OB rats who were paired together in *experiment 1* in Chapter 3, there was no significant difference in bodyweight gain between sham-operated and OB rats over the course of this study; as can be seen with the lack of significant differences between surgery groups at five weeks post-surgery. A lack of change in bodyweight is a positive finding, as it indicates that the animals are not experiencing any negative effects as a result of surgery and this finding could be due in part to the improved and additional surgical techniques that have been implemented as part of the surgical procedure.

With regards the behaviours in the forced swim stress, classic swimming, climbing and immobility behaviours were examined by a number of methods, using both manual and automated approaches. These classic behaviours scored were also adapted, such that a new strict immobility could be classified; as that of no movement at all, rather than the minimum required by the rat to stay afloat; immobility-lenient. In addition to this, all behaviours also were broken down into 15-minute timebins per scoring technique, and per calculated formulation, to see if there was any effect on behaviours over time. Therefore, with regards total duration in the forced swim, regardless of which scoring technique was used (continuous, count, or automated activity analysis), there was consistently no significant difference in swimming,

climbing or immobility between sham-operated and OB rats in this study. The automated method of scoring that was introduced, explored a novel method that evaluating the behaviour which examined the activity of the rats and displayed a much higher level of reduced variance in both the total duration and temporal profile, highlighting the importance of a method that shows consistent findings that could be further developed for wider use.

Our findings of no differences in behaviours in the forced swim test are at variance with those seen in the majority of previous investigations with the OB rat, as indicated by the meta-analysis for this test in Chapter 2. However, there are a number of differences between this study and these other studies mentioned above. Firstly, our approach was to expose the animals to a single acute swim exposure in the form of the pre-swim, not in the classic FST, where animals are exposed to the apparatus twice, and with the second exposure being typically shortened to 5 minutes; the behaviour from this second exposure is that which is usually reported in papers. As a result, the results in these studies are not very comparable to the results in this OB study. Although most of the OB-FST studies did not include pre-swim data, those that do report conflicting results, with some studies showing an increase in immobility in OB animals (Morales-Medina et al., 2012a; 2012b; 2012c; 2013, Yang et al., 2014), and other showing a decrease in immobility in OB animals (Kalshetti et al., 2015b, Linge et al., 2013). The timepoint at which animals were exposed to the swim in these studies when compared to ours must also be taken into consideration. Morales-Medina et al. (2012a; 2012b, 2012c, 2013) tested animals at four weeks post-surgery, Kalshetti et al. (2015b) did not state the timepoint at which animals were tested, and although Yang et al. (2014) tested rats at 31 days post-surgery, the closest timepoint to ours, they only scored the last four minutes of a six minute exposure, and so the animals total time spent immobile for the duration of testing is unknown. Nevertheless, this experiment is not the only study to find a lack of behavioural effect, with a number of papers having shown no significant changes in immobility in OB rats when tested in the forced swim apparatus (Healy et al., 1999, Kelly and Leonard, 1999, Pudell et al., 2014, Stepanichev et al., 2016, Vieyra-Reyes et al., 2008). Stepanichev et al. (2016) also tested rats over five weeks post-surgery (day 36), and found this lack of behavioural effect between sham-operated and OB rats. However, it must be remembered that these papers are all second exposure results and as such, are less comparable.

With regards to swimming and climbing behaviours, again no significant differences were found between sham-operated and OB rats in this study. Interestingly, of the mass amount of OB studies examined, very few have reported on swimming and climbing behaviour after forced swim exposure. One study reported similar findings to this experiment in that there was no significant difference in climbing or swimming behaviours in OB rats when compared to sham-operated rats, but this was in a 5-minute second exposure (Pudell et al., 2014). In contrast, Kalshetti et al. (2015b), reported that during a single 5 minute exposure OB rats displayed a significant increase in swimming when compared to sham-operated rats, with no significant difference in climbing behaviours. Some studies use modified terms and parameter conditions by which they measure behaviours similar to swimming and/or climbing *per se*. For example, Padilla et al. (2018) examined 'struggling' time in OB rats, indicative of the front paws breaking the surface of the water, similar to that of climbing, and found that OB rats exhibited reduced 'struggling' time when compared to sham-operated rats. In a similar fashion, Shin et al. (2017) examined 'fast' behaviour, described as a rat in an active vertical motion with its forepaws above the water, again similar to climbing, and found that OB rats spent less time exhibiting 'fast' behaviour when compared to sham-operated rats. In contrast, Maturana et al. (2015) found that OB animals showed a significant increase in climbing behaviour, and a significant decrease in swimming behaviour in the FST. Taking all of this into account, there is quite a range of variability in swimming and climbing behaviour in OB rats, and a lack of consistency in these measures and the way they are often specifically defined. As a result, it is difficult to explain what these active behaviours might mean in the OB syndrome as no one explicit effect in climbing or swimming behaviour is consistently seen.

Similar to Padilla et al. (2018) and Shin et al. (2017), in this study we decided to redefine the FST behaviour and categorise immobility under two separate branches; immobility-strict and immobility-lenient. The aim was to define explicit immobility as an unequivocal static movement, rather than that which is necessary for the rat to remain floating above the water. No matter which way immobility-strict, or indeed inactive behaviour, was analysed in the study, it was consistently shown that there was no significant difference in sham-operated and OB rats with regards this parameter. In itself, this is a steadfast and reliable finding. Classic immobility scored in the FST can often be subjective, and in this regard immobility-lenient can be bracketed in a similar

subjective fashion. Time spent immobile in *sham-operated* rats tested in the FST has been shown to unveil a range of scores, with a study by Smaga et al. (2012) showing a score of 122.34 ± 15.12 seconds immobile, and a study by Rinwa and Kumar (2013) finding a score of 72.85 ± 24.15 seconds immobile. Both studies used a continuous method of scoring, used Wistar rats, tested the rats twice, and summed up data from the second 5-minute exposure. Even though these findings are not directly comparable to ours, they demonstrate that a subjective scoring approach can result in divergent results across laboratories. These findings show that scoring parameters can be subjective, and more importantly, that similar to humans and regardless of treatment or syndrome, animals do not always act the same.

Of the three methods of scoring that were used; continuous, count or automated, there was no significant difference in behaviours across any of the categories analysed. As a whole, this again reveals a consistency in our OB rats and this model, in that when exposed to the acute swim stress, these OB syndrome rats displayed no behavioural adaptations or differences to sham-operated rats. The most commonly used scoring technique still appears to be the original continuous method (See Table 4.1), although some studies are beginning to utilise the count method, and automated tracking-techniques. Regardless, in this experiment, irrespective of method used, OB rats did not differ from sham-operated rats in any behavioural manner. Whether this is argued as a strength and consistency in our OB animals and model, or indeed a lack of difference in the method of scoring used and so an indication that all scoring techniques, though quite different, appear equally as efficacious, is left to be decided.

On a separate note it should be noted that a limitation of this study is that the OB animals were not exposed to the OF test. This was not possible as part of this particular Chapter as we only wanted the animals to be exposed to one acute stressor, and had the animals been exposed to the OF test after the swim stress, this would not have been in line with our aims. This particular experiment was undertaken as part of a collaboration whereby the impact of acute stress to the opioid system was examined in a number of animal models of disease. The forced swim apparatus was choosen as the common stressful paradigm to use and examine in all four animal models, including the OB model. Future studies could employ a similar experimental design as this experiment but with the OF test as the acute stressor. In this manner, the hyperactive profile in the OB model could be more accurately verified.

To examine and confirm that exposure to the swim stress induced neuronal activation in the hypothalamus, c-fos mRNA expression was examined as a putative marker. c-Fos is a marker for neuronal activity and is classically associated with clarifying the effects of stress and validating that neurochemically a stress effect has been observed (Bullitt, 1990). The hypothalamus is a region in the brain involved with the regulation of stress that activates regions and glands in the brain that secrete factors in response to stress such as ACTH, CRF and cortisol. The paraventricular nucleus (PVN) in the hypothalamus is dense with neurons containing CRF which have been shown to be activated by stress (Lin et al., 2018). Rats exposed to a 10-minute pre-swim have been shown to have increased c-fos mRNA expression (Cullinan et al., 1995), and Fos-like expression (Cullinan et al., 1996) in a number of areas of the hypothalamus. Fos-like expression has also been shown to be increased in the hypothalamus of mice after exposure to a 10-minute pre-swim (Yanagida et al., 2016). In addition to this, Fos-like expression has been shown to be increased in the PVN after exposure to other stressful paradigms such as restraint stress, electric foot shock stress and multi-modal stress (Lin et al., 2018, Maras et al., 2014). Activity of c-fos mRNA expression was examined to validate that animals had actively experienced stress-related neuronal activation. Rats exposed to the swim stress, regardless of surgery group, were shown to have significantly increased levels of c-fos mRNA expression when compared to non-swim rats, indicating that all swim rats had increased neuronal activity as a result of swim exposure, in comparison to rats that were not swum.

The opioid system is another system well-validated for its involvement in the regulation of mood and affect and as such has opposing effects when it comes to the modulation of stress and stress-related functioning (Valentino and Bockstaele, 2015). In this study, a number of changes to opioid receptor and prepropeptide mRNA expression were examined after exposure to acute and chronic stressors in several brain regions associated with stress and depression (Table 4.12).

Group	Hippocampus	Amygdala	Hypothalamus	Prefrontal Cortex
MOP	–	–	–	–
DOP	–	–	–	↓ OB effect
KOP	↓ OB and swim effect	–	–	↓ OB and swim effect
POMC	–	–	–	↑ OB effect
PENK	↓ OB and swim effect	–	–	↓ OB effect
PDYN	–	–	–	↑ OB swim effect

Table 4.12 Summary of opioidergic receptor and peptide mRNA expression in the hippocampus, amygdala, hypothalamus and prefrontal cortex after exposure t to acute (forced swim) and chronic (OB) stressors. DOP=delta opioid receptor, KOP=kappa opioid receptor, MOP=mu opioid receptor, OB=olfactory bulbectomy, PENK=pre-proenkephalin, PDYN=pre-prodynorphin, POMC=pro-opiomelanocortin, – =no significant difference, ↓=decreasing ↑=increasing.

As mentioned in Chapter 3, there are very few studies that have examined the opioid system in the OB model of depression so the majority of the findings in this chapter are relatively novel. In the hypothalamus and the amygdala, we found no significant differences in the mRNA expression of any of the opioidergic receptors or endogenous peptides examined between sham-operated and OB rats, regardless of the presence or absence of swim exposure. Similarly, Hirsch (1980), found that opioid binding of MOP, KOP and DOP was initially increased in the hypothalamus of OB mice at one week post-surgery, and decreased in the amygdala of OB mice at the same time-point, but that all three receptors mentioned had returned to baseline by 4 weeks post-surgery. This is closely in-line with our results which were observed at 5 weeks post-surgery. Although the method of molecular analysis used by Hirsch (1980) differs from that of the method we employ, it still provides implications for the opioid system and these two regions in this particular model, with our experiment adding to the knowledge of a lack of change in these two regions, or indeed perhaps a return to baseline in these two regions over time.

In the hippocampus, a decrease in KOP mRNA expression and PENK mRNA expression observed in both sham-operated swim rats, and OB non-swim rats. The decrease in these two experimental sub-groups, both of which experienced a single

stressor; either acute or chronic, exemplifies the link between the opioid system and stress-related function. This being said, the majority of studies that have examined links between KOP activity, stress and depression, have shown that acute stress exposure is associated with an increase in KOP and DYN mRNA expression in a number of regions (Van't Veer and Carlezon, 2013, Knoll et al., 2011, Shirayama et al., 2004, Chartoff et al., 2009), or indeed that blockade of KOP activity with a KOP antagonist causes antidepressant-like behaviour (Shirayama et al., 2004). As a result, the significant *decrease* seen in KOP mRNA in this study in the hippocampus after exposure to two differing stressors requires further investigation. In a study by Haj-Mirzaian et al. (2019), a decrease in KOP mRNA was seen in the hippocampus of socially-isolated rats after exposure to a number of stress-induced test. Indeed, a brief literature search revealed no specific instances of KOP *reductions* following acute swim stress, with one study conducted by Flaisher-Grainberg et al. (2012), showing that KOP mRNA was shown to be increased in the hippocampus of rats after exposure to a repeated force swim test paradigm. One argument for this decrease in KOP mRNA, could be that the reduction in KOP mRNA in this region may be as a result of an adaptive response to increased KOP activation by endogenous DYN release (Van't Veer and Carlezon, 2013). However, it must be noted that no changes were found in PDYN mRNA expression in this region in this experiment. Interestingly, OB swim rats showed an increase in KOP mRNA expression in the hippocampus when compared to relevant sham-operated swim rats, bearing in mind that these were still both below the non-swim sham-operated controls. Nevertheless, this was unexpected, as with the decrease in KOP mRNA in this region as a result of the swim in sham-operated rats, and then again as a result of the surgery in OB non-swim rats, a further decrease when these two stressors were combined could have been hypothesised. However, perhaps a 'floor' effect was in action here, with the receptor having reached its lowest level possible with exposure to each stressor alone. Having said this, when these two stressors were combined, OB swim rats experienced a significant magnitude of increase in KOP activity. KOP opioid activity mediates dysphoria and so increased KOP activity would exemplify increased dysphoric arousal (Lutz and Kieffer, 2013). Under stressful conditions, this logically would mean that KOP activity would be heightened or increased and so perhaps the combination of both stressors, rather than just one stressor alone, is that which was needed in order to cause an elevated change in expression in this region (Lutz and Kieffer, 2013).

With regards PENK mRNA expression in the hippocampus, similar effects to KOP mRNA expression were seen, with significant decreases in expression in the sham-operated swim and OB non-swim groups but with no changes seen in the OB swim rats. Perhaps a 'ceiling' effect occurred here, in that the combination of the two stressors was not strong enough to displace this peptide any further. Overall, PENK mRNA in the hippocampus has been less explored. ENK mRNA has been shown to be increased in the hippocampus of rats after exposure to chronic immobilization stress (Chen et al., 2004), and in contrast PENK mRNA has been shown to be no different in CMS-exposed mice in the hippocampus when compared to wild-type mice (Melo et al., 2014). Activating the DOP system is considered to be antidepressant-like in nature, and so a decrease in DOP functioning has been shown to increase anxiety and depressive-like behaviour (Bérubé et al., 2014). With this in mind, the significant decrease in this peptide in this study, after exposure to each form of stressor, is in accordance with this theory.

Lastly, the prefrontal cortex was the region most affected after exposure to both swim stress and OB surgery. With regards the DOP system, OB surgery caused a significant decrease in both DOP and its endogenous peptide PENK mRNA expression in this region. Prefrontal cortical DOP protein levels have been shown to be decreased in mice after exposure to social defeat stress, single forced swim testing, and repeated forced swim testing, all when compared to naïve mice (Rosa et al., 2018a; 2018b). Met-enkephalin has also been shown to be down-regulated in the prefrontal cortex of rats after exposure to chronic stress via the peripubertal stress protocol, followed by acute restraint stress in adulthood (Li et al., 2018). As only the surgery effected this system, perhaps a chronic stressor that caused longer-lasting adaptations in these animals was explicitly necessary in order to cause a change in this system. The decrease in both receptor and pre-propeptide mRNA expression in the DOP system as a result of exposure to this particular chronic model of depression, strengthens the knowledge of the DOP system as a modulatory system in stress and depression, whereby agonism of this system should therefore reduce stress-related effects.

With regards the KOP system, similar findings to the hippocampus were found, in that KOP mRNA expression in the prefrontal cortex was decreased in sham-operated swim rats, and in OB non-swim rats. Again a single stressor reduced KOP mRNA expression in a region associated with cognitive functioning. The hippocampus and prefrontal cortex are linked via the corticolimbic pathway whereby learning and

memory in the hippocampus, effect motivation and executive function in the prefrontal cortex, and vice versa. The similar findings in these two regions suggests that KOP plays an important role in the functioning of memory and cognitive function. Unlike other KOP antagonism studies, in a study by Takahashi et al. (2018), intracerebroventricular administration of two different KOP agonists restored cognitive function in OB mice in the PA task (Takahashi et al., 2018). This finding regarding KOP agonists in this study, and particularly in the OB model, indicates that the KOP system and the pathways it activates still has much left to be deciphered. Interestingly, unlike the hippocampus, there was no significant difference in the KOP mRNA expression of OB swim rats when compared to OB non-swim rats, indicating that unlike the hippocampus, the prefrontal cortex was not affected by the combination of an acute and chronic stressful stimuli. Unlike the prefrontal cortex, the ventral hippocampus is involved in the processing of conditioned fear and anxiety, so perhaps the effects in the OB swim rats seen in this region were due to further KOP adaptations in this region as a result of fear processing in combination with longer term effects of OB surgery. Interestingly, the peptide PDYN mRNA expression was increased in the prefrontal cortex of OB swim rats when compared to OB non-swim rats. Examination of the protein levels in these regions via enzyme-linked immunosorbent assay (ELISA) or mass spectrometry may have helped to provide more insight as to why these increases in expression are only seen in *either* the receptor and pre-propeptide in each region.

OB surgery also affected POMC mRNA expression, with an increase in the pre-propeptide observed in the prefrontal cortex, but only in non-swim rats. With this in mind, only the effects of the chronic stress were enough to cause a change in this region. In contrast to the increase found in this study, a reduction in POMC mRNA expression in the prefrontal cortex of rats has previously been shown after exposure to chronic immobilisation stress (Chen et al., 2008), and chronic social instability stress (Nowacka-Chmielewska et al., 2017). Again both these studies indicate that a chronic stressor was necessary in order to cause a change, but both studies show a change in the opposite direction to ours. Having said this, the OB model is a surgical model and so more long-term downstream adaptations as a result of the surgery may be causing the increase in our experiment rather than the decrease seen in the other experiments. The MOP system is probably the most researched of all three opioid systems with regards depression and stress, and drugs with MOP agonistic activity have shown

antidepressant efficacy within the clinic (Serafini et al., 2018, Wagstaff et al., 2001), but interestingly this system also has converse effects in some preclinical studies (Lutz and Kieffer, 2013). Unlike the acute euphoric effects of MOP activation, chronic MOP activation has been shown to be have the reverse effects, promoting depressive-like and anxiety-like behaviours, particularly in MOP-KO mice (Filliol et al., 2000, Lutz and Kieffer, 2013). The increase in mRNA in the pre-propeptide in this study could indicate that the majority of MOP receptors have been activated and are bound as a result of exposure to the chronic (OB) stressor, causing an increase in POMC mRNA as there is more POMC being stimulated as a result of longer term adaptations from OB surgery. Another justification for this increase could be the link between POMC and the HPA-axis. POMC stimulates CRF secretion and as a result is a marker for the activation of stress-related function (Chen et al., 2008). The increase seen in POMC mRNA expression could be as a result of stress functioning, and in this manner POMC stimulation of other stress markers upon the actual stimulus of the chronic (OB) stress. As to why this effect in this system is only seen in the prefrontal cortex, and particularly not in the hypothalamus, is unknown, but it must be noted that as POMC is also the precursor for ACTH, it is difficult to say whether it may be transcribed to ACTH rather than to the endorphins.

In conclusion, acute forced swim stress induces swimming, climbing or immobility behaviour which can be assessed using a variety of different methods. The behavioural phenotype on exposure to the forced swim stress did not differ between sham-operated and OB rats. Regardless of what scoring technique was used, manual or automated, the OB rats in this study behaved consistently similar to sham-operated rats in the forced swim apparatus. Although no behavioural changes occurred, all rats exposed to the 15-minute swim stress had significantly increased c-fos mRNA expression in the hypothalamus when compared to non-swim rats, indicating that the presence of the swim still caused increased neuronal activation in these animals. The prefrontal cortex and the hippocampus were the two regions that we examined that were affected as a result of acute and chronic stress exposure; two regions that are executive sources in the control and processing of cognitive function, a feature in depression which has been proven to be dysfunctional. Both acute (forced swim) and chronic (OB) stressors produced qualitatively similar effects to the KOP system, substantiating the role that this system has in regulating stress-related function. Exposure to the chronic (OB) stressor, was primarily seen to effect the DOP system

suggesting that longer-term OB-related alterations are needed in order to cause activation and manipulation of this opioid receptor system. As such, these two opioid receptor systems could be considered when developing methods and tools for coping with stress, with manipulations to the hippocampus and prefrontal cortex presenting potential modulating channels through which to do so.

Chapter 5:

Pharmacological intervention with opioid modulating drugs in the olfactory bulbectomised (OB) rat model of depression

5.1 Introduction

The opioid system has attracted renewed interest as a potential candidate for antidepressant treatment in the last 15-20 years (Berrocoso et al., 2009, Peciña et al., 2019). Widely distributed throughout the central nervous system (CNS) and primarily associated with the treatment and alleviation of pain, the opioid system has been shown to regulate mood and emotion, and is an important feature in the modulation of stress (Lutz and Kieffer, 2013). As the opioid system is an endogenous system that is co-expressed and distributed with the monoamine system, whose targeting has yielded most of the currently-marketed antidepressants, the potential use of the opioid system to combat disorders of mood and stress has promising prospects (Berrocoso et al., 2009). The use of the opioid system as a target for antidepressant therapy actually predates the monoamine-derived treatments produced in the 1950s (Peciña et al., 2019). However, with the emergence of the monoaminergic antidepressant classes such as the tricyclic antidepressants and the monoamine oxidase inhibitors, the use of opioid drugs declined due their less favourable safety profile and as a result of their potential risk for addiction and abuse. Having said this, as a result of the inadequacy of currently marketed antidepressants and the standstill of progression with regards depression therapy, a considerable diversity of targets are being considered for their antidepressant efficacy (as demonstrated in Chapter 2 when we examined the targets examined in the OB rat model). In addition, the recent advances in our understanding of opioid receptor pharmacology have reinvigorated such targets in order to facilitate the current gap in treatment and create new therapeutic strategies for MDD, and treatment-resistant patients (Browne and Lucki, 2019).

In clinical studies, a number of pharmaceutical companies have reached phase III with opioid compounds and with many having shown that opioid modulating drugs have proven efficacious in reducing the symptoms of MDD (Ehrich et al., 2015, Serafini et al., 2018, Thase et al., 2019, Zajecka et al., 2019). In preclinical studies, opioid manipulation has been shown to modulate anxiety and depressive-like behaviour in rodents, as described in the General Introduction in Chapter 1. In summary, MOP and DOP agonists have been shown to reduce depressive-like behaviour in animals, whereas in direct contrast, KOP agonists have been shown to induce depressive-like behaviour in rodents (Berrocoso et al., 2013, Carlezon et al., 2006, Carr et al., 2010, Lutz and Kieffer, 2013, Saitoh et al., 2008, Torregrossa et al., 2006, Zhang et al., 2006; 2007). Opioid KO mice have also presented interesting and

often complex findings with regards depression research. DOP KO mice show increased depressive-like behaviour, with DOP agonists showing the opposite effects (König et al., 1996, Lutz and Kieffer, 2013). In comparison, MOP KO mice show decreased depressive-like behaviour, which is in contrast with the effects of MOP agonists (Ide et al., 2010, Lutz and Kieffer, 2013, Noble et al., 2008).

When considering the development of drugs for treating psychiatric disorders, the tolerability, selectivity, and addiction potential are all important factors that are particularly pertinent when considering drugs that have the opioid system as their site of action. We have shown that the OB rat model is well-established as an animal model of depression, with locomotor and anxiety-like effects seen in the OF, and with cognitive deficits and social dysfunction seen in the 3-chamber sociability test. Classified as a model with strong predictive validity for detecting antidepressant efficacy, it is highly regarded as a suitable paradigm for assessing novel antidepressant compounds. In this chapter, the aim was to assess behavioural and molecular endpoints in the OB rat model after pharmacological manipulation with opioid modulating drugs, and to see what potential these opioid compounds might have to attenuate any of the behavioural and molecular adaptations seen in the OB rat model. As such, the aim was to evaluate the effects of chronic administration of opioid modulators on OF, EPM and 3-chamber sociability test behavioural effects in sham and OB rats. With this in mind, the experimental design of the housing study in Chapter 3 was replicated, but using opioid modulating drugs and on a much larger scale. In the housing study presented in Chapter 3, we found a characteristic habituation effect and social cognitive deficit in the OB rats that interestingly could not be attenuated with chronic exposure to two different classes of conventional antidepressants. As a result, in conjunction with examining the effects of opioid modulation on cognitive decline using this paradigm in this model, it was decided that a positive control would also be examined in tandem. Donepezil is an acetylcholinesterase inhibitor that is used in the treatment of Alzheimer's disease to help alleviate the burden of cognitive deficit and decline that is experienced in the disease (Birks and Harvey, 2018, Knowles et al., 2006). In research using animal models, donepezil has been shown to increase cognitive function and enhance memory performance in the novel objection recognition task and novel object location task, and has been classed and used as a positive control in cognitive research (de Bruin et al., 2011). A number of marketed and experimental

opioid compounds (agonists and antagonists) would also be employed in this study (Table 5.1).

	MOP	KOP	DOP
Agonist	Buprenorphine (functional antagonist at kappa), RDC 2944	U50,488	SNC80
Antagonist	Cyprodime	DIPPA	–

Table 5.1 Mechanism of action of opioid modulating drugs used for experiments in Chapter 5. – =not applicable, as not used in these experiments.

Buprenorphine, a partial agonist at the MOP which acts as a functional antagonist at the KOP, has shown antidepressant efficacy in the clinic (Ehrich et al., 2015, Serafini et al., 2018) and antidepressant-like activity in a number of behavioural tests in rodents, including the FST, OF test and NIHY test (Burke et al., 2016, Falcon et al., 2015; 2016, Robinson et al., 2017, Smith et al. 2019). In conjunction with this, buprenorphine has also been shown to have antidepressant-like effects in the OB rat model (Burke et al., 2019b). RDC 2944 is a selective partial agonist at the MOP, and is a compound that was provided by Alkermes Inc., to examine the effects of MOP antagonism in the OB model. Previously examined in IFN-α treated rats (translational model of depression), acute administration of RDC 2944 was shown to have no effect on immobility in the FST (Callaghan et al., 2019). However, the effect of chronic administration of this compound on depression-related behaviour has yet to be assessed. The effects of an MOP antagonist in this model also warranted examination. Cyprodime, a MOP antagonist, has been shown to decrease approach latency and increase food consumption in the NIHY test (Robinson et al., 2017). As stated previously, KOP agonists have been shown to induce depressive-like behaviour and this has been seen with the KOP agonist U50,488 which has been shown to increase immobility time in the FST, decrease sociability, and reduce sucrose preference (Dogra et al., 2016). Interestingly, U50,488, has been shown to improve cognitive deficits in the PA test in OB mice (Takahashi et al., 2018), so this compound was included to elucidate its effects on the OB model in the 3-chamber sociability test. In contrast, KOP antagonists have been shown to have antidepressant-like effects, with the KOP antagonist DIPPA inducing a decrease in immobility in Wistar-Kyoto (Carr et al., 2010) and IFN-α treated rats (Callaghan et al., 2019). SNC80, an agonist at the DOP

has been shown to have efficacy in a number of depressive-like behavioural tests (Haj-Mirzaian et a., 2019, Saitoh et al., 2007, Perrine et al., 2006), whilst also being shown to decrease immobility in the FST, decrease HE, and increase open arm entries in the EPM in the OB rat model (Saitoh et al., 2008). The combination of targeting two opioid receptors, rather than just one receptor, is also beginning to be investigated, and has also been proven efficacious in a number of preclinical studies with regards depression. This could provide a more beneficial strategy to targeting the opioid system, whereby lower doses of each compound could be employed, aiding in attenuation of adverse effects. The use of a DOP agonist in combination with a KOP antagonist (Callaghan et al., 2019, Huang et al., 2016), a MOP agonist in combination with a KOP antagonist (Almatroudi et al., 2015, Falcon et al., 2015; 2016, Rosa et al., 2018a; 2018b), or a MOP agonist (with functional antagonism for kappa) in combination with a MOP antagonist (Bidlack et al., 2018, Burke et al., 2019a; 2019b, Smith et al., 2019) have all been shown to present antidepressant-like effects in preclinical testing. It was decided these combination pairings would also be encompassed and examined for their effects in the OB rat model.

Lastly, and as stated previously, chronic dosing is a much more translational approach as currently-marketed antidepressants have a therapeutic lag, with significant symptomatic improvements reported four-to-six weeks post-treatment. With this in mind, and in direct contrast, most of the studies that have examined the effects of opioid drugs on depression *in vivo* use acute dosing regimens in animals, with very few examining the effects of chronic dosing. These studies therefore, miss out on the importance of tolerability over time that might a cause of concern with repeated administration of drugs. As a result, a chronic dosing regimen was employed that was similar to that used previously with the OB rat model and which is usually necessary to attenuate the OF alterations (Kelly et al., 1997) and to that used with desipramine and fluoxetine in Chapter 3. In order to assess such a diversity of treatments in both sham-operated and OB rats, two large-scale chronic dosing experiments in the OB rat model were undertaken; the first study focusing on the effects of the KOP antagonist DIPPA and the DOP agonist SNC80, alone or in combination, and with the second study primarily examining the MOP agonist buprenorphine and RDC 2944, alone or in combination with the KOP antagonist DIPPA or the MOP antagonist cyprodime.

Taking all of this into consideration, it was hypothesised that opioid modulating drugs, given alone or in combinations, will reverse the social cognitive deficits in the OB rat model, with examination of the opioid receptor and pre-propeptide profile providing a complimentary feature.

Therefore, the specific aims of the experiments in this chapter were as follows:

Experiment 1:

- To assess the effects of chronic dosing with opioid modulating drugs, DIPPA (a KOP antagonist) and SNC80 (a DOP agonist), given alone or in combination, on behavioural responses and opioid expression in the hippocampus in the OB rat model of depression
    - To assess the effects of chronic dosing with opioid modulating drugs in OB rats in the OF test
    - To assess the effects of chronic dosing with opioid modulating drugs on anxiety-like behaviour in the EPM in OB rats
    - To assess the effects of chronic dosing with opioid modulating drugs on social cognition in the 3-chamber sociability test in the OB rat
    - To assess the effects of donepezil, an acetylcholinesterase inhibitor, on social cognition, OF behaviour and EPM behaviour in the OB rat model
    - To analyse opioid receptor and pre-propeptide mRNA expression in the hippocampus after chronic dosing with opioid modulating drugs and an acetylcholinesterase inhibitor in OB rats

      Note: The hippocampus was chosen as the reason for examination due to the results seen in this region in Chapter 4. Although changes were observed in both the hippocampus and the prefrontal cortex, for logistical reasons, i.e. due to the volume and number of brains, only one region could be chosen to be analysed

Experiment 2:

- To assess the effects of chronic dosing with opioid modulating drugs, RDC 2944 (a MOP agonist), DIPPA (a KOP antagonist), U50,488 (a KOP agonist), buprenorphine (a MOP agonist and KOP antagonist) and cyprodime (a MOP antagonist), given alone and/or in different combinations, on behavioural

responses and opioid expression in the hippocampus in the OB rat model of depression

- To assess the effects of chronic dosing with opioid modulating drugs in OB rats in the OF test
- To assess the effects of chronic dosing with opioid modulating drugs on anxiety-like behaviour in the EPM in OB rats
- To assess the effects of chronic dosing with opioid modulating drugs on social cognition in the 3-chamber sociability test in the OB rat
- To analyse opioid receptor and pre-propeptide mRNA expression in the hippocampus after chronic dosing with opioid modulating drugs in OB rats

5.2 Materials and Methods

5.2.1 Materials

See Chapter 3, Section 3.2. All materials for animal sourcing, husbandry and monitoring were kept consistent with *experiment 1* in Chapter 3. All materials needed for OB surgery were kept consistent with Chapter 3, with additional adjustments made to surgery kept consistent with Chapter 4. All materials for behavioural recording, and analyses were kept the same as in Chapter 3. All qRT-PCR kits and reagents used were kept consistent with those used in Chapter 3.

5.2.2 Animals

Experiments were carried out on a total of 140 (*experiment 1*) and 164 (*experiment 2*) male Sprague Dawley rats (100-150 g on arrival, obtained from Charles River, UK). A lower weight range was chosen on arrival, as in each experiment in this chapter two separate cohorts were used, with surgeries for each cohort performed one week after each other. As a result, the acclimatisation period was extended for one cohort, and so that the animal weights at surgery would be similar to that as in the previous experiments, rats were ordered in at a lower weight on initial arrival. In *experiment 1*, 100 rats were used as test subjects and 40 rats served as conspecifics for the 3-chamber sociability test. In *experiment 2*, 120 rats were used as test subjects and 44 rats served as conspecifics for the 3-chamber sociability test. On arrival rats were housed in groups of 4 per cage, in plastic cage bottoms (42 cm x 25.5 cm x 13 cm) with a metal grated cage top with plastic water bottles (North Kent Plastics, Coalville, Leicestershire, UK). Cages contained 3Rs paper bedding (Fibrecycle Ltd., Scunthorpe, Lincolnshire, UK). Prior to surgery, cages also contained sizzle-nesting material for environmental enrichment (LBS Biotechnology, Horley, UK), and rats were given nutritional enrichment once a week (CocoPops, Tesco PLC, Ireland). All test rats were pair-housed one week prior to surgery (based on bodyweights) and were randomly allotted to their surgery and treatment groups. These allotted groups were checked for statistical significance using IBM SPSS Statistics Version 24 software to make sure that there was no significant difference between groups prior to the commencement of the study. The conspecific rats were randomly housed in pairs based on bodyweights at the same time as the test rats, and conspecific rats continued to receive sizzle nesting material and environmental enrichment throughout the duration of the study. All rats were housed in an environment with controlled temperature (20-24°C) and humidity

(45-65%) (Monitor, Radionics Ltd, Dublin, Ireland), in a 12:12 h light-dark cycle, lights on from 08:00 h. Animals had access to food (*Exp. 1 and 2*: 20% protein rodent diet Advanced Protocol® Verified 75 IF Irradiated (5V75), LabDiet®, Brentwood, MO, USA) and water *ad libitum*. Bodyweight, food, and water consumption were monitored daily (weighing scales, Mason Technology, Dublin, Ireland) from one week pre-surgery until sacrifice.

All animal procedures were carried out under the approval of the Animal Care and Research Ethics Committee (ACREC), National University of Ireland, Galway (NUIG) (12/NOV/07). All procedures for this project were approved for authorisation from the Health Products Regulatory Authority (HPRA) and in compliance with EU Directive 86/609 (HPRA Authorisation ID: AE19125/P069).

5.2.3 Experimental Design

Male Sprague Dawley rats (200-250 g) underwent surgery (sham or OB) under isoflurane anaesthesia (*Experiment 1* and *2*) ten days after initial arrival. In both experiments, rats were pair-housed (a sham-operated rat paired with an OB) a week prior to surgery (*n*=10/group), see Figure 5.1. In *experiment 1*, two weeks post-surgery rats received daily subcutaneous injections of vehicle (0.01M hydrochloric acid (HCl) 0.9% saline solution + 20% dimethyl sulfoxide (DMSO) in distilled water (H_2O)), DIPPA (1 mg/kg), SNC80 (1 mg/kg), SNC80/DIPPA (1 mg/kg each), or donepezil (5 mg/kg) for three weeks (*n*=10/group) (Figure 5.1). In *experiment 2*, rats received daily subcutaneous injections of vehicle (0.01M HCl 0.9% saline solution + 20% DMSO in distilled water), RDC 2944 (0.1 mg/kg), RDC 2944/DIPPA (0.1 mg/kg/1 mg/kg), U50,488 (1 mg/kg), buprenorphine (0.1 mg/kg), or buprenorphine/cyprodime (0.1 mg/kg/1 mg/kg) two weeks post-surgery for three weeks (*n*=10/group) (Figure 5.1). In both experiments, rats were tested in the 3-chamber sociability test (four weeks post-surgery), followed by the EPM and then OF test (five weeks post-surgery). In both experiments, immediately after exposure to the OF at five weeks post-surgery, rats were euthanized via decapitation (Figure 5.1).

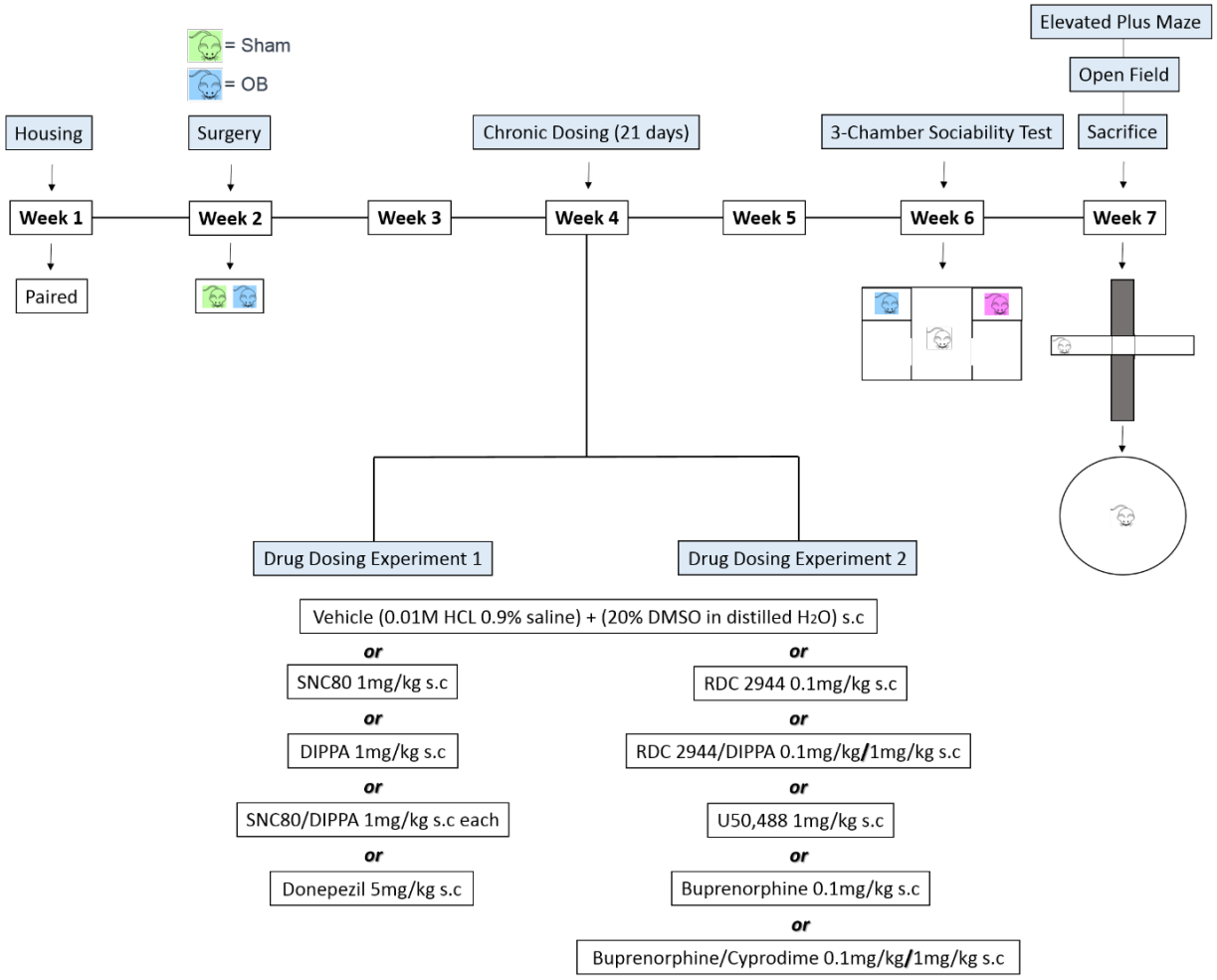


Figure 5.1 Experimental design for experiment 1 and 2.

5.2.4 OB Surgery

This was carried out as described in Chapter 4, section 4.2.4.

5.2.5 Drugs

The chemical name and source for each compound is summarised in Table 5.2. The selection of drug doses for RDC 2944, cyprodime, SNC80 and DIPPA was based upon pharmacodynamic and pharmacokinetic studies and as recommended by Alkermes Inc., (Alkermes Inc., Internal data, Callaghan et al., 2019, Nozaki et al., 2012). The dose selection of the other drugs (buprenorphine, donepezil and U50,488) were based upon Alkermes Inc., internal data, literature review, or previous findings within this lab group (Alkermes Inc., Internal data, Bidlack et al., 2018, Burke et al., 2019b, De Bruin et al., 2011, Dogra et al., 2016, McLaughlin et al., 2004, Smith et al., 2019).

Compound	Source
(+)-4-[(α*R*)-α-((2*S*,5*R*)-4-Allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethylbenzamide **(SNC80)**	Cat# 0764/10, Tocris Bioscience, Bristol, UK
2-(3,4-Dichlorophenyl)-*N*-methyl-*N*-[(1*S*)-1-(3-isothiocyanatophenyl)-2-(1-pyrrolidinyl)ethyl]acetamide hydrochloride **(DIPPA)**	Cat# 0794/10, Tocris Bioscience, Bristol, UK
2-(1-Benzyl-4-piperdylmethyl)-5,6-dimethoxy-1-indanone hydrochloride **(Donepezil hydrochloride)**	Cat# D4099-1G, Tokyo Chemical Industry (TCI) Ltd., Belgium
trans-(±)-3,4-Dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide hydrochloride **[(±)-U-50,488 hydrochloride]**	Cat# 0495/25, Tocris Bioscience, Abingdon, UK
Buprenorphine hydrochloride **(Buprenorphine hydrochloride)**	Cat# RP0003, Chanelle Veterinary Galway, Ireland
RDC002944-01 **(RDC 2944)**	Provided by Alkermes Inc. Waltham, Massachusetts, USA
17-(Cyclopropylmethyl)-4,14-dimethoxymorphinan-6-one hydrochloride **(Cyprodime hydrochloride)**	Cat# 2601/10, Tocris Bioscience, Bristol, UK

Table 5.2 Chemical name and source for each compound. Inc.=incorporated, Ltd.=Limited, UK=United Kingdom, USA=United States of America.

For a clear and detailed explanation of drug doses, solubility, other vehicles received, and drug effect, see Table 5.3. For *experiment 1 and 2*, different drugs were made-up in different vehicles, and so all rats received a second injection of the 'other' vehicle that their drug was not dissolved in. In this manner, all rats were injected with both of the vehicles; 20% DMSO (Cat# D8418-250ML, Sigma-Aldrich Ireland Ltd., Ireland) in distilled water, and 0.01M HCl (Cat# 10316380, Thermo Fischer Scientific Inc., Dublin, Ireland) dissolved in 0.9% saline solution (Sodium Chloride, Cat# 10428420, Thermo Fischer Scientific Inc., Dublin, Ireland). It should be noted that a limitation of this study could be both the high level of DMSO and HCL, two chemicals that are both quite antagonistic and irritable in nature. Having said this, these were the exact concentrations that these compounds were soluble in (Alkermes Inc., Internal data). For all combination groups, drugs were made-up separately and rats received an injection of each drug, i.e. two injections. In both experiments, salt was accounted for in all compounds. It must be noted that for the buprenorphine/cyprodime treatment group, both of these compounds were made-up in the 0.01M HCl 0.9% saline solution; so the other vehicle would be the 20% DMSO solution. However, as these animals

were already receiving two injections, it was decided and deemed that it would be unethical for these rats to receive a third procedural injection, and so this group just received the two experiments drugs and did not receive the 20% DMSO solution treatment (Table 5.3). For both experiments, all drugs were injected via subcutaneous (s.c) administration. All drugs were injected at the same time each day, in the afternoon between 14:00-18:00 h. If a rat was to be behaviourally tested that day, that animal was injected after testing. This would allow for a washout of drugs for 20-24 hours before animals were exposed to behavioural testing, so as not get the effects of acute drug administration. All drugs were made up in batch quantities and aliquoted with the amount of millilitres needed per day calculated previously. Aliquots were frozen at -20°C, and were thawed and sonicated in a water bath on the morning of the day they were needed. These instructions for drug preparation were given by Alkermes Inc., whereby a freeze/thaw cycle was shown to have no effect on the efficacy of the drug (Alkermes Inc., Internal data). Most of the OB studies examined report chronic dosing with antidepressants for 21 days, with behavioural testing commencing after at least two weeks of drug administration. Thus, the 21 day dosing regime was selected for the study.

Experiment 1				
Drug	**Dose and Volume**	**Soluble**	**Other Vehicle**	**Effect**
SNC80	1 mg/kg @ 1 ml/kg	0.01M HCl 0.9% saline solution	20% DMSO in distilled H_2O	DOP agonist
DIPPA	1 mg/kg @ 1 ml/kg	20% DMSO in distilled H_2O	0.01M HCl 0.9% saline solution	KOP antagonist
SNC80/DIPPA	1 mg/kg @ 1 ml/kg each	0.01M HCl 0.9% saline solution and 20% DMSO in distilled H_2O	–	DOP agonist and KOP antagonist
Donepezil	5 mg/kg @ 1 ml/kg	0.01M HCl 0.9% saline solution	20% DMSO in distilled H_2O	Acetylcholinesterase Inhibitor
Experiment 2				
Drug	**Dose and Volume**	**Soluble**	**Other Vehicle**	**Effect**
RDC 2944	0.1 mg/kg @ 1 ml/kg	0.01M HCl 0.9% saline solution	20% DMSO in distilled H_2O	MOP agonist
RDC 2944/DIPPA	0.1 mg/kg @ 1 ml/kg / 1 mg/kg @ 1 ml/kg	0.01M HCl 0.9% saline solution and 20% DMSO in distilled H_2O	–	MOP agonist and KOP antagonist
U50,488	1 mg/kg @ 1 ml/kg	0.01M HCl 0.9% saline solution	20% DMSO in distilled H_2O	KOP agonist
Buprenorphine	0.1 mg/kg @ 1 ml/kg	0.01M HCl 0.9% saline solution	20% DMSO in distilled H_2O	MOP agonist
*Buprenorphine/Cyprodime	0.1 mg/kg @ 1 ml/kg / 1 mg/kg @ 1 ml/kg	0.01M HCl 0.9% saline solution	*	MOP agonist and MOP antagonist

Table 5.3 Breakdown of the drugs, dose, solubility, drug effects and other vehicles used in experiment 1 and 2. *The decision was made to not have a 20% DMSO injection for this group as that would mean these rats would receive three injections, see Section 5.2. DMSO=dimethyl sulfoxide, DOP=delta opioid receptor, HCl=hydrochloric acid, H_2O=water, kg=kilograms, KOP=kappa opioid receptor, mg=milligrams, ml=millilitres, MOP=mu opioid agonist, – =not applicable.

5.2.6 Behavioural Testing

5.2.6.1 3-Chamber Sociability Test

This was carried out as described in Chapter 3, section 3.2.6.3. Testing was performed at four weeks post-surgery. A total of 4 rats were excluded from the analysis due to tracking issues.

5.2.6.2 Elevated Plus Maze

This was carried out as described in Chapter 3, section 3.2.6.2. If the rat fell off the EPM for any reason, the timer was paused, the experimenter entered the room and placed the rat, as before, with their head and front two paws in the centre of the EPM, facing the open arm. The experimenter left the room, the door was closed and the timer was restarted from where it had been paused and the trial was continued. Testing was performed at five weeks post-surgery. In the housing study in Chapter 3, the EPM was examined directly before the first OF exposure and so we wanted to maintain consistency with this test pairing. Although in the housing study in Chapter 3 the testing was examined at two weeks post-surgery for both parameters, the EPM was examined at the same time point as the OF in this chapter, i.e. five weeks post-surgery. Again, this later time-point also allowed for the assessment of the effects of opioid modulating drugs on this parameter.

5.2.6.3 Open Field Test

This was carried out as described in Chapter 3, section 3.2.6.1. Testing was performed at five weeks post-surgery. The OF was only tested once in these experiments because of the habituation effect that was seen on the second OF exposure in the housing study in Chapter 3 and as a result of similar habituation results examined by Gigliucci et al., (2014) and by previous experiments in our lab (Doherty, unpublished). As a result, it was decided that OB rats would be examined in the OF once and that this would be at the five weeks post-surgery time-point in order to maintain a consistency of timing with the previous housing study in Chapter 3 and in order to assess the effects of opioid modulating drugs on OF exposure.

5.2.7 Animal Sacrifice and Brain Extraction

This was carried out as described in Chapter 3, section 3.2.7.

5.2.8 Brain Dissection and Tissue Collection

This was carried out as described in Chapter 3, section 3.2.8. Brains were thawed and dissected from fresh frozen. The hippocampus was dissected from both sides of the brain, weighed and placed into an eppendorf tube. The left and right side of the brain were randomised for each region so that equal amounts of left and right sides were used for PCR analysis per group. 708 µl of RA1 buffer containing 1% β-mercaptoethanol was added to each tube and homogenised. This larger quantity was added to fully homogenise whole left/right larger brain regions, rather than to cut them in half. 354 µl of the homogenised sample was then taken for PCR analysis. The other half of the homogenised sample was frozen at -80°C in case it would be needed for later use.

5.2.9 Detection of MOP, DOP, KOP gene expression using real-time quantitative polymerase chain reaction (qRT-PCR)

5.2.9.1 RNA Isolation

This was carried out as described in Chapter 3, section 3.2.9.1.

5.2.9.2 RNA Quantification and Equalisation

This was carried out as described in Chapter 3, section 3.2.9.2. After quantification, all samples were equalised to the same RNA concentration for each region (Table 5.4) by the addition of RNase-free water (supplied in the kit).

Region	Mean RNA concentration (µg/µl)	Quality (260:280)	Purity (260:230)	Equalised RNA concentration (µg/µl)
Experiment 1				
Hippocampus	165 ± 53	1.9-2.2	1.5-2.3	60
Experiment 2				
Hippocampus	187 ± 45	2.0-2.2	1.8-2.2	85

Table 5.4 The RNA concentration, quality, purity, and equalised RNA concentration, in the hippocampus after RNA isolation.

5.2.9.3 Complimentary DNA (cDNA) Synthesis

This was carried out as described in Chapter 3, section 3.2.9.3.

5.2.9.4 Quantitative Real-Time PCR (qRT-PCR) Analysis of Gene Expression

This was carried out as described in Chapter 3, section 3.2.9.4. Target genes and assay ID's were the same as in Chapter 4, section 4.2.8.4 and are listed in Table 5.5. VIC-labelled β-actin was used as the endogenous control gene. All samples were run in multiplex assays. Preparation for Taqman master mix is seen in Table 5.6.

Target Gene	**Assay ID**	**Fluorescent Label**
MOP *(Oprm1)*	Rn01430371_m1	FAM
KOP *(Oprk1)*	Rn00567737_m1	FAM
DOP *(Oprd1)*	Rn00561699_m1	FAM
POMC *(Pomc)*	Rn00595020_m1	FAM
PDYN *(Pdyn)*	Rn00571351_m1	FAM
PENK *(Penk)*	Rn00567566_m1	FAM
Endogenous Control Gene	**Assay ID**	**Fluorescent Label**
β-Actin *(Actb)*	Rn00667869_m1	VIC

Table 5.5 Assay ID's and fluorescent labels of target genes and the endogenous control gene.

Taqman Master Mix	
Taqman Reagent	5 µl
Taqman Primer *(ex. Oprm1 for MOP)*	0.5 µl
Endogenous Control Gene *(ex. β-actin)*	0.5 µl
RNase-free water	1 µl
Total volume per sample	**7.5 µl**

Table 5.6 Reagents and corresponding volumes used to make-up Taqman master mix.

5.2.9.5 Analysis of qRT-PCR Data

This was carried out as described in Chapter 3, section 3.2.9.5. Figure 5.2 shows amplification plots for all opioid peptides and receptors in the hippocampus. The control group in this case was the sham-operated vehicle-treated group.

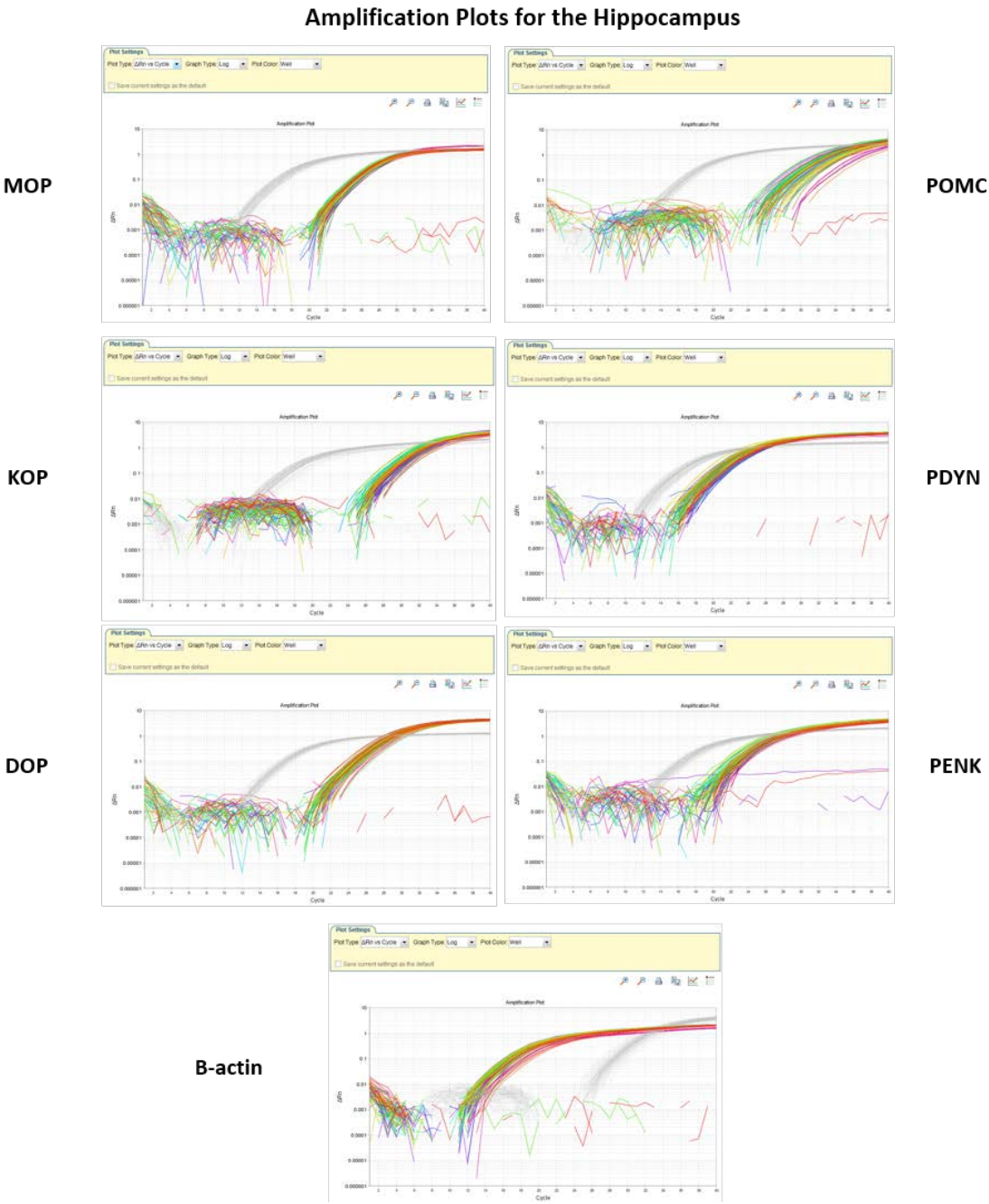


Figure 5.2 Sample amplification plots for β-Actin, MOP, KOP, DOP, PENK, PDYN and POMC in the hippocampus from experiment 2. Images taken from Applied Biosystems 7500 system SDS Software 1.3.1. DOP=delta opioid receptor, KOP=kappa opioid receptor, MOP=mu opioid receptor, PDYN=prodynorphin, PENK=preproenkephalin, POMC=proopiomelanocortin.

5.2.10 Statistical Analysis

All statistical analysis was performed using IBM SPSS Statistics Version 24 software package. In all datasets, the presence of possible outliers was checked by assessing the distribution of data. In case a data point fell out of the range of (mean-2*standard deviation) to (mean+2*standard deviation), it was considered an outlier and excluded from subsequent analysis. Data were expressed as mean ± standard deviation, unless they are deemed non-parametric, in which case the data were expressed as median ± interquartile range. All data was tested for normality using Shapiro-Wilks test for normality, and all data was also tested for Levene's test for homogeneity of variance. If data was deemed parametric, it was analysed using two-way ANOVA, followed by *post-hoc* Tukey's Honest Significance Test (HSD); $p<0.05$ was deemed statistically significant. If data was non-parametric, Kruskal-Wallis, followed where appropriate by *post-hoc* multiple Mann-Whitney U tests, with Bonferroni corrections were used. All graphs were prepared using GraphPad Prism Version 8.

5.3 Results

5.3.1 *Experiment 1:* Effects of KOP antagonists and DOP agonists on social cognition and depressive-like behaviour in the OB rat model of depression

5.3.1.1 Mortality rates and verification after OB surgery

In *experiment 1*, of the 100 rats that had undergone surgery, eight rats died in the post-operative and/or recovery period. Of the eight rats that died, all deaths occurred as a result of olfactory bulbectomy surgery, with deaths occurring in the recovery cage, or within 4 hours after being removed to their homecage. Autopsy revealed that five rats had slight damage to the PFC (left hemisphere), one rat had damage to the back of the PFC (left and right hemisphere) and the two remaining rats had no damage to the cortex, with complete bilateral bulb removal and as such cause of death was unconfirmed. Upon completion and verification of OB removal at the end of the study, there were ten animals excluded from further analysis for incomplete or excessive bulb removal.

5.3.1.2 Bodyweight gain after week one, two and three of chronic dosing

After one week of dosing, there was a significant effect of drug [$F_{(4,77)}$=19.869, p<0.001], but no significant effect of surgery [$F_{(1,77)}$=1.241, p=0.269] or any significant surgery x drug interaction effect [$F_{(4,77)}$=0.439, p=0.780] (Figure 5.3A). *Post-hoc* analysis revealed that regardless of surgery group, DIPPA caused a significant decrease in bodyweight gain in both sham-operated and OB rats when compared to their appropriate vehicle counterparts after one week of chronic dosing (p<0.05). Donepezil, and the combination of DIPPA/SNC80, caused a significant decrease in bodyweight gain in OB rats when compared to their appropriate vehicle counterparts after one week of chronic dosing (p<0.05). After two weeks of treatment, there was no drug [$F_{(4,77)}$=1.686, p=0.162], surgery [$F_{(1,77)}$=2.580, p=0.112] or surgery x drug interaction [$F_{(4,77)}$=0.961, p=0.434] (Figure 5.3B). However, after three weeks of treatment, there was a significant effect of drug [$F_{(4,77)}$=3.289, p=0.015], and a significant effect of surgery [$F_{(1,77)}$=4.634, p=0.034] but no significant surgery x drug interaction effect [$F_{(4,77)}$=1.737, p=0.150] (Figure 5.3C). However, *post-hoc* analysis revealed that there was no significant difference between any of the groups.

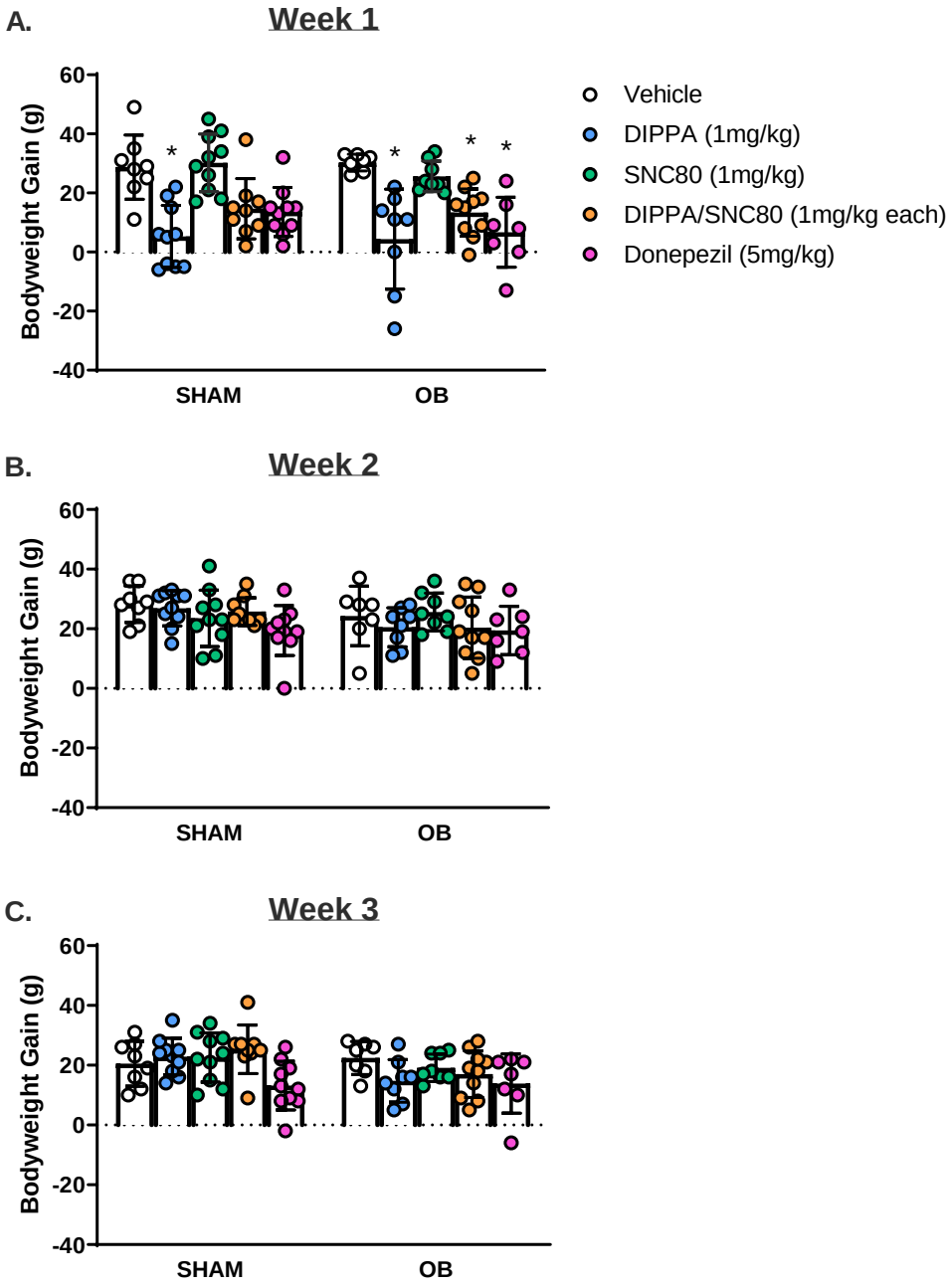


Figure 5.3 Bodyweight gain in sham-operated and OB rats after one, two and three weeks of chronic dosing. DIPPA caused a significant decrease in bodyweight gain at one week post-dosing, regardless of surgery group (A). Donepezil, and the combination treatment of DIPPA/SNC80, caused a significant decrease in bodyweight gain at one week post-dosing in OB rats (A). There was no significant difference in bodyweight gain among the treatment groups at two weeks (B) or three weeks (C) post chronic dosing commencement. Data are expressed as mean ± standard deviation (n=7-10 per group). *p<0.05 vs. appropriate vehicle counterpart.

5.3.1.3 3-Chamber Sociability Test Results

5.3.1.3.1 Distance moved in the 3-chamber sociability test

With regards distance moved in the habituation trial, two-way ANOVA revealed that there was no significant effect of surgery [$F_{(1,72)}$=0.411, p=0.524], drug [$F_{(1,72)}$=0.590, p=0.671], or any surgery x drug interaction effect [$F_{(4,72)}$=0.519, p=0.722] (Figure 5.4).

With regards distance moved in the sociability trial, two-way ANOVA revealed that there was no significant effect of surgery [$F_{(1,72)}$=0.867, p=0.355], drug [$F_{(1,72)}$=1.641, p=0.173], or any surgery x drug interaction effect [$F_{(4,72)}$=0.979, p=0.425] (Figure 5.4).

With regards distance moved in the social preference trial, two-way ANOVA revealed that there was a significant effect of surgery [$F_{(1,72)}$=11.108, p=0.001], but no significant effect of drug [$F_{(4,72)}$=0.707, p=0.589], or surgery x drug interaction effect [$F_{(4,72)}$=1.344, p=0.262] (Figure 5.4). However, *post-hoc* analysis revealed that there was no significant difference between any of the groups.

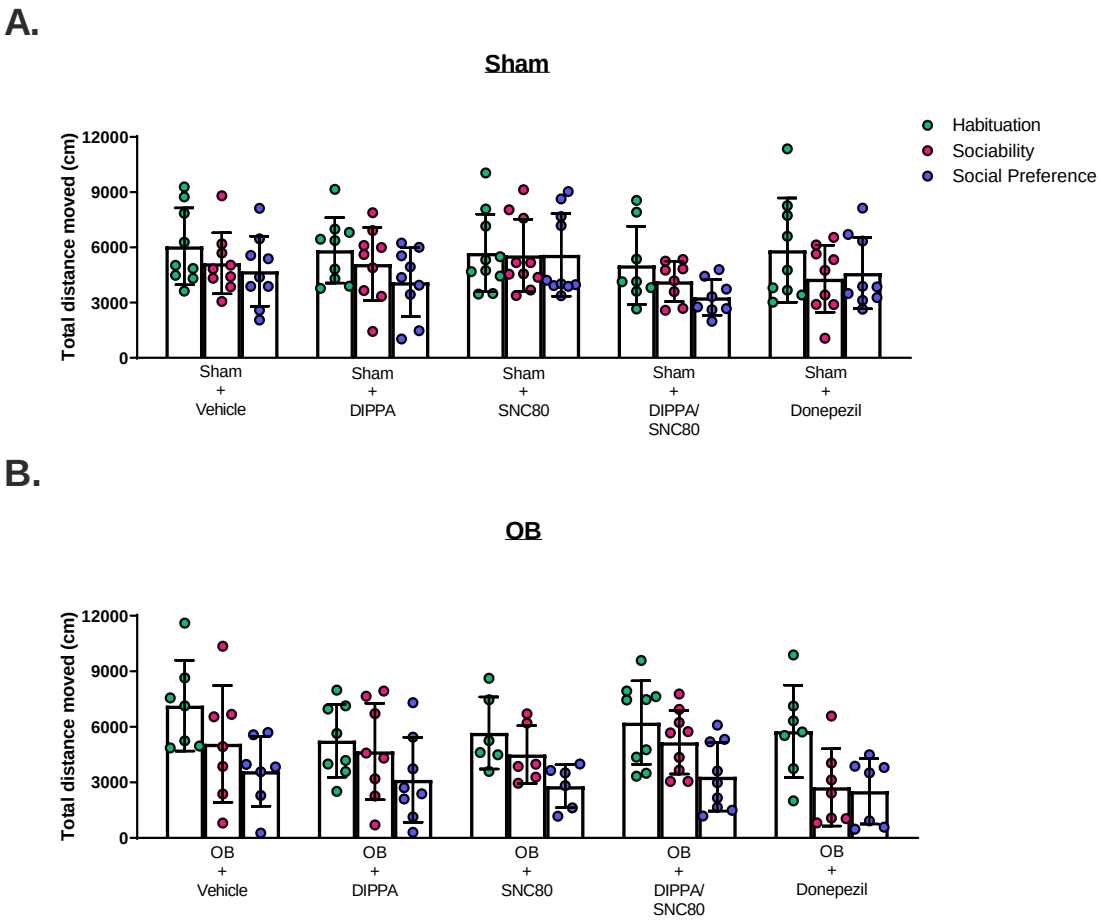


Figure 5.4 Distanced moved in the habituation, sociability and social preference trial in the 3-chamber sociability test. The three white bars for each experimental group represent the distance moved per group per trial with the coloured circles in each bar representing a different trial. The circular shape for the distance moved in the habituation trial is coloured in green, the sociability trial in pink, and the social preference trial is in blue. There was no significant difference in the distance moved in the habituation, sociability or social preference trials between experimental sham-operated and OB groups. Data are expressed as mean ± standard deviation (habituation, sociability trials and social preference trial) (*n*=6-10 per group).

5.3.1.3.2 % Habituation in the 3-chamber sociability test

As a result of the pattern for OB rats to display a decreased distance moved across the 30 minute period of testing in the 3-chamber sociability test in the housing study in Chapter 3, it was decided to examine and express the data in terms of % habituation to the arena again in this study, i.e. to see if there was a difference in % habituation to the arena across surgery groups and drug conditions. The % habituation score was calculated as described in the methods of Chapter 3, section 3.2.9.

With regards % habituation in the 3-chamber sociability test, two-way ANOVA revealed that there was a significant effect of surgery [$F_{(1,72)}$=17.484, p<0.001], but no significant effect of drug [$F_{(4,72)}$=0.611, p=0.656], or surgery x drug interaction effect [$F_{(4,72)}$=0.675, p=0.612] (Figure 5.5). However, *post-hoc* analysis revealed that there was no significant difference between any of the groups.

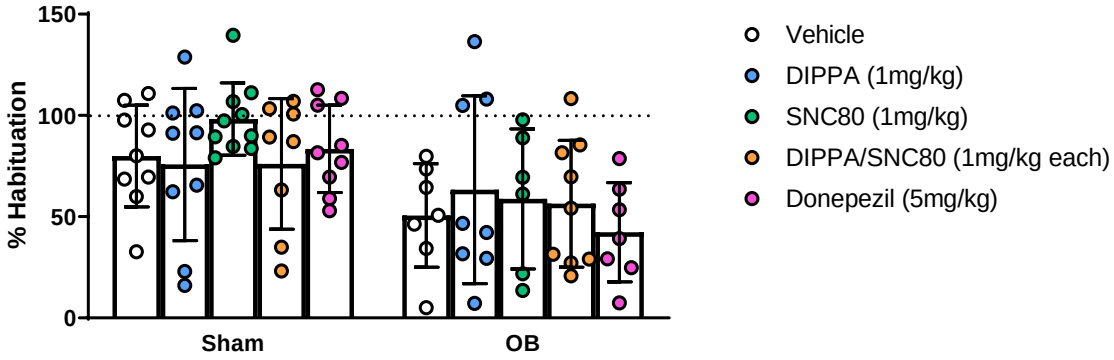


Figure 5.5 % Habituation of sham-operated and OB rats in the 3-chamber sociability test after chronic dosing with opioid modulating drugs. There was no significant difference between sham-operated and OB rats in % habituation scores in the 3-chamber sociability test, regardless of treatment. Data are expressed as mean ± standard deviation (n=6-10 per group).

5.3.1.3.3 *Sociability Trial:* Time spent interacting with the empty cage and novel animal

With regards time spent interacting with the empty cage in the sociability trial, two-way ANOVA revealed that there was a significant effect of drug [$F_{(4,72)}$=3.292, p=0.016], and of surgery [$F_{(1,72)}$=14.220, p<0.001], but there no surgery x drug interaction effect [$F_{(4,72)}$=1.750, p=0.148] (Figure 5.6). *Post-hoc* analysis revealed that OB rats treated with donepezil spent less time interacting with the empty cage than sham-operated counterparts that had been treated with donepezil (p<0.05).

With regards time spent interacting with the novel animals in the sociability trial, two-way ANOVA revealed that there was a significant effect of surgery [$F_{(1,72)}$=40.087, p<0.001], but no drug [$F_{(4,72)}$=1.463, p=0.222], or surgery x drug interaction effect [$F_{(4,72)}$=0.705, p=0.591] (Figure 5.6). *Post-hoc* analysis revealed that OB rats treated with donepezil and SNC80 alone spent less time interacting with the novel animal in the sociability trial than sham-operated counterparts that had been treated with donepezil (p<0.05).

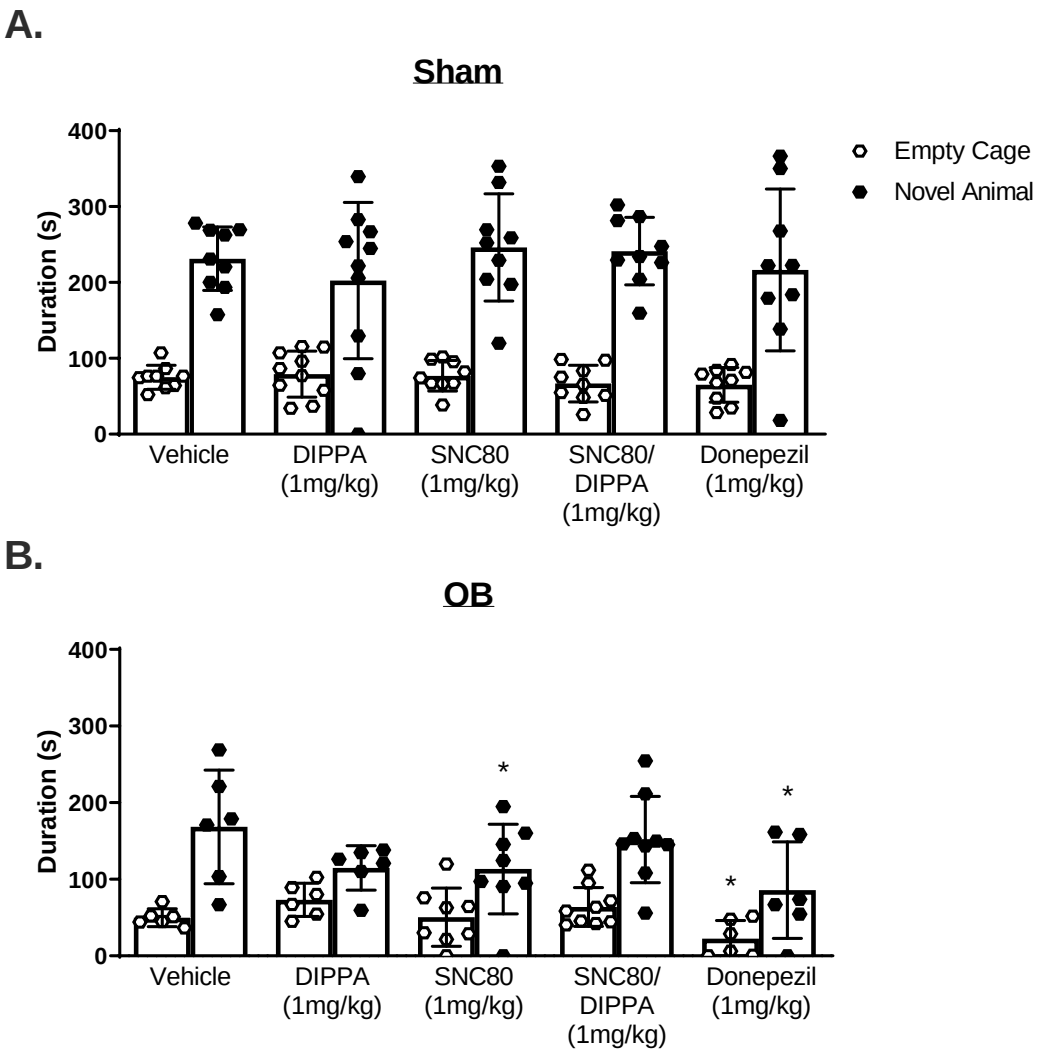


Figure 5.6 Duration spent interacting with the empty cage and the novel animal in the sociability trial. The circles that are shaded in white represent time spent interacting with the empty cage and the circles that are shaded in black represent time spent interacting with the novel animal. Sham-operated results are in graph A. and OB results in graph B. Donepezil-treated OB rats spent significantly less time interacting with the empty cage than sham-operated rats that had been treated with donepezil. OB rats that were treated with SNC80 alone, or, donepezil, spent significantly less time interacting with the novel animal in comparison to their sham-treated counterparts. Data are expressed as mean ± standard deviation (n=6-10 per group). *p<0.05 vs. appropriate sham-treated counterpart.

5.3.1.3.4 *Social Preference Trial:* Time spent interacting with the familiar animal and novel animal

With regards time spent interacting with the familiar animal in the social preference trial, two-way ANOVA revealed that there was a significant effect of surgery [$F_{(1,72)}$=12.545, p=0.001], and of drug [$F_{(4,72)}$=2.568, p=0.045], but no surgery x drug interaction effect [$F_{(4,72)}$=1.286, p=0.283] (Figure 5.7). *Post-hoc* analysis revealed that there was no significant difference between experimental groups.

With regards time spent interacting with the novel animal in the social preference trial, two-way ANOVA revealed that there was a significant effect of surgery [$F_{(1,72)}$=35.754, p<0.001], but no significant effect of drug [$F_{(4,72)}$=2.338, p=0.063], and no surgery x drug interaction effect [$F_{(4,72)}$=0.291, p=0.883] (Figure 5.7). *Post-hoc* analysis revealed that OB donepezil-treated rats interacted significantly less with the novel animal than their sham-treated counterparts (p<0.05).

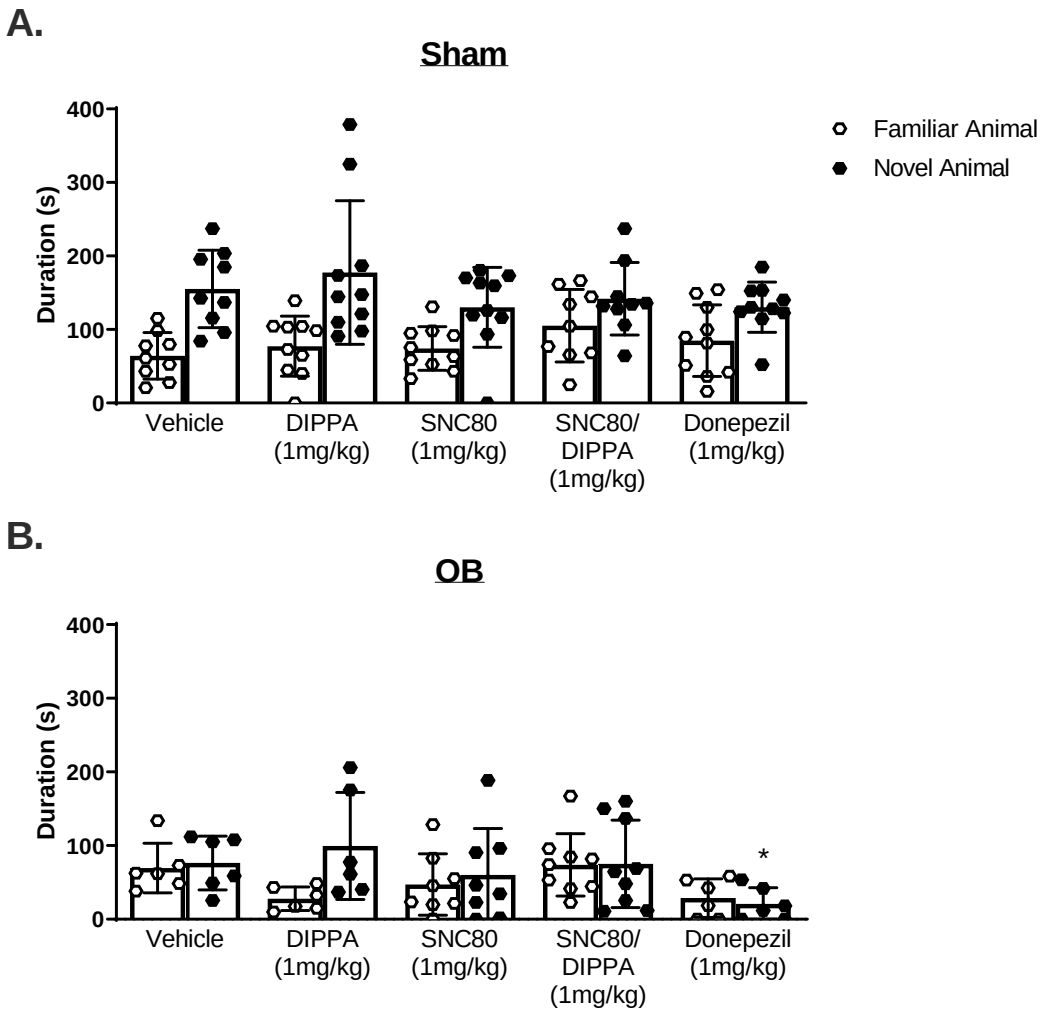


Figure 5.7 Duration spent interacting with the familiar animal and the novel animal in the social preference trial. The circles that are shaded in white represent time spent interacting with the familiar animal and the circles that are shaded in black represent time spent interacting with the novel animal. Sham-operated results are in graph A and OB results in graph B. OB rats that were treated with donepezil, spent significantly less time interacting with the novel animal in comparison to their appropriate sham-treated counterparts. There was no significant difference between groups in the time spent interacting with the familiar animal. Data are expressed as mean ± standard deviation (n=6-10 per group). *p<0.05 vs. appropriate sham-treated counterpart.

5.3.1.4 Elevated Plus Maze Results

5.3.1.4.1 Distance moved in the elevated plus maze

With regards distance moved in the EPM, two-way ANOVA revealed that there was no significant effect of surgery [$F_{(1,76)}$=2.534, p=0.116], drug [$F_{(4,76)}$=0.303, p=0.875], or any effect of surgery x drug interaction [$F_{(4,76)}$=1.322, p=0.269] (Figure 5.8).

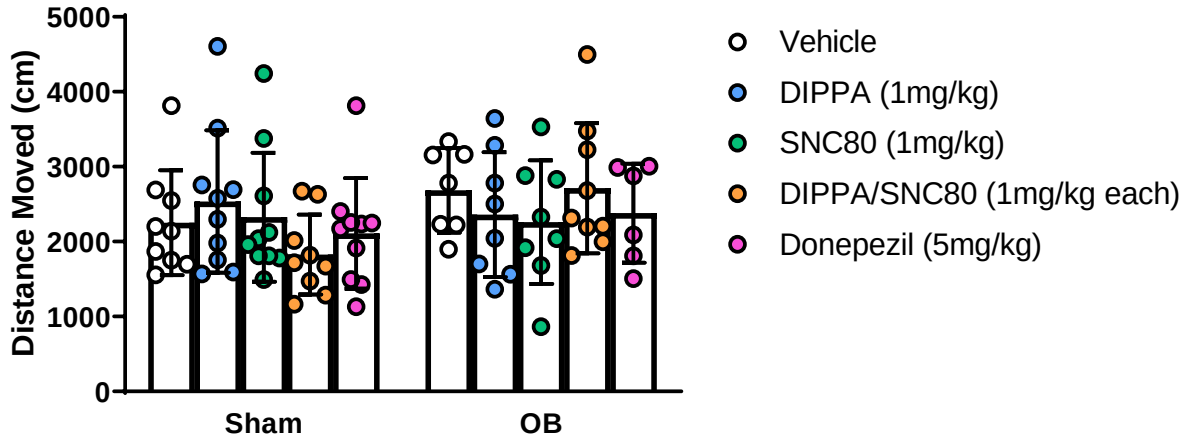


Figure 5.8 Distance moved in the elevated plus maze by sham-operated and OB rats after three weeks of chronic dosing. There was no significant difference in distance moved in the EPM between sham-operated and OB rats, regardless of drug treatment. Data are expressed as mean ± standard deviation (n=6-10 per group).

5.3.1.4.2 Open arm entries and % open arm duration in the elevated plus maze

With regards % open arm entries in the EPM, two-way ANOVA revealed that there was no significant effect of surgery [$F_{(1,76)}$=0.224, p=0.637], drug [$F_{(4,76)}$=2.365, p=0.060], or any effect of surgery x drug interaction [$F_{(4,76)}$=0.714, p=0.585] (Figure 5.9A).

With regards % open arm duration in the EPM, two-way ANOVA revealed that there was no significant effect of surgery [$F_{(1,76)}$=0.007, p=0.934], drug [$F_{(4,76)}$=1.965, p=0.108], or any effect of surgery x drug interaction [$F_{(4,76)}$=0.653, p=0.627] (Figure 5.9B).

5.3.1.4.3 Open arm entries and open arm duration in the elevated plus maze

With regards open arm entries in the EPM, two-way ANOVA revealed that there was no significant effect of surgery [$F_{(1,76)}$=1.435, p=0.235], drug [$F_{(4,76)}$=1.385, p=0.247], or any effect of surgery x drug interaction [$F_{(4,76)}$=0.274, p=0.894] (Figure 5.9C).

With regards the time spent in the open arms of the EPM, two-way ANOVA revealed that there was no significant effect of surgery [$F_{(1,76)}$=0.080, p=0.778], drug [$F_{(4,76)}$=1.699, p=0.159], or any effect of surgery x drug interaction [$F_{(4,76)}$=0.874, p=0.484] (Figure 5.9D).

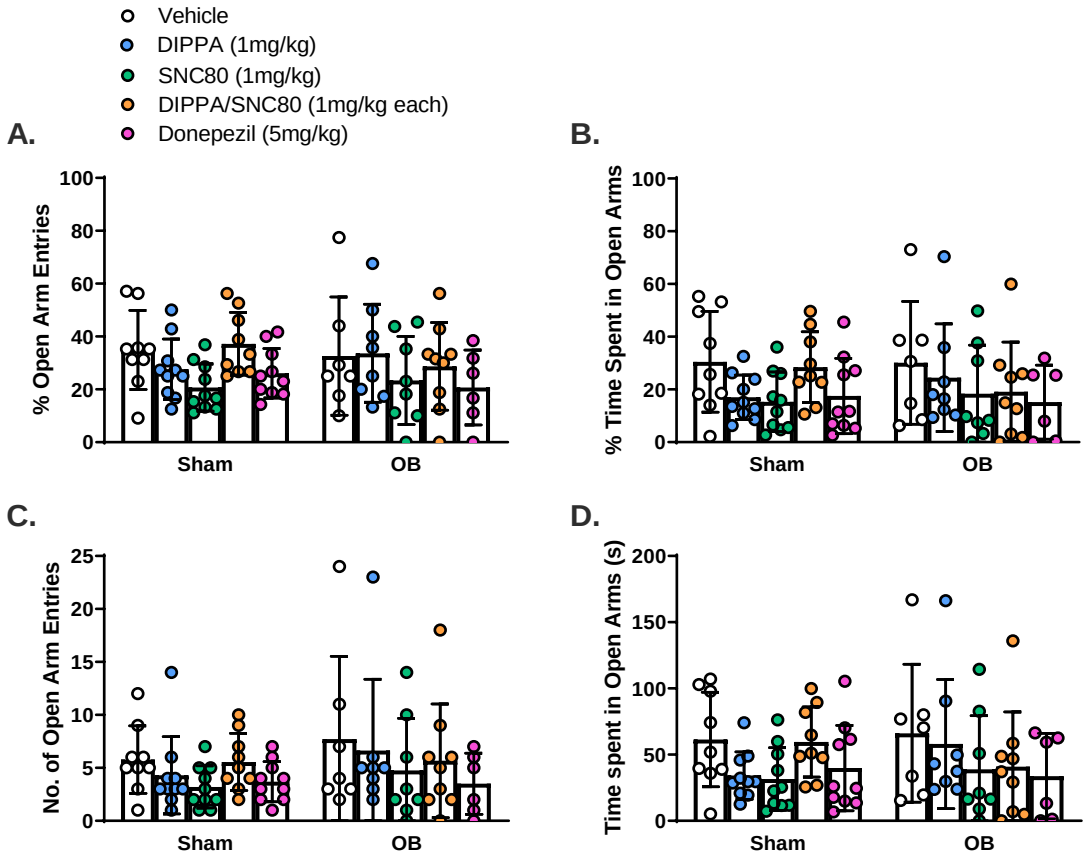


Figure 5.9 Open arm entries and duration spent in the open arms (percentage and raw values) of the elevated plus maze made by sham-operated and OB rats after three weeks of chronic dosing. There was no significant difference in % open arm entries (A), % open arm duration (B), the number of open arm entries (C) or the duration spent in the open arms (D). Data are expressed as mean ± standard deviation (*n*=6-10 per group).

5.3.1.5 Open Field Results

5.3.1.5.1 Distance moved in the open field

With regards distance moved in the OF, two-way ANOVA revealed that there was a significant effect of surgery [$F_{(1,76)}$=7.464, p=0.008], and of drug [$F_{(4,76)}$=5.080, p=0.001], but no surgery x drug interaction effect [$F_{(4,76)}$=1.586, p=0.187] (Figure 5.10A). *Post-hoc* analysis revealed that OB rats that were treated with DIPPA moved significantly more in the OF than sham-treated counterparts (p<0.05).

5.3.1.5.2 Time spent in the inner circle of the open field

With regards time spent in the inner circle of the OF, two-way ANOVA revealed that there was a significant effect of surgery [$F_{(1,76)}$=31.483, p<0.001], and of drug [$F_{(4,76)}$=3.018, p=0.023], but no surgery x drug interaction effect [$F_{(4,76)}$=1.346, p=0.261] Figure 5.10B). *Post-hoc* analysis revealed that OB rats that were treated with the combination of DIPPA and SNC80 spent significantly less time in the inner circle of the OF than their appropriate sham-treated counterparts (p<0.05).

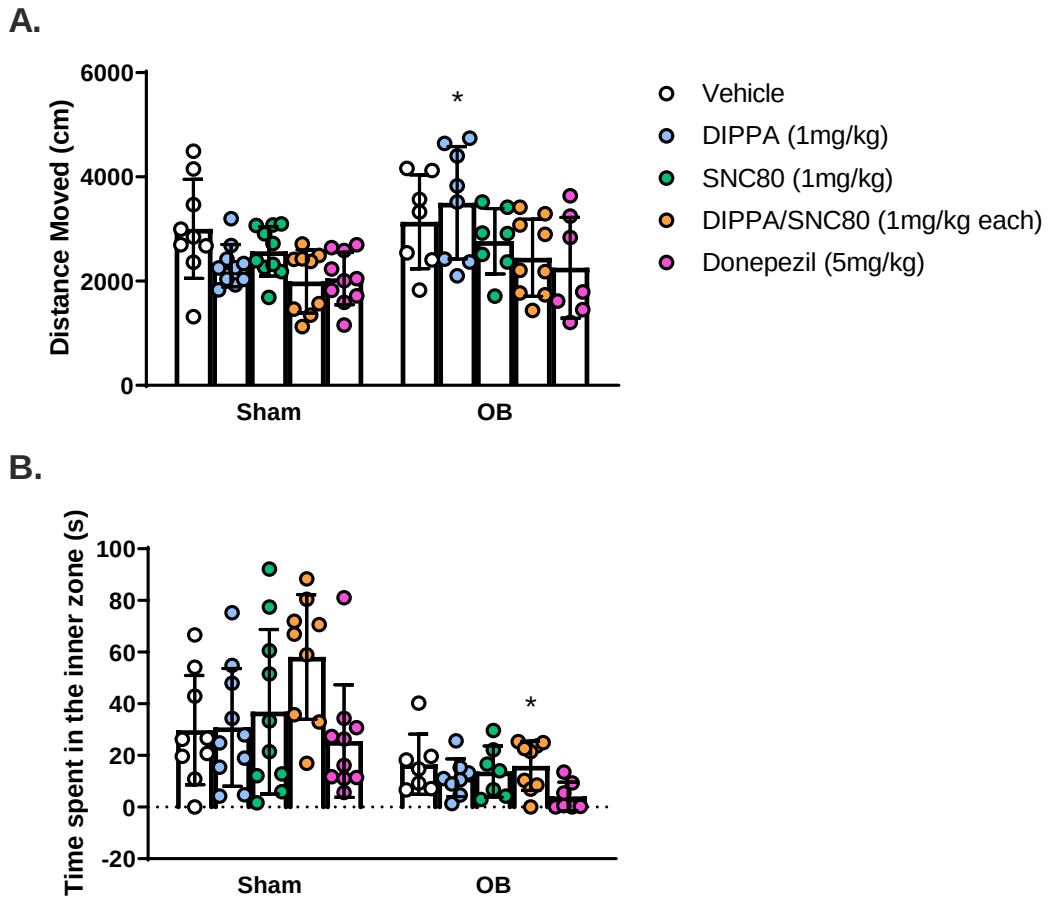


Figure 5.10 Distance moved in the open field (A) and duration spent in the inner zone (B) of the open field by sham-operated and OB rats after three weeks of chronic dosing. OB rats that were treated with DIPPA moved significantly more than sham-treated counterparts (A). OB rats treated with the combination of DIPPA/SNC80 spent significantly less time in the inner zone of the OF than their appropriate sham-treated counterparts (B). Data are expressed as mean ± standard deviation (n=7-10 per group). *p<0.05 vs. appropriate sham-treated counterpart.

5.3.1.6 Opioid receptor and pre-propeptide mRNA expression in the hippocampus

5.3.1.6.1 MOP system

5.3.1.6.1.1 MOP (*Oprm1*) and pre-propeptide POMC (*Pomc*) mRNA expression in the hippocampus

With regards *Oprm1* mRNA expression in the hippocampus, two-way ANOVA revealed that there was no significant difference (surgery, drug, and surgery x drug interaction) between sham-operated and OB experimental groups (Table 5.7).

With regards *Pomc* mRNA expression in the hippocampus, two-way ANOVA revealed that there was no significant difference (surgery, drug, and surgery x drug interaction) between sham-operated and OB experimental groups (Table 5.7).

Group	MOP	POMC
Sham		
Vehicle	1 ± 0.19	1 ± 0.60
DIPPA (1 mg/kg)	1.15 ± 0.21	0.68 ± 0.49
SNC80 (1 mg/kg)	1.13 ± 0.28	0.80 ± 0.39
DIPPA/SNC80 (1 mg/kg each)	1.21 ± 0.25	1.02 ± 0.31
Donepezil (5 mg/kg)	0.91 ± 0.09	0.45 ± 0.24
OB		
Vehicle	1.08 ± 0.24	1.35 ± 1.02
DIPPA (1 mg/kg)	1.19 ± 0.36	1.04 ± 0.51
SNC80 (1 mg/kg)	1.22 ± 0.31	0.75 ± 0.35
DIPPA/SNC80 (1 mg/kg each)	1.07 ± 0.18	0.60 ± 0.33
Donepezil (5 mg/kg)	1.15 ± 0.31	0.79 ± 0.40
Effect		
Surgery	F(1,71)=0.431, p=0.514	F(1,71)=0.903, p=0.345
Drug	F(4,71)=1.687, p=0.163	F(4,71)=2.226, p=0.075
Surgery x Drug	F(4,71)=0.724, p=0.578	F(4,71)=1.820, p=0.135

Table 5.7 MOP (*Oprm1*) and POMC (*Pomc*) mRNA expression in the hippocampus. *Oprm1* and *Pomc* mRNA expression is expressed as a percentage of the sham-operated vehicle-treated group. There was no significant difference in *Oprm1* and *Pomc* mRNA expression between sham-operated and OB rats in the hippocampus. Data are expressed as mean ± standard deviation (n=6-10 per group).

5.3.1.6.2 KOP system

5.3.1.6.2.1 KOP (*Oprk1*) and propeptide PDYN (*Pdyn*) mRNA expression in the hippocampus

With regards *Oprk1* mRNA expression in the hippocampus, two-way ANOVA revealed that there was no significant effect of surgery, drug or any surgery x drug interaction effect (Table 5.8).

With regards *Pdyn* mRNA expression in the hippocampus, two-way ANOVA revealed that there was no significant difference (surgery, drug, and surgery x drug interaction) between sham-operated and OB experimental groups (Table 5.8).

Group	KOP	PDYN
Sham		
Vehicle	1 ± 0.30	1 ± 0.26
DIPPA (1 mg/kg)	0.93 ± 0.37	1.08 ± 0.36
SNC80 (1 mg/kg)	0.92 ± 0.26	1.25 ± 0.60
DIPPA/SNC80 (1 mg/kg each)	1.20 ± 0.47	0.82 ± 0.28
Donepezil (5 mg/kg)	0.76 ± 0.15	0.81 ± 0.21
OB		
Vehicle	1.14 ± 0.44	0.82 ± 0.15
DIPPA (1 mg/kg)	1.10 ± 0.39	1.21 ± 0.44
SNC80 (1 mg/kg)	0.84 ± 0.26	0.84 ± 0.25
DIPPA/SNC80 (1 mg/kg each)	1.05 ± 0.29	1.08 ± 0.45
Donepezil (5 mg/kg)	0.91 ± 0.24	1.16 ± 0.52
Effect		
Surgery	F(1,71)=0.993, *p*=0.322	F(1,70)=0.092, *p*=0.762
Drug	F(4,71)=1.120, *p*=0.354	F(4,70)=0.884, *p*=0.478
Surgery x Drug	F(4,71)=1.036, *p*=0.395	F(4,70)=2.340, *p*=0.063

Table 5.8 KOP (*Oprk1*) and PDYN (*Pdyn*) mRNA expression in the hippocampus. *Oprk1* and *Pdyn* mRNA expression is expressed as a percentage of the sham-operated vehicle-treated group. There was no significant difference in *Oprk1* or *Pdyn* mRNA expression between sham-operated and OB rats in the hippocampus. Data are expressed as mean ± standard deviation (*n*=6-10 per group).

5.3.1.6.3 DOP system

5.3.1.6.3.1 DOP (*Oprd1*) and pre-propeptide PENK (*Penk*) mRNA expression in the hippocampus

With regards *Oprd1* mRNA expression in the hippocampus, two-way ANOVA revealed that there was no significant effect of surgery, drug or any surgery x drug interaction effect (Table 5.9).

With regards *Penk* mRNA expression in the hippocampus, two-way ANOVA revealed that there was no significant effect of surgery, drug or any surgery x drug interaction effect (Table 5.9).

Group	DOP	PENK
Sham		
Vehicle	1 ± 0.17	1 ± 0.52
DIPPA (1 mg/kg)	0.95 ± 0.23	1.23 ± 0.84
SNC80 (1 mg/kg)	1.02 ± 0.19	1.02 ± 0.48
DIPPA/SNC80 (1 mg/kg each)	1.09 ± 0.13	0.95 ± 0.49
Donepezil (5 mg/kg)	0.90 ± 0.18	1.19 ± 0.34
OB		
Vehicle	1.03 ± 0.22	0.87 ± 0.34
DIPPA (1 mg/kg)	0.99 ± 0.14	0.91 ± 0.52
SNC80 (1 mg/kg)	1.05 ± 0.20	0.98 ± 0.76
DIPPA/SNC80 (1 mg/kg each)	1 ± 0.09	1.04 ± 0.52
Donepezil (5 mg/kg)	0.90 ± 0.14	1.09 ± 0.45
Effect		
Surgery	F(1,71)=0.003, *p*=0.960	F(1,73)=0.558, *p*=0.457
Drug	F(4,71)=1.613, *p*=0.180	F(1,73)=0.273, *p*=0.894
Surgery x Drug	F(4,71)=0.373, *p*=0.827	F(1,73)=0.301, *p*=0.877

Table 5.9 DOP (*Oprd1*) and PENK (*Penk*) mRNA expression in the hippocampus. *Oprd1* and *Penk* mRNA expression is expressed as a percentage of the sham-operated vehicle-treated group. There was no significant difference in *Oprd1* or *Penk* mRNA expression between sham-operated and OB rats in the hippocampus. Data are expressed as mean ± standard deviation (*n*=6-10 per group).

5.3.2 *Experiment 2:* Effects of MOP agonists, alone or in combination with other opioid modulating drugs, on social cognition and depressive-like behaviour in the OB rat model of depression

5.3.2.1 Morality rates and verification after OB surgery

In *experiment 2*, of the 120 rats that had undergone surgery, six rats died in the post-operative and/or recovery period. Of the six rats that died, all deaths occurred as a result of olfactory bulbectomy surgery, with deaths occurring in the recovery cage, or within 4 hours after being removed to their homecage. Autopsy revealed that four rats had slight damage to the PFC (left hemisphere) and the two remaining rats had no damage to the cortex, with complete bilateral bulb removal and as such cause of death was unconfirmed. Upon completion and verification of OB removal at the end of the study, there were nine animals excluded from further analysis for incomplete or excessive bulb removal

5.3.2.2 Bodyweight gain after week one, two and three of chronic dosing

After one week of dosing, there was a significant effect of drug [$F_{(5,100)}$=9.881, p<0.001] and of surgery [$F_{(1,100)}$=12.658, p=0.001], but there no significant surgery x drug interaction effect [$F_{(5,100)}$=0.508, p=0.770] (Figure 5.11A). *Post-hoc* analysis revealed that the combination of RDC 2944/DIPPA treatment caused a significant decrease in the bodyweight gain of sham-operated rats after one week of chronic dosing when compared to their vehicle counterparts (p<0.05). After two weeks of treatment, there was a significant effect of drug [$F_{(5,101)}$=2.557, p=0.032] and of surgery [$F_{(1,101)}$=15.853, p<0.001], but no significant surgery x drug interaction effect [$F_{(5,101)}$=0.428, p=0.828] (Figure 5.11B). However, *post-hoc* analysis revealed that there was no significant difference between any of the groups. After three weeks of treatment, there was a significant effect of surgery [$F_{(1,101)}$=9.274, p=0.003], but there was no significant effect of drug [$F_{(5,101)}$=1.457, p=0.211] or surgery x drug interaction [$F_{(5,101)}$=0.826, p=0.534] (Figure 5.11C). However, *post-hoc* analysis revealed that there was no significant difference between any of the groups.

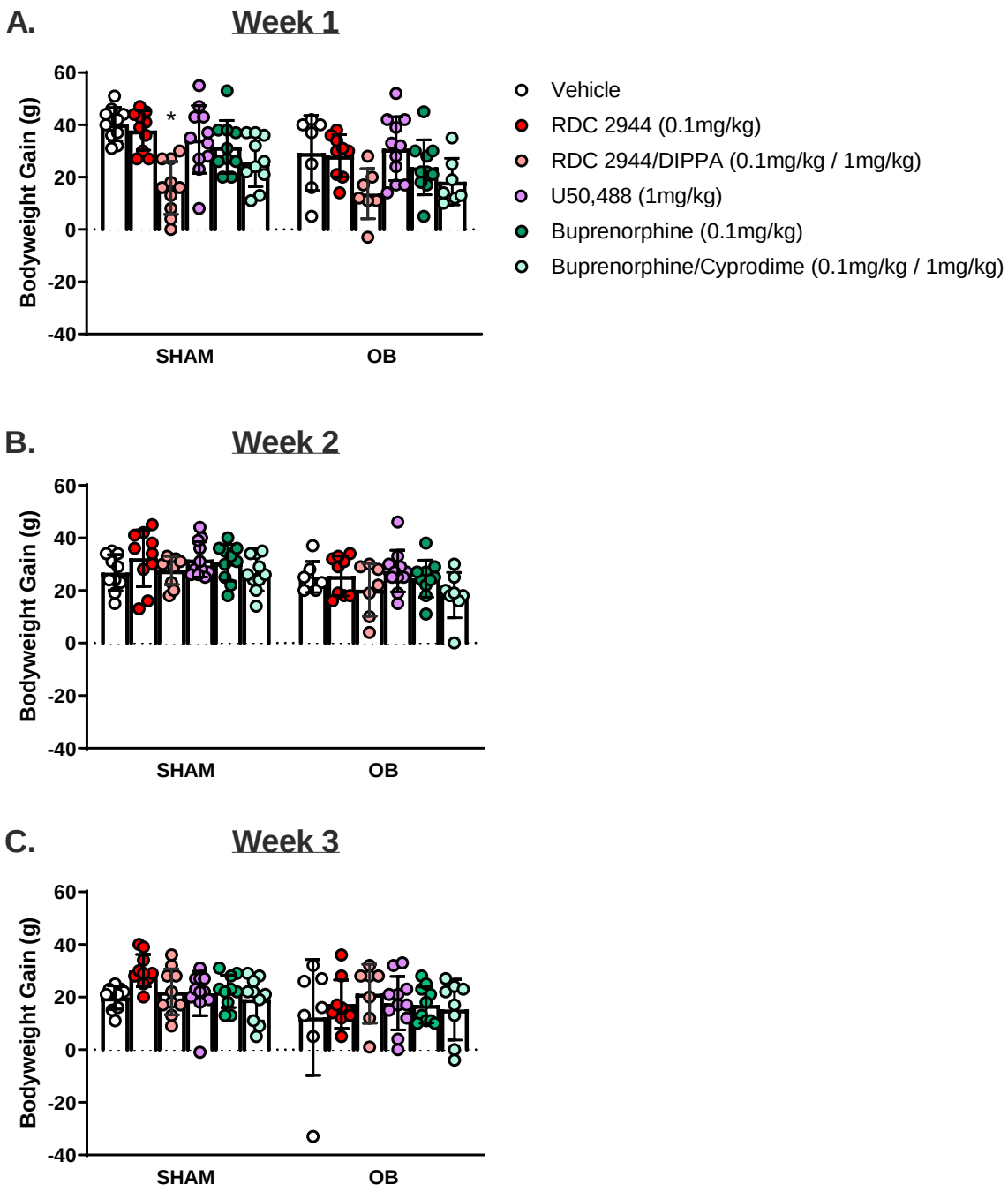


Figure 5.11 Bodyweight gain in sham-operated and OB rats after one, two and three weeks of chronic dosing. The combination treatment of RDC 2944/DIPPA, caused a significant decrease in bodyweight gain at one week post-dosing in sham-operated rats when compared to vehicle-treated sham-operated rats (A). There was no significant difference in bodyweight gain among the treatment groups at two weeks (B) or three weeks (C) post chronic dosing commencement. Data are expressed as mean ± standard deviation (n=7-11 per group). *p<0.05 vs. appropriate vehicle counterpart.

5.3.2.3 3-Chamber Sociability Test Results

5.3.2.3.1 Distance moved in the 3-chamber sociability test

5.3.2.3.1.1 Modulation of the MOP

With regards distance moved in the habituation trial for animals treated with compounds acting on the MOP, two-way ANOVA revealed that there was a significant effect of surgery [$F_{(1,81)}$=85.687, p<0.001], drug [$F_{(4,81)}$=10.256, p<0.001], and a surgery x drug interaction effect [$F_{(4,81)}$=4.354, p=0.003] (Figure 5.12). *Post-hoc* analysis revealed that OB rats that were treated with RDC 2944 alone, RDC 2944 in combination with DIPPA, and buprenorphine in combination with cyprodime, moved significantly more than their sham-treated counterparts in the habituation trial (p<0.05). OB rats treated with RDC2944 alone, or in combination with DIPPA, moved significantly more than vehicle-treated OB rats in the habituation trial (p<0.05).

With regards distance moved in the sociability trial for animals treated with compounds acting on the MOP, two-way ANOVA revealed that there was a significant effect of drug [$F_{(4,81)}$=6.423, p<0.001] and a significant surgery x drug interaction effect [$F_{(4,81)}$=3.370, p=0.013], but no effect of surgery [$F_{(1,81)}$=1.958, p=0.166] (Figure 5.12). *Post-hoc* analysis revealed that OB rats that were treated with buprenorphine alone moved significantly less than their sham-treated counterparts in the sociability trial (p<0.05).

With regards distance moved in the social preference trial for animals treated with compounds acting on the MOP, two-way ANOVA revealed that there was a significant effect of surgery [$F_{(1,79)}$=32.741, p<0.001], and of drug [$F_{(4,79)}$=9.873, p<0.001], but no surgery x drug interaction effect [$F_{(4,79)}$=1.730, p=0.152] (Figure 5.12). *Post-hoc* analysis revealed that OB rats that were treated with buprenorphine alone, or in combination with cyprodime moved significantly less than their sham-treated counterparts in the social preference trial (p<0.05). OB rats treated with buprenorphine alone also moved significantly less than vehicle-treated OB rats in the social preference trial (p<0.05).

5.3.2.3.1.2 Modulation of the KOP

With regards distance moved in the habituation trial for animals treated with compounds acting on the KOP, two-way ANOVA revealed that there was a significant effect of surgery [$F_{(1,50)}$=48.463, p<0.001], and a surgery x drug interaction effect [$F_{(2,50)}$=8.398, p=0.001], but there was no effect of drug [$F_{(2,50)}$=3.060, p=0.056] (Figure 5.12). *Post-hoc* analysis revealed that OB rats that were treated with U50,488 and RDC 2944 in combination with DIPPA, moved significantly more than their sham-treated counterparts in the habituation trial (p<0.05). OB rats treated with RDC2944 alone in combination with DIPPA, moved significantly more than vehicle-treated OB rats in the habituation trial (p<0.05).

With regards distance moved in the sociability trial for animals treated with compounds acting on the KOP, two-way ANOVA revealed that there was no significant effect of surgery [$F_{(1,50)}$=0.001, p=0.981], drug [$F_{(2,50)}$=0.863, p=0.428], or any surgery x drug interaction effect [$F_{(2,50)}$=1.817, p=0.173] (Figure 5.12).

With regards distance moved in the social preference trial for animals treated with compounds acting on the KOP, two-way ANOVA revealed that there was a significant effect of surgery [$F_{(1,50)}$=9.837, p=0.003], but no significant effect of drug [$F_{(2,50)}$=0.765, p=0.471], or any surgery x drug interaction effect [$F_{(2,50)}$=0.559, p=0.575] (Figure 5.12). *Post-hoc* analysis revealed that there was no significant differences between any of the groups.

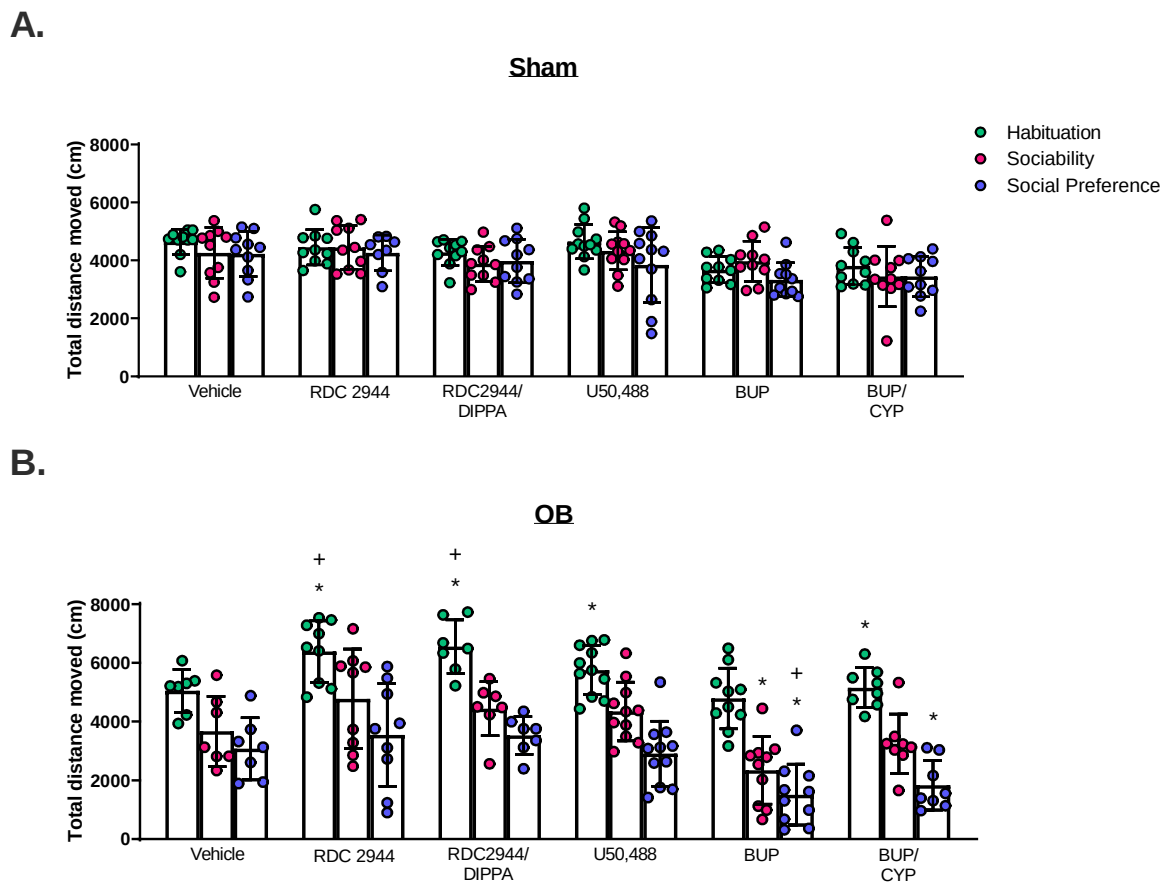


Figure 5.12 Distance moved in the habituation, sociability and social preference trial of the 3-chamber sociability test. The three white bars for each experimental group represent the distance moved per group per trial with the coloured circles in each bar representing a different trial. The circular shape for the distance moved in the habituation trial is coloured in green, the sociability trial in pink, and the social preference trial is in blue. In the habituation trial, OB rats that were treated with U50, 488, RDC 2944 alone, RDC 2944 in combination with DIPPA, and buprenorphine in combination with cyprodime, moved significantly more than their sham-treated counterparts. OB rats treated with RDC 2944 alone, or in combination with DIPPA, moved significantly more than vehicle-treated OB rats in the habituation trial. In the sociability trial, OB rats that were treated with buprenorphine alone moved significantly less than their sham-treated counterparts. In the social preference trial, OB rats that were treated with buprenorphine alone, and in combination with cyprodime, moved significantly less than their sham-treated counterparts. OB rats treated with buprenorphine alone also moved significantly less than vehicle-treated OB rats in the social preference trial ($p<0.05$). Data are expressed as mean ± standard deviation (n=7-11 per group). *$p<0.05$ vs. appropriate sham-treated counterpart, +$p<0.05$ vs. appropriate vehicle counterpart.

5.3.2.3.2 % Habituate in the 3-chamber sociability test

As a result of the pattern for OB rats to display a decreased distance moved across the 30 minute period of testing in the 3-chamber sociability test in the housing study in Chapter 3, it was decided to examine and express the data in terms of % habituation to the arena again in this study, i.e. to see if there was a difference in % habituation to the arena across surgery groups and drug conditions. The % habituation score was calculated as described in the methods of Chapter 3, section 3.2.9.

With regards % habituation, two-way ANOVA revealed that there was a significant effect of surgery [$F_{(1,99)}$=137.936, p<0.001], but there was no effect of drug [$F_{(5,99)}$=1.978, p=0.088], or any surgery x drug interaction effect [$F_{(5,99)}$=1.838, p=0.112] (Figure 5.13). *Post-hoc* analysis revealed that all OB rats, regardless of drug treatment, scored significantly lower in % habituation, indicating that OB rats habituate to the arena over time in comparison to sham-operated rats (p<0.05).

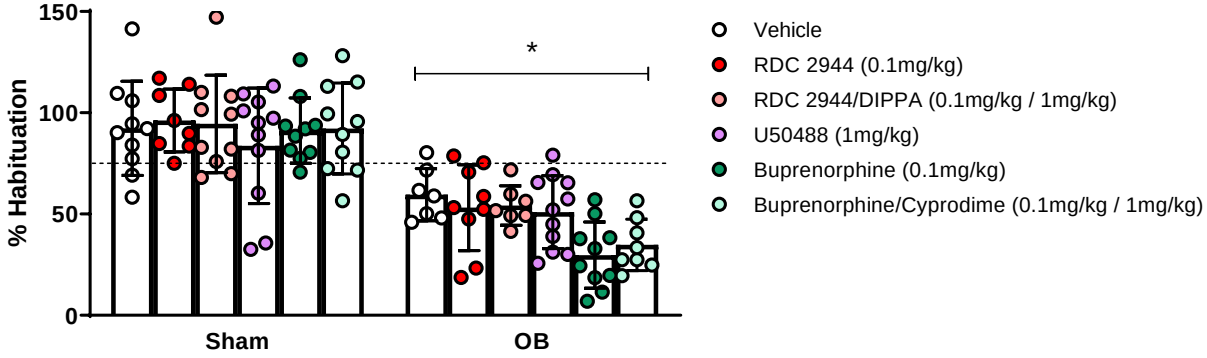


Figure 5.13 % Habituation of sham-operated and OB rats in the 3-chamber sociability test after chronic dosing with opioid modulating drugs. OB rats, regardless of drug treatment, had significantly lower % habituation scores in the 3-chamber sociability test in comparison to sham-treated rats. Data are expressed as mean ± standard deviation (n=7-11 per group). *p<0.05 vs. appropriate sham-treated counterpart.

5.3.2.3.3 *Sociability Trial:* Time spent interacting with the empty cage and novel animal

5.3.2.3.3.1 Modulation of the MOP

With regards time spent interacting with the empty cage in the sociability trial for animals treated with compounds acting on the MOP, two-way ANOVA revealed that there was a significant effect of surgery [$F_{(1,81)}$=5.645, p=0.020], but there was no significant effect of drug [$F_{(4,81)}$=1.238, p=0.301], or any surgery x drug interaction effect [$F_{(4,81)}$=0.389, p=0.816] with regards to time spent interacting with the empty cage in the sociability trial (Figure 5.14). *Post-hoc* analysis revealed that there was no significant differences between any of the groups.

With regards time spent interacting with the novel animal in the sociability trial for animals treated with compounds acting on the MOP, two-way ANOVA revealed that there was a significant effect of surgery [$F_{(1,81)}$=45.728, p<0.001], but there was no significant effect of drug [$F_{(4,81)}$=0.961, p=0.433], or any surgery x drug interaction effect [$F_{(4,81)}$=0.584, p=0.675] with regards to time spent interacting with the novel animal in the sociability trial (Figure 5.14). *Post-hoc* analysis revealed that OB rats that had been treated with RDC 2944 alone spent significantly less time interacting with the animal in the sociability trial than their sham-treated counterpart (p<0.05).

5.3.2.3.3.2 Modulation of the KOP

With regards time spent interacting with the empty cage in the sociability trial for animals treated with compounds acting on the KOP, two-way ANOVA revealed that there was no significant effect of surgery [$F_{(1,50)}$=2.706, p=0.106], drug [$F_{(2,50)}$=1.354, p=0.268], or any surgery x drug interaction effect [$F_{(2,50)}$=0.033, p=0.967] with regards to time spent interacting with the empty cage in the sociability trial (Figure 5.14).

With regards time spent interacting with the novel animal in the sociability trial for animals treated with compounds acting on the KOP, two-way ANOVA revealed that there was a significant effect of surgery [$F_{(1,50)}$=28.613, p<0.001], but there was no significant effect of drug [$F_{(2,50)}$=0.204, p=0.816], or any surgery x drug interaction effect [$F_{(2,50)}$=0.099, p=0.906] with regards to time spent interacting with the novel animal in the sociability trial (Figure 5.14). *Post-hoc* analysis revealed that OB rats that had been treated with vehicle, and RDC 2944 alone spent significantly less time interacting with the animal in the sociability trial than their sham-treated counterpart (p<0.05).

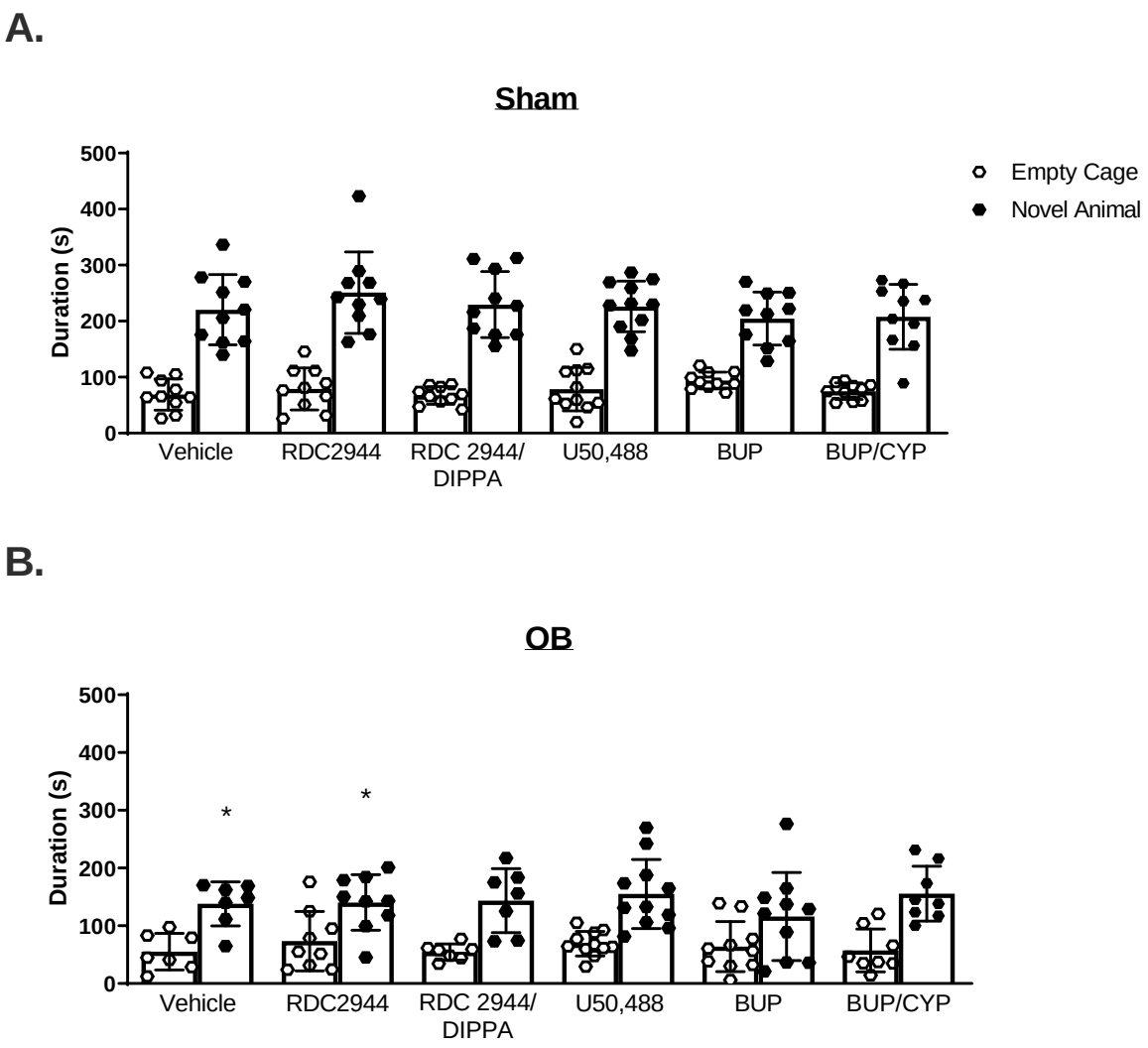


Figure 5.14 Duration spent interacting with the empty cage and the novel animal in the sociability trial. The circles that are shaded in white represent time spent interacting with the empty cage and the circles that are shaded in black represent time spent interacting with the novel animal. Sham-operated results are in graph A and OB results in graph B. OB rats that were treated with vehicle, and RDC 2944 alone spent significantly less time interacting with the novel animal in comparison to their sham-treated counterparts. There was no significant difference between groups in the time they spent interacting with the empty cage. Data are expressed as mean ± standard deviation (n=7-11 per group). *p<0.05 vs. appropriate sham-treated counterpart.

5.3.2.3.4 *Social Preference Trial:* Time spent interacting with the familiar animal and novel animal

5.3.2.3.4.1 Modulation of the MOP

With regards time spent interacting with the familiar animal in the social preference trial for animals treated with compounds acting on the MOP, two-way ANOVA revealed that there was a significant effect of surgery [$F_{(1,79)}$=7.143, p=0.009], but there was no significant effect of drug [$F_{(4,79)}$=0.668, p=0.616], or any surgery x drug interaction effect [$F_{(4,79)}$=0.751, p=0.560] (Figure 5.15). *Post-hoc* analysis revealed that there was no significant differences between any of the groups.

With regards time spent interacting with the novel animal in the social preference trial for animals treated with compounds acting on the MOP, two-way ANOVA revealed that there was a significant effect of surgery [$F_{(1,79)}$=65.892, p<0.001], drug [$F_{(4,79)}$=3.114, p=0.020], and a significant surgery x drug interaction effect [$F_{(4,79)}$=3.178, p=0.018] (Figure 5.15). *Post-hoc* analysis revealed that OB rats that were treated with RDC 2944 alone, buprenorphine alone, or buprenorphine in combination with cyprodime, spent significant less time interacting with the novel animal in the social preference trial in comparison to their appropriate sham-treated counterparts (p<0.05)

5.3.2.3.4.2 Modulation of the KOP

With regards time spent interacting with the familiar animal in the social preference trial for animals treated with compounds acting on the KOP, two-way ANOVA revealed that there was no significant effect of surgery [$F_{(1,50)}$=1.619, p=0.209], drug [$F_{(2,50)}$=0.906, p=0.411], or any surgery x drug interaction effect [$F_{(2,50)}$=0.733, p=0.486] (Figure 5.15).

With regards time spent interacting with the novel animal in the social preference trial for animals treated with compounds acting on the KOP, two-way ANOVA revealed that there was a significant effect of surgery [$F_{(1,50)}$=6.710, p=0.013], but no effect of drug [$F_{(2,50)}$=0.344, p=0.711], or any significant surgery x drug interaction effect [$F_{(2,50)}$=0.069, p=0.933] (Figure 5.15). *Post-hoc* analysis revealed that there was no significant differences between any of the groups.

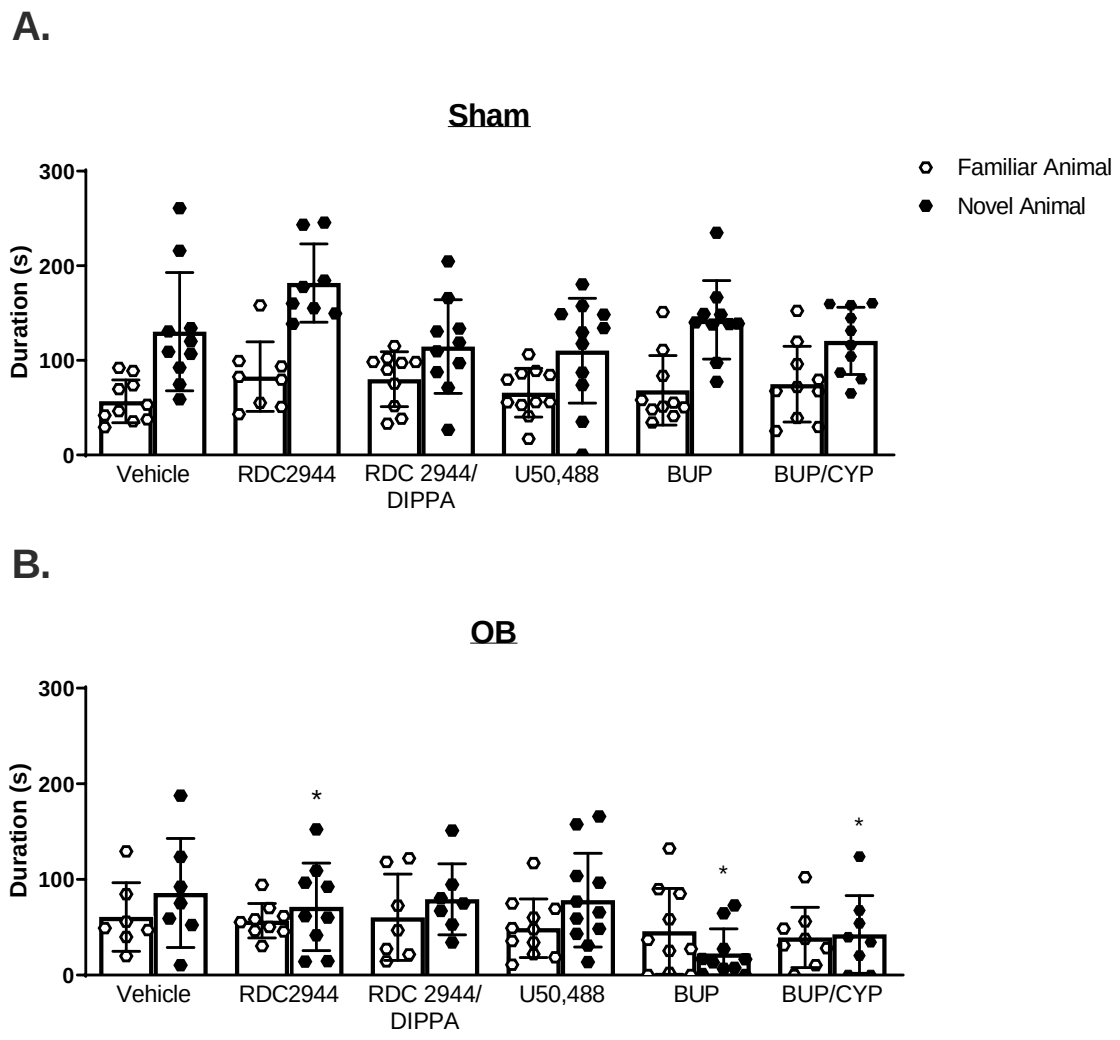


Figure 5.15 Duration spent interacting with the familiar animal and the novel animal in the social preference trial. The circles that are shaded in white represent time spent interacting with the familiar animal and the circles that are shaded in black represent time spent interacting with the novel animal. OB rats that were treated with RDC 2944 alone, buprenorphine alone or in combination with cyprodime, spent significantly less time interacting with the novel animal in comparison to their appropriate sham-treated counterparts. There was no significant difference between groups in the time spent interacting with the familiar animal. Data are expressed as mean ± standard deviation (n=7-10 per group). *p<0.05 vs. appropriate sham-treated counterpart.

5.3.2.4 Elevated Plus Maze Results

5.3.2.4.1 Distance moved in the elevated plus maze

With regards distance moved in the EPM, two-way ANOVA revealed that there was a significant effect of surgery [$F_{(1,99)}$=56.047, p<0.001], and drug [$F_{(5,99)}$=4.617, p=0.001], but no surgery x drug interaction effect [$F_{(5,99)}$=0.949, p=0.453] (Figure 5.16). *Post-hoc* analysis revealed that OB rats that had been treated with buprenorphine alone, RDC 2944 alone, or RDC 2944 in combination with DIPPA, had significantly increased distance moved in the EPM in comparison to appropriate sham-treated counterparts (p<0.05).

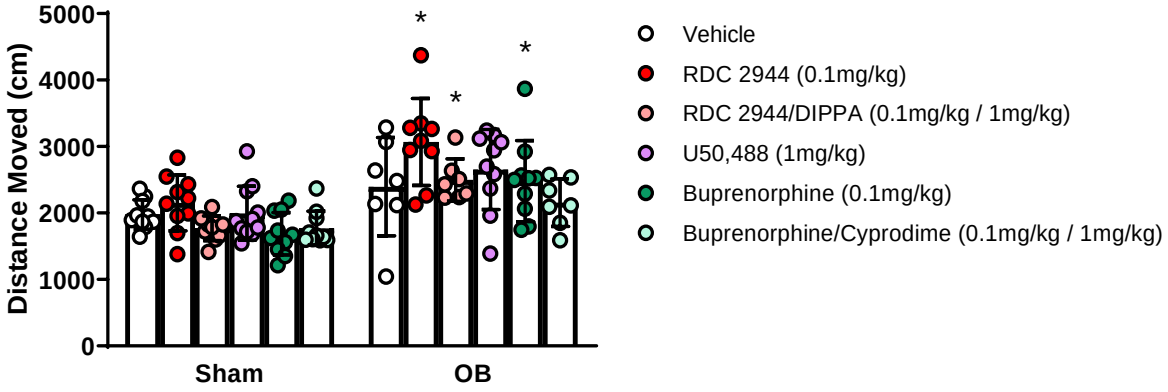


Figure 5.16 Distance moved in the elevated plus maze by sham-operated and OB rats after three weeks of chronic dosing. Buprenorphine alone, RDC 2944 alone, and RDC 2944/DIPPA, caused a significant increase in distance moved in OB rats in the EPM, in comparison to appropriate sham-treated counterparts. Data are expressed as mean ± standard deviation (n=7-11 per group). *p<0.05 vs. appropriate sham-treated counterpart.

5.3.2.4.2 % Open arm entries and % open arm duration in the elevated plus maze

With regards % open arm entries in the EPM, two-way ANOVA revealed that there was a significant effect of surgery [$F_{(1,98)}$=4.131, p=0.045], but there was no significant effect of drug [$F_{(5,98)}$=1.250, p=0.292], or any surgery x drug interaction effect [$F_{(5,98)}$=1.634, p=0.158] (Figure 5.17A). However, *post-hoc* analysis revealed that there was no significant difference between the groups.

With regards the % time spent in the open arms of the EPM, two-way ANOVA revealed that there was a significant effect of surgery [$F_{(1,98)}$=11.991, p=0.001], but there was no significant effect of drug [$F_{(5,98)}$=1.627, p=0.160], or any surgery x drug interaction effect [$F_{(5,98)}$=1.992, p=0.086] (Figure 5.17B). However, *post-hoc* analysis revealed that there was no significant difference between the groups.

5.3.2.4.3 Open arm entries and open arm duration in the elevated plus maze

With regards the number of open arm entries in the EPM, two-way ANOVA revealed that there was a significant effect of surgery [$F_{(1,98)}$=34.362, p<0.001], and a significant effect of drug [$F_{(5,98)}$=3.348, p=0.008], but no surgery x drug interaction effect [$F_{(5,98)}$=1.802, p=0.119] (Figure 5.17C). *Post-hoc* analysis revealed that OB rats that were treated with U50,488 and RDC 2944 alone had significantly more open arm entries in the EPM in comparison to their appropriate sham-treated counterparts (p<0.05).

With regards the time spent in the open arms of the EPM, two-way ANOVA revealed that there was a significant effect of surgery [$F_{(1,98)}$=9.573, p=0.003], but there was no significant effect of drug [$F_{(5,98)}$=1.502, p=0.196], or any surgery x drug interaction effect [$F_{(5,98)}$=1.614, p=0.164] (Figure 5.17D). However, *post-hoc* analysis revealed that there were no significant differences between the groups.

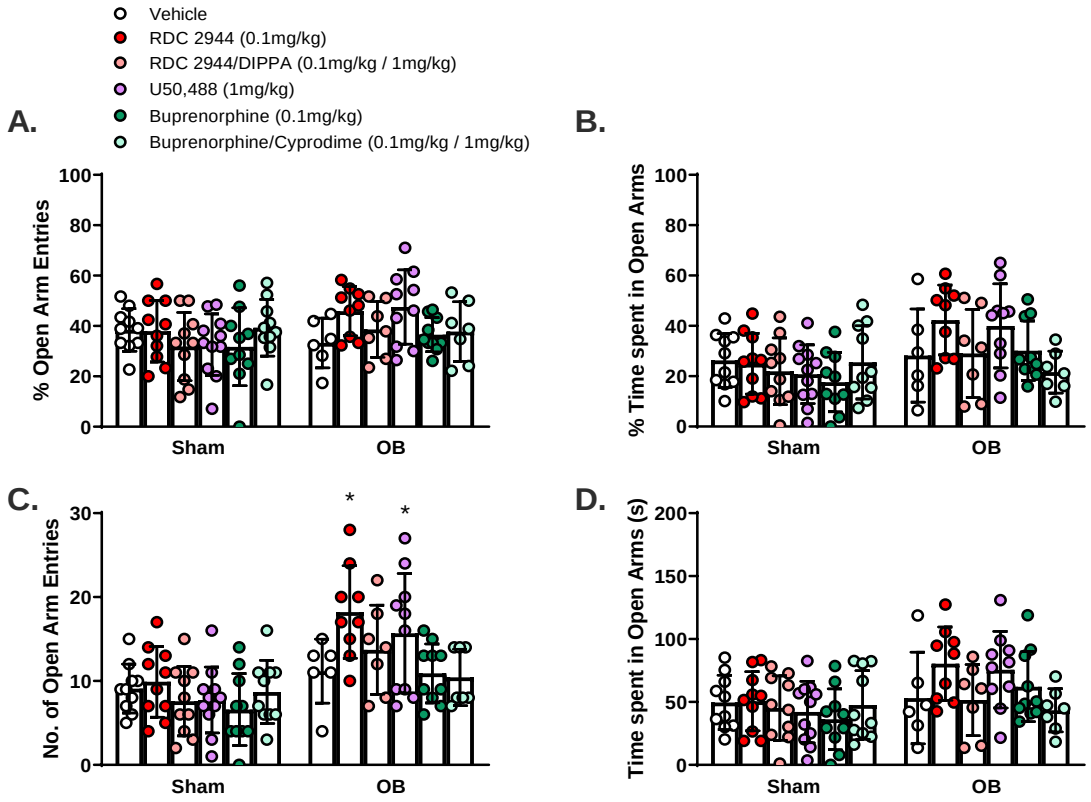


Figure 5.17 Open arm entries and duration spent in the open arms (percentage and raw values) of the elevated plus maze made by sham-operated and OB rats after three weeks of chronic dosing. OB rats that were treated with U50, 488 and RDC 2944 alone had significantly more open arm entries in the EPM in comparison to their appropriate sham-treated counterparts (C). There was no significant difference in % open arm entries (A), % open arm duration (B), or the duration spent in the open arms (D). Data are expressed as mean ± standard deviation (A, B, C, D) (n=6-11 per group). *p<0.05 vs. appropriate sham-treated counterpart.

5.3.2.5 Open Field Results

5.3.2.5.1 Distance moved in the open field

With regards distance moved in the OF, two-way ANOVA revealed that there was no significant effect of surgery [$F_{(1,83)}$=0.075, p=0.785], drug [$F_{(5,83)}$=1.725, p=0.138], or any surgery x drug interaction effect [$F_{(5,83)}$=1.684, p=0.147] (Figure 5.18A).

5.3.2.5.2 Time spent in the inner circle of the open field

With regards time spent in the inner circle of the OF, two-way ANOVA revealed that there was a significant effect of surgery [$F_{(1,83)}$=44.350, p<0.001], but no effect of drug [$F_{(5,83)}$=0.460, p=0.805], or any surgery x drug interaction effect [$F_{(5,83)}$=0.894, p=0.489] (Figure 5.18B). *Post-hoc* analysis revealed that OB rats that were treated with vehicle spent significantly less time in the inner circle of the OF than their sham vehicle-treated counterparts (p<0.05).

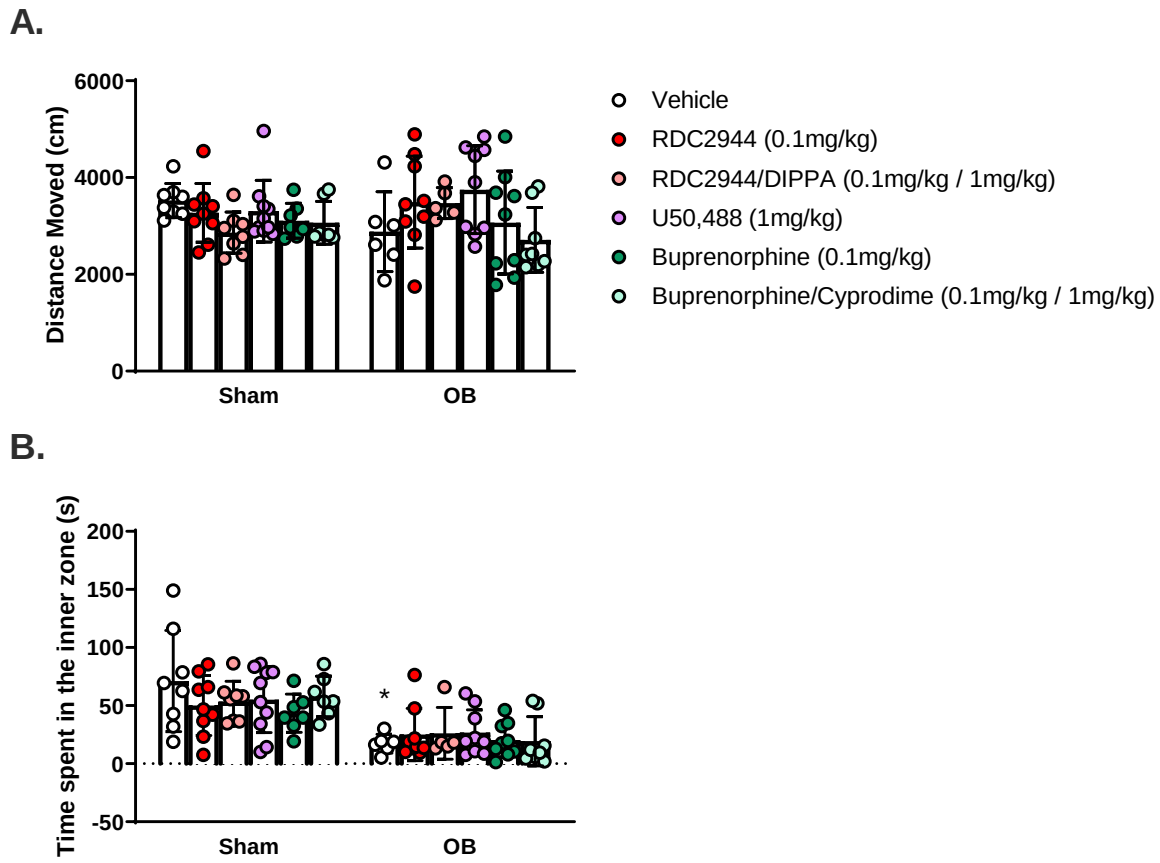


Figure 5.18 Distance moved in the open field (A) and duration spent in the inner zone (B) of the open field by sham-operated and OB rats after three weeks of chronic dosing. There was no significant difference between groups with regards the distance moved in the OF (A). OB rats that were treated with vehicle spent significantly less time in the inner zone of the OF than their vehicle-treated sham counterparts (B). Data are expressed as mean ± standard deviation (n=5-10 per group). *p<0.05 vs. appropriate sham-treated counterpart.

5.3.2.6 Opioid receptor and peptide mRNA expression in the hippocampus

5.3.2.6.1 MOP system

5.3.2.6.1.1 MOP (*Oprm1*) and pre-propeptide POMC (*Pomc*) mRNA expression in the hippocampus

With regards *Oprm1* mRNA expression in the hippocampus, two-way ANOVA revealed that there was no significant effect of surgery, drug, or any surgery x drug interaction effect (Table 5.10).

With regards *Pomc* mRNA expression in the hippocampus, two-way ANOVA revealed that there was no significant effect of surgery, drug, or any surgery x drug interaction effect (Table 5.10).

Group	MOP	POMC
Sham		
Vehicle	1 ± 0.33	1 ± 0.73
RDC 2944 (0.1 mg/kg)	1.11 ± 0.29	1.44 ± 0.80
RDC 2944/DIPPA (0.1 mg/kg / 1 mg/kg)	1.10 ± 0.23	1.10 ± 0.58
U50,488 (1 mg/kg)	1.09 ± 0.38	1.42 ± 1.12
Buprenorphine (0.1 mg/kg)	1.20 ± 0.46	1.33 ± 1.40
Buprenorphine/Cyprodime (0.1 mg/kg / 1 mg/kg)	1.09 ± 0.34	1.63 ± 1.23
OB		
Vehicle	1.26 ± 0.32	1.08 ± 0.74
RDC 2944 (0.1 mg/kg)	1.28 ± 0.55	1.67 ± 1.31
RDC 2944/DIPPA (0.1 mg/kg / 1 mg/kg)	1.24 ± 0.29	1.11 ± 0.69
U50,488 (1 mg/kg)	1.08 ± 0.25	0.71 ± 0.51
Buprenorphine (0.1 mg/kg)	1.05 ± 0.39	1.23 ± 0.83
Buprenorphine/Cyprodime (0.1 mg/kg / 1 mg/kg)	1.26 ± 0.44	0.90 ± 0.63
Effect		
Surgery	F(1,97)=1.739, *p*=0.190	F(1,95)=1.123, *p*=0.292
Drug	F(5,97)=0.207, *p*=0.959	F(5,95)=0.703, *p*=0.622
Surgery x Drug	F(5,97)=0.670, *p*=0.647	F(5,95)=0.817, *p*=0.541

Table 5.10 MOP (*Oprm1*) and POMC (*Pomc*) mRNA expression in the hippocampus. *Oprm1* and *Pomc* mRNA expression is expressed as a percentage of the sham-operated vehicle-treated group. There was no significant difference in *Oprm1* or *Pomc* mRNA expression between sham-operated and OB rats in the hippocampus. Data are expressed as mean ± standard deviation (*n*=6-11 per group).

5.3.2.6.2 KOP system

5.3.2.6.2.1 KOP (*Oprk1*) and propeptide PDYN (*Pdyn*) mRNA expression in the hippocampus

With regard *Oprk1* mRNA expression in the hippocampus, two-way ANOVA revealed that there was a significant effect of drug, but no significant effect of surgery, or any surgery x drug interaction effect (Table 5.11). *Post-hoc* analysis revealed that there was no significant difference between the groups.

With regards *Pdyn* mRNA expression in the hippocampus, two-way ANOVA revealed that there was no significant difference (surgery, drug, and surgery x drug interaction) between sham-operated and OB experimental groups (Table 5.11).

Group	KOP	PDYN
Sham		
Vehicle	1 ± 0.25	1 ± 0.31
RDC 2944 (0.1 mg/kg)	1.05 ± 0.22	1.08 ± 0.37
RDC 2944/DIPPA (0.1 mg/kg / 1 mg/kg)	1.03 ± 0.22	0.86 ± 0.22
U50,488 (1 mg/kg)	0.92 ± 0.20	0.83 ± 0.19
Buprenorphine (0.1 mg/kg)	0.75 ± 0.17	0.96 ± 0.30
Buprenorphine/Cyprodime (0.1 mg/kg / 1 mg/kg)	0.70 ± 0.08	0.77 ± 0.19
OB		
Vehicle	0.87 ± 0.19	0.99 ± 0.56
RDC 2944 (0.1 mg/kg)	0.88 ± 0.28	0.91 ± 0.27
RDC 2944/DIPPA (0.1 mg/kg / 1 mg/kg)	0.91 ± 0.18	0.71 ± 0.91
U50,488 (1 mg/kg)	0.93 ± 0.26	0.87 ± 0.23
Buprenorphine (0.1 mg/kg)	0.66 ± 0.09	1.03 ± 0.36
Buprenorphine/Cyprodime (0.1 mg/kg / 1 mg/kg)	0.84 ± 0.10	0.77 ± 0.23
Effect		
Surgery	F(1,93)=2.048, p=0.156	F(1,94)=0.386, p=0.536
Drug	**F(5,93)=4.841, p=0.001**	F(5,94)=2.101, p=0.072
Surgery x Drug	F(5,93)=1.185, p=0.323	F(5,94)=0.486, p=0.786

Table 5.11 KOP (Oprk1) and PDYN (Pdyn) mRNA expression in the hippocampus. *Oprk1* and *Pdyn* mRNA expression is expressed as a percentage of the sham-operated vehicle-treated group. There was no significant difference in *Oprk1* or *Pdyn* mRNA expression between sham-operated and OB rats in the hippocampus. Data are expressed as mean ± standard deviation (n=6-11 per group).

5.3.2.6.3 DOP system

5.3.2.6.3.1 DOP (*Oprd1*) and pre-propeptide PENK (*Penk*) mRNA expression in the hippocampus

With regards *Oprd1* mRNA expression in the hippocampus, two-way ANOVA revealed that there was no significant effect of drug, but no significant effect of surgery, or any surgery x drug interaction effect (Table 5.12).

With regards peptide *Penk* mRNA expression in the hippocampus, two-way ANOVA revealed that there was no significant effect of surgery, drug or any surgery x drug interaction effect (Table 5.12).

Group	DOP	PENK
Sham		
Vehicle	1 ± 0.14	1 ± 0.24
RDC 2944 (0.1 mg/kg)	1.18 ± 0.11	0.93 ± 0.22
RDC 2944/DIPPA (0.1 mg/kg / 1 mg/kg)	1.03 ± 0.11	1.05 ± 0.28
U50,488 (1 mg/kg)	1.05 ± 0.07	0.96 ± 0.29
Buprenorphine (0.1 mg/kg)	0.92 ± 0.24	1.12 ± 0.33
Buprenorphine/Cyprodime (0.1 mg/kg / 1 mg/kg)	0.95 ± 0.11	0.93 ± 0.24
OB		
Vehicle	1.15. ± 0.18	1.04 ± 0.27
RDC 2944 (0.1 mg/kg)	1 ± 0.17	1.03 ± 0.44
RDC 2944/DIPPA (0.1 mg/kg / 1 mg/kg)	0.94 ± 0.21	0.88 ± 0.21
U50,488 (1 mg/kg)	1.01 ± 0.25	0.97 ± 0.34
Buprenorphine (0.1 mg/kg)	0.85 ± 0.09	0.83 ± 0.14
Buprenorphine/Cyprodime (0.1 mg/kg / 1 mg/kg)	0.94 ± 0.23	1 ± 0.08
Effect		
Surgery	F(1,93)=1.310, *p*=0.255	F(1,92)=0.436, *p*=0.511
Drug	**F(5,93)=3.288, *p*=0.009**	F(5,92)=0.091, *p*=0.993
Surgery x Drug	F(5,93)=1.422, *p*=0.224	F(5,92)=1.224, *p*=0.304

Table 5.12 DOP (Oprd1) and PENK (Penk) mRNA expression in the hippocampus. *Oprd1* and *Penk* mRNA expression is expressed as a percentage of the sham-operated vehicle-treated group. There was no significant difference in *Oprd1* or *Penk* mRNA expression between sham-operated and OB rats in the hippocampus. Data are expressed as mean ± standard deviation (*n*=6-11 per group).

5.4 Discussion

The involvement of the opioid system in the modulation of stress and emotion is long established, such that the usage of opioids preceded current monoamine-derived antidepressant therapies and was one of the original sources used to treat 'melancholia' or depression. In recent years, the use of opioid strategies for targeting the treatment of depression has been rekindled (Berrocoso et al., 2009, Lutz and Kieffer, 2013). The lack of efficacy of currently-marketed antidepressants to treat all patients with depression, and the development of safer pharmacological profiles for opioid compounds has reignited the idea of utilising the opioid system as a novel approach or therapy for the treatment of depression. Examining opioid modulating compounds *in vivo*, aids in the assessment and detection of compounds that are deemed selective, safe, efficacious and tolerable prior to human assessment. In this Chapter, a range of opioid modulating drugs were examined for their antidepressant-like efficacy in the OB rat model. Bodyweight, tolerability, cognitive functioning, anxiety-like behaviour and psychomotor retardation were all assessed in two large chronic dosing experiments and results are summarised in Table 5.13.

Firstly, bodyweight gain was examined after weeks one, two and three of dosing in both experiments. In *experiment 1*, DIPPA alone caused a significant decrease in bodyweight gain after one week of chronic dosing in both sham-operated and OB rats. The combination of DIPPA/SNC80, and donepezil alone, also caused a significant decrease in the bodyweight gain of OB rats, with a trend for the same reduction in sham-operated rats but not to a statistically significant level. Taking this into account, it is clear to see that DIPPA appears to be having a negative effect on bodyweight after acute exposure to chronic dosing. Chronic dosing with SNC80 (1 mg/kg) for eight days has been previously shown to have no negative effect on bodyweight gain in OB rats (Saitoh et al., 2008). Similarly, subchronic administration with donepezil (1 mg/kg) has also been reported to have no negative effects of bodyweight in an animal model of autism (Kim et al., 2014). To the best of our knowledge, no other papers reporting the effects of DIPPA have examined bodyweight, such that no paper has reported or published any results on bodyweight change. Having said this, many papers do not report bodyweight changes unless a drug is being examined for chronic exposure, and the majority of DIPPA papers published use the drug acutely so perhaps this is why there is no available data on bodyweight changes.

Test/Parameter	Experiment 1		Experiment 2	
Bodyweight gain post-dosing	**Sham**	**OB**	**Sham**	**OB**
Week 1	BW ↓ DIPPA	BW ↓ DIPPA, SNC80/DIPPA and Donepezil	BW ↓ RDC 2944/DIPPA	–
Week 2	–	–	–	–
Week 3	–	–	–	–
3-Chamber Sociability Test				
DM in Habituation Trail	–	–		↑ RDC 2944, RDC 2944/DIPPA, and Buprenorphine/Cyprodime
DM in Sociability Trial	–	–	–	↓ Buprenorphine
DM in Social Preference Trial	–	–	–	↓ Buprenorphine and Buprenorphine/Cyprodime
% Habituation	–	–	–	↓ All OB groups
Sociability Trial: Interaction with Empty Cage	–	↓ Donepezil	–	–
Sociability Trial: Interaction with Novel Animal	–	↓ SNC80 and Donepezil	–	↓ RDC 2944
Social Preference Trial: Interaction with Familiar Animal	–	–	–	–
Social Preference Trial: Interaction with Novel Animal	–	↓ Donepezil	–	↓ RDC 2944, Buprenorphine, and Buprenorphine/Cyprodime
Elevated Plus Maze Test				
DM	–	–	–	↑ RDC 2944, RDC 2944/DIPPA, and Buprenorphine
% Open Arm Entries	–	–	–	–
% Open Arm Duration	–	–	–	–
Open Arm Entries	–	–	–	↑ RDC 2944 and U50,488
Open Arm Duration	–	–	–	–
Open Field Test				
DM	–	↑ DIPPA	–	–
Time spent in Inner Circle	–	↓ DIPPA/SNC80	–	↓ Vehicle

Table 5.13 Summary of behavioural effects from opioid modulating drugs used in experiment 1 and 2. BW=bodyweight, DM=distance moved (cm), ↓=decreasing, ↑=increasing, – =no significant effect, OB=olfactory bulbectomy.

Nevertheless, it must be noted that exposure to DIPPA is the common factor across all experimental groups, affecting both surgery groups. Having said this, there was no changes in bodyweight gain in any sham or OB groups at two and three weeks post-dosing in these DIPPA-treated groups, suggesting theses changes are transient. Similarly, there was no significant difference in bodyweight gain in any of the other drugs after one, two and three weeks post-dosing, indicating that the drugs were well-tolerated over time. A similar scenario was seen in *experiment 2*, in that sham-operated rats treated with the RDC 2944/DIPPA combination experienced a significant decrease in bodyweight gain after one week of dosing.

With regards social cognition and the 3-chamber sociability test, distance moved in all three trials was examined and data were represented as % habituation to examine whether any habituation effects were seen in OB rats similar to that which was seen in both experiments in Chapter 3. In *experiment 1*, there was no significant differences in distance moved in any trial between sham-operated and OB rats. Having said this, there was a trend for all OB animals to have reduced distance travelled over time in the three trials, and although not statistically significant across the OB drug-treated groups, there was again a specific trend for lower % habituation scores. In *experiment 2* in this chapter, all of the previous significant OB changes were reproduced. A pronounced development for a reduced distance moved in OB rats over time in all three trials was observed, with sham-operated groups consistently exploring the arena to the same degree in each trial, where OB rats did not. In conjunction with this and as was shown in Chapter 3, in *experiment 2* a significant difference in % habituation scores was observed in all OB groups regardless of treatment.

With regards drug effects and distance moved in the 3-chamber sociability test, RDC 2944 alone, RDC 2944/DIPPA and buprenorphine/cyprodime all increased distance moved in OB rats when compared to sham-operated counterparts during the habituation trial. The two RDC 2944 groups also showed an increase in movement in OB rats when compared to vehicle-treated OB counterparts. Buprenorphine alone also decreased distance moved in OB rats in the sociability trial and social preference trial, with the combination of buprenorphine/cyprodime decreasing distance moved in the social preference trial in OB rats, all when compared to appropriate sham-operated counterparts. MOP agonists have been shown to increase locomotor activity (Smith et al., 2019), especially after repeated treatment (Burke et al., 2019a). Buprenorphine (0.1 mg/kg), a partial MOP agonist and KOP antagonist, has been shown to decrease

immobility in the FST, hypothesising increased mobility and movement as a result (Burke et al., 2019a; 2019b, Smith et al., 2019), and the same dose has been shown to increase locomotor activity in the OF (Smith et al., 2019). Having said this, the majority of these effects are after acute administration, and are also in naive rats; and so are more applicably comparable to the sham-operated rats than to the OB rats. Burke et al. (2019b) looked at chronic dosing with 0.1 mg/kg buprenorphine for 14 days in OB rats, and showed that chronic treatment decreased distance moved in OB rats in the OF, and also reduced immobility in the FST. KOP antagonist DIPPA is hypothesised to have low KOP agonism with more long-lasting KOP antagonist effects (Terner et al., 2005), and has been shown to significantly decrease immobility (5 mg/kg) and increase swimming behaviour (10 mg/kg) in the FST (Carr et al.,. 2010). RDC 2944 and cyprodime are less well characterised, with previous reports only examining acute administration and observing no differences in locomotor activity *per se* after RDC 2944, or cyprodime exposure (Callaghan et al., 2019, Sikora et al., 2019). Having said this, each of these drugs is given in combination with another drug; a drug that has been shown to increase locomotor activity, so perhaps this accompanying drug is in action and this is why an increase in distance moved is seen in these groups in this study. As to why these effects are seen in the OB rat and not sham-operated rats is interesting. It is hard to explain it as an OB model effect of hyperactivity since the same effect is not statistically significant in OB vehicle-treated rats. Opioid agonists, particularly at the KOP and MOP, have been shown to affect dopaminergic neurotransmission. MOP agonism has been shown to increase the release of dopamine which would indicate increased movement (Smith et al., 2019), with KOP agonists shown to decrease the release of dopamine, would should indicate reduced movement (Spanagel et al., 1990). In conjunction with this, dopaminergic pathways have been shown to be affected after OB removal, with reduced dopamine, DOPAC and HVA concentrations being shown in limbic regions (Thakare et al., 2017, Ruda-Kucerova et al., 2015). Perhaps, different compensatory mechanisms may be mediating processes in the corticolimbic regions and dopaminergic system of OB rats in comparison to sham-operated rats, explaining these differing results in surgery groups. More interesting, are the effects of such a low dose of buprenorphine, which is shown to enhance movement in OB rats in the habituation trial, and cause a decrease in movement in OB rats on subsequent trials. Given the extent of research that has been shown above that buprenorphine at this dose (0.1 mg/kg) can increase locomotor

activity (Burke et al., 2019a; 2019b, Smith et al., 2019), these findings are difficult to fathom. Nevertheless, it must be noted that the habituation observed over the subsequent two trials could be due to longer time of testing compared with published works (30 minutes vs. 5-10 minutes), and it could also depend on the conditions under which testing happened, i.e. very bright lighting in the OF vs. relatively low lighting in the 3-chamber sociability test. The other difference across these trials is contextual, i.e. the addition of a social stimulus. This may indicate that in OB animals, this significant decrease in movement in these contextually social trials is as a result of MOP mechanisms that selectively effect sociability. Comparing the 'social interaction' results in OB buprenorphine-treated rats to these findings could indicate more with regards this theory, and this will be discussed shortly.

In both experiments, interaction times in the sociability and social preference trials were examined. Firstly it must be noted that only three other papers have published results using opioid drugs in the 3-chamber sociability test (Dogra et al., 2016, Smith et al., 2015; 2018). Their findings will be discussed in detail and in relation to our results in the next paragraph. In this Chapter, regardless of drug treatments, sham-operated rats showed no differences in interaction times in the sociability or social preference trial in either experiment. In OB rats, a number of differences were seen with regards the wide variety of drugs and combinations used, but the primary characteristic of both studies was that once again, none of the opioid modulating drugs, nor the acetylcholinesterase inhibitor, attenuated the social cognitive deficit recorded in our OB rats. In the sociability and social preference trials in both experiments, and similar to the housing experiment in Chapter 3, our OB animals appeared to spend less time exploring the novel animal in both trials, with an effect of surgery seen in all examinations (*post-hoc* analysis revealed no differences between the groups). Any significant drug effects that were seen in the OB rat were only seen to deepen or heighten the pre-existing trend for social cognitive dysfunction in these rats, rather than to attenuate it. In *experiment 1*, OB rats treated with donepezil spent significantly less time exploring the empty cage and the novel animal in the sociability trial than their sham-treated counterparts. In the social preference trial, OB rats treated with donepezil also spent significantly less time exploring the novel conspecific animal than their sham-treated counterparts. This was the direct opposite effect to that which would be hypothesised for this drug; a drug which is used to therapeutically treat cognitive decline in Alzheimer's disease. Less time exploring the

empty cage in the sociability trial could suggest more time investigating the novel animal and so could suggest an enhancement of motivation. However, this was not the case as donepezil treated OB rats also spent less time exploring the novel animal in this trial when compared to their OB vehicle-treated counterpart. As a result, these results suggest that they moved less and were less motivated to engage in any exploration of either the cage or animal. Chronic administration (15 days) with 0.75 mg/kg of donepezil, two weeks prior to testing, has been examined previously in the 3-chamber sociability test in an Alzheimer's disease rat model (Cutuli et al., 2013). Pre-treatment with donepezil failed to have any effect on social cognitive functioning in a model of Alzheimer's disease (Cutuli et al., 2013), which is in line with the findings in this experiment. In the OB model, donepezil did not improve social cognitive functioning, seeming to amplify the lack of stimulus and non-stimulus interaction in the OB rat rather than to retrieve it. This is very similar to what was seen with the conventional antidepressant drugs used in Chapter 3, perhaps indicating that the depth of this deficit or effect of change in this model will need further investigation before a therapy to restore this trend in social cognitive functioning can be reached.

It should be noted that donepezil was chosen as the positive control because it has been proven to improve cognitive deficits in both animals (de Bruin et al., 2011, Narimatsu et al., 2009) and humans (Rogers et al., 1998). Nevertheless, the use of donepezil to treat *social* cognitive functioning has been less explored. It was only on retrospect of this experiment that the above paper by Cutuli et al. (2013) was found, whereby donepezil failed to improve social cognitive processing in the 3-chamber sociability test in an animal model of Alzheimer's disease. In conjuction with this, it should be mentioned that given the loss of cholinergic activity that has been shown in the OB model and was discussed in Chapter 1, perhaps the choice of an acetylcholinesterase inhibitor was not the appropriate candidate of choice for a positive control. Memantine was also initially considered as a positive control, and is a compound that is used to improve cognitive functioning in Alzheimer's disease (Orgogozo et al., 2002), by means of blocking the NMDA receptor, and as such decreasing excessive glutamate from causing cell death (Orgogozo et al., 2002). In hindsight perhaps memantine may have been the more appropriate choice of positive control.

With this in mind, the anatomical and neurotransmitter substrates involved in social cognitive functioning are crucial to the treatment of social cognitive decline and

dysfunction. Anatomically, the prefrontal cortex, hippocampus, amygdala and hypothalamus are the main regions of the brain involved in social cognitive functioning (Amodio and Frith, 2006).The medial prefrontal cortex has been shown to be involved in socially related action-and-outcome measures such as goal-directed social behaviour and decision-making behaviour with regards social context (Amodio and Frith, 2006, Eslinger et al., 2004). Indeed, patients with lesions to the medial prefrontal cortex have been shown to exhibit altered social cognitive function including malfunctioning social and moral reasoning (Anderson et al., 1999). The hippocampus is involved in social memory functioning. The ventral hippocampus has been shown to be integral to the facilitation of storage and retrieval of social memories, with activation of CA1 neurons in mice shown to enable the retrieval of memories and response to a familiar mouse in a social interaction task (Okuyama et al., 2016). The hypothalamus is responsible for social behaviours such as arousal, aggression and affiliation (Amodio and Frith, 2006, Shelley et al., 2006). The amygdala is closely linked with the prefrontal cortex and both regions are highly correlated with social cognitive functioning (Amodio and Frith, 2006). Lesions to the amygdala in rhesus macaque monkeys in infancy causes several social cognitive deficits in adulthood including a reduction in social interaction time with familiar animals, a reduction in exploration of novel animals, and a decrease in vocalisations (Bliss-Moreau et al., 2013). The amygdala is also responsible of the release of several neurotransmitters and neuropeptides involved in regulating social cognitive behaviour including vasopressin, oxytocin, 5-HT and DA (Amodio and Frith, 2006). Indeed, two of the main neurotransmitters that have been shown to be involved in social cognition and social behaviour are that of estrogen and the neuropeptide oxytocin (Shelley et al., 2006, Young, 2002). For example, both estrogen and oxytocin are important for social recognition (Shelley et al., 2006). Estrogen regulates the release of oxytocin in the hypothalamus, which projects to the amygdala which is the region that ultimately enables social recognition (Shelley et al., 2006). Mice lacking the oxytocin gene have been shown to display social amnesia with a failure to develop social memory, with administration of oxytocin retrieving this deficit (Ferguson et al., 2000). Another neuropeptide, vasopressin has also been shown to modulate social cognitive functioning. Administration of arginine vassopressin, a vassopressin agonist, enhances social memory functioning in rat's prexposed to a conspecific juvenile rat, with a vasopressor antagonist in contrast impairing recognition of a familiar juvenile rat

(Dantzer et al., 1987). The neurotransmitters 5-HT and DA are also substrates of social cognitive functioning, with serotonin input shown to regulate and reinforce social reward behaviour via oxycotin innervations (Dölen et al., 2013). DA is also involved in social reward behaviour and is increased during social play behaviour (Robinson et al., 2011), with DA antagonists shown to reduce play (Niesink and Van Ree, 1989). Lastly, the endogenous opioid system in shown to promote positive social affect (Siviy and Panksepp, 2011), and therefore has been shown to increase social play behaviour via cognitive motivational behaviour to play in the rats (Normansell and Panksepp, 1990, Siviy and Panksepp, 2011).

SNC80 alone and RDC 2944 alone caused a significant reduction in time spent with the novel animal in the sociability trial in OB rats, with RDC 2944 alone, buprenorphine alone and buprenorphine/cyprodime all seen to cause a significant reduction in time spent with the novel animal in the social preference trial in these rats. As mentioned previously, there are only three other papers that have published results using opioid drugs in the 3-chamber sociability test (Dogra et al., 2016, Smith et al., 2015; 2018). To the best of our knowledge this is the first experiment that reports the effects of a DOP agonist on the 3-chamber sociability test, so these findings are novel. Intracranial administration of a MOP antagonist (CTAP) into the nucleus accumbens has been shown to decrease sociability time and social novelty preference time in juvenile rats (Smith et al., 2015). In contrast, MOP blockade in the basolateral amygdala has been shown to have no effect of social novelty preference (Smith et al., 2018). MOP agonist DAMGO has been shown to increase social novelty preference in rats that are susceptible to social isolation, with no effect on rats that are not susceptible (Smith et al., 2018). In this study, the direct opposite to the majority of previous literature was found. MOP partial agonist RDC 2944 reduced sociability in OB rats, with both MOP partial agonist's buprenorphine and RDC 2944 shown to reduce social preference in OB rats. This shows that partial MOP agonism using two different drugs impairs social motivation and cognition in these animals, unlike previous reports that it increases social play in rats susceptible to social isolation (Smith et al., 2018). This reduction in social preference in OB rats was still seen when buprenorphine was given in combination with the MOP antagonist cyprodime, highlighting that these MOP agonists have a strong effect in the social mechanisms and circuitry in OB animals. Interestingly, this matches the effects of buprenorphine that were seen with regards distanced moved in the sociability and social preference

trials, and the reduction it caused in OB rats in these two socially contextual trials. As many of the drugs used modulate the MOP, much more work is needed in order to fully ascertain the nature of these effects, especially in regards to changes in the brain after OB. The experiments in this chapter differ from the three studies cited above with regards route of administration, selectivity and affinity's of drugs used, lower dosages, and acute verses chronic administrations. Therefore, these considerations need to be taken into account as these previous studies act more as a guide than as directly comparable.

In the EPM, there was no significant differences found between sham-operated and OB groups in *experiment 1* in any parameter assessed. However in *experiment 2*, distanced moved was increased in OB rats that were treated with RDC 2944 alone and in combination with DIPPA, and in OB rats that were treated with buprenorphine alone, when compared to sham-treated counterparts. Again, the same MOP agonists and KOP antagonistic drugs are affecting the locomotor activity of OB rats as was seen in the habituation trial of the 3-chamber sociability test, but not affecting that of the sham-operated rats. Chronic dosing with RDC 2944 and with U50,488 was shown to increase the number of open arm entries in OB rats, demonstrating anxiolytic-like effects in these compound in this model. Previous studies have shown that acute administration of U50,488 produces anxiety-like behaviour in the EPM in both rats and mice (Privette and Terrian et al., 1995, Smith et al., 2012). Given that this effect of producing anxiogenic behaviour was not seen in our sham-operated rats, and that the opposite was seen in our OB rats, there appears to be some different mechanism of action by this KOP agonist in the OB model, highlighting that the KOP circuitry in this model must be adapted in some manner. As very few papers have reported results using RDC 2944, this examination of the compound in the EPM may be the first experiment to do so. In the housing study in Chapter 3, OB rats were all seen to move significantly more than sham-operated rats in the EPM, an attribute which was not found in both of the OB vehicle-treated groups in the two experiments in this chapter. Having said this, in the housing study, the EPM was the first behavioural test OB rats were exposed to and this test was also examined at the earlier time-point of three weeks, so both of these factors must be taken into consideration.

The last behavioural parameter assessed was that of the OF. As stated in previous chapters and as indicated with our meta-analysis, the OF is the most reliable and reproducible test used in the OB rat model of depression. A pronounced

hyperactivity is found in these animals when placed into an aversive brightly lit arena, but in both of the experiments in this chapter, neither of the OB vehicle-treated groups were found to display this classic increase in locomotor activity in the OF when compared to sham-operated rats. There was also no effect of any drug on locomotor activity in the OF. In our housing experiment in Chapter 3, hyperactivity in our OB rats was displayed at two weeks post-surgery, but not at five weeks post-surgery. This was thought to be attributed to a habituation effect, as has been shown by previous researchers (Gigliucci et al., 2014, Holubova et al., 2016, Zueger et al., 2005). In the housing experiment, the OF test was first assessed immediately after the EPM and as such, these can nearly be regarded as a joint-first exposure to behavioural testing, with the idea of running them directly after each other heightening the strength of the novelty of both tests. The loss of hyperactivity in the second OF exposure was accounted for at five weeks post-surgery in the housing experiment, and after each animal had gone through a thirty minute exposure to the 3-chamber sociability trial, including the habituation phase. One explanation for the loss of hyperactivity in OB rats in both experiments in this Chapter could be the pre-exposure to the 3-chamber sociability test. The OF was the last parameter assessed in both experiments, and this was done in order to keep with the timing of the OF testing in the housing study and also in order to assess the effects of chronic dosing with these drugs. As the 3-chamber sociability test and the EPM were both assessed prior to the OF, perhaps the OB animals experienced a habituation effect to behavioural testing in general after exposure to the 3-chamber sociability trial, with regards duration of the test and exposure to a different type of novel arena in the habituation trial. This is strengthened by the knowledge that the OB rats show a reduction in % habituation to the arena over the thirty minutes in comparison to the sham-operated rats. It should also be noted here that a lack of increased locomotor activity in OB rats in the EPM was also seen in these experiments, and again in contrast to the housing study. This could be indicating another effect of attenuation to distance moved after first behavioural exposure in this model. The exact reason for the lack of hyperactivity in both studies is hard to explain but given that the OB rats tested after the 3-chamber sociability trial in the housing study in Chapter 3 did not exhibit an increase in locomotor activity either, it could be coherent to think that pre-exposure to previous behavioural testing and paradigms may be having an effect.

Lastly, the time spent in the centre or inner circle of the OF was also examined for anxiety-like behaviour. In *experiment 2*, OB vehicle-treated rats spent significantly less time in the centre of the OF than vehicle-treated sham-operated rats, displaying an anxiogenic-like phenotype within the OB model. Although the same effect was not seen to be significant in *experiment 1*, a direct and similar trend was observed for vehicle-treated OB rats, as well as all other drug-treated OB rats. In the housing study in Chapter 3, the time spent in the inner circle or zone of the OF at five weeks post-surgery was also found to be significantly decreased in paired OB rats, with a similar reduction in centre time seen in single and paired (different condition) OB rats as well but not to a statistically significant level. As a result, it is clear that a pattern for a reduction in the time spent in the centre of the OF by OB rats can be seen across all studies at this time point, suggesting an anxiogenic-like characteristic in the model. This has also been shown in OB rats within our lab previously (Burke et al., 2013; 2015). The combination of DIPPA/SNC80 significantly decreased time spent in the centre of the OF in OB rats, indicating that this drug caused anxiogenic-like effects in OB rats, rather than having anxiolytic-like effects. Few papers examine the effects of opioid exposure on anxiety-like behaviour in the OF, with most examining locomotor activity. Nevertheless, acute administration of KOP antagonists, NorBNI and JDTic, have been shown to have no effect on the time spent in the centre of the OF in rats (Knoll et al., 2007). In contrast to the DOP agonist in our study, microdialysis injection of DOP agonist, KNT-127, has been shown to increase time spent in the centre of the OF arena in mice (Saitoh et al., 2018). Acute injection of SNC80 (1 mg/kg) has been shown to increase the time in the centre of the OF in socially isolated rats but not in non-isolated rats (Haj-Mirzaian et al., 2019). In this experiment, SNC80 when given in combination appeared to have a trend for increasing the time spent in the centre of the OF in sham-operated rats, with no effect in OB animals, showing that chronic treatment with this drug does not alter the anxiogenic profile in this particular model.

Finally, the mRNA expression for the three opioid receptors and their endogenous peptides in the hippocampus were examined. Overall, there was a lack of significant difference found in the mRNA expression of all opioid peptides and receptors in this region in both experiments, with the exception of a drug effect seen for the mRNA expression of KOP and DOP in *experiment 2*. Taking this drug effect on both genes into account, it was noted that the buprenorphine alone treatment displayed a trending decrease in receptor mRNA expression in both systems, and

perhaps with particular respect to OB animals. It must be noted that buprenorphine is an opioid modulating drug with high affinity for the MOP and KOP receptors, acting as an agonist at the MOP and an antagonist at the KOP. It has also been shown to have affinity for the DOP, and to act as an antagonist at the DOP. Buprenorphine has been found to decrease MOP binding and have no effect on DOP binding in the hippocampus of rats (Belcheva et al., 1996). Acute injection of buprenorphine (0.25 mg/kg) has also been shown to have no effect on KOP mRNA expression in the hippocampus of control mice (Falcon et al., 2016). Few papers report or examine mRNA changes to the opioid system after buprenorphine treatment and of those that have, they tend to examine regions other than the hippocampus, and MOP mechanisms rather than DOP or KOP. Due to the number of samples and the necessity of time, it was decided that only one region would be examined. The hippocampus was examined over other regions due to the changes found as a result of stress exposure in the model in Chapter 3, with changes seen in both the KOP and DOP systems. The hippocampus is a region that is involved in the retention of learning and memory, and the processing of emotions, and has been shown to be reduced in volume in patients with depression (Videbech and Ravnkilde, 2004). Given that all three opioid receptors are co-expressed in the hippocampus and have been shown to modulate neurogenesis and BDNF in the hippocampus (Lutz and Kieffer, 2013), the hippocampus seemed the appropriate first-choice for examination. Nevertheless, it must be said that in hindsight a limitation of this Chapter is that the prefrontal cortex was not examined. Indeed in Chapter 4, the KOP and DOP systems in the prefrontal cortex were both altered as a result of the OB model and exposure to the acute swim stress, and it must be stated that this was to an even greater extent than in the hippocampus. The prefrontal cortex is a region in the brain that is associated with executive function, as well as social processing (Billeke and Aboitiz, 2013). The medial prefrontal cortex is responsible for social attribution and perception, with the orbitalfrontal cortex responsible for social reward and motivational behaviour, and the dorsolateral prefrontal cortex involved in the processing of social behavioural adaptations (Billeke and Aboitiz, 2013). Taking all of this into account, the prefrontal cortex is involved in the complex processing of social behaviour and responding, and as such, would have been a plausible region to examine in these experiments as a consequence of the social behaviour investigated in the 3-chamber sociability test. Therefore, in hindsight it must be said that the prefrontal cortex would have been the more appropriate choice for examination in this Chapter

and it is this researcher's recommendation that future studies that examine the opioid system after OB removal focus on post-mortem analysis in this region.

In conclusion, in this study opioid modulating drugs were examined in a number of behavioural tests to assess their potential to act as antidepressant-like in function, and lessen any depressive-like, anxiety-like and cognitive dysfunctional behaviours in the OB rat model. A number of drug effects were seen, but most importantly, none of these opioid compounds were found to attenuate any behaviour phenotypes in the OB model. Similar to the antidepressants used in Chapter 3, many drugs actually heightened or worsened pre-existing behavioural deficits in the model. Many drugs that were given in combination, often appeared to have increased or more potent effects as opposed to when given alone, perhaps positing a synergistic effect (Huang et al., 2016). Overall, no opioid receptor or peptide mRNA differences were found in either study, regardless of group or drug. Although a number of the behavioural features of the OB rat model previously seen to be significant in Chapter 3 were not seen as significant in this chapter, it must be noted that a strong trend for a number of these behavioural phenotypes in this model still existed. The depth and pattern of the social cognitive deficit seen in the OB rat is pronounced, and perhaps a deficit this deep in magnitude, which has not been attenuated with chronic administration with antidepressants, an acetylcholinesterase inhibitor, or opioid modulating drugs, is in need of a different type of therapy completely in order for cognitive functioning to be retrieved. The selectivity of opioid compounds is pivotal to understanding the effects that they might have in a mood disorder such as depression. The non-selective or dual affinities of many of the drugs used, with compounds having high affinity for one receptor and low affinity or shared affinity for others, adds a level of neurochemical functioning that may need to be more extensively assessed in naïve animals, before it gets to the stage of models of mood disorder. Of the drug effects that were seen, these were mainly significant in the OB rat and not as often in the sham-operated counterparts, highlighting that different mechanisms are at play in this model of depression. Whether these effects in the model are exclusively to the opioid system, or are as a result of a secondary or subsequent pathway that is altered or changed, will need further investigation.

Chapter 6:

General Discussion

6.1 Discussion

The central opioid system has been shown to be altered in patients with MDD, such that utilisation of opioid strategies to treat depression predates the conventionally derived monoaminergic therapies that are most commonly used today. Due to the standstill in progression of therapies to treat depressive disorders, with particular respect to pharmacological strategies, the opioid system has attracted renewed interest, particularly due to the development of selective targeting and agents for this system. The central opioid system is densely populated throughout regions of the brain that are involved in the regulation of emotion, stress, social functioning and cognition (Brown and Lucki et al., 2019, Le Merrer et al., 2009, Peciña et al., 2019), and has been shown to be co-localised with the monoaminergic system (Lutz and Kieffer, 2013). As such, the opioid system presents itself as a plausible candidate for the treatment of mood disorders. In order to assess whether targeting the opioid system for the treatment of psychiatric disorders such as depression is a viable option, preclinical research must first be undertaken.

As GPCRs, the opioid receptors and their peptides have the ability to be involved in a lot of 'cross-talk', and as such can affect many downstream pathways and mechanisms in both the peripheral and central nervous systems. This can present difficulties, as the receptor system that is targeted can cause differential effects; with activation of some receptors causing negative effects to mood and others causing up-regulation of mood. Modulation of the central opioid system in rodents and its effects on cognition, social functioning, stress regulation and 'depressive-like' behaviours, provides an opportunity to understand the mechanisms involved in opioid circuitry, in addition to elucidating which subsystems to target in order to alleviate the symptoms of psychiatric illness. However, it is most important that the rodent model or paradigm that is chosen for use has strong face, construct and predictive validity, and that this model can be replicated across different laboratories, with behavioural phenotypes that can be readily reproduced. This is a feature of preclinical research that is receiving much criticism in recent years as many animal models that have been developed, particularly for psychiatric illness, fail to fully tick all of the domains of disease pathology and symptomatology. It must be restated that challenges are met when trying to model psychiatric illnesses in animals, particularly with how closely they can resemble the clinical condition, with many models only implementing some facets to these disorders, and struggling to employ all. Nevertheless, the OB rat model is an

animal model that exhibits adaptations in endocrine, physiological, behavioural and neuroinflammatory functioning, with these changes resembling the alterations that are seen in these systems in depression (Kelly et al., 1997). As such, this paradigm is often employed as a tool used to investigate the efficacy of novel compounds and/or interventions. Bearing this in mind, this model has begun to be used to evaluate antidepressant potential in modulators of the opioid system (Saitoh et al., 2008, Gotoh et al., 2017, Takahashi et al., 2018). Therefore, the overall aim of this project was to add to this research being conducted, by examining the role of the central opioid system in the OB rat model, with a focus on social cognition, a feature of depression that has not been examined in this model before.

Firstly, it must be noted that an appropriate method by which to validate the use of an animal model or intervention, is to undertake a systematic review and meta-analysis of the model in question. It was noted in the literature that such an approach in the OB rat model had never been employed, and would add to the knowledge and validation of its use as a model in preclinical science. The systematic review and meta-analysis of the literature spanning the 20 years between 1999 and 2018 yielded 133 primary papers that used the model for evaluating antidepressant properties. The results from the systematic review indicated that the OB rat model has been most explored in male Albino rats and that singly-housing the rats is the most traditional form of housing used. The majority of papers examined were shown to conduct and report thorough experimental designs, however, a number of papers failed to be as transparent, with a full lack of disclosure seen with regards the housing of rats after surgery; an important feature when examining social functioning. The meta-analysis revealed that multiple behavioural tests have been investigated in the OB rat model and that the behavioural responses in a number of these paradigms displayed robustly reproducible results across a number of different laboratories, with particular respect to the OF test. In contrast, the behavioural responses of OB rats in the EPM test, a test used to explore anxiety-like behaviour, were found to be much less consistent, indicating that the behaviour between sham-operated and OB rats did not reliably differ. Interestingly, this is a result in OB rats in the EPM that was also seen in this project in both Chapter 3 and Chapter 5, with different behavioural effects being seen across experiments and in this case appearing to be dependent upon housing regimes. Overall, the multiple meta-analyses performed revealed that the OB rat displays a number of behavioural deficits that can be reliably reproduced. These include

psychomotor retardation, cognitive dysfunction, learned helplessness, abnormal emotional regulation and deficits in social functioning, all deficits that mirror symptomatic traits observed in patients with MDD. As such, the investigation and scientific tools utilised in Chapter 2, highlight the validity of the OB rat as an animal model in preclinical depression research, and also highlight the importance and relevance of conducting a meta-analysis in preclinical research.

Moving on, Chapter 3-5 focused on behavioural attributes in the OB rat and changes to the opioid system in this model, as a result of exposure to stress, and/or chronic pharmacological manipulation. Table 6.1 depicts the behavioural effects that were seen in OB rats in each experiment in this project, with Table 6.2 depicting the changes that were seen in the opioid system in OB rats when compared to sham-operated rats throughout the course of the project. These will now be discussed in detail in order of the main findings for the behavioural results, molecular results, with the implications of pharmacological modulation being discussed last.

With regards to behaviour, the most striking finding was the behavioural deficit in social cognitive functioning displayed in the OB rat, through the introduction of a new behavioural investigation in the form of the 3-chamber sociability test. Throughout this project, the OB rat was observed to display a reduction in the motivation to interact with a social stimulus, in addition to having a reduced preference for a novel social interaction upon presentation of a second novel animal. In addition to this, the OB rat, regardless of pharmacological manipulation, was shown to habituate to this test arena over time, with reduced movement upon each subsequent trial when compared to sham-operated counterparts. OB rats display a reduced preference for the novel animal in the social preference trial when compared to sham-operated rats, spending around the same amount of time with both the familiar and second novel conspecific animals. Whether this behavioural effect can be regarded as a reduction in motivation to interact or exhibits a loss in cognitive functioning and memory, is difficult to definitely say but it is evident that a deficit in social cognitive functioning is present after OB removal. Given that this feature was seen across the OB rat in several experiments, it presents itself as an attribute that is deeply engrained in these animals and cannot be regarded as solely a loss in olfactory functioning. It must be remembered that these animals can also use their others senses to process the social stimulus in front of them, such as visual and auditory cues (Crawley, 2004, Moy et al., 2004). These characteristics are also critical for the animals social and cognitive

functioning and again show that this deficit in theses animals cannot be regarded as solely a loss in olfactory functioning alone. Nevertheless, in order to replicate these findings and fully accertain the mechanism of this social cognitive deficit, future studies could potentially employ a positive control to reaffirm this apparent adaptation in social memory functioning in OB animals. As discussed previously, $ZnSO_4$ is a chemical used to destroy the olfactory epithelium and cause a loss in olfactory functioning (Mayer and Rosenblatt, 1993, McBride et al., 2003, Thor et al., 1976, van Riezen et al., 1977). Indeed, animals treated with $ZnSO_4$, have been shown to exhibit a loss in olfactory functioning but do not act in the same manner that OB animals do, particularly with regards cognitive functioning as has been shown in the PA test (Borre et al., 2014, van Riezen et al., 1977), T-Maze (Borre et al., 2014) and MWM test (van Rijzingen et al., 1995). These findings shows that memory functioning is changed after OB surgery, and that this is due to a more complex reorganisation of the regions with the brain, than as a result of olfactory ablation (Kelly et al., 1997). Although social memory has not been examined across these two types of models, examiniation of $ZnSO_4$ animals along with OB animals in future studies could potentially facilitate and help to further explain this social cognitive adaption in the OB model.

Behavioural Parameters	**Chapter 3**				**Chapter 4**	**Chapter 5**	
	Experiment 1			*Experiment 2*		*Experiment 1*	*Experiment 2*
Housing	*Single*	*Paired (same)*	*Paired (different)*	*Single*	*Paired (different)*	*Paired (different)*	*Paired (different)*
Bodyweight Gain	–	↓ OB (2 and 5 week)	–		–	–	–
Open Field Test – 2 Weeks							
Distance Moved	↑ OB	–	↑ OB				
Time in Centre Zone	–	–	–				
Open Field Test – 5 Weeks							
Distance Moved	–	–	–			–	–
Time in Centre Zone	TREND ↓ OB	↓ OB	TREND ↓ OB			TREND ↓ OB	↓ OB
Elevated Plus Maze							
Distance Moved	↑ OB	↑ OB	–			–	–
OA Duration	–	–	–			–	–
OA Entries	↑ OB	↑ OB	–			–	–
3 Chamber Sociability Test							
DM in Habituation	–	–	–	–		–	–
DM in Sociability	–	–	–	–		–	–
DM in Social Preference	–	↓ OB	↓ OB	–		–	–
% Habituation	↓ OB	↓ OB	↓ OB	↓ OB		TREND ↓ OB	↓ OB
Sociability – Novel Animal	↓ OB	↓ OB	–	↓ OB		–	TREND ↓ OB
Social Preference – Novel Animal	↓ OB	↓ OB	↓ OB	–		TREND ↓ OB	TREND ↓ OB
Forced Swim Stress					–		

Table 6.1 Behavioural effects in OB rats in each experiment conducted in this project. The results are divided by chapter, experiment number and condition of housing. Changes to behaviour in the form of bodyweight gain, OF testing, EPM testing, 3-chamber sociability testing, and exposure to an acute forced swim stress are summarised for OB rats by a reduction or increase in activity. The shaded boxes indicate that the behavioural test was not examined in this experiment/chapter. DM=distance moved, EPM=elevated plus maze, OA=open arm, OB=olfactory bulbectomy, OF=open field, ↓=decrease, ↑=increase, – = no difference from sham–counterparts.

Molecular Parameters	**Chapter 3**		**Chapter 4**		**Chapter 5**	
	Experiment 1		*Experiment 2*		*Experiment 1*	*Experiment 2*
Housing	*Single*	*Paired (different)*	*Paired (different)*		*Paired (different)*	*Paired (different)*
			OB Surgery	*OB Surgery and Swim*		
Hippocampus						
MOP System	–	–	–	–	–	–
KOP System	–	–	KOP ↓ OB	KOP ↓ OB	–	*KOP Drug effect*
DOP System	–	–	PENK ↓ OB	–	–	*DOP Drug effect*
Prefrontal Cortex						
MOP System			POMC ↑ OB	–		
KOP System			KOP ↓ OB	PDYN ↑ OB		
DOP System			DOP ↓ OB and PENK ↓ OB	DOP ↓ OB and PENK ↓ OB		
Amygdala						
MOP System	MOP ↑ OB	–	–	–		
KOP System	KOP ↑ OB	–	–	–		
DOP System	*DOP Surgery effect*	–	–	–		
Hypothalamus						
MOP System			*MOP Surgery x Swim effect*	–		
KOP System			–	–		
DOP System			*DOP Surgery x Swim effect*	–		
Nucleus Accumbens						
MOP System	–	–				
KOP System	–	–				
DOP System	–	–				

Table 6.2 Changes to opioid receptor and pre-propeptide mRNA expression in OB rats in each experiment conducted in this project. The results are divided by chapter, experiment number and condition of housing. Changes to mRNA expression is described as a reduction or increase in expression per region per receptor system. Surgery, drug, and surgery x swim effects are also shown. The shaded boxes indicate that the region/gene was not examined in this experiment/chapter. DOP=delta opioid receptor, KOP=kappa opioid receptor, MOP=mu opioid receptor, OB=olfactory bulbectomy, PDYN=prodynorphin, PENK=preproenkephalin, POMC=proopiomelanocortin ↓=decrease, ↑=increase, – = no difference from sham–counterparts.

As discussed previously, OB animals have been proven to have a much more extensive remodelling of the brain following lesioning, with neurochemical restructuring of the brain and behavioural deficits observed that are not present when compared to animals that have had their olfactory epithelium destroyed, i.e. $ZnSO_4$ animals (Borre et al., 2014, van Riezen et al., 1977, van Rijzingen et al., 1995). In conjunction with this, although olfaction is indeed a primary sense for the rodent, it must not be forgotten that in this behavioural paradigm these animals can still receive auditory and visual sensory cues from the conspecifics surrounding them (Moy et al., 2004). The 3-chamber sociability test has value over other social tests such as the social interaction test, in that it allows animals to interact with two novel conspecifics rather than just one conspecific, and this is performed in a consecutive manner, allowing for the added assessment of memory functioning. In this manner, the 3-chamber sociability test provides a more elaborate method for assessing two of the features of functioning that are affected in MDD; social functioning and cognition, moving forward from some of the older cognitive behavioural paradigms that solely examine cognition and that are less examined today, i.e. PA test. The social cognitive dysfunction observed in the OB rat in this project provides an additional behavioural feature to the OB rat model, introducing another symptom and facet of behaviour that is seen to be altered in patients with MDD (Knight and Baune, 2019, Weightman et al., 2014).

Having said all this, the positives of providing a new behavioural paradigm in the form of the 3-chamber sociability test did not come without its consequences as was demonstrated with the behaviour of our OB rats in the OF test. As discussed previously, the OF test is by far the most extensively established behavioural test that the OB model has been assessed in, with a characteristic hyperactivity seen in OB animals that is attenuated with chronic but not acute antidepressant therapy (Kelly et al., 1997). As such, this behavioural test and response in OB animals has become an iconic ‘gold standard’ of use in the OB model. This was also demonstrated with the volume of papers that assessed this behavioural response in the OB rat model in the meta-analysis performed in Chapter 2, which indicated a robust behavioural response of increased locomotor activity in these syndrome animals. That being said, this behavioural feature of the OB rat was seen to be variable throughout this project, dependent upon three experimental features; housing, repeated exposure, and pre-exposure to other behavioural tests. The details and impact of these factors have been discussed in detail in the discussion of each Chapter. To summarise the main findings

with regards to this topic in this project, the hyperactive profile in the OB rat could be regarded as a profile that is fragile to signalling; thus indicating that this behavioural phenotype in the model is sensitive or vulnerable to modification through environmental manipulations. This is a feature of the OB model that has been found by other researchers (Gigliucci et al., 2014, Holubova et al., 2016), and is also a finding that we have found previously in our laboratory (Hayley, unpublished). In Chapter 5, it was acknowledged upon implementation of the experimental design that pre-exposure to the 3-chamber sociability test may affect the behaviour of OB rats on subsequent exposure to the OF but this decision was made in order to focus on the social cognitive feature of this project. As a result, less of a focus was placed upon hyperactivity and the OF test, with more emphasis placed upon social cognition and the 3-chamber sociability test, as the aim was to explore a behaviour that had more relevance to MDD. In conclusion, the 'gold standard' of hyperactivity in the OF arena in OB rats was not consistently displayed in this project, given the subtle manipulations within the experiments and the added reasons outlined above, nonetheless, this is a limitation of the experimental design and work presented.

With regards to molecular changes, it was established in the thesis that there was a role for the central opioid system in the OB rat model, but that this was dependent upon context and situation. For example, the majority of changes to the central opioid system in OB rats were seen in the swim stress study in Chapter 4, where animals also had limited handling and no exposure to injection stress, in comparison to Chapters 3 and 5. The opioid system has been shown to be altered in patients with depression; with low opioid receptor availability in depressed patient's *in vivo* and low levels of endorphins in the brains of depressed patient's post-mortem (Bernstein et al., 2002, Hsu et al., 2015, Kennedy et al., 2006, Prossin et al., 2011; 2016, Scarone et al., 1990). The primary method used to examine opioid receptor and pre-propeptide changes was qRT-PCR. In this project, we showed that OB rats that had undergone singly-housing and a number of stress-induced behavioural test exposures showed increases in MOP and KOP mRNA expression, with a surgery effect also seen in DOP mRNA expression (although no differences in the groups were shown upon *post-hoc* analysis). In Chapter 3, these effects were not seen when an OB rat was paired with a sham. Interestingly, in Chapter 4, OB rats that were paired with shams were shown to have changes to both the DOP and KOP systems in the hippocampus and prefrontal cortex, as a result of the surgery alone, and in conjunction with exposure to an acute

swim stress. Surgery x swim effects were also seen in the MOP and DOP systems in the hypothalamus (although there were no differences in groups upon *post-hoc* analysis). The findings in Chapter 4 indicate that the opioid system in the OB rat is altered upon surgery alone, and is further altered as a result of housing and of acute stress exposure. No differences in opioid mRNA expression in the hippocampus were found after chronic administration with opioid modulating compounds in Chapter 5. Due to the confines of time, only one region could be examined. To conclude, the neurochemical changes seen in the OB rat in this project showed that the opioid system is altered as a result of OB ablation, and further consequences occur to this system in this model upon further stress-induced exploitation. However, it also appeared that activation of the opioid system has a great extent of lability in the OB rat; as it does not take much for an activation or change in the system to be easily lost upon manipulation of the situation or environment.

Lastly, the pharmacological manipulations will be discussed. The main aim of the pharmacological manipulation in this project was to assess whether opioid modulating drugs would attenuate any 'depressive-like' or 'anxiety-like' behaviours that were seen in the OB rat model. The discovery of the social cognitive dysfunction displayed by OB rats in the 3-chamber sociability test added another promising and clinically relevant feature. Chronic administration with two different classes of conventional antidepressants; a TCA and SSRI, or donepezil, an acetylcholinesterase inhibitor used to treat the cognitive decline that is seen in neurodegenerative disorders such as Alzheimer's disease, all failed to attenuate this deficit in OB rats. In fact, some of the social cognitive deficits in the OB rat were actually shown to be worsened with these drugs, surprisingly indicating that they appeared to be more detrimental. Chronic administration of opioid modulating drugs that target the three receptor systems as either agonists or antagonists, alone and in combination, in a manner that reflected the antidepressant-like properties of opioid compounds in previous preclinical research were assessed (Brown and Lucki, 2019, Lutz and Kieffer, 2013). However, regardless of which system was targeted, chronic administration with opioid modulating compounds failed to restore the social cognitive function in OB rats. To summarise, no matter which pharmacological mechanism was targeted in this project, the social cognitive dysfunction in OB rats was refractory to normalisation by chronic dosing with any of the drugs used, representing an additional behavioural phenotype in the

model, which represents a dysfunctional characteristic of MDD, and which may need other strategies to be rescued.

Chronic administration with opioid modulating drugs caused differential effects to locomotor functioning. Partial MOP agonists, given alone or in combination with KOP antagonists were generally shown to increase locomotor behaviour in OB rats, dependent upon the context of the environment that the activity was assessed in. The regulation of the dopaminergic system by MOP and KOP receptors, with increases in DA neurotransmission having been previously shown as a result of activating MOP (Smith et al., 2019) and blocking KOP mechanisms (Mague et al., 2003), causing increases in locomotor activity and as such may explain and account for these effects. However, it must be noted that these studies use acute administration whereas the administration in the experiments in this project were chronic. The partial MOP agonist RDC 2944 given alone, in addition to the KOP agonist U50,488, were shown to decrease anxiety-like behaviour in the EPM in OB rats. This was surprising as KOP agonists have been shown to induce dysphoria in animals (Lutz and Kieffer, 2013). Given that no effects of dysphoria were shown in our sham animals in the EPM, the KOP system in the CNS of the OB rat may be modified in some way. By far the most prominent attribute of Chapter 5, was the fact that any of the opioid modulating drugs effects that were seen were only evident in OB animals. However, ascertaining the direct mechanisms by which these interactions occur in these animals is much more complex and deserves further investigation.

Chronic administration was chosen as the method of administration in this project, as it more appropriately represents the repeated administration that is received by patients in the clinic. Although acute administration of opioid modulating compounds has presented a vast array of findings that depict antidepressant-like properties, there is a lack of research on the effects of chronic administration with these compounds. Given the current opioid epidemic, tolerance of these compounds and their addictive potential are important facets that must be addressed in preclinical research if opioid modulating drugs are ever to reach and pass phase IV trials. Relatively low doses of these compounds were chosen in order to assess their tolerance over time and in general, the drugs and doses that were chosen were well-tolerated by all rats with any negative effects to bodyweight being transient in manner. As these particular doses did not have any major effects on behavioural deficits in OB animals,

subsequent investigations could employ slightly higher doses that have been shown not to be detrimental when given acutely or subacutely.

6.2 Conclusion

In conclusion, the overall aim of this thesis was to examine the role of the central opioid system in the OB rat model, with particular emphasis placed upon social cognitive functioning. The novelty of this project was the discovery of a new behavioural deficit in social cognitive dysfunction in the OB rat, which was demonstrated upon exposure to the 3-chamber sociability test. This behavioural adaptation in the OB rat adds another feature to the model, one which replicates the disruptions to social cognitive functioning that is seen in MDD. This alteration in social cognitive processing in these bulbectomised animals could not be attenuated with chronic treatment of two classes of conventional antidepressants, an acetylcholinesterase inhibitor, or by a range of opioid modulating compounds that acted at all three opioid receptor systems. As a result, the deficit exhibited by OB rats in social cognitive function may need a different pharmacological modification in order for it to be rescued, or indeed it may be a deeply rooted phenotype that is refractory to normalisation and that may need more consideration before another method of resolution is applied or attempted. Emphasis was placed upon chronic administration rather than acute administration as this method is much less explored (particularly with opioid modulation) and more appropriately resembles the timecourse of treatment in MDD. Although a number of behavioural effects were seen after administration of opioid modulating compounds, chronic administration with these compounds failed to rectify the majority of behavioural phenotypes in the OB rat. In addition to this, although administered chronically, the selection of low doses used may have been too small to alter behavioural phenotypes. Nevertheless, the central opioid system itself was shown to be altered in the OB rat, particularly after exposure to swim stress, in regions such as the hippocampus and prefrontal cortex, and after exposure to minimal housing and handling, in regions such as the amygdala, highlighting another neurochemical system and feature that is shown to be altered in MDD. To finalise, the work in this thesis has added to the body of knowledge for the role of the central opioid system in the OB rat model. Although only modest changes to the opioid system were seen in this model, the opioid system as a target for

antidepressant-like potential, and/or as a target for treatment for psychiatric illness, should continue to be investigated.

6.3 Limitations and future recommendations

There were a number of limitations to the behavioural, molecular and pharmacological interventions in this thesis and so there are a number of recommendations that can be made for future research on the role of the opioid system in the OB rat model. These limitations and recommendations will now be discussed.

A limitation of the work presented was the experimental design as upon subsequent experiments in Chapter 5 classic behavioural phenotypes of the OB rat were not evident, such as hyperactivity in the OF. Future work could consider monitoring homecage locomotor activity with software that allows for two animals to be easily tracked. The current software used in this lab, unfortunately, did not allow for assessment of this additional locomotor parameter in these experiments, but this would also allow for the assessment of the locomotor profile over time.

Another suggestion would be to use a separate cohort of animals that are just examined in the OF test. In this manner, activity in the OF could be evaluated and the hyperactive profile in this test ascertained, without animals having undergone prior exposure to the 3-chamber sociability test. Nevertheless, this would endeavour the use of a much larger number of animals, from an experiment that is already large in scale, and so may have ethical implications.

Although there was no change to opioid mRNA or peptide expression in regions on a consistent basis, not all receptor and pre-propeptides were explored consistently throughout the five regions examined during the course of this project, and indeed this is a drawback of the work presented. As a result, future work could fill in these gaps in this project and measure the pre-propeptide and receptor mRNA expression in any of these five regions that they were not previously examined in, for example pre-propeptide expression in the nucleus accumbens. Lastly with to regards neurochemical endpoints, methods such as immunohistochemistry or autoradiography could be used to examine downstream signalling mechanisms, as using additional techniques such as these were beyond the scope of this project. In addition, perhaps earlier timepoints for sacrifice and therefore neurochemical processing could be employed in future work in order to ascertain if changes may have occurred earlier in the model, which disappeared over time.

With regards to the opioid modulating compounds employed, future recommendations could include employing higher doses. As mentioned in the discussion, lower doses were utilised in this project as we wanted to make sure that these drugs were well-tolerated by the animals, as chronic dosing with the majority of these drugs was a novel strategy being employed. Due to the lack of attenuating effect seen in many compounds in this model, perhaps higher doses may need to be used in order for an effect to be observed. A limitation of the work presented is that due to the large number of compounds trialled, only one dose of each compound could be chosen, therefore future experiments could employ a dose response paradigm.

The only opioid receptor compound not employed was a delta antagonist. Upon literature review, delta antagonists have been less explored for their antidepressant-like properties due to the substantial evidence for the effects seen with delta agonists. Nevertheless, chronic administration with a delta antagonist could be employed in future work to complete the opioid agonist/antagonist profile examined in this project.

It was beyond the scope of this project to examine the opioid receptor and pre-propeptide mRNA expression in more than one region in the two experiments in Chapter 5 due to the volume of samples that needed to be analysed. The hippocampus was chosen because of the alterations that were seen in opioid receptor and pre-propeptide mRNA expression in Chapter 4. Future work could have employed analysis of the prefrontal cortex, the other region that was shown to be affected in Chapter 4, to see if chronic treatment with opioid compounds have changed the levels of expression in this region.

Chapter 7:

Appendix

7.1 Appendix A

LABORATORY ANIMALS
STANDARD OPERATING PROCEDURES
TITLE: Aseptic Surgery: Guiding Principles and Protocols

SOP No: GR-VC-009.01

Author: Tatiana Doroshenkova	**Date: 24/01/2018**
Reviewed by AWB Date: N/A	**Effective from:**
Approved by the BRU Director: Yolanda Garcia	
Review Dates:	

OBJECTIVES:
This document provides the information, guidance and protocols for the performance of surgical procedures under aseptic conditions. It is an essential guide to aseptic technique for both, new and experienced authorised personnel who intend to undertake surgical procedures under the S.I. No 543 of 2012 as amended. This document does not address pre-, peri- and post- procedural care, anaesthesia and/or analgesia; which are normally associated to the principles of good aseptic technique. This document applies to the procedures associated to the recovery of experimental animals that should survive more than 6 hours post surgically. The main aims of any surgical procedure are that it is carried out skilfully with the minimum risk of acquiring infections, of tissue damage and minimum disturbance to the animal's homeostasis while producing high quality scientific output.

SCOPE:
All personnel dealing directly with experimental animals undergoing aseptic surgery in the NUIG-BRU must read, understand and adhere to the practices and procedures described in this SOP. And to this effect all users involved must sign this SOP, Appendix I, before implementing any of the protocols/practices set out in it. Furthermore, before signing, special attention should be paid to the risk assessment of this task. If for any specific reason this assessment does not match the users individual needs/condition, the user should alert the Unit's ACWO or the BRU Director before engaging in the task or being allowed to perform it. Once the agreement in Appendix I is signed, it is understood that the user will adhere to the contents of this SOP in full.

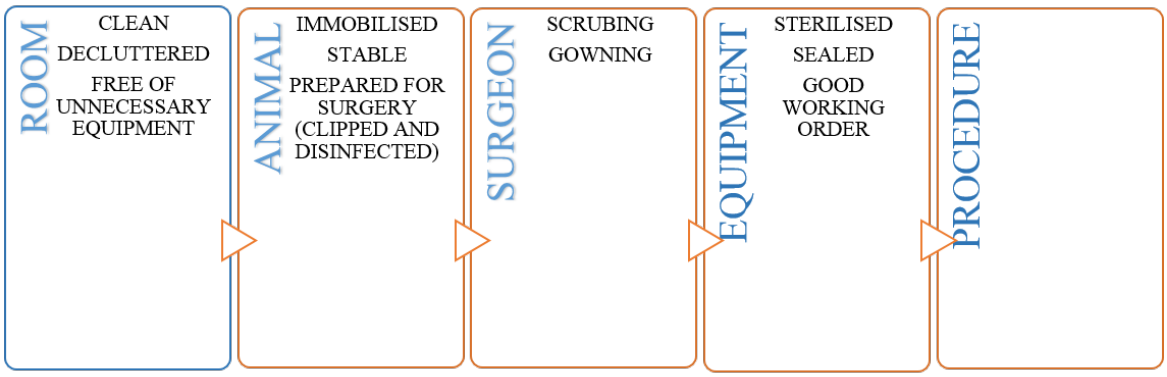
BACKGROUND:
What is asepsis? By definition, asepsis is the absence of bacteria, viruses or other microorganisms that are harmful contaminants for a subject undergoing surgery. Therefore, aseptic technique compiles all the practices and procedures applied in surgical setups to avoid this contamination by using different sterilisation methods. Aseptic technique is essential when undertaking recovery surgery on any lab animal species. Infection frequently leads to wound breakdown, pain and delayed healing and recovery, while compromising the reliability of the scientific data gathered under non-aseptic conditions. There is extensive published evidence that subclinical infections can become clinical diseases following stress or following co-infections when asepsis

is ignored. Even when infections are not clinically evident, they affect the animal's homeostasis (blood cell counts, plasma fibrinogen, hormone levels, glucose in serum), causing profound effects on the physiology, behaviour, immune system and response to therapies. The misconception that lab rodents are resistant to infection does not help the development and implementation of basic aseptic techniques and therefore robust science. However, the lack of reproducibility and reliability of the published data with the associated waste in time, money and resources should encourage any researcher in this arena to implement refined approaches ergo aseptic technique when breaching the dermal barrier. To this effect the Competent Authority, the HPRA, has provided the guidelines in one of their updates as the standards to follow providing also the background information for this SOP. Forward planning and attention to detail are integral parts of all surgical procedures. Every step in the process should be carefully considered, from the checking of authorised procedures and the conditions, under which those were approved, to the preparation/examination of instruments, consumables, facilities, the surgeon and the animal.

PRE-REQUISITES

1. Generate your own check-list of consumables (sutures/medicines/equipment) required and check that they are within date for this particular procedure.
2. Ensure that all equipment required is serviced, in good working order and available for use.
3. Consider extra requirements for things that could go wrong while performing the procedure. Be ready with a plan B.
4. Check that the sterilisation process (e.g. autoclaving, irradiation or other suitable method) has been effective, by checking the indicator/mark on packaged instruments, that they are within date and that the outer packaging is intact.
5. Ensure that sufficient support staff is available for both surgery and peri/postoperative care (animal care, veterinary staff, anaesthesiologist and scientists).
6. Animal should be examined the day before and cleared for the surgery the day after.

ASEPTIC TECHNIQUE PREPARATIONS FLOW CHART



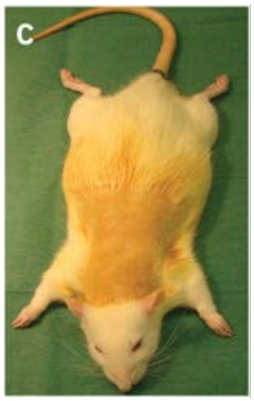
ROOM PREPARATION

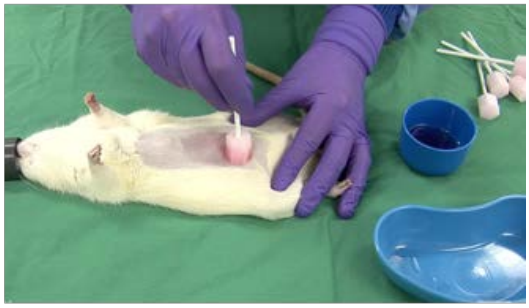
1. Ensure that the area acting as the operating theatre is clean and ready for use.
2. Ensure only the necessary equipment is in the area and additional clutter has been removed.
3. Use good disinfecting protocol of surfaces (detergent for organic material and chlorinated disinfection) where surgery will take place.

4. Avoid using the surgical area as a storage. Shelving and sharp corners (furniture-floor and wall floor) do not allow effective/thorough cleaning and disinfection.
5. After completing surgery make sure that the room/area in which you have been working is left clean and tidy.

ANIMAL PREPARATION

1. Animals should be prepared for surgery in a room adjacent to the surgery to avoid contamination of the surgical area with air-borne hair, dust and dander or even bedding particles.
2. Regarding anaesthesia please see RD/SP/010 and RD/SP/011 for further reference.
3. **Hair removal should be done outside the operating theatre, or in an area from which airborne particulates cannot escape** (hood/negative ventilated room). User should wear suitable masks to prevent lab animal allergies.
4. Detailed instruction on how to prepare small rodents for surgery can be found in this link:**http://www.procedureswithcare.org.uk/aseptic-technique-in-rodent-surgery-tutorial/aseptic-technique-in-rodent-surgery-preparation-of-the-animal/**
5. Following the induction of anaesthesia, sufficient hair must be removed to frame the surgical site. Allow adequate skin preparation to prevent hair ingress into the incision during surgery and facilitate wound healing.
6. The clipped area depends on the incision size required and the size of the animal. This should be kept to a minimum to reduce the risk of hypothermia, see image C above.
7. Hair removal should be done with care because inappropriate preparation of the incision area can make the skin more susceptible to infection through disturbance to the delicate skin microfloral ecosystem, grazes and other skin surface damage. For this reason careful shaving must be done to maintain the skin intact.
8. If epilation cream is to be used, it needs to be tested beforehand as some can be quite aggressive and induce local oedema.
9. Preliminary skin preparation should also be done in an ante-room (prep-room) or an area such as a containment cabinet and not in the surgical theatre/area.
10. The skin must be cleaned and then prepared with a suitable topical solution (e.g. diluted chlorhexidine or povidone-iodine). These solutions should be used sparingly to avoid soaking the animal and focused on the surgical area as this could affect thermoregulation.
11. Where possible, solutions should be pre-warmed to body temperature. This pre-treatment increases their effectiveness and reduces the risk of causing hypothermia. The use of warm solutions is especially important in small animals, such as rodents.





SURGEON PREPARATION

SCRUBBING:

1. All hair should be covered; a suitable cap should be used to cover the hair on the head (this should be applied first).
2. The mask is applied before scrubbing, gowning and gloving. Bearded individuals should use masks which are large enough to cover their facial hair. A face mask is worn primarily to protect the individual from allergy and, for some species, the animal from catching contaminants from the individual. Immunocompromised animals may be particularly susceptible to infection via this route.
3. Locate scrub brushes, antimicrobial soap, nail cleaners.
4. The surgeon must remove all jewellery from hands and wrists and perform a thorough scrub of hands and nails. Fingernails should be cut short before starting to "scrub up" for surgery. Please see this video for further instructions: **https://www.atdove.org/video/scrubbing**
5. Turn on the water and adjust the flow so that the water is warm. Warm water removes less of the protective oil of the skin than hot water.
6. Open the disposable brush with antimicrobial soap, lay the brush on the back of the scrub sink. If no disposable brush is available; used autoclaved brushes with dispenser of disinfectant soap that can be operated with your elbows.
7. Wet hands and arms for an initial pre-scrub wash. Use 2-3 pumps of surgical detergent. Typically, a commercial iodine or chlorhexidine - containing skin disinfectant, such as Betadine® or Hibiscrub®, is used.
8. Work the soap on the fingertips and clean the spaces under the fingernails and subungual areas of both hands under running water with a file. Once finished, discard the file.
9. Rinse the hands and arms to a point about two inches above the elbow. Do not retrace or shake the hands and arms; let the water drip from them. (Rationale: Movement of water and dirt will flow from hands to less clean areas thus preventing contamination of hands during scrubbing).
10. Apply 2-3 pump of surgical detergent to hand and arm.
11. Take the sterile brush, moisten it and add 2-3 pumps of surgical detergent.
12. When scrubbing, bend slightly forward, holding the hands and the arms above the elbow, and keeping the arms away from the body to minimise the risk of contact with contaminated surfaces.
13. **A 5- to 7-minute scrub for the first case of the day, followed by a 2- to 3-minute scrub between subsequent operations, is generally adequate.**

Anatomic Timed Method	**Counted Brush Stroke Method**
Note starting time; scrub each side of each finger (4 sides), between fingers and back and front of the hand for 2 minutes	Apply 30 strokes (one stroke consists of up and down or back and front motion) to the very tip of your fingers and thumb.
Proceed to scrub the arms, keeping the hands higher than the arm.	Divide each finger and thumb into four parts and apply 20 strokes to each of the four surfaces, including the finger webs.
Scrub each side of the arm (4 sides) to 8cm above the elbow for 1 minute.	Scrub from the tip of the finger to the wrist when scrubbing the thumb, index and small fingers.
Total scrub time is 2-3 minutes per hand and arm.	Divide your forearm into four planes and apply 20 strokes to each surface.

14. Rinse the scrub brush well under the running water, and transfer the brush to your scrubbed hand. Do not rinse the scrubbed hand and arm at this time.
15. Repeat the process on your other hand and arm.
16. Ehen both hands and arms have been scrubbed; drop the scrub brush in the sink.
17. Starting with the fingertips of one hand, rinse under water by moving your fingertips up and out of the water stream and allowing the rest of your arm to be rinsed off on the way out of the stream.
18. Always allow the water to run from your fingertips to your elbows.
19. Never allow your fingertips to come below the level of your elbows.
20. Never shake your hands to get rid of excess water; allow the water to drip from your elbows.
21. Rinse your hands similarly.
22. Hold your hands upright and in front of you so they can be seen and proceed to the gowning and gloving area.
23. Rinse thoroughly and dry. For other products, follow the ‘’manufacturers‟ instructions for volumes and contact time. Hands should be dried using a sterile paper towel starting with the hands and finishing with the elbows.
24. Alternatively, instead of an aqueous scrub, an alcohol rub containing additional active ingredients (e.g. chlorhexidine) could be used, as a recent report has demonstrated that this produces an equivalent level of antisepsis.

GOWNING:

1. A sterile, clean, long-sleeved operating gown should be worn during surgery.
2. The gown should be put on by the “scrubbed’’ surgeon and the gown ties passed carefully to an assistant to tie it at the back.
3. An assistant will open the pack that contains the sterile gown and paper towels to dry your hands (dry from finger to elbows).
4. Pick up the gown and step back so the gown does not touch bench or other surfaces nearby.
5. The surgeon places the arms into the sleeves of the gown with care that the out part of the gown does not touch the surgeon or any other non-sterile surface
6. The assistant dresses the surgeon into a comfortable position avoiding touching the front part of the gown. Ties the strings at the back and fastens the Velcro of the neck piece.
7. Note that the hands of the surgeon do not come out of the sleeves.

GLOVING:

1. Sterile surgical gloves must be worn.
2. The outer packet of gloves must be opened either by the surgeon before scrubbing up or by an assistant.
3. To prevent contamination, the internal sterile packing containing the gloves should be touched only by the ‘scrubbed‟ surgeon and the gloves unpacked without touching their outer surface, preferably using a „closed‟ gloving technique. See video here attached: **https://www.youtube.com/watch?v=_WqVHPxmfFo**
4. Once the surgeon is wearing sterile clothing and gloves, care must be taken, throughout the procedure, to avoid touching non-sterile items such as the table, animal, anaesthetic equipment or operating lights.
5. Ideally an assistant should be available, throughout surgery, to move the animal, adjust the table and any non-sterile equipment and to assist with monitoring the depth of anaesthesia and making adjustments, as required
6. For these reasons the presence of an assistant is strongly recommended but if an assistant is not available, or if the surgeon needs to make fine adjustments to equipment, such as the vaporiser or operating microscope, the adjustable knobs should be covered with suitable sterile material, such as foil or plastic covering, before surgery starts.
7. If working alone cannot be avoided, the surgeon must take particular care not to touch non-sterile materials. If this cannot be avoided or happens accidentally, the surgeon must at least change their contaminated gloves for a new sterile pair.
8. Where it is suspected or has the certainty that instruments have been accidentally contaminated, these **must be replaced** with sterile ones before continuing or, if the surgeon believes only the tips of the instrument have been contaminated, a „bead sterilizer‟ may be used.
9. ***“Non-scrubbed” surgical assistants must not touch sterile instruments, drapes or consumables.***

EQUIPMENT AND MATERIALS PREPARATION

1. All surgical instruments must be sterilised (appropriately packed and autoclaved for 15 minutes at 134 °C) before use. See this video for further instructions **http://www.procedureswithcare.org.uk/aseptic-technique-in-rodent-surgery-tutorial/aseptic-technique-in-rodent-surgery-instruments/**
2. Similarly, all consumables (e.g. swabs, needles, suture materials) and other materials coming into contact with the surgical equipment must be sterile and should be of an appropriate size and packaged in suitable quantities. Please see this video for further instructions on how to open this materials and consumables: **http://www.procedureswithcare.org.uk/aseptic-technique-in-rodent-surgery-tutorial/aseptic-technique-in-rodent-surgery-other-materials/**
3. Aluminium foil can be packed into autoclave pouches and sterilised. This can be used to cover any items that can't be sterilised, such as hand-held drills, microscope and stereotactic frame knobs, etc.
4. It is good practice to use a new set of sterile instruments for each animal to avoid cross-contamination between animals. Therefore, if multiple animals are undergoing surgery in one session, ensure that you have a sufficient number of sterile instruments as well as consumable kits before you start.
5. Disposable sterile instruments are readily available and may present an economical option.
6. A bead steriliser may be used during surgery to sterilise the tips of instruments. Instruments must be previously cleaned with sterile toothbrush or similar and sterile water not saline to remove rests of blood and organic material before it is dipped into the hot beads.
7. Note that, since only the tips of the instruments get sterilised, their use is not considered best practice, as the handle remains untreated.
8. The use of alcohol/disinfectant dipping is ineffective in ensuring asepsis and are therefore not considered good practice.
9. The preparation for a surgery is clearly described in this video: **http://www.procedureswithcare.org.uk/aseptic-technique-in-rodent-surgery-tutorial/aseptic-technique-in-rodent-surgery-preparation-for-surgery/**
10. Note: the following key points are literally transcribed for reference from the same website:

a. An assistant should open the outer wrapping of instrument packs, sutures, and scalpel blades.
b. The surgeon needs to take care when unwrapping the pack to drop the corners of the drapes so that the back of their hand does not touch the surface of the table.
c. The surgeon drapes the animal, once again taking care not to touch any non-sterile surface.
d. Using a drape prevents sterilised items touching the animals' fur, and becoming contaminated.
e. When using a paper drape, a suitable-sized hole can be cut to access the surgical site – avoid cutting along one of the pre-folded sections as this can prevent the drape conforming to the animal.
f. The sterile field can be extended by using an additional paper or cloth drape.
g. Drapes may need to be cut to size, or positioned carefully so that the position of the animal's nose in the face mask can be monitored – alternatively a transparent drape can be used.

h. Several mask designs (such as the one used to anaesthetise the mouse) provide much more secure placement of the animal's nose.
i. The position of the head can be fixed using tape, but care must be taken not to interfere with respiratory movements, or to fix the animal's limbs in an abnormal position.

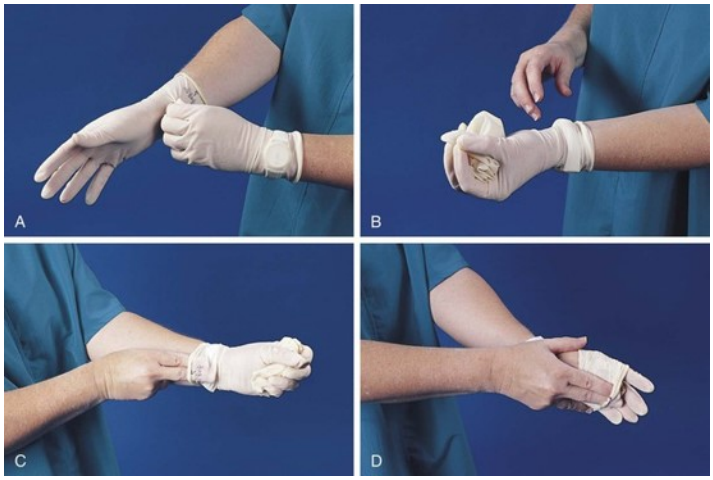
PROCEDURE:
http://www.procedureswithcare.org.uk/aseptic-technique-in-rodent-surgery-tutorial/aseptic-technique-in-rodent-surgery-the-surgical-procedure/

1. The animals should be now on the operating table over a heating source (homeothermic blanket or heating blanket) and the thermometer probe in place to check any fluctuation in temperature.

2. Final skin cleansing and preparation of the animal should be completed in the surgical theatre, just prior to draping.
3. Avoid the excessive application of alcohol, especially to small rodents, because loose of body temperature will occur as the alcohol evaporates.
4. Sterile drapes of a sufficient size must be used to cover unprepared parts of the animal and adjacent surfaces. Proper draping will provide sufficient space for the surgeon to use instruments and suture materials without accidentally contacting non-sterile items or surfaces.
5. The use of transparent disposable drapes over the animal can aid anaesthetic monitoring (STERILIZED GLAD® PRESS'N SEAL®).
6. Instruments should be placed either on a sterile surface (e.g. plastic sterile drape) or on a sterile tray.
7. During surgery it is preferable and highly beneficial for the surgeon to have an assistant on hand to pass him/her extra materials, to adjust equipment (e.g. operating microscope) and to help with the surgery (e.g. retraction of skin edges and organs).
8. A „scrubbed" surgical assistant must be surgically attired (e.g. sterile gown and gloves) if assisting with the surgery. If it is not possible to have a „non-scrubbed" assistant present, sufficient consumables must be laid out on a sterile drape/tray prior to the surgeon commencing the procedure. A means of handling non-sterile items (e.g. vaporiser, drill, stereotactic frame) must be provided, such as sterilised aluminium foil. Post-surgical cleaning of the surgical tools can be viewed here: https://www.atdove.org/video/cleaning-surgical-instruments

DEGOWNING:
How to remove gloves:

1. Using one gloved hand, grasp the outside of the opposite glove near the wrist, pull and peel the glove away from the hand (A-B).
2. Slide one or two fingers of the ungloved hand under the wrist of the remaining glove (C).
3. Peel the glove off from the inside, creating a bag for both gloves (D).
4. Discard in waste contained.



Removing goggles or face shield:

1. Using ungloved hands, grasp the ‘’clean’’ ear of head pieces and lift away from face.
2. If reusable, place them in a designated receptacle for subsequent reprocessing. Otherwise, discard.

Removing surgical gown:

1. Unfasten ties, peel gown away from neck and shoulder.
2. Turn contaminated outside toward the inside.
3. Fold or roll into a bundle.
4. Discard.

Removing a mask:

Untie the bottom, then top tie, remove from face then discard.

Chapter 8:

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