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Title	Characterisation of sex differences in behavioural domains and antidepressant response in two rat models of depression
Author(s)	Doherty, Hayley
Publication Date	2018-10-17
Publisher	NUI Galway
Item record	<a href="http://hdl.handle.net/10379/14617">http://hdl.handle.net/10379/14617</a>

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# **Characterisation of sex differences in behavioural domains and antidepressant response in two rat models of depression**

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A thesis submitted to the National University of Ireland, Galway for the degree of  
Doctor of Philosophy

**July 2018**



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## **Abstract**

Although the rate of depression is known to be almost twice as high in women compared to men, most evaluation of antidepressant efficacy involved only males until the early 1990s. In pre-clinical depression research male subjects are still preferred over females due to the perception that females' responses are more variable than those of males. The aim of this thesis is to characterise two rodent models of depression, the olfactory bulbectomy (OB) model and the Wistar Kyoto (WKY) model, in regards to sex differences in anxiety- and depressive-like behaviours, cognitive ability as well as responsiveness to chronic antidepressant treatment, namely fluoxetine, an SSRI, and desipramine, a tricyclic antidepressant. We also aim to evaluate the reproductive ability of these models as well as their maternal and offspring characteristics.

Irrespective of sex, a hyperactive response was observed in the elevated plus maze (EPM) in the OB rat, but not in the open field test (OFT) where a hyperactive response is usually associated with this model. However only male OB rats displayed increased open arm entries in the EPM and decreased time in the target zone of the Morris water maze (MWM). In the WKY rats, both sexes displayed a hypoactive phenotype in the EPM and OFT but males exhibited a heightened freezing response compared to females upon exposure to these arenas. Again only males displayed impairments in the MWM and neither sex exhibited increased immobility time in the FST. We were unable to detect an antidepressant response in either male or female OB rats due to habituation to the OFT arena causing an absence of hyperactivity in the vehicle treated groups. As for the WKY rats, we found that both sexes were unresponsive to chronic fluoxetine treatment and desipramine caused a reduction in immobility time in the FST in male WKY rats only. We hypothesised that BDNF mRNA levels could be used as an index associated with antidepressant response however we did not find any effect of drug treatment on hippocampus or frontal cortex levels in either OB or WKY rats. We did not find impaired

sexual activity in male rats in either model however female OB rats were less likely to give birth following mating compared to their controls. No developmental delays could be detected in pups born to OB mothers although small litter size could have had confounding effects in this regard. Pups born to WKY mothers exhibited delays in somatic and cognitive development however we did not observe any differences in maternal behaviour in WKY dams.

From our results we can see that female OB rats exhibit the main behavioural characteristic of the OB syndrome but they fail to display other aspects that have been previously reported in males, including learning and memory deficits and altered behaviour in the EPM. This suggests the need for further evaluation of the OB syndrome in females perhaps with the use of alternative tests to measure different aspects of depressive-like behaviour such as anhedonia. A similar inference could be made for the WKY females; they exhibited the hypoactive, anxious phenotype associated with the WKY model but failed to display cognitive deficits, depressive-like behaviour or antidepressant responsiveness. To conclude, these findings highlight the need for investigating sex differences in models of psychiatric disease as such evaluations could shed light on novel therapeutic approaches.

### **Author's 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:

- Olfactory bulbectomy studies in chapters 3-5 were performed with assistance from Dr. Zara McAleavey, Kelly McHugh and Prof. John Kelly
- WKY antidepressant dosing study was carried out with assistance from Prof. John Kelly

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: \_\_\_\_\_

## **Acknowledgements**

Firstly I would like to thank my supervisor Prof John Kelly for taking a chance on an unknown physiology student and offering me the PhD and also for the guidance and support you has provided over the past 4 years.

To the Kelly girls, both past and present but especially Kelly, Natalie and Zara, I don't think I would have made it this far without all of the support you have given me, both practical and moral. I couldn't have asked for a better group of people to spend the last four years with, your kindness won't be forgotten.

To the rest of CNS, thank you for making the lab such a friendly and welcoming place to work! The spirit of comradery in CNS has made it a great place to spend the past four years and the lunchtime crosswords will be missed! Your willingness to help others is something not found in many work environments and will hopefully remain for years to come.

To Danny, I want to thank you for all of your support especially for checking my drug calculations and making sure I didn't overdose all my rats and of course your crossword clues!

To Silke, thank you for all of your help over the past few years, from all of the animal checks you did to keeping my m-drive up to date! You do so much in the lab and I am so grateful for all of the support you have given me.

To Amby, without your help I don't think I would have made it. You were always the first person we would call on when there was a problem and you always found a way to solve it. You wouldn't accept our thanks stating that you were just doing your job but you went above and beyond to help in whatever way you could and I will always be grateful.

To my mum and dad, I cannot express how grateful I am to have the two of you as my parents, thank you for all of the love and support you have given me over the past few years. It hasn't always been easy but I know you would have supported me with whatever I decided to do.

## List of Abbreviations

3 CT	3 Chamber test
ACTH	Adrenocorticotrophic hormone
APA	American Psychological Association
ARRIVE	Animal Research: Reporting of In Vivo Experiments
BDI	Beck depression index
BDNF	Brain derived neurotrophic factor
CAE	Closed arm entries
CBT	Cognitive behavioural therapy
cDNA	complimentary DNA
CIDI	Composite International Diagnostic Interview
CMS	Chronic mild stress
CRH	Corticotropin-releasing hormone
CSF	Cerebro-spinal fluid
Ct	Copy threshold
CTFPHC	Canadian Task Force on Preventative Health Care
DA	Dopamine
DALY	Disability adjusted life years
DH <sub>2</sub> O	Distilled water
DMI	Desipramine
DRN	Doral raphe nucleus
DSM	Diagnostic and Statistical Manual of Mental Disorders
EPM	Elevated plus maze
FLX	Fluoxetine

FST	Forced swim test
GBD	Global burden of disease
GR	Glucocorticoid receptor
HAM-D	Hamilton rating scale for depression
HPA	Hypothalamic- pituitary axis
HRT	Hormone replacement therapy
ICD	International Classification of Diseases
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IL	Interleukin
IPT	Interpersonal psychotherapy
KYN	Kynurenine
LPS	Lipopolysaccharide
MADRS	Montgomery-Åsberg depression rating scale
MAOI	Monoamine oxidase inhibitor
MBCT	Mindfulness-based cognitive therapy
MDD	Major depressive disorder
MR	Mineralocorticoid receptor
mRNA	messenger RNA
MS	Maternal separation
MWM	Morris water maze
NCS	National Comorbidity Study
NE	Noradrenaline
NHP	Non-human primates

NICE	National Institute for Health and Care Excellence
NIH	Novelty-induced hypophagia
NOR	Novel object recognition
NTF	Neurotrophic factor
o.g.	Oral gavage
OAE	Open arm entries
OB	Olfactory bulbectomy
OFT	Open field test
PDD	Premenstrual dysphoric disorder
PFC	Pre-frontal cortex
PHQ	Patient Health Questionnaire
PMDD	Pre-menstrual dysphoric disorder
PND	Postnatal day
PPD	Postpartum depression
PST	Problem solving therapy
PUFA	Polyunsaturated fatty acids
RCT	Randomised controlled trial
RDoC	Research Domain Criteria
s.c.	Subcutaneous
SD	Sprague-Dawley
SERT	Serotonin transporter
SHR	Spontaneously hypertensive rat
SNRI	Serotonin-noradrenaline reuptake inhibitor
SPT	Supportive psychotherapy

SSRI	Selective serotonin reuptake inhibitor
TCA	Tri-cyclic antidepressant
TNF	Tumour necrosis factor
TPH	Tryptophan hydroxylase
TRD	Treatment resistant depression
VEGF	Vascular endothelial growth factor
WHO	World Health Organisation
WKY	Wistar Kyoto
WLI	WKY less immobile
WMH	World Mental Health
WMI	WKY more immobile
YLD	Years lived with disability
YLL	Years of life lost

## List of Publications and Conference Proceedings

### Published Abstracts from Conference Proceedings

**Doherty, H.**, Kelly, J.P. 2015. Are there Gender Differences in the Behavioural Responses in the Olfactory Bulbectomy Model of Depression? *E-journal of the British Pharmacological Society*, 13 (3).

**Doherty, H.**, Kelly, J.P. 2016. The effect of olfactory bulbectomy on reproduction and offspring development in the rat *E-journal of the British Pharmacological Society*, 16 (1).

McHugh, K.L., **Doherty, H.**, Deaver, D., Roche, M., Finn, D.P., Kelly, J.P., 2016. An assessment of social cognition in the olfactory bulbectomised rat model of depression. *E-journal of the British Pharmacological Society*, 16 (1).

**Doherty, H.**, Kelly, J.P. 2016. Investigation of reproductive and littering parameters in the olfactory bulbectomy rodent model of depression. *Journal of Psychopharmacology*, Issue 30 (8). Supplement Page A68, Abstract C06.

**Doherty, H.**, Kelly, J.P. 2017. The Wistar Kyoto rat displays a number of cognitive deficits, some of which are sex-specific. *Journal of Psychopharmacology*, Issue 31 (8). Supplement Page A77, Abstract D04.

### Unpublished Abstracts from Conference Proceedings

**Doherty, H.**, Allen, J., Kelly, J.P., 2015. Do gender differences exist in the olfactory bulbectomised rat model of depression? *Neuroscience Ireland, Dublin City University, Ireland*. Poster presentation.

**Doherty, H.,** Kelly, J.P., 2015. Are there gender differences present in the rodent olfactory bulbectomy rat model of depression? *Galway Neuroscience Centre research Day, NUI Galway*. Poster presentation.

**Doherty, H.,** Kelly, J.P., 2016. An investigation into the effects of olfactory bulbectomy surgery on anxiety and depressive-like behaviours and reproductive parameters in the rat. *Biomedical Sciences Section Annual Meeting*. Oral presentation.

**Doherty, H.,** Kelly, J.P., 2017. An examination of whether they are sex-specific cognitive deficits in the Wistar Kyoto (WKY) rat model of depression. *Neuroscience Ireland, NUI Galway, Ireland*. Poster presentation.

**Doherty, H.,** Kelly, J.P., 2017. An investigation into sex differences in cognitive ability in the Wistar Kyoto (WKY) rat model of depression. *EBPS Biennial Meeting, Crete, Greece*. Poster presentation

# ***Chapter 1: Introduction***

## 1.1. Epidemiology of Depression

Mental illness, including Major Depressive Disorder (MDD) accounts for 7.4% of the disease burden worldwide (Papakostas and Ionescu, 2014). According to the World Health Organisation (WHO), depression is the world's leading causes of disability and is a large contributor to the overall global burden of disease. Depression affects people in all socio-economic levels but rates have been shown to be slightly higher in high-income countries compared to low- to middle-income countries (Bromet et al., 2011).

Epidemiological studies carried out to investigate the prevalence of depression often report large variability in rates between different countries. This may be due to a combination of different methods of measurement and factors involved in study design. In order to combat this problem the WHO launched an international World Mental Health (WMH) Survey Initiative with the aim to collect accurate data from 27 countries which included both the developed and developing world regarding the prevalence and treatment of mental disorders worldwide (<https://www.hcp.med.harvard.edu/wmh/index.php>). In order for the data collected to be comparable, all surveys undertaken as part of the WMH Initiative used the WHO Composite International Diagnostic Interview (CIDI). This interview can be carried out by trained lay people which generates diagnoses of mental disorders according to the definitions and criteria of both the Diagnostic and statistical Manual of Mental Disorders (DSM) and the International Classification of Diseases (ICD) systems that are used by the scientific community (Kessler et al., 2009).

Results from the WMH Survey did not show any reduction in variability in 12 month prevalence rates of mental disorders between regions of the world compared with previous reports. The lowest rates were observed in Shanghai (4.3%) and the highest in the US (26.4%). Reasons for the variability in rates of depression across the world may

be due to methodological concepts such as the wording of the interview questions and concepts used to describe mental disorders being more in line with Western culture and not necessarily with the culture in which the interviews were taking place, for example stigma may be attached to mental disorders in particular countries meaning people are unwilling to admit suffering from them. Anxiety disorders were the most common disorders in all but one country (Ukraine, in which mood disorders were more prevalent), with prevalence rates between 2.4-18.2%. Mood disorders, including MDD, were the second most common form of mental disorder in all countries (Nigeria and Beijing had equal or higher prevalence rates of substance abuse disorders) with a prevalence range of 0.8% to 9.6% (Demyttenaere et al., 2004). This supports previous observations regarding data collected from the National Comorbidity Survey Replication (NCS-R), that also reported MDD as being the second most prevalent mood disorder in the US general population between 2001 and 2003 (Kessler et al., 2012). Similarly in the EU, MDD was found to affect ~30.3 million people in 2011 which is the second most frequent mental disorder, second only to anxiety disorders which affected ~69.1 million people (Wittchen et al., 2011). The prevalence rate for any mental disorder was higher in EU (38.2%)

countries compared the estimated worldwide range reported by the WMH study described above (4.3-26.4%).

**Figure 1.1.** Representation of the number of people affected by mental disorders in the EU in 2010 (Wittchen et al., 2011).

Comparing prevalence rates of MDD reported in the National Comorbidity Survey (NCS) and NCS-R, in the US, rates of depression did not change between 1990-1992 and 2001-2003, however the percentage of those receiving treatment increased from 20.3% to 32.9% (Kessler et al., 2005). In agreement with this finding, the results from Global Burden of Disease (GBD) Study 2010, suggest that the prevalence of MDD has remained stable between 1990 and 2010 (Ferrari et al., 2013).

As well as studies investigating the prevalence of diseases worldwide, another marker used to measure and compare disease epidemiology is the burden caused by a disease. In

order to quantify this burden, disability adjusted life years (DALYs) are used. Each healthy year of life lost to disability is measured by one DALY. DALYs are calculated by combining the years of life lived with disability (YLD) and the years of life lost (YLL) due to premature death. The Global Burden of Disease (GBD) Study 2010 used these measures to compare the burden of 291 diseases and injuries. They found that MDD was the second leading cause YLDs, accounting for 8.2% of all YLDs, with lower back pain being the leading cause of YLDs. YLDs caused by depression were consistently higher for females compared to males (Ferrari et al., 2013).

## **1.2. Diagnostic Criteria for Psychiatric Disease**

The Diagnostic and Statistical Manual of Mental Disorders (DSM) and the International Statistical Classification of Diseases (ICD) are the main classification systems used for the categorization of mental health disorders. They are also tools used by physicians when diagnosing psychiatric disorders, including depression. The DSM, which is now in its fifth edition was first published in 1952 by the American Psychiatric Association (APA). In 1968 the second edition, DSM-II, was released. It listed 182 different disorders. More drastic changes were made in the third edition, published in 1980, in response to criticism that mounted during the early 1970's. A study published that described such criticisms compared diagnoses given by American and British psychiatrists based on videotapes of patients describing their symptoms. The American psychiatrists came up with varying, incorrect, diagnoses which led to the conclusion that they were mediocre diagnosticians compared to their British counterparts (Kendell et al., 1971). Another paper reporting criticisms of the DSM concluded that inpatient facilities were unable to differentiate between the sane and insane using the DSM-II diagnostic criteria (Rosenhan, 1973). Changes introduced to DSM-III involved the addition diagnostic criteria, typical demographic profiles and information about the onset and

course of the disorder as well as the inclusion of many new categories with the number of disorders increasing to 265. DSM-IV was published in 1994 and contained 297 disorders. DSM-5 is the most recent edition of the manual which was published in 2013. It contains considerably edited diagnoses, deletion of subtypes of schizophrenia and deletion of the subsets of autism spectrum disorder.

DSM-5 contains several updates to the Depressive Disorders section including the addition of new disorders, namely disruptive mood dysregulation disorder (DMDD) and premenstrual dysphoric disorder (PDD). DMDD is a diagnosis for children who present with persistent irritability and episodes of extreme and uncontrollable behaviour. It was created in order to prevent overdiagnosis of bipolar disorder in children. PDD which was included in DSM-IV in the appendix section, has been moved to the main body in DSM-5. No changes to the core criterion symptoms or the duration of symptoms were made but the coexistence of a depressive episode with 3 manic symptoms is acknowledged by the specifier “with mixed features”. Another change is the removal of the bereavement exclusion from DSM-5. Finally, the specifier “with anxious distress” was added as research has shown the relevance of anxiety when it comes to decision making regarding treatment as well as patient prognosis (APA).

The ICD was designed as a medical classification system which uses a system of diagnostic codes for classifying diseases and providing assistance in the diagnosing of diseases. Its purpose has been to improve comparability of medical data collected internationally for the use in identifying health trends and generating global health statistics. The first edition, called the International List of Causes of Death, as the title implies, described injuries and diseases that led to death only. ICD-6 which was published in 1949 by the WHO was the first version to include classifications of morbidity, including mental health disorders. Few changes were introduced to the next

version, ICD-7, published in 1955. Three more versions were released, ICD-8, ICD-9 and the most current ICD-10, which was published in 1990. ICD-11 for Morbidity and Mortality Statistics (ICD-11-MMS) is due for release in 2018. According to the WHO the latest ICD revision was conducted in order to “better reflect progress in health sciences and medical practice”.

### **1.2.1. Diagnosis criteria for major depression**

The three broad categories of depression are mild, moderate and severe. These are distinguished based on the number and severity of symptoms presented. The criteria to be met for the diagnosis of depression vary slightly between DSM-5 and ICD-10.

According to DSM-5, depression is diagnosed when a person experiences a period of at least 2 weeks in which they suffer from at least 5 out of the following 9 key symptoms:

- depressed mood most or all of the day, nearly every day,
- diminished interest or pleasure in all or almost all activities most days,
- significant weight loss or gain or changes in appetite,
- insomnia or hypersomnia nearly every day,
- psychomotor agitation or retardation,
- fatigue or loss of energy,
- feelings of hopelessness or excessive or inappropriate guilt,
- diminished ability to think or indecisiveness,
- recurrent thoughts of death or suicidal ideation.

Either of the first two symptoms must be present every day. The symptoms must cause clinically significant impairment in all aspects of the patients functioning, they must not

be caused by drug use nor meet the criteria for another mental disorder and the symptoms should not be better explained by mourning/bereavement (DSM-5).

In relation to ICD-10 criteria, a diagnosis of ‘mild’ depression is given based on the presentation of at least 2 of the 3 following typical symptoms:

- depressed mood
- loss of interest and enjoyment
- increased fatigability

and at least 2 of the following common symptoms:

- reduced concentration and attention
- reduced self-esteem and self-confidence
- ideas of guilt and unworthiness
- bleak and pessimistic views of the future
- ideas or acts of self-harm or suicide
- disturbed sleep
- diminished appetite

Again the symptoms must be present for at least 2 weeks in order to be diagnosed with depression. None of the symptoms should be present to an intense degree, and the patient will experience some difficulty with work and social aspect of life but will not experience significant impairment.

Depression is classed as ‘moderate’ if the individual experiences 2 of the 3 typical symptoms as well as 3-4 of the other common symptoms. An individual diagnosed with moderate depression will experience considerable difficulty in all aspects of daily functioning.

All 3 of the typical symptoms should be present as well as at least 4 of the other symptoms during a severe depressive episode. It is unlikely that an individual experiencing a severe episode will be capable to continue with routine functioning including work. It is also assumed that the somatic syndrome is present in a severe episode; this entails symptoms such as decreased emotional reactivity, early wakening from sleep, symptoms worse in the morning, psychomotor agitation or retardation, loss of appetite and loss of libido. Four symptoms need to be present for the somatic syndrome to be regarded as present.

There are no diagnostic tests currently available for depression. Diagnosis relies solely on the patients' willingness to describe how they are feeling and the clinicians' ability to recognise the symptoms. Diagnosis of depression can be difficult as it is associated with a broad clinical presentation and individuals are often unwilling to admit how they are feeling (Aragones et al., 2004). A meta-analysis study has shown that recognition of depression by non-psychiatric physicians is low and as many as half of depressed patients are incorrectly diagnosed by their GPs (Cepoiu et al., 2008).

Depression has diagnostic heterogeneity due to its polythetic definition, meaning there are multiple ways to meet the criteria for diagnosis. As calculated by Zimmerman et al (2015), there are 227 ways to meet the diagnostic criteria for MDD. Based on the symptom profile presented by 1566 patients diagnosed with MDD based on the DSM-IV criteria, they found that the patients met this criteria in 170 different ways, i.e. there were 170 different symptom profiles (Zimmerman et al., 2015). This heterogeneity of symptom profiles may cause the mis- or under-diagnosis of depression.

A range of screening tools to aid diagnosis are available for use in both a primary care setting as well as in clinical research. These screening tools include but are not limited to, the Patient Health Questionnaire (PHQ)-2 and PHQ-9, the Beck Depression Inventory

(BDI), the Hamilton Rating Scale for Depression (HAM-D) and the Montgomery Åsberg Depression Rating Scale (MADRS).

The PHQ-2 and -9 are used mainly by clinicians in a primary care setting. Although consisting of only 2 questions, the PHQ-2 has been proven to be as effective as longer screening instruments (Whooley et al., 1997, Meader et al., 2011). The PHQ-9 is often administered following a positive PHQ-2 result. It has been shown to be 61% sensitive and 94% specific for mood disorders in adults (Maurer, 2012).

<b>Over the past 2 weeks, how often have you been bothered by any of the following problems?</b>	<b>Not at all</b>	<b>Several days</b>	<b>More than half the days</b>	<b>Nearly every day</b>
<b>1. Little interest or pleasure in doing things</b>	0	1	2	3
<b>2. Feeling down, depressed or hopeless</b>	0	1	2	3

**Table 1.1.** An example of the PHQ-2 used in primary care as a tool in the diagnosis of depression.

The BDI is a 21-question multiple choice questionnaire designed for the self-reporting of depression symptomology and severity (Beck et al., 1961). In 1996, BDI-II was developed as a revision to the original in order to comply with the newly changed diagnostic criteria for MDD as set out in DSM-IV. Answers are scored on a scale of 0-3 with higher total scores indicating a more severe depressive episode.

The HAM-D scale was published “for use in assessing the symptoms of patients diagnosed as suffering from depressive states” (Hamilton, 1960). Individuals are rated on 17-21 items and each item is scored on a 3 or 5 point scale with higher total scores related to more severe depression.

The MADRS consists of a 10 item questionnaire designed to be sensitive to antidepressant treatment effects. It was found to be more reliable in differentiating between those who do and do not respond to antidepressant treatment (Montgomery and Asberg, 1979). The answers for each item are scaled from 0-6, again with higher scores indicating more severe depression.

The UK National Health Service (NHS) recommends against screening the general population for depression due to concerns over the reliability of screening tests (UK, NSC). The Canadian Task Force on Preventative Health Care (CTFPHC) also recommended to not screen for depression in primary care, instead advising clinicians to be alert to signs of depression symptomatology. Their reasons for this stance were due to a lack of ‘any randomized controlled trials (RCTs) that have shown depression screening to be beneficial’ and due to concerns over high numbers of false positive diagnoses (Thombs and Ziegelstein, 2013). A study has shown that 12 out of 21 meta-analyses that examine the diagnostic accuracy of depression screening tools were found to have deficiencies in the completeness and transparency of their results (Rice et al., 2016). This suggests that caution should be taken when using the results from these meta-analysis studies to infer the accuracy of screening tools.

### **1.3. Treatment of depression**

Depression cannot be treated with a ‘one size fits all’ approach as the optimal treatment varies from patient to patient. Presently, the only method of identifying the most effective treatment is through a process of trial and error. As discussed further below, antidepressants have a lag time of ~2-3 weeks before therapeutic effects are seen which means that a sufficient time must be given for each treatment trialling period, meaning it may take a prolonged time before the optimal course of treatment is uncovered.

Medication algorithms are used to aid decision making in regards to treatment approaches. Algorithms for the treatment of depression exist and usually consist of a series of steps to be followed in the event of non-responsiveness to first line antidepressants. These steps involve either an increase in medication dosage, a switch in medication type or the augmentation of the failed medication with a second drug (Spijker and Nolen, 2010).

The UK National Institute for Health Care Excellence (NICE) guidelines for the use of antidepressant medication recommend using a selective serotonin reuptake inhibitor (SSRI) as a first line treatment for depression. If there is an inadequate response to this treatment, the next recommended step is switching to a different SSRI and subsequently to switch to a different pharmacological class if there is still an unsatisfactory response. They only recommend the combining and augmenting of medications upon discussion with a consultant psychiatrist.

Treatment options for depression consist of pharmacotherapy, psychotherapy or a combination of both. As mentioned, it generally it takes multiple attempts to find the correct course of treatment with studies showing that up to two thirds of patients do not respond to the first antidepressant prescribed to them (Trivedi et al., 2006). In the UK, prescriptions for antidepressants rose by more than 100% from 1995 to 2011 with the majority (51%) for selective serotonin reuptake inhibitors (SSRIs). The rise in the number of prescriptions is thought to be due to an increase in duration of treatment rather than an increase in the number of people starting the medication (Mars et al., 2017, Ilyas and Moncrieff, 2012). Similarly in the US, the rate of antidepressant treatment rose, with the use of SSRIs increasing and the use of tricyclic antidepressants (TCAs) decreasing (Olfson and Marcus, 2009, Mojtabai and Olfson, 2014).

Although antidepressant drugs cause an increase in monoamine levels in the brain almost immediately, there is a lag of about 2-3 weeks before any therapeutic effect is observed. The reason for this is not completely understood but it confirms the theory that other factors rather than/as well as altered monoamine levels as being the cause of depression.

Depression is associated with a high level of non-recovery. It is reported that ~20% of patients receiving appropriate antidepressant treatment fail to respond and are said to suffer from treatment resistant depression (TRD) (McIntyre et al., 2014). There is no concrete definition for TRD, with some classifying it as depression that persists after 1 failed course of treatment (Nierenberg and DeCecco, 2001) while others define it as 2 or more failed treatment attempts (Malhi et al., 2005, Souery et al., 1999) and so it is hard to determine the precise percentage of people with this form of chronic depression.

There are various forms of psychotherapy that can be used in the treatment of depression including (but not limited to) cognitive behavioural therapy (CBT), interpersonal therapy (IPT), mindfulness-based cognitive therapy (MBCT), supportive psychotherapy (SPT) and problem-solving therapy (PST); the most common forms are CBT and IPT. Briefly, CBT focuses on the development of coping strategies and changing damaging patterns in thoughts and behaviours; IPT involves resolving interpersonal problems and aims to improve communication skills within relationships.

Studies show that psychotherapy is effective in the treatment of depression, with evidence suggesting that interpersonal therapy is more efficacious than other forms, particularly as an adjunct to pharmacological treatment (Cuijpers et al., 2011, Corruble et al., 2016).

In studies of peoples attitude to depression treatment, psychotherapy was found to be the preferred and thought of as the most acceptable form of treatment, with antidepressant medication perceived as being the least acceptable (Hanson et al., 2016, Raue et al., 2009, van Schaik et al., 2004). Sex, level of education and family history were found to

influence the preferred type of treatment in patients with newly diagnosed depression. Females are more likely to favour psychotherapy compared to males whose preference was more evenly split between psychotherapy and medication. People with a university degree and those with a family history of depression were also more likely to prefer psychotherapy (Houle et al., 2013).

#### **1.4. Discovery of Antidepressant medications**

Serendipity played a large role in the discovery of the first pharmacological treatment for depression with the ‘side-effects’ of a drug called iproniazid, which was being trialled for the treatment of tuberculosis, being described as causing euphoria and psychostimulation in patients receiving it. A clinical study was carried out on depressed patients and it was reported that 70% of those receiving iproniazid reported significant improvements which led to its off-label use as an antidepressant (Hillhouse and Porter, 2015). Iproniazid is a monoamine oxidase inhibitor (MAOI) which acts by blocking the activity of the enzyme monoamine oxidase preventing the breakdown and thus increasing the levels of monoamines such as serotonin and noradrenaline.

Tricyclic antidepressants (TCAs) were discovered in the 1950’s when the search for antipsychotics by pharmaceutical companies grew following the success of chlorpromazine for the treatment of schizophrenia which was the first antipsychotic discovered. The first TCA, imipramine, was derived from a modification of the classic antihistamine structure. Following testing in psychiatric patients, the drug was found to improve depressive symptoms with no serious side-effects which was an improvement over iproniazid that was eventually removed from the market over safety concerns (Hillhouse and Porter, 2015). TCAs produce an antidepressant effect by blocking the reuptake of serotonin and noradrenaline which causes elevated levels of these

monoamines in the synapse resulting in enhanced neurotransmission. Current clinical practice is for TCAs to be reserved for individuals who do not respond to first-line antidepressants.

Selective serotonin reuptake inhibitors (SSRIs) were the first rationally designed psychotropic medication and are now the first line pharmacotherapy for depression due to their improved side-effect profile compared to older classes of antidepressants. As the name suggests, SSRIs work by inhibiting the reuptake of serotonin and thus increasing serotonin levels at the synapse resulting in increased binding to the receptor. Examples of commonly prescribed SSRIs include fluoxetine (Prozac), citalopram (Celexa) and its S-enantiomer escitalopram (Lexapro). Serotonin-noradrenaline reuptake inhibitors (SNRIs) work in a similar fashion to SSRIs only they also inhibit the reuptake of noradrenaline, increasing the levels of both monoamines. Venlafaxine (Effexor) is the most commonly prescribed SNRI.

**Figure 1.2.** The 10 most commonly prescribed antidepressants in Ireland in 2013, compared to their prescription rate in 2004. All drugs were the same for both years except trazodone (2004) was replaced with duloxetine (2013). Data provided by Garvey and Kelly, 2015.

### **1.5. Sex difference in rates of depression**

Neuropsychiatric disorders account for a large proportion of the total disability burden suffered in the EU. Many neuropsychiatric disorders are reported to be more prevalent in females than in males and it has been reported that these disorders account for 30.1% of the total disease burden in females compared to just 23.4% for males (Wittchen et al., 2011). Depression is one such neuropsychiatric disorder that has been consistently reported to affect roughly twice as many females than males (Parker and Brotchie, 2010, Kuehner, 2003, Marcus et al., 2005, Mattisson et al., 2005, Boyd et al., 2015). This finding is true for both high-income and low- to middle-income countries (Bromet et al., 2011). The increased prevalence rate of depression in females is believed to first appear in adolescence (Oldehinkel and Bouma, 2011) and it has also been shown that the rate of newly diagnosed depression did not differ between postmenopausal women and age matched men (Faravelli et al., 2013). The timing of this increase and then decline in rate of depression in females would suggest that ovarian hormones play a role in the onset of depression in women; this theory is explained in further detail in a later section. YLDs caused by depression were consistently higher for females compared to males (Ferrari et al., 2013).

**Figure 1.3:** Years lived with disability (YLDs) by age and sex for MDD and dysthymia (Ferrari et al., 2013).

### **1.5.1. Sex differences in disease characteristics and symptoms**

There is debate as to whether the clinical characteristics of depression differ between the sexes with some studies reporting significant differences and others finding none. It has been shown that females often report an earlier onset of MDD (Kornstein et al., 2000, Schuch et al., 2014), a longer current episode, more past suicide attempts and a greater symptom severity; although no differences in severity of depression has also been reported (Parker et al., 2014). A large epidemiological study found that in 8 out of 10 European countries, women were significantly more likely to have had suicidal thoughts however they reported higher rates of fatal suicide in males (Boyd et al., 2015). However contradictory reports suggest males experience more frequent suicidal ideation and also

a greater amount of past major depressive episodes (Marcus et al., 2005, Smith et al., 2008, van Noorden et al., 2010).

A differential symptomatic profile is also commonly reported between males and females, with females more likely to report increased weight and appetite (Carter et al., 2000) hypersomnia, loss of energy, worthlessness, feelings of guilt, crying (van Noorden et al., 2010) somatic complaints, gastrointestinal symptoms and interpersonal sensitivity (Marcus et al., 2008, Schuch et al., 2014). Males are more likely to report sad mood, decreased reactivity of mood, decreased pleasure or enjoyment and psychomotor slowing or agitation (Schuch et al., 2014) and are also more likely to commit suicide (Webster Rudmin et al., 2003). Males have been found to often report more externalising symptoms such as aggression, irritability, risk-taking behaviours and substance abuse and when these symptoms were included in diagnostic criteria, more men were identified as being depressed than when traditional symptom criteria was used (Martin et al., 2013, Rice et al., 2015). These findings suggest that there may be a subtype of depression more common in males than females that may not be diagnosed when only the more traditional symptoms of depression are considered. Further research in this area should be conducted to determine if this is the case.

Multiple studies have reported that females are more likely to present with atypical features of depression which are listed in DSM-5 as being hypersomnia, leaden paralysis, interpersonal hypersensitivity and weight or appetite increases (van Noorden et al., 2010, Schuch et al., 2014, Angst et al., 2002b) (DSM-5). Males have been found to have an increased risk of melancholia, a severe typical subtype of depression (Rodgers et al., 2014, Xiang et al., 2012).

There is also contradicting data reporting no differences in the majority of symptoms (Wagener et al., 2016), and no difference in the level of disability caused by depression

experienced between males and females (Gili et al., 2014), and no difference in the severity of depression experienced by males and females (Parker et al., 2014).

Due to the high level of contradictory data, it is difficult to definitively conclude the exact differences in disease characteristics and symptoms experienced by males and females. It is fair to accept that males are more likely to commit suicide, report more externalising symptoms and also report higher levels of substance abuse. Females more commonly experience appetite and weight effects, somatic features and comorbid anxiety.

### **1.5.2. Sex differences in response to antidepressant treatment**

Although much research has been carried out in order to address this question, it is still unclear as to whether there are any sex differences in response to antidepressant treatment. Whilst many studies do report some sex differences there are also many that contradict these findings. One of the most commonly suggested sex differences is that females respond more favourably to SSRIs and males to TCAs. The table below lists the studies that report that males and females respond differentially to antidepressant treatment.

Reference	Drug	Subjects	Findings
<b>Kornstein et al 2000</b>	SERT, IMI	235 Males 400 Females aged 21-65	Females respond better to SERT based on BDI and HAM-D scores Males responded better to IMI than SERT
<b>Martenyi et al 2001</b>	FLX, MAP	42 Males 59 Females	Females responded better to FLX (SSRI) compared to an SNRI- no difference in males
<b>Quitkin et al 2002</b>	TCA's, SSRIs, MAOIs	1746 patients	Females had better response to MAOIs than males and post-menopausal females responded better to TCA's than pre-menopausal females
<b>Berlanga and Flores-Ramos, 2006</b>	CTP (SSRI), RBX (SNRI)	86 patients ages 18-40	Females responded better to SSRIs than SNRI based on HDRS point reduction with no difference in male groups
<b>Young, 2009</b>	CTP	2876 outpatients aged 18-75	Females more likely to remit with CIT than males based on HRSD

**Table 1.2.** Studies reporting sex differences in response to treatment with different classes of antidepressant drugs. SERT: Sertraline; IMI: Imipramine; FLX: Fluoxetine; MAP: Maprotiline; TCA: Tricyclic antidepressant; SSRI: Selective serotonin reuptake inhibitor; MAOI: monoamine oxidase inhibitor; CTP: Citalopram; RBX: Reboxetine.

These studies suggest that females respond better to SSRIs compared to males and post-menopausal females. There is also evidence to suggest that males respond more favourably to TCAs than to SSRIs (Kornstein et al., 2000).

A research group reported that premenopausal women prescribed TCAs were more likely to drop out from the study due to adverse effects compared to postmenopausal women also taking TCAs (Kornstein et al., 2000). The same study also found that premenopausal women responded better to SSRI treatment compared to that with TCAs, whereas there

was no difference in response rate between the two drug classes in postmenopausal women (Kornstein et al., 2000). These findings suggest a role of ovarian hormones in the action and efficacy of antidepressant treatment. In support of this statement it has been shown that hormone replacement therapy (HRT), particularly transdermal E<sub>2</sub> treatment, is a promising line of antidepressant therapy for pre- and peri-menopausal women (Gordon and Girdler, 2014). The data for the effectiveness of HRT as an antidepressant therapy in late postmenopausal women is contradictory with one study showing its effectiveness as a part of combined therapy with an antidepressant drug in postmenopausal women (Liu et al., 2004) and another concluding that oestradiol has no efficacy as an antidepressant therapy in postmenopausal women (Morrison et al., 2004). It was suggested that the difference in effectiveness of HRT as an antidepressant therapy between different stages of menopause may be due different levels of oestrogen observed between peri- and post-menopause. During the perimenopause oestrogen levels fluctuate greatly and stabilisation of these levels by HRT may be the mechanism by which the antidepressant effects are caused. In the postmenopausal state, oestrogen levels are consistently low and so HRT will not have the same effects.

As mentioned above there are also studies that did not find any sex differences in response to treatment with different classes of antidepressants (Thiels et al., 2005, Parker et al., 2003). No differences were reported between males and females in remission rates or time to remission when treated with either venlafaxine, an SNRI, or an SSRI (Entsuah et al., 2001). A large retrospective study found that there were also no differences between males and females in response to treatment with the TCAs imipramine and desipramine, although older females (>50) did respond better compared to younger females. They also found no sex differences in response to the SSRI fluoxetine (Quitkin et al., 2002). A secondary analysis of a large multicentre, multi-phase study in which MDD patients were

treated with either venlafaxine or fluoxetine, showed no effects of sex or menopausal status on treatment outcome (Kornstein et al., 2014).

Reasons for the inconsistent findings may be down to differences in therapeutic agents used as well as different dosages, dosing regimes and duration of treatment. Another important factor which may account for some of the variability in results is the level of adherence to treatment by the subjects (Sramek et al., 2016). The criteria for determining a significant response to treatment also varied across studies making comparisons difficult. For example, one study used paired t-test to analyse the difference in HAM-D-17 score from baseline to assessment (Martenyi et al., 2001), whereas another study classed response as at least a 50% decrease in HAM-D-17 score (Entsuah et al., 2001).

Another possible reason for the lack of consistency of results in treatment response studies may be due to differential pharmacokinetic profiles of antidepressants in the sexes. When it comes to absorption of antidepressants, females have lower levels of gastric acid secretion than males which could increase antidepressant absorption (Kokras et al., 2011). It has been shown that female sex hormones affect the rate of gastric emptying which could also affect the rate of antidepressant absorption (Hutson et al., 1989). Physiological differences in body composition between males and females can also cause differences in antidepressant pharmacokinetics. Females tend to have higher percentage body fat, lower organ blood flow and less muscle which can all interfere with the volume of distribution of antidepressants which is thought to be higher in women (Kokras et al., 2011). Antidepressants are metabolized by, inhibit and/or induce a range of cytochrome P450 (CYP) enzymes. Sex differences have been reported in CYP 3A4 and CYP1A2 isozymes both of which have effects in antidepressant clearance (Bigos et al., 2009). Drug substrates of CYP3A4, including several SSRIs and TCAs, clear faster in women

than men (Meibohm et al., 2002), possibly caused by higher activity levels of CYP3A4 in females than males (Hunt et al., 1992).

Female reproductive hormones are believed to play a role in the effectiveness of antidepressant treatment. Oestrogen interacts with the serotonergic system which can affect the action of SSRI drugs. This can be seen in one study which found that depressed postmenopausal women treated with supplemental oestrogen in combination with an SSRI exhibited improved responses compared to depressed postmenopausal women receiving the SSRI alone (Schneider et al., 1997).

## **1.6. Reasons for sex differences in prevalence and treatment responses**

### **1.6.1. Non-biological factors**

There are various theories, both biological and non-biological, as to why the rates of depression are almost twice as high in females compared to males.

One explanation could be that females have an increased willingness to seek mental health care, particularly from GPs (Kovess-Masfety et al., 2014). There may be many males suffering from depression but only a small proportion seek help for it and so this causes a bias in the prevalence rates towards females. As well as this a number of large studies show that females tend to report more symptoms compared to males (van Noorden et al., 2010, Marcus et al., 2005, Angst et al., 2002a), shown by higher scores in diagnostic/severity assessments and females have a better ability to recall previous MDD episodes which would also increase rates of diagnosis (Wilhelm and Parker, 1994). This difference in symptom reporting may be a contributor to the difference in rates of depression because if females report more symptoms they will be more likely to reach the threshold of 5 symptoms needed for a diagnosis of MDD, according to DSM-5, compared to males who may not report enough symptoms needed to get a diagnosis. This theory is

supported by the finding that the gender difference in minor depression, which is diagnosed upon presentation of 2-4 symptoms, is much lower than that of MDD at 1.2-1 for females compared to males (Angst et al., 2002a).

It is thought that the difference in rates of depression may be due to women reporting more somatic symptoms such as fatigue and sleep and appetite disturbances compared to males (Angst et al., 2002b, Blanco et al., 2012). A study found that when depression was divided in to 'somatic depression' and 'pure depression' based on symptom profiles (appetite and sleep disturbances included in somatic depression only), the prevalence of 'pure depression' was similar in males and females (Silverstein, 1999).

Childhood sexual abuse has been linked to a multitude of psychiatric disorders in adulthood including anxiety disorders, eating disorders and depression (Chou, 2012, Lindert et al., 2014, Comijs et al., 2013). The rates for childhood sexual abuse are consistently found to be higher in females than males, with a rate of 18% for females and 8% for males (Stoltenborgh et al., 2011). It is unclear whether this is due to the under-reporting of abuse in males, however if it is simply due to higher rates of abuse in females it could be a cause of the increased rates of depression in females.

It has been suggested that retrospective assessment of abuse yielded stronger associations between childhood abuse and depression in later life when compared with prospective studies. This may cause concern as there can be doubts about the validity of results as recall ability and bias can influence the data retrieved. However a large study carried out to specifically address this issue found no difference in the strength of interaction between childhood abuse and depression, between retrospectively and prospectively collected data (Scott et al., 2012).

As reported in the review by Hankin and Abramson (2001), studies have found that after the age of 13 females report more negative life events as well as greater cognitive

vulnerability in response to these negative events (Hankin and Abramson, 2001, Rubenstein et al., 2015). One such cognitive vulnerability is rumination, the obsessive focus on bad feelings and experiences from the past, and it was reported that ruminative responses could predict further major depressive episodes. This may be due to people focusing their attention on negative memories instead of trying to improve their current situation or looking positively into the future (Nolen-Hoeksema, 2000, Rubenstein et al., 2015).

### **1.6.2. Biological factors**

The increase in rate of depression in females begins at puberty (~13 years and older) (Parker and Hadzi-Pavlovic, 2004), with studies showing that the stage of pubertal development characterised by Tanner stage III, the start of ovarian cycling, corresponds better to the rise in depression rates amongst females compared to using age alone (Angold et al., 1998). It is at this time that females start to experience fluctuations in reproductive hormones. However as all females experience this and only a proportion will develop depression, the cause of the female preponderance in depression beginning at this time cannot simply be explained by the increasing and decreasing levels of these hormones.

**Figure 1.4.** Hormonal fluctuation observed during the female menstrual cycle (Hassan et al., 2014).

Parker and Brotchie (2010) put forward the theory of a diathesis stress model for the cause of the gender difference in rates of depression. This model proposes that females are more biologically vulnerable to depression as well as to the social factors which often precipitate the onset of a depressive episode (Parker and Brotchie, 2010).

Ovarian hormones are believed to play a role in the aetiology of depression in women. Evidence for this comes from the fact that women are roughly twice as likely as men to suffer from depression and many women suffer from specific depressive disorders that are closely related to fluctuations in ovarian hormone levels, i.e. premenstrual dysphoric disorder (PMDD) and peripartum depression (PPD) both of which will be looked at in further detail below. More evidence for the involvement of ovarian hormones in depression comes from studies showing that oestrogen affects antidepressant treatment outcomes, specifically, treatment with SSRIs. Postmenopausal women treated with a combination of oestrogen and fluoxetine responded better to treatment compared to postmenopausal women treated with fluoxetine alone (Westlund Tam and Parry, 2003).

### 1.6.2.1. Premenstrual dysphoric disorder (PMDD)

PMDD is a mood disorder that is characterised by cognitive-affect and physical symptoms in the week before menses that has only been recently recognised as a disorder in the DSM-5. PMDD is thought to affect 3-8% of women of reproductive age (Dennerstein et al., 2012, Epperson et al., 2012). According to DSM-5, there are criteria to be met for the diagnosis of PMDD:

Criteria A is that in most menstrual cycles in the past year, at least 5 of the following symptoms were present:

- Depressed mood, feelings of hopelessness or self-deprecating thoughts
- Marked anxiety, tension
- Marked affective lability (e.g. feeling suddenly sad or tearful or increased sensitivity to rejection)
- Persistent anger or irritability or increased interpersonal conflicts
- Decreased interest in usual activities
- Subjective sense of difficulty concentrating
- Lethargy, easy fatigability or lack of energy
- Change in appetite, overeating or specific food cravings
- Hypersomnia or insomnia
- Sense of being overwhelmed or out of control
- Physical symptoms such as breast tenderness or swelling, headaches, joint or muscle pain, bloating or weight gain

Criteria B is that the symptoms must be severe enough to interfere significantly with everyday functioning.

Criteria C is that the symptoms must be discretely related to the menstrual cycle and not merely represent an exacerbation of symptoms of another disorder.

Criteria D is that all above criteria must be confirmed by prospective daily ratings during at least 2 consecutive symptomatic menstrual cycles.

The timing of onset of symptoms of PMDD suggests that hormonal fluctuations play a role in the pathogenesis of the disorder. The levels of ovarian hormones in females with PMDD are similar to females without the disorder, indicating that perhaps PMDD is caused by an increased sensitivity to normal hormonal fluctuations (Hantsoo and Epperson, 2015). Progesterone and its main metabolite allopregnanolone levels are low during menses and the follicular phase. In the luteal phase levels rise significantly before a rapid decrease at menses. This rapid withdrawal is thought to play a role in PMDD aetiology. Preclinical studies have developed a model of PMDD based on progesterone withdrawal which exhibits similar characteristics compared to clinical symptoms, for example, social withdrawal and anhedonia (Smith et al., 2006).

Research shows that oestrogen can have effects on different aspects of the serotonergic system including increased 5-HT<sub>2A</sub> receptor binding in the PFC in humans (Kugaya et al., 2003), increases expression of genes for the 5-HT<sub>2A</sub> receptor and serotonin transporter (SERT) in rats (Fink et al., 1998) and low oestrogen levels reduced SERT density in the hippocampus and the nucleus accumbens in mice (Bertrand et al., 2005). These effects of oestrogen in relation to PMDD are not understood but seem likely to play a significant role in the disorder.

### **1.6.2.2. Perinatal/Postpartum depression**

Postpartum depression (PPD) is thought to affect about 19.2% of new mothers (Gavin et al., 2005). Depression occurring around pregnancy was not classified as being distinct from depression occurring at any other time in life until the release of the DSM-IV in 1994 which outlined a 'postpartum onset specifier' which was defined as onset of a depressive episode within 4 weeks of childbirth. In DSM-5 the postpartum onset specifier was changed to 'peripartum onset' defined as the most recent episode which occurred during pregnancy or within the 4 weeks following childbirth. The criteria for diagnosis of PPD are the same in DSM-5 as in DSM-IV individuals must exhibit 5 of 9 symptoms of depression in the same 2 week period. The DSM-5 requires that the onset of symptoms occurs either during pregnancy or within the first 4 weeks postpartum and similarly the ICD-10 states that depressive symptoms must appear within 6 weeks postpartum. However, this criteria contradicts what is observed clinically as data shows that the peak time period for the onset of depressive symptoms is 2-3 months postpartum (O'Hara and Wisner, 2014, Wisner et al., 2010).

Factors that have been found to be associated with perinatal depression include having a history of depression, stressful life events, poor marital status and poor social support (Lancaster et al., 2010, O'Hara and Wisner, 2014). A history of past negative obstetrical outcomes was also a significant factor for predicting postpartum depression (Koleva et al., 2011). Research has also shown that a history of PMDD increases the risk of PPD almost two-fold (Buttner et al., 2013). Evidence of a genetic component is described in a study which found that out of 31 new mothers who had a sister with previously diagnosed postpartum depression, 29% of these were themselves diagnosed with postpartum depression, compared to 59 new mothers who did not have a sister previously diagnosed with PPD, 12% of these were followed with a diagnosis of PPD (Forty et al., 2006).

As with PMDD, ovarian hormones are also thought to play a role in the aetiology of PPD. During pregnancy, the levels of oestrogen and progesterone are higher than normal and then rapidly decline following childbirth. It is believed that that severe withdrawal of these hormones may contribute to the development of PPD (Bloch et al., 2003). In support of this, it was shown that in healthy female volunteers, pharmacologically induced fluctuations in oestradiol were associated with increased SERT levels, lowering serotonergic tone, and causing depressive symptoms, thus increasing the evidence for a causal role of hormone fluctuations in the aetiology of PPD (Frokjaer et al., 2015, Brummelte and Galea, 2016). As well as a possible role of ovarian hormones in PPD, the glucocorticoid cortisol, the main stress hormone in humans, is elevated during pregnancy which is similar to what is observed in MDD patients (Glynn et al., 2013).

Perinatal depression has been linked to multiple negative effects in pregnancy as well as in the neonate, including preeclampsia (Kurki et al., 2000), increased foetal activity (Field et al., 2008), preterm birth (Jesse et al., 2003, Orr et al., 2002), low birth weight (Grote et al., 2010a, Greene et al., 2015), as well as impaired mother-child bond and adverse effects later in childhood. Elevated cortisol is a common feature of depression and it is believed that maternal cortisol crosses the placenta, entering the foetus resulting in foetal HPA hyperactivation, which then affects foetal growth (Diego et al., 2006). Foetuses with mothers exhibiting high levels of cortisol were more likely to display growth delays at mid-gestation, including reduced head and abdominal circumference and weight (Field et al., 2006). It has also been reported that neonates born to depressed mothers show decreased response to face/voice stimuli and on alertness items suggesting they are less attentive (Field et al., 2009).

A meta-analysis of studies reporting on the early interactions between postpartum depressed mothers and their infants found that these mothers were more likely to show

irritability and hostility, to have lower rates of play interaction and show less loving emotions towards their infants (Lovejoy et al., 2000). Mothers with PPD have been shown to be less likely to engage in enriching activities with their infant such as talking to, showing picture books to and playing with their infant (McLearn et al., 2006). Data also shows that children whose mothers had depression, had increased number of A and E hospital visits in the first year of life and these children had decreased receipt of preventative services, such as age-appropriate GP visits and up-to-date vaccinations (Minkovitz et al., 2005).

Decreased levels of breastfeeding initiation has been reported in depressed mothers (Grigoriadis et al., 2013), however a more common finding in the literature is early cessation of breastfeeding. This difference in breastfeeding rates has been shown as early as 4 weeks post-partum (Dennis and McQueen, 2007) and persists at 2 and 4 months post-partum (McLearn et al., 2006).

There is limited and often conflicting information regarding associations between perinatal depression and infant mortality. Two large prospective cohort studies, one carried out in the low-income setting of rural Ghana and the other in the relatively high-income setting of Amsterdam, found no links between maternal depression and perinatal deaths, including miscarriages and stillbirths (Weobong et al., 2014, Goedhart et al., 2010). Contrasting data found that postnatal depression was associated with almost a threefold increased risk of infant mortality up to 6 months of age; reported by the same group that 1 year previous found no effects of antenatal depression on survival rates of infants. They suggest that antenatal depression is associated with infant morbidity whereas postnatal depression is linked to infant mortality (Weobong et al., 2015). In support of this finding, a large analysis of 3 nationwide datasets in Taiwan showed that

infant death was 1.47-fold higher when postnatal depression was present (Chen et al., 2011).

The effects of PPD can also be seen later in infant life with infants at greater risk of negative affectivity (Rouse and Goodman, 2014), as well as increased sleep disturbances in toddlers, including waking in the night, nightmares, difficulty getting to sleep and waking after only a few hours of sleep (O'Connor et al., 2007). There is a 50% increase in the odds of childhood developmental delay (Deave et al., 2008), and it has been shown that at 16 years old, sons born to mothers who suffered PPD showed cognitive deficits (Murray et al., 2010). A 20 year follow up study revealed that these children were also more likely to suffer from MDD and substance use disorders (Pilowsky et al., 2006).

### **1.7. Aetiological theories of depression**

The underlying neurobiology of depression is still poorly understood. There are many theories as to the aetiology of this disease however it is unlikely that any one of them is the sole cause. Depression is most likely caused by dysregulation/dysfunction of multiple systems (some of which are described below) thought to be a result of both a genetic and environmental contribution. Much of the information we have learned so far about the neurobiology of depression comes from drug discoveries. The first to be discovered were the monoamine altering drugs, MAOIs, TCAs and SSRIs, which lead to the monoamine theory of depression, and more recently the discovery of atypical antidepressants which suggest different pathways are involved in depression. Current research into the pathophysiology of depression is taking a more holistic approach, incorporating multiple factors which are believed to play a role in the onset of depression rather than solely studying brain neurocircuitry.

### 1.7.1. The monoamine theory of depression

The monoamine theory of depression is based on the assumption that dysregulation of the brain monoamine systems, particularly the serotonergic, noradrenergic and dopaminergic systems, are the cause of depression. This theory was based on the discovery that drugs which increased levels of monoamines also elevated mood and also on the finding that reserpine, a drug used to treat hypertensive vascular disease, caused a depressed mood due to inhibition of the vesicular monoamine transporter and that decreased brain levels of monoamines.

As described previously, the first class of antidepressant drugs was discovered accidentally after the finding that a drug being used to treat tuberculosis elevated the mood of the patients given it. The drug, called iproniazid, was found to have monoamine oxidase inhibitory (MAOI) properties (Deverteuil and Lehmann, 1958) which increased brain levels of monoamines via the inhibition of the enzyme monoamine oxidase, responsible for the metabolism of monoamines. Around the same time a different class of drug, tricyclic antidepressants, was developed from the antihistamine structure. The first TCA, which would be called imipramine, was originally tested as an antipsychotic, however a 'side-effect' of the drug was an elevated mood in patients (Kuhn, 1957). It had a much better side-effect profile compared to MAOI drugs, which led to its approval by the FDA for the treatment of MDD in 1959.

Following the discovery and use of these drugs for the treatment of depression, two separate monoamine theories emerged, each supporting a different monoamine system as being responsible for depression. An American psychiatrist, Joseph Schildkraut, believed that altered brain catecholamine levels were the cause of the depression (Schildkraut, 1965), whereas a British psychiatrist published his theory that serotonin was the main monoamine involved in depression pathogenesis (Coppen, 1967). Popularity for the

belief that decreased serotonin levels in the brain was the cause for depression grew in the 1960's leading to race between pharmaceutical companies to develop a molecule based on this theory. In 1987 fluoxetine was the first of the SSRI drugs to be approved (Hillhouse and Porter, 2015). SSRIs are now the most commonly prescribed form of antidepressant medication (Mars et al., 2017).

**Figure 1.5.** Prevalence of antidepressant prescriptions by drug class. Image taken from (Mars et al., 2017).

The monoamine theory came about through accidental discovery that increasing monoamine levels in the brain causes antidepressant effects. However a problem with the theory is that a period of at least 2-3 weeks is necessary before antidepressant drugs cause a reduction in depressive symptoms even though monoamine levels are increased shortly after taking them, i.e. within a couple of hours. This leads to the conclusion that depression is not solely caused by altered monoamine levels but perhaps by downstream consequences. Antidepressant treatment has been shown to increase neurogenesis, which is the formation of new neurons, in the hippocampus of adult rats which may explain the

mechanism by which treatment with antidepressant drugs exert their effects (Dranovsky and Hen, 2006, Malberg et al., 2000). In support of this, a study has shown that ablation of neurogenesis blocks the effects of antidepressant drugs on decreasing anhedonic- and depressive-like behaviours in rodents (David et al., 2009). Chronic antidepressant treatment has also been shown to modulate glutamatergic signalling in a neuroprotective manner (Pittenger et al., 2007, Witkin et al., 2007) as well as increasing neuroplasticity-related signalling pathways including the upregulation of the cAMP–PKA–CREB cascade (Donati and Rasenick, 2003) and the MAPK signalling cascade (Tiraboschi et al., 2004).

**Figure 1.6.** The human serotonergic system. The cell bodies of serotonergic neurons are found in the raphe nuclei which project to various brain areas including the cerebellum, thalamus, cortex, prefrontal cortex, basal ganglia and the components of the limbic system. The various 5-HT receptor subtypes have specific distributions in the brain. Depicted above are the most prominent 5-HT receptor subtypes in the brain regions innervated by serotonergic neurons; the receptors can be present as either pre- or post-synaptic receptors. Image from (Visser et al., 2011).

There is a growing body of research on the effects of oestrogen on the serotonergic system in multiple brain regions associated with depression. The raphe nuclei is a major source of serotonergic neurons in the brain and research has shown the oestrogen elicits great

effects on serotonin and its metabolites in the raphe nuclei. Both oestrogen receptors (ER)  $\alpha$  and  $\beta$  are found in the raphe nucleus (Sheng et al., 2004). ER- $\beta$  has been identified on serotonergic neurons in non-human primates (Gundlah et al., 2001). In pre-clinical studies it has been shown that oestradiol administration increased tryptophan hydroxylase (TPH), the rate limiting enzyme for serotonin synthesis, mRNA in the dorsal raphe nucleus (DRN) and reduced depressive-like behaviour in the forced swim test (Donner and Handa, 2009). Peripheral administration of oestradiol has been found to increase TPH protein levels in the DRN of ovariectomised macaques (Bethea et al., 2000). Oestradiol also has modulatory effects on the serotonin transporter (SERT) in the DRN, although the exact effects are inconclusive with some showing upregulation of SERT mRNA in ovariectomised rats (Charoenphandhu et al., 2011, McQueen et al., 1997) but yet downregulation in ovariectomised macaques (Pecins-Thompson et al., 1998).

**Figure 1.7.** Simplified diagram of the effects of oestradiol on the serotonergic system and BDNF. Image: (Borrow and Cameron, 2014).

The effects of oestradiol in the serotonergic system in the hippocampus are unclear with much contradicting data. Models of low oestradiol levels in mice show decreased density of SERT in the hippocampus (Bertrand et al., 2005). However levels of SERT have also been shown to not be impacted by oestradiol in rats (Charoenphandhu et al., 2011). Oestradiol given at a dose similar to that seen in the proestrous stage of the oestrous cycle caused inhibition of the SSRI fluvoxamine's effect on 5-HT clearance and decrease depressive-like behaviour in the FST and local administration of oestradiol alone was shown to decrease SERT function measured by slower 5-HT clearance (Benmansour et al., 2012). While a supraphysiological dose of oestradiol decreased SSRI binding in the hippocampus in female rats (Mendelson et al., 1993).

The amygdala, which is involved in stimulus and emotional processing, can be affected by changing levels of oestrogen, with amygdalar activity highest during the luteal phase of the menstrual cycle, when oestradiol is also highest (Ossewaarde et al., 2010). The amygdala responds to both aversive and appetitive stimuli with evidence pointing to greater involvement in the processing of aversive stimuli. Amygdalar activation is heightened in women with PMDD, as it was shown they have an increased amygdala response to negative stimuli compared to asymptomatic women (Protopopescu et al., 2008). In preclinical research, oestradiol appears to decrease 5HT<sub>1A</sub> receptor binding (Osterlund et al., 2000) while it increases the number of cells that express SERT mRNA (McQueen et al., 1997). These effects of oestrogen on the serotonergic system in brain regions associated with depression suggest a role of oestrogen in the pathophysiology of depression.

### **1.7.2. The neurotrophic hypothesis for depression**

The neurotrophic theory of depression suggests that a loss/decrease in neurotrophic factors (NTFs) may play a role in the onset of depression. NTFs are biomolecules that promote the survival, growth and differentiation of both mature and developing neurons. Brain-derived neurotrophic factor (BDNF) is the most studied NTF with regards to links with depression. Briefly, it is thought that decreased levels of BDNF caused by stress leads to neuronal atrophy and decreased neurogenesis in the hippocampus, a key limbic region implicated in depression. Neurogenesis is the formation of new neurons which in the adult occurs primarily in the hippocampus (Eriksson et al., 1998). Decreased numbers of granule cells and reduced overall hippocampal volume in depressed patients support the theory of reduced neurogenesis (Boldrini et al., 2013).

Decreased hippocampal volume is a common finding in imaging studies of patients with depression (Gerritsen et al., 2015, Videbech and Ravnkilde, 2004, McKinnon et al., 2009). Smaller hippocampal volume has been associated with decreased rates of remission with traditional antidepressant drugs (MacQueen et al., 2008, Frodl et al., 2008). The hippocampus has important roles in learning and memory and regulation of the HPA axis. It also has connections to the amygdala and prefrontal which are involved in emotion and cognition and are linked to major symptoms in depression.

The method by which stress causes a reduction in BDNF has not yet been fully elucidated although it is thought that activation of the HPA axis and release of glucocorticoids mediates the effects on BDNF. This has been investigated in animal studies that have manipulated the levels of glucocorticoids. Adrenalectomy increased BDNF mRNA levels in the rat hippocampus (Chao et al., 1998) and exogenous corticosterone administration decreased expression of BDNF mRNA and protein in the rat hippocampus (Schaaf et al., 1998).

Many animal models of depression use stress, both acute and chronic, as a method of inducing a depressive-like phenotype. This is relevant clinically as depression is often caused or worsened by stress and negative life events. It is important to note that rats exposed to a restraint stress exhibit a reduction of BDNF mRNA levels in the hippocampus (Smith et al., 1995, Molteni et al., 2016).

Clinically it is difficult to investigate the role of BDNF in depression because hippocampal levels cannot be measured unless taken from post-mortem samples. BDNF can be measured in the serum, however it is unclear what pathophysiological relevance this serum BDNF has in depression. Nevertheless, a decreased level of BDNF in the serum of depressed patients is a common finding (de Azevedo Cardoso et al., 2014, Pallavi et al., 2013, Fornaro et al., 2015).

Both physical activity and antidepressant treatment have been shown to increase levels of BDNF mRNA in the hypothalamus of rats (Russo-Neustadt et al., 2000, Nibuya et al., 1996). Further to this, it was found that mice expressing a truncated form of the BDNF receptor TrkB protein did not exhibit decreased depressive-like behaviour following antidepressant treatment suggesting BDNF signalling is required for the functional effects of antidepressant treatment (Duric and Duman, 2013). Further evidence for this comes from a study that reported no alteration in depressive-like behaviour in a BDNF knockout mouse, however loss of BDNF in the dentate gyrus and CA1 region of the hippocampus attenuated the effects of both desipramine and citalopram in the FST (Adachi et al., 2008).

This finding has been replicated in humans with increased levels of BDNF reported in post-mortem hippocampal samples taken from patients diagnosed with depression and being treated with anti-depressants at the time of death (Chen et al., 2001). In addition to this, it has been found that only patients that respond to antidepressant treatment exhibit increased plasma levels of BDNF following 12 weeks of treatment while non-responders

actually displayed decreased levels between week 8 and 12 of treatment (Kurita et al., 2012). This observation is supported by other findings which show increased plasma levels of BDNF following ketamine administration in those who responded to the treatment but not non-responders (Haile et al., 2014).

Oestrogen receptors are expressed in the hypothalamus and are thought to modulate BDNF activity in this brain region. Ovariectomised female rats treated with proestrous levels of oestradiol exhibited increased BDNF levels as well as increased serotonin turnover (Kiss et al., 2012). Similar to the above study, treatment with proestrous levels of oestradiol increased vascular endothelial growth factor (VEGF) levels; VEGF being another neurotrophic factor (Barouk et al., 2011). High doses of oestradiol have been shown to increase BDNF mRNA expression in the amygdala (Zhou et al., 2005). Clinically, while it is unknown if oestradiol affects neurotrophin expression in the brain, it has been documented that plasma levels of BDNF vary across the menstrual cycle (Begliuomini et al., 2007).

### **1.7.3. The neuroinflammatory theory of depression**

Although research into the effects of the immune system in the aetiology of depression has gained much support in recent years, it was first proposed in the early 1990's when Smith suggested depression was caused by increased IL-1 levels (Smith, 1991). IL-1 is a cytokine which are a family of large proteins that are released by immune cells. Cytokines' roles are to signal between immune cells and act as immunomodulating agents. Cytokines are generally divided into pro-inflammatory and anti-inflammatory cytokines depending on whether they exert facilitatory or inhibitory actions. Anti-inflammatory cytokines regulate the activity of pro-inflammatory cytokines, however if

the balance between them becomes unregulated it can lead to excessive or chronic inflammation.

Levels of pro-inflammatory cytokines, such as IL-1 $\beta$ , TNF- $\alpha$ , and IL-6 are reported to be increased in depressed patients (Dowlati et al., 2010, Felger and Lotrich, 2013). Symptom severity has also been shown to be positively correlated to an overexpression of pro-inflammatory cytokines (Suarez et al., 2003).

IL-6 and TNF- $\alpha$  have also been reported to be elevated in rats following both an acute and chronic stress (Himmerich et al., 2013). Peripheral administration of lipopolysaccharide (LPS), a component of bacteria that induces an immune response, or of pro-inflammatory cytokines, induces a phenotype in rats termed ‘sickness behaviour’ that mimics the behavioural phenotype of depression that is observed clinically, including decreased food consumption, anhedonia and increased sleep (Dunn et al., 2005).

Another strand of evidence which would suggest that a role for inflammation in the aetiology of depression is that depression is common in people suffering from autoimmune diseases, e.g. multiple sclerosis (MS) (Feinstein et al., 2014), type-1 diabetes (Holt et al., 2014) and rheumatoid arthritis (Matcham et al., 2013).

The role of microglia in depression has become common thread of research with evidence for both microglial activation and suppression contributing to the pathogenesis of depression. Briefly, microglial activation has been reported in animal models of stress (Sugama et al., 2009, Sugama et al., 2007, Frank et al., 2007), and antidepressant drugs, including SSRIs (Hashioka et al., 2007) and TCAs (Obuchowicz et al., 2014) were found to have microglia-suppressive properties. Microglia become activated via signals sent from peripheral immune cells in response to detection of bacterial or viral threats. Normally these signals between peripheral immune cells and microglia are mediated by the blood brain barrier and the choroid plexus, however under pathological conditions

these barriers do not function correctly and immune cells enter the brain parenchyma and modulate the immune response from within (Yirmiya et al., 2015). Stress can also cause microglial activation via glucocorticoid action (Sorrells and Sapolsky, 2007). A mechanism by which it is thought microglia cause depression is through the suppression of neurogenesis (Sierra et al., 2014, Ekdahl, 2012) mediated through secretion of IL-1. Chronically stressed mice exhibited reduced hippocampal neurogenesis which was not observed in IL-1 receptor knock out mice (Goshen et al., 2008).

It is known that gonadal steroids have the ability to modulate immune functions. Pre-clinical work has demonstrated the ability of oestradiol to decrease cytokine production by decreasing NF- $\kappa$ B binding in the dorsal raphe nucleus of macaque monkeys (Bethea et al., 2006). Ovariectomy in rats resulted in increased levels of IFN- $\gamma$  and IL-6 production as well as increased levels of indoleamine-2,3-dioxygenase (IDO), an enzyme that degrades tryptophan to kynurenine (KYN) and causes decreased production of 5-HT, in the hippocampus. Treatment with oestradiol resulted in decreased inflammation and IDO levels, which suggests that oestrogen plays a role in regulating inflammation and serotonin levels (Xu et al., 2015). Again in ovariectomised rats, treatment with omega-3 polyunsaturated fatty acids (PUFA) and oestradiol resulted in increased BDNF levels and decreasing hippocampal expression of IL-6 and TNF- $\alpha$  (Jin and Park, 2015).

#### **1.7.4. The HPA Axis theory of depression**

In times of stress, the first reaction of the immune system is to activate the hypothalamus-pituitary adrenal (HPA) axis which is the body's ventral stress response system. The hypothalamus secretes corticotrophin releasing hormone (CRH) which bind to receptors in the pituitary and stimulates the release of adrenocorticotrophic hormone (ACTH). This hormone travels via the systemic circulation to the adrenal glands where it causes the release of glucocorticoids, cortisol in humans and corticosterone in rodents. These

glucocorticoids bind to glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs) to exert their physiological actions. When corticosteroid levels are chronically elevated, a GR-mediated negative feedback loop is activated which prevents the release of more corticosteroids at the level of the hypothalamus by preventing the release of CRH. In depression, it is believed that this negative feedback loop is impaired leading to chronically elevated levels of corticosteroids. This has been supported by the finding that MDD patients exhibit elevated levels of plasma, urine, salivary and CSF cortisol levels (Nemeroff and Vale, 2005, Murphy, 1991, Bhagwagar et al., 2005). People who have recovered from depression also have increased waking salivary cortisol levels (Bhagwagar et al., 2003) as well as those who have never presented symptoms but have a family history of depression (Mannie et al., 2007). Antidepressants may enhance GR sensitivity in rat brains which may restore the negative-feedback inhibition (Peiffer et al., 1991).

Early-life stress including childhood physical, sexual and emotional abuse are risk factors for the development of depression in later life (Dube et al., 2001, Heim and Nemeroff, 2001). Studies have shown that early-life stress can have lasting effects on the ability of the HPA axis to respond to stress, possibly causing a susceptibility to depression in later life (Von Werne Baes et al., 2012, Nemeroff et al., 2003).

When activated by stress, the HPA axis exerts an inhibitory effect on the female reproductive system. CRH and CRH-induced proopiomelanocortin peptides, inhibit GnRH secretion from the hypothalamus, and glucocorticoid administration has been shown to reduce the response of luteinising hormone to GnRH (Kalantaridou et al., 2004). These effects of the HPA axis on the reproductive endocrine system are responsible for amenorrhea often seen clinically in patients with anxiety and depression.

## **1.8. Animal models in preclinical research**

The use of animals in preclinical research has been essential in allowing for progress in biomedical research, with uses in both investigating pathophysiology of diseases as well as the search for and development of new treatments. Animal research formed the basis of our understanding of normal physiology however these results are only valid under the assumption that the mechanisms are conserved across evolution (Insel, 2007). An ideal animal model is one which replicates the human disease phenotype as well as the underlying mechanism of action. Multiple species are used in preclinical animal research and the area of research affects the choice of species used as certain species have benefits for their use in particular fields of research, e.g. humans and swine share similar cardiovascular systems and so swine species are used in this topic of research more commonly than research into neurodegenerative diseases for instance (Vandamme, 2015). As non-human primates (NHPs) are our closest living relative, it could be argued that these species are the best for use in preclinical research as they would best replicate human subjects, however, use of NHPs is limited due to expense and ethical considerations.

Studying animal models has advantages over clinical studies when it comes to experimental design as background variables including genetics can be controlled to a much greater extent in lab animals. However, there are also a number of limitations that arise from the use of animals in preclinical research (described later in more detail), which include a lack of reproducibility of results, an inability to create more accurate animal models of disease and low predictive validity for human response to drugs (Greek and Menache, 2013). Rodents and fish are estimated to make up 95% of all animals used in preclinical research (Vandamme, 2015).

Genetic manipulation may be the greatest advantage in animal studies. Different strains of a species can be produced by controlled breeding which can allow for certain disease models to be produced. An example of a model produced this way is the Wistar Kyoto strain that was originally bred from the Wistar rat as a normotensive control strain for the spontaneously hypertensive rat. It was later found to exhibit certain behavioural and physiological differences from other rat strains that resembled clinical symptoms of depression leading to its use as a model of depression (described in more detail in later chapters).

Recent advances in genetic engineering allow for the investigation into specific genes and their function. Animal models can be created which have had specific gene/s knocked out, i.e. the apolipoprotein-E (apoE) knockout mouse model of atherosclerosis (Stevens and Vaccarino, 2015). On the other hand, knockin and transgenic animals are created by engineering specific sequence changes that cause gain of function mutations. This involves one-for-one replacement of sequences in the genetic locus of interest allowing for the introduction of specific genetic anomalies based on clinical scenarios or previous work (Doyle et al., 2012). Knockin disease models for long QT syndrome (Nilles and London, 2007) and 5-HT deficiency (Jacobsen et al., 2012), have been developed.

Before new drug compounds can be trialled clinically they must first be tested on animals in order to determine the safety margins, toxicity and efficacy. In recent years, there has been a gradual decline in drug discovery research being carried out by pharmaceutical companies, particularly in neuropharmacology, with one reason for this thought to be due to the inability to develop valid models of diseases on which to accurately test drug compounds (McGonigle and Ruggeri, 2014). The value of animal models in testing new drug compounds is being doubted. Out of all animal studies published in seven high impact factor journals over the course of one year, only one-third were taken to the stage

of clinical randomised trial and of these only one-tenth of the treatments studied were approved for use in humans (Hackam and Redelmeier, 2006).

A large survey investigating the reliability of animal studies carried out in the UK and US found that many publications ‘fail to report important information regarding experimental and statistical methods’. The most common pieces of missing information included sex, strain, age and weight of animals used, the number of subjects used and statistical methods used. Worryingly only a small number of studies reported using random allocation of animals to treatment groups and blinded assessment, without which bias can be introduced which will decrease the validity of findings (Kilkenny et al., 2009). One way to increase the reliability and reproducibility of animal research studies is to ensure that all necessary and relevant information about how the experiment/s were performed is published. Guidelines have been put in place in order to inform researchers on what information should be included when describing their research. The ARRIVE guidelines (Animals in Research: Reporting *In Vivo* Experiments) are one such set of guidelines consisting of a 20 item checklist of the minimum level of information that all publications reporting experiments using animal subjects should report, e.g. the number, species/strain, sex of animals used, details of animal housing and husbandry, experimental design and procedures and statistical analysis used (Kilkenny et al., 2010).

Another way to increase reproducibility of preclinical studies is to standardise testing procedures to minimise the number of different protocols used in labs. This will allow for experiments carried out in different labs to be compared more easily and will also allow for systematic reviews to be more readily performed on preclinical data. Systematic reviews on animal research can aid in design and implementation of future studies (de Vries et al., 2014).

### **1.8.1. Animal models of depression**

There is a limit to the amount and type of information that can be obtained solely through clinical trials because of the many ethical objections to human testing. Most clinical trials investigate the epidemiology of MDD and responses to current treatments as it is difficult to examine the underlying neurological causes of the disorder. Neuroimaging is one of the most used methods for studying depression clinically. Magnetic resonance imaging (MRI) studies are used for analysing brain structure and function in depressed patients and reports have revealed altered prefrontal cortex and hippocampus structure in depressed patients (Dusi et al., 2015). MR Spectroscopy can be used in patients to measure the concentration of compounds in the brain; it has been used to study the neurobiology of depression and to predict treatment response (Lee et al., 2014). Post-mortem human brain tissue has been studied in the past however the information that can be obtained from this type of investigation is limited. Animal models of neuropsychiatric disorders are used to get a better understanding of the human disorder and to attempt to elucidate the aetiology of the disease being studied. They allow for the examination of neural circuitry and cellular and molecular pathways that may play a role in the pathogenesis of depression. However as there are significant anatomical differences between human and rodent brains, as can be seen in figure 1.8., data obtained from preclinical research must be appropriately translated to the clinical scenario.

There have been few breakthroughs in the research of neuropsychiatric illnesses in the recent past which has led to the development of a new Research Domain Criteria (RDoC) by the National Institute of Mental Health (NIMH) which aims to integrate different aspects of basic research in order to develop our understanding of the underlying processes and pathophysiology of mental disorders (Clark et al., 2017). Overreliance on symptom-based diagnostic systems, such as the DSM and ICD are thought to be contributing to this slow progress in understanding the aetiology of psychiatric disease.

The RDoC aims to classify mental disorders based on fundamental behavioural and brain mechanisms and to provide a framework for conducting research on psychopathology without the use of categorical, symptom-based diagnostic systems (Cuthbert, 2014). This may imply that we will see a move away from classical models of disease to the modelling and research of behaviours and cognition without making conclusions about specific psychiatric diseases.

**Figure 1.8.** A diagram comparing the mature human and rat brain structure ([learn.genetics.utah.edu](http://learn.genetics.utah.edu)).

Currently animal models are generated by attempting to replicate factors believed to cause human conditions, for example chronic stress as a model for depression. They can also

be created by modelling a known disease mechanism, e.g. gene knockout models. The second method works well for creating animal models of neurological diseases of which the aetiologies are known, however as the underlying neurological causes of depression are still unclear which makes generating valid models of the disease much harder.

Animal models of depression aim to reproduce only a small number of the clinical characteristics of depression. The characteristics modelled must be easily measurable and so tend to be based on the physical symptoms of depression, i.e. activity levels, response to reward. As it is not possible to ascertain what the animals are feeling, tests are also used to infer the levels of anxiety and depressive-like behaviours exhibited by the animal.

The first to develop minimum requirements that an animal model of depression should meet were McKinney and Bunney in 1969. They proposed 5 criteria which were:

1. “The symptoms of the depression so induced should be reasonably analogous to those seen in human depression
2. There should be observable behavioural changes which can be objectively evaluated
3. Independent observers should agree on objective criteria for drawing conclusions about the subjective state
4. The treatment modalities effective in reversing depression in humans should reverse the changes seen in animals
5. The system should be reproducible by other investigators” (McKinney and Bunney, 1969).

These criteria were then condensed into the measures that are now used to determine the legitimacy of animal models of depression: face, construct and predictive validity. Face validity refers to the ability of the model to replicate the clinical features of depression,

construct validity examines whether the basis the model is developed on follows a plausible explanatory theory for human depression. Predictive validity refers to the responsiveness of the model to antidepressant treatment (Willner, 1984).

In order to assess the validity of a model different behavioural tests must be carried out in order to determine if they possess a depressive-like phenotype (face validity) and if they respond to antidepressant treatment (predictive validity). There are many behavioural tests used in preclinical research, each assessing different aspects of cognition and behaviour. The most commonly used tests of depressive- and anxiety-like behaviours will be described in the next few paragraphs.

The forced swim test (FST), developed by Porsolt et al, is the most common test for screening antidepressant activity and also measuring depressive-like behaviour in animals. The most widely used protocol consists of two exposures to an inescapable swim. The rat is placed into a cylindrical vessel filled with warm water so that the rat is unable to touch the bottom. This initial swim lasts for 15 minutes (pre-swim) and 24 hours later they are re-exposed for a further 5 minutes (test swim). The behaviour in the test swim is scored based on whether the rat exhibits an immobile posture or active behaviours. Immobility is interpreted as a hopelessness and lack of motivation in the animal, termed 'behavioural despair' (Porsolt et al., 1977, Porsolt et al., 1978). When testing for antidepressant efficacy, typically a 3 dose regime is used which are administered 24 hours, 5 hours and 1 hour before the test swim. A reduction in the time spent immobile by the rat is indicative of antidepressant activity.

The open field test (OFT), first described in 1932 by Hall, examines locomotor activity as well as anxiety-like behaviours in rats. The apparatus used for the OFT can vary between laboratories but usually consists of an open circular or square arena surrounded by high walls which are typically made of a reflective material. Bright lighting is also

commonly used during testing to create a more stressful environment and so make it easier to detect anxiety-like behaviours. The rat is placed into the centre of the arena and behaviours such as distanced moved and rearing are measured to score locomotor activity and time spent in the centre of the arena and defaecation are scored to measure anxiety-like behaviour. The trials commonly last 5 minutes but can range from 3-10 minutes. Rats will spend most of the trial against the walls of the arena as the bright, open space of the centre is anxiogenic for the animals and so animals that spend more time in the centre are thought to be less anxious than those that spend little time there. Similarly, drugs that cause rats to spend more time in the centre of the arena are considered to have anxiolytic effects.

<b>Ref.</b>	<b>Arena</b>	<b>Duration</b>	<b>Lighting</b>
<b>(Rinwa and Kumar, 2014)</b>	Circular, 80cm diameter	5 mins	No info. given
<b>(Yang et al., 2014)</b>	Not stated	5 mins	Bright
<b>(Kalshetti et al., 2015)</b>	Square, 50 cm x 50 cm	5 mins	Bright
<b>(Pochwat et al., 2015)</b>	Circular	3 mins	Bright
<b>(Borre et al., 2012b)</b>	Square	5 mins	Low lighting
<b>(Wang et al., 2012)</b>	Square	5 mins	Low lighting

**Table 1.3.** Differences in OFT procedure used in various laboratories when testing OB rats.

The elevated plus maze (EPM) is another common test of anxiety-like behaviour. It was first validated as a measure of anxiety in the rat by Pellow et al in 1985 (Pellow et al., 1985). The apparatus consists of a raised plus shaped platform with two open and two closed arms. The rat is placed in the crossing point of the arms and allowed to freely

explore. Typically rats will spend more time in the closed arms as the open arms are anxiogenic. By scoring the time spent in the open versus closed arms you can get an indication of whether the model exhibits anxiety-like behaviours. The EPM can also be used to screen for anxiolytic drugs as there will increase the time spent in the open arms by the rat.

Below is a table summarising some of the different behaviours that can be measured in animal models of depression and how they are correlated to symptoms of depression observed clinically.

<b>Test</b>	<b>Response</b>	<b>Clinical correlate</b>
<b>Forced Swim Test</b>	Immobility	Despair
<b>Open Field Test</b>	Less time in centre	Anxiety
<b>Open Field Test</b>	Freezing	Anxiety
<b>Open Field Test</b>	Hyperactivity	Psychomotor Agitation
<b>Sucrose Preference Test</b>	Less sucrose intake	Anhedonia
<b>Female urine sniffing test</b>	Reduced sniffing time	Anhedonia
<b>Elevated Plus Maze</b>	Less time in open arms	Anxiety
<b>Elevated Plus Maze</b>	More time in open arms	Less anxious
<b>Marble Burying test</b>	Increased burying	Anxiety
<b>Novelty Induced Hypophagia</b>	Less food intake	Anxiety
<b>Stress induced Hypothermia</b>	Decrease in body temperature	Physiological symptoms assoc. with anxiety

**Table 1.4.** Tests of rodent anxiety-like and depressive-like behaviour and their clinical correlate.

The tests described above should not be confused with models of depression. These tests are performed on animal models to enable us to get a measure of the depressive and anxiety-like phenotype of the model being studied. There are different types of models

of depression based on the phenotype inducing conditions used to generate the model. There are three main classes of models of depression, stress models, genetically modified models and drug/surgically induced models.

As stress is believed to play a role in the onset of clinical depression, animal models of depression have been developed by inducing a depressive-like phenotype following exposure to various forms of stress. An example of a clinically relevant type of stress that can be replicated preclinically is the early-life stress model of depression. As mentioned previously, in the clinical scenario, early life stress such as abuse and being raised by parents suffering from depression, can increase the risk of developing depression later in life. Preclinically, methods for inducing early life stress in pups involve a reduction in the time or quality of maternal care. Maternal separation (MS) is a common model for early life stress in rats. There are different variations of this model carried out with the most commonly used method involving the removal of the mother from the litter of pups for a number of hours during the neonatal period. Variations include separating rat pups from their mothers and litter mates and isolating them for a period of time daily (Wang et al., 2015c), and there are also different protocols for the length of time of separation and at what point in the neonatal period the separation is performed (Freund et al., 2013). Exposure to stress in children in the prepubertal period is related to depression and suicidality later in life whereas stresses suffered after puberty are linked to post traumatic stress disorder (PTSD) (Schoedl et al., 2010). A prolonged MS protocol involving removing the litter from the dam for three hours twice daily from PND 2 to PND 20 found that corticosterone was significantly increased in these pups and concentrations of 5-HT and expression of 5-HT<sub>1</sub> receptor mRNA were altered in discrete brain regions at multiple time points during MS (Ohta et al., 2014). A less severe MS protocol involving removal of the litter from the dam for one three-hour period per day from PND 3-15 found no effect of this stress on the pups' HPA axis and also concluded

that it protected against chronic stress exposure later in life (Biggio et al., 2014). Another approach which used a slightly different protocol involving the removal of pups from the dam for three hours per day from PND 2-14, showed depressive-like behaviour in the FST and deficits in spatial memory in adulthood which were reversed by chronic antidepressant treatment (Couto et al., 2012). Differences in results and methods used in these studies highlight the need for correct reporting of all information regarding how the study was carried out. It could also be suggested that a standard protocol for MS is adopted across lab groups in order to make comparisons between results easier.

One of the most common stress-induced animal models of depression is the chronic mild stress (CMS) model which was first developed by Willner et al in the early 1990s. Earlier work by Katz et al showed severe stressors elicit a depressive-like profile in rats, with their main behavioural finding being reduced locomotor activity as measured using the OF test (Katz et al., 1981). Willner created a stress model similar to the one Katz et al. worked on but with 2 major differences, first, the stressors were much milder and second, the main behavioural output to be examined was sucrose preference which is correlated to the clinical symptom, anhedonia (Willner, 1997). The CMS model is produced by carrying out a series of mild stressors over a sustained period of time, at least 2-3 weeks. The stressors are unpredictable, short in duration and are varied regularly, common stressors include periods of food and water deprivation, tilted cage and reversal of light cycle. After 2-3 weeks of the stress protocol, there is a reduction in consumption of a weak sucrose solution in the stressed group (Willner et al., 1987, Willner et al., 1992) which lasts as long as the stressors are applied. Another test that has been used to measure anhedonia in the CMS model is conditioned place preference (Bergstrom et al., 2008). This decrease in sucrose and conditioned place preference in the rat are correlated to anhedonia, a major symptom of clinical depression. The chronic and episodic nature of the model make it one of the more realistic animal models of depression.

The CMS model does not exhibit anxiety-like behaviours in two commonly used tests of anxiety and social anxiety, the EPM and social interaction test (D'Aquila et al., 1994). This would suggest that this is a model of depression only and not depression with comorbid anxiety-like features.

As well as face and construct validity, the CMS model exhibits predictive validity as tricyclic antidepressants, including amitriptyline, desipramine and imipramine, have been shown to be effective at reversing CMS induced anhedonia (Orsetti et al., 2007, Willner et al., 1987, Muscat et al., 1990, Duda et al., 2016). The SSRIs fluoxetine, sertraline and citalopram have also been shown to be effective at reversing the CMS-induced decrease in sucrose intake (Muscat et al., 1992, Marona-Lewicka and Nichols, 1997, Przegalinski et al., 1995).

The CMS model is regarded to be the model of depression with greatest validity and translational potential, however there have been criticisms that the model is unreliable as it can be difficult to establish the phenotype. Even the group that first described the model had difficulty re-establishing the stressor-induced depressive-like phenotype following a move of the animal laboratory (Willner, 1997). Even with these criticisms, the CMS model has been validated in 180 labs in over 30 countries by 2015 suggesting that the problem of unreliability is not as serious as once thought (Willner, 2017).

### **1.8.2. The Wistar Kyoto genetic model of depression**

The Wistar Kyoto (WKY) rat strain was originally bred as a normotensive control strain for the spontaneous hypertensive line rat but is now widely used as a genetic model of depression.

One of the first groups to describe the depressive-like phenotype of the WKY was Paré et al in 1989. They described a 'behavioural despair' exhibited by this rat strain as well

as a susceptibility to the development of stress ulcers (Pare, 1989). This lab group went on to characterise the depressive-like phenotype of the WKY strain, with their results being highly replicated leading to the WKY strain becoming a commonly used model of depression and anxiety.

### **1.8.2.1. Face validity of WKY model**

Paré et al. first described an increased immobility in the WKY strain in 1989, with numerous studies replicating this finding of increased immobility in WKY compared to both Sprague-Dawley controls (Lopez-Rubalcava and Lucki, 2000, Carr et al., 2010) and Wistar controls (Tejani-Butt et al., 2003, Nagasawa et al., 2015). This behaviour in the FST is considered to be a depressive-like behaviour in rodents which is paralleled to depressed mood and feelings of hopelessness in human patients. It has also been found that male but not female WKY rats exhibit an anhedonic-like behaviour in the sucrose preference test (Burke et al., 2016), which again is indicative of a depressive-like phenotype.

As depression and anxiety are often clinically comorbid, animal models of depression may also exhibit increased anxiety-like behaviours in various behavioural paradigms. A common finding with the WKY strain is a decreased locomotor activity in the open field test which involves being exposed to a brightly lit, aversive arena (Ferguson and Gray, 2005, Luo et al., 2015, Chen et al., 2014). This hypo-locomotive response is not apparent in a familiar environment as Burke et al. found no difference in locomotor activity when monitored over a 24h period in the animals home cage (Burke et al., 2016). The control strain used in studies should also be carefully chosen as this can affect the results obtained, for example Browne et al. found a difference in locomotor activity only when comparing WKY against Wistar rats and not SDs (Browne et al., 2015). This study highlights the importance of choosing the appropriate control strain for comparison

against the WKY rat; while the Wistar strain has advantages as it is the original strain from which the WKY was derived, the SD strain is the more commonly used rat strain in research and so could be considered as more of a normal baseline strain to which comparisons can be made.

Differences in behaviour in the EPM have also been found in the WKY strain. A common finding is decreased entries into and time spent in the open arms of the EPM arena (Langen and Dost, 2011, Shepard and Myers, 2008) as well as increased time spent in the centre zone of the arena (Nam et al., 2014). These results indicate increased anxiety-like behaviours in the WKY model.

#### **1.8.2.2. Construct validity**

As mentioned above, the WKY strain was originally bred as a control strain to the spontaneously hypertensive rat (SHR) strain, meaning that the depressive- and anxiety-like behaviours in this model occurred by chance and not by a particular construct design. Even so, the model does present with certain neurotransmitter and neuroendocrine abnormalities that are observed in the clinical scenario. WKY rats were found to have lower serotonin (5-HT) concentrations in various discrete brain regions including the lateral hypothalamus, dorsal hypothalamus, nucleus accumbens shell, dorsal raphe nucleus and the substantia nigra. Levels of noradrenaline were also found to be lower in the WKY strain in the ventral hippocampus and lateral hypothalamus compared to Sprague-Dawley and Wistar strains respectively (Scholl et al., 2010, Yamada et al., 2013, Pare and Tejani-Butt, 1996). These alterations in neurotransmitter systems in the WKY model are important as the monoamine theory of depression is what most antidepressant drugs are based on and there is much evidence pointing to dysfunction in the serotonergic system in particular in clinical depression.

The genetics underlying the anxiety- and depressive-like phenotype of the WKY model have not been fully elucidated, however a number of studies have attempted to shed light on the genes/genetic mutations in WKY rats that cause these behaviours. Quantitative trait locus (QTL) analysis is a statistical method that links phenotypic and genotypic data in an attempt to explain the genetic basis of variation in traits. Several QTL have been identified for the FST behaviours- swimming, climbing and immobility, in the WKY rat which share overlapping candidate regions for emotionality QTL in rodents and human loci for depression (Solberg et al., 2004).

Another genetic component of the WKY model is that in the locus coeruleus (LC), genes encoding for the synthesis and catabolism of norepinephrine, i.e. tyrosine hydroxylase and monoamine oxidase, were more highly expressed in WKY compared to SD rats (Pearson et al., 2006). As well as this, WKY rats show a 4-7 fold increase in levels of catechol-O-methyltransferase (COMT) mRNA, COMT being the enzyme involved in catecholamine degradation, in the cerebral and frontal cortices compared to SD rats (Walker et al., 2004). As NE is one of the neurotransmitter systems thought to be involved in human depression, these findings may suggest that a dysfunction in NE turnover leads to the phenotype observed in this rat strain.

Brain levels of BDNF have been shown to be decreased in the WKY rat, particularly in the frontal cortex and the hippocampus (Vinod et al., 2012). Chronic stress in the form of restraint stress has been shown to cause a significant decrease in serum BDNF in WKY rats compared to similarly stressed SDs (Karege et al., 2002). This is similar to the clinical scenario as mentioned previously, serum BDNF is also decreased in depressed patients (de Azevedo Cardoso et al., 2014, Pallavi et al., 2013, Fornaro et al., 2015).

At a cellular level, the serotonergic neurons of the dorsal raphe nucleus are implicated in mediating behavioural responses to stress. In WKY rats, there is a decrease in basal

excitability of these serotonergic neurons and it was also shown that corticotrophin-releasing factor (CRF) did not increase GABAergic activity in the serotonergic neurons in the WKY rat as was seen in the SD rat (Lemos et al., 2011). Disruption of 5-HT neurotransmission and modulation by CRF may play a role in pathological stress states and could possibly cause the stress-hyper responsiveness observed in the WKY rat.

### **1.8.2.3. Predictive validity**

The WKY strain has been shown to respond variably to the different classes of antidepressants. Notably neither acute nor chronic treatment with the SSRIs fluoxetine or paroxetine affect time spent immobile in the FST even at high doses (Lopez-Rubalcava and Lucki, 2000, Griebel et al., 1998, Tejani-Butt et al., 2003). In contrast, the TCA drugs desipramine and imipramine were both found to decrease immobility in the FST when administered both acutely and chronically, desipramine even causing a significant reduction in immobility time in the WKY at a dose that had no response in the SD control (Lopez-Rubalcava and Lucki, 2000, Lahmame et al., 1997). Furthermore, nomifensine, a noradrenaline (NE) and dopamine (DA) reuptake blocker also decreased immobility time and increased swimming time (Tejani-Butt et al., 2003). A study examining the effects of subacute treatment with three different classes of antidepressants in two WKY sub-strains developed by selective breeding based on immobility time in the FST, found that the WKY more-immobile (WMI) substrain male rats exhibited a significant reduction in immobility time following desipramine treatment, while WKY least-immobile (WLI) males only showed a non-significant decrease. Phenzelzine, an MAOI drug, significantly reduced immobility in WMI males with a smaller effect observed in WLI males and fluoxetine had no effect on FST behaviours in either WMI or WLI males (Will et al., 2003).

Due to the seeming resistance of WKY rats to SSRI drugs, this may mean that the WKY model could be used to detect novel antidepressant compounds that are not reliant on altering serotonin levels. Ketamine, which has been proven to have short term clinical benefit for depression, causes a rapid and lasting antidepressant effect in the WKY rat (Tizabi et al., 2012, Akinfiresoye and Tizabi, 2013).

Another avenue of research into antidepressant compounds is kappa-opioid receptor antagonists. An increased expression of  $\kappa$ -opioid receptors was found in the locus coeruleus of WKY rats compared to SD controls (Pearson et al., 2006), which led a research group to investigate the effect of a  $\kappa$ -opioid receptor antagonist on antidepressant activity in this model. They found that the antagonist nor-BNI resulted in significantly reduced immobility time in the FST not caused by a general increase in locomotor activity (Carr et al., 2010). The same research group also reported anxiolytic activity of another  $\kappa$ -opioid receptor antagonist 2-(3,4-dichlorophenyl)-*N*-methyl- *N*-[(1*S*)-1-(3-isothiocyanatophenyl)-2-(1-pyrrolidinyl) ethyl]acetamide hydrochloride (DIPPA) in the WKY model (Carr and Lucki, 2010).

#### **1.8.2.4. Weaknesses of the WKY model**

Even though the WKY strain is classed as an inbred rat strain, there is evidence of genetic heterogeneity between commercial sources of WKY rats. This may result in biologic variability in WKY rats from different suppliers which can affect research and the comparability of experiments. The most likely cause of this genetic variability is that WKY breeding rats were distributed to supplier companies before the strain was fully inbred, with evidence to suggest the breeding stock may have been sent to suppliers as early as the F 10 generation (Kurtz et al., 1989).

It has been shown that WKY rats obtained from Harlan, WKY/NHsd, display a three times greater behavioural variability compared to other in- and out-bred rat strains (Will et al., 2003). Evidence of variability in depressive-like behaviours have been reported in WKY rats obtained from different suppliers (Pare and Kluczynski, 1997), however rats from all suppliers are used equally and compared against each other which may affect how data is interpreted. In the last 5 years an equal number of studies used WKY rats from Harlan and Charles River as a model for depression (n= 17 each) (Zhang-James et al., 2013).

Another weakness of the WKY strain as a model of depression is that it has been found to respond to subacute antidepressant treatment which is not representative of the clinical scenario as treatment with these drugs only provide relief after at least 2-3 weeks of treatment in depressed patients (Will et al., 2003). Animal models of depression should preferably only respond to antidepressant treatment following chronic dosing regimes.

### **1.8.3. The olfactory bulbectomy model of depression**

In rodents, the olfactory bulbs are bilateral projections rostral to the frontal cortex and make up roughly about 4% of the total brain volume. The olfactory bulbs are organised in two levels for the processing and transfer of information. The outermost level contains mostly glomeruli and external tufted cells which are dopaminergic and GABAergic containing cells, while the inner level is made up of mostly middle and internal tufted cells and mitral cells which are acetylcholine-, serotonin-, noradrenaline-containing cells. The mitral cells project to the amygdala, the posterior pyriform cortex and the entorhinal cortex, and the tufted cells project to the anterior pyriform cortex (Kelly et al., 1997).

The OB model was developed based on the idea that lesions to areas in the limbic system/ or areas projecting to or from the limbic system may result in a depressive phenotype due to the high involvement of emotional processing in this area. The OB model gained

interest due to the reversal of its behavioural profile following chronic administration of antidepressant drugs which does not occur with acute dosing regimes (Jancsar and Leonard, 1981). OB results in anterograde and retrograde degeneration of neurons that connect the bulbs with other brain regions, demonstrated by lowered glucose utilisation in the amygdala, hypothalamus, ventral hippocampus and ventral tegmental area (Skelin et al., 2008). This is relevant as hypoactive metabolism has been described in the limbic system in depression patients (Su et al., 2014).

(B)



**Figure 1.9.** (A) Illustration representing where holes are drilled to allow removal of olfactory bulbs and (B) a defrosted brain with no olfactory bulbs.

### 1.8.3.1. Face validity

The most commonly reported behavioural alteration following OB surgery is hyperactivity, particularly in the open field test (van Riezen and Leonard, 1990). However, it has been reported that the conditions in which the test is carried out affect whether the OB operated animals exhibit this hyperactivity or not. According to results from Kelly and Leonard, an aversive arena with a bright light and reflective walls is the

best way to elicit the hyperactive behaviour (Kelly and Leonard, 1995). As rats have a dislike for open, bright areas, this finding by Kelly et al has led to the hypothesis that the increase in activity of OB operated animals is due to a decrease in defensive freezing behaviour as found in one such study that reported decreased immobility and increased locomotion in the open field test in OB operated animals (Primeaux and Holmes, 1999). Another theory is that OB rats exhibit a reduced ability to habituate to a novel environment (Song and Leonard, 2005).

Abnormal learning and memory has been investigated in the OB model using a variety of tests looking at different aspects of cognition. OB operated animals exhibit a deficit in fear memory, commonly investigated using passive avoidance tests. This alteration in behaviour expressed by OB operated animals has been shown to appear as early as 3 days following surgery (Douma et al., 2011). Spatial memory deficits in the OB model have also been reported. This finding has been described in multiple tests for assessing spatial memory, such as the holeboard test (Borre et al., 2012a), the Morris water maze (Song et al., 2009) and the 8 arm radial maze (Hall and Macrides, 1983). Impaired object memory is another feature of the cognitive impairment observed in OB model to be investigated (Douma et al., 2011).

Anhedonia is another aspect of the depressive phenotype presented by the OB model. It has been shown the OB operated animals exhibit a long lasting reduction in sucrose intake compared to sham animals, beginning 10 days after surgery and still present 1 month following surgery. The same study also reported a blunted response to amphetamine-induced activation of reward circuitry (Romeas et al., 2009).

The OB model is also used to model neurodegeneration which is believed to have a causal role in various neurological diseases including Alzheimer's disease and OB mice have even been used as models of this disease (Ostrovskaya et al., 2007). OB in mice causes

neurodegenerative processes in the temporal lobe observed via signs of apoptosis including pyknosis, karyolysis and vacuolysis (Bobkova et al., 2004). OB in mice was also shown to cause increased levels of  $\beta$ -amyloid protein in the neocortex and hippocampus and these mice also exhibited impaired spatial memory which are both relevant to clinical presentation of Alzheimer's disease (Aleksandrova et al., 2004). Following removal of the olfactory bulbs, neurodegeneration occurs along the neural pathways that once connected the bulbs to various brain regions including cortical regions, the hippocampus and the amygdala (Song and Leonard, 2005).

### **1.8.3.2. Construct validity**

Neuronal degeneration occurs in many areas receiving projections from the olfactory bulbs, including the cortex, amygdala and hippocampus. MRI studies have been carried out on the OB model and have found that the lateral and 3<sup>rd</sup> ventricles are significantly larger in OB operated rats (Wrynn et al., 2000). Enlargement of the ventricles has been associated with impaired hippocampal function in human depression patients (Kellner et al., 1986) and so it may also explain the impaired learning and memory often observed in this model. Reduced volume in the prefrontal cortex (PFC) (Salvadore et al., 2011, Grieve et al., 2013), hippocampus (Colla et al., 2007, Bearden et al., 2009), basal ganglia (Husain et al., 1991) and there are mixed reports for volume differences in the thalamus (Grieve et al., 2013) and amygdala of depressed patients.

Neurotransmitter systems are altered in the OB model. There is evidence for disruption of the noradrenaline, serotonin, glutamate, acetylcholine and GABA systems, with the most relevant to depression being serotonin. It has been shown that levels of serotonin in the OB rat are reduced (Lumia et al., 1992). Hyper-innervation of the frontal cortex by serotonergic cells occurs following OB surgery (Zhou et al., 1998, Huether et al., 1997). Another anomaly of the serotonergic system in OB rats is an increase in density of 5-HT2

receptors on platelet membranes which has also been shown to occur in patients of depression (Healy et al., 1985). An imbalance in 5HT synthesis rates in discrete brain regions has also been reported following OB (Watanabe et al., 2003). Levels of noradrenaline in the telencephalon are decreased following OB (van Riezen and Leonard, 1990) as are levels of glutamate in the olfactory tract (Collins, 1984).

Components of the HPA axis have been shown to be disrupted in the OB model of depression which is comparable to the clinical scenario. Specifically, increased levels of CRH in the hypothalamus and elevated serum corticosterone (CORT) levels in OB animals indicate HPA axis hyperactivity (Yang et al., 2014, Jindal et al., 2015b, Rinwa and Kumar, 2013). Another result of OB is decreased levels of BDNF in the brain (Jindal et al., 2015b). A reduced circulating level of BDNF is commonly reported in depressed patients (Fornaro et al., 2015) and has led to the neurotrophin theory of depression.

### **1.8.3.3. Predictive validity**

An important feature of any animal model of depression, as mentioned before, is a response to chronic antidepressant treatment, known as predictive validity. Chronic antidepressant treatment, with both TCAs and SSRIs, normalises the increased locomotor activity observed in the OB model (Pandey et al., 2014, Jarosik et al., 2007, Machado et al., 2012, Breuer et al., 2007); this effect is not elicited by acute treatment (Wang et al., 2012). Chronic treatment with SSRIs also results in a decrease in thigmotaxis, rearing and attenuates the increase in defecation exhibited by OB animals in the open field test (Pandey et al., 2014). It has also been shown to increase neurogenesis in the dentate gyrus (Jaako-Movits et al., 2006).

Both TCAs and SSRIs have been shown to produce long term effects in the attenuation of the depressive-like phenotype observed in the OB model, lasting up to 10 weeks

(Breuer et al., 2007). It has been reported that the co-administration of ketanserin with the SSRI escitalopram leads to an earlier onset of antidepressant effects compared to treatment with escitalopram alone. Reversal of the behaviours caused by OB surgery was found after 7 days of treatment with the combination therapy versus 14 days with the SSRI alone (Pandey et al., 2014).

#### **1.8.3.4. Weaknesses of the OB model**

As the mechanism behind the resolution of hyperactivity by currently available antidepressant treatments in the OB model is not understood, new compounds that do have antidepressant properties but exert their actions differently and so don't reduce hyperactivity levels, may be missed (Hendriksen et al., 2015).

From studies conducted in our lab, we have concluded that there is a relatively high mortality rate when performing OB surgery, about 5-10%. This means that more animals may have to be bought to account for this loss during surgery or else some group numbers may be lower than what is needed. A majority of studies fail to report how many (if any) deaths occurred in their studies.

Another weakness of this model is that you cannot confirm that the surgery has been performed properly, i.e. all of the bulbs removed with no damage caused to the prefrontal cortex, until the study is complete and the brains have been removed and inspected. Again some studies do not report whether or not the data presented is from animals that received successful surgeries or if all animals were included regardless of how well the surgery was carried out.

Below is a table comparing the CMS, WKY and OB models in regards to ease of use and their ability to display face, construct and predictive validities

Model	Face Validity	Construct Validity	Predictive Validity	Etiological Validity
<b>Chronic Mild Stress (CMS)</b>	Anhedonic-like behaviour-reduced sucrose preference (Willner, 2005, Willner et al., 1987)	Increased circulating levels of corticosterone (Fortunato et al., 2010) Decreased BDNF levels (Ibarguen-Vargas et al., 2009)	Responds to chronic treatment with TCAs and SSRIs as well as with experimental strategies including ketamine and deep brain stimulation (Willner, 2017)	Inducing conditions closely mimic naturalistic setting and ability to cause successive episodes adds etiological value (Czeh et al., 2016)
<b>Wistar Kyoto (WKY)</b>	Depressive-like behaviour-higher immobility in FST (Pare, 1989, Carr et al., 2010) Anhedonia- (Burke et al., 2016) Freezing- (DaSilva et al., 2011)	Altered neurotransmitter systems (Pare and Tejani-Butt, 1996, Scholl et al., 2010) Decreased BDNF levels (Vinod et al., 2012) Increased levels of CORT (Braw et al., 2006b)	Responds to TCAs (both acute and chronic) (Lopez-Rubalcava and Lucki, 2000) Unresponsive to SSRIs (Griebel et al., 1999)	Poor etiological validity as was originally bred as normotensive strain and genetics/mechanisms underlying depressive-like behaviour poorly understood
<b>Olfactory Bulbectomy (OB)</b>	Poor as main behavioural output (hyperactivity) does not correlate with MDD Learning and memory deficits (Douma et al., 2011)	Altered neurotransmitter systems (Lumia et al., 1992, Zhou et al., 1998) Elevated CORT levels (Yang et al., 2014)	Good predictive validity as only responds to chronic TCA and SSRI treatment	Poor etiological value as inducing conditions do not correlate with clinical scenario

**Table 1.5.** Summary of validities displayed by the models of depression described in this chapter. BDNF-Brain derived neurotrophic factor; TCA- Tricyclic antidepressant; SSRI- Selective serotonin reuptake inhibitor; FST- Forced swim test; CORT- corticosterone; MDD- Major depressive disorder.

### **1.9. The use of male and female animals in preclinical research**

Historically, males were considered to be the representative of a species and so all clinical and non-clinical studies used only males as subjects, presuming that the results would be the same for females and this pervasive attitude that most physiological systems are the same in males and females still persists in preclinical research today. In 1993, the US National Institute of Health (NIH) released an act (The NIH Revitalisation Act) requiring the enlistment of females in clinical trials. This resulted in an increased proportion of clinical studies using both male and female subjects (Beery and Zucker, 2011, Soldin and Mattison, 2009) and currently almost half of all subjects in NIH funded clinical research are females. However, preclinical research studies continue to rely heavily on the use of male animals only or else often neglect to report the sex of the subjects studied. The fields of research with the most skewed use of male only: female only studies are pharmacology, endocrinology, physiology and neuroscience (Beery and Zucker, 2011).

(A)

(B)

**Figure 1.6.:** Distribution of studies by sex and field. Percent of articles describing (A) human and (B) animal research that used male, female, both male and female subjects or did not specify the sex of the subjects. Graphs courtesy of (Beery and Zucker, 2011).

This under-representation of females is particularly concerning when it comes to pharmacological studies as vital information on drug pharmaco-kinetics/dynamics could be missed in females. In most studies of physiology and disease, the focus on a single sex threatens to restrict the impact of the results as these may not be applicable to half of the population. The reason for the lack of use of females in preclinical research is most likely due to concerns that the oestrous cycle causes increased variability in the data collected from females and that costs of studies would be increased; however it has been shown that female mice are no more variable than males in a wide range of behavioural, morphological, physiological and molecular traits regardless of oestrous cycle stage (Prendergast et al., 2014). This finding has also been extended to rats with Becker *et al* reporting in a meta-analysis of studies published between August 1 2010 and July 31 2014

that found that female rats are no more variable than male rats in a range of neuroscience related measures; this finding held regardless of oestrous cycle stage (Becker et al., 2016).

In 2014, the NIH announced plans to rectify the over-reliance of males in preclinical research. The new approach requires research groups applying for NIH funding to account ‘for sex as a biological variable’ (SABV) in their application or else they must provide ‘strong justification from the scientific literature, preliminary data, or other relevant considerations’ if they wish to only include only one sex in their research (NIH Guide Notice NOT-OD-15-102). As the NIH is a tax payer funded institution, they have a responsibility to ensure they fund the most unbiased, reliable and complete studies possible and by ignoring one half of the population in preclinical research, as has been done until now, has led to an incomplete understanding of basic physiological systems and pathophysiology of diseases. This policy change will hopefully lead to an increase in research using both sexes as was seen when they introduced a similar policy for clinical research (McCullough et al., 2014).

Of course a certain amount of resistance is to be expected with the introduction of this policy because as mentioned above, there is reluctance to the use of female subjects in research. However this policy does not require that all future studies contain an equal number of male and female subjects but that investigators address the possibility that sex could have an influence on the variables being studied in the experiment and only if this is the case would this then need to be investigated in both sexes (Guizzetti et al., 2016).

The SABV policy is also part of a plan to increase reproducibility of preclinical research which is currently the subject of much critique (Baker, 2016, Collins and Tabak, 2014). The omission of important information regarding how a research study is performed decreases the possibility of the results being replicated by others. The sex of the subjects used in a research study is an important piece of information that can affect the success

of an experiment however it is one of the more often omitted details which can lead to difficulty when others attempt to replicate the results of the study. By helping to enforce the proper reporting of sex in research studies, the guidelines set out by the NIH hope to improve the reproducibility of preclinical research.

Other groups promoting the use of females in preclinical research include the European Association of Science Editors (EASE) who in 2012 established the Gender Policy Committee (GPC). In 2016 the GPC released the Sex and Gender Equity in Research (SAGER) guidelines to encourage a more complete approach to researching and reporting on sex and gender differences in preclinical research. In 2020, the EU's largest Research and Innovation programme, Horizon 2020, will have gender as one of its main research themes (Lee, 2018).

#### **1.10. Sex differences in models of depression**

Sex differences in various behaviours have been previously reported and so it is wise to assume that sex differences are present in animal models of disease. Investigations should be carried out in order to identify these possible sex differences because if a female's behaviour is so different to that of the males in certain tests of anxiety and depression they may not possess the necessary validity in order to be classed as a model of depression. Previous studies have shown some behavioural differences between females and males in tests of anxiety and depression however further investigation is needed in order to fully elucidate these differences. The table below shows what behaviours have been found to differ and at what stage of the oestrous cycle this difference was found. A limitation with some of the findings presented below are that they compare females in the different oestrous stages against each other but not against male animals and so it is not clear if the behaviours are different to those in the male animals.

Findings	Reference
Rats in metestrous stage spent a greater percentage of time in open arms of EPM test compared to rats in oestrus (no comparison made against males)	(Mora et al., 1996)
Female rats in proestrous spent a greater percentage of time in open arms of EPM compared to rats in diestrous, no difference compared to males	(Marcondes et al., 2001, Frye et al., 2000)
Female rats in both proestrous and oestrous moved more in the OFT compared to male rats; female rats in proestrous entered arms in EPM more than males	(Frye et al., 2000)
Females in proestrous were more immobile in FST vs. females in diestrous but no females group were different to males	(Frye and Walf, 2002)
Administration of progesterone to ovariectomised rats caused a significant decrease in burying behaviour and caused increased open arm entries in the EPM (vs. ovariectomised rats not treated with progesterone)	(Fernandez-Guasti and Picazo, 1992, Mora et al., 1996)

**Table 1.6.** Sex differences previously reported in anxiety and depressive-like behaviours. EPM- elevated plus maze; OFT- open field test; FST- forced swim test.

Even though it is widely accepted that rates of depression are twice as high in women compared to men, due to the lack of use of female animals in preclinical research, models of depression have been validated using male subjects and so it is not clear whether these models accurately represent a depressive model in female subjects.

Little research has been carried out on potential sex differences in the WKY model of depression. An early paper that did report on sex differences in the WKY model in response to stress and found that males had a greater response to acute stressors compared to females however they were able to adapt to chronic stress which females were more vulnerable to (Pare et al., 1999). Will et al (2003) described baseline sex differences in the WKY strain, and even went further by looking at the sex differences in the WMI and

WLI sub-strains. They found that overall male WKYs were more immobile in the FST compared to females and females spent more time in the centre of the OF test compared to males (Will et al., 2003). These results would indicate that male WKY rats show a more depressive phenotype compared to females. However, as the tests used in this study acute stressors we may not be seeing the full picture as reported by Pare that female WKY rats only showed a greater depressive phenotype when chronically stressed (Pare et al., 1999). A more recent study which also compares gender differences in the two WKY sub-strains reported that adult male WMI exhibited increased floating (immobility) in the FST compared to adult female WMI. They also looked at FST behaviours in adolescent rats and found that adolescent male WMI were more immobile than their WLI counterparts but there was no difference in immobility time between female adolescent WMIs and WLIs. Behaviour in the OF test also differed between sexes and sub-strains with male and female adolescent WLIs spending less time in the anxiety producing centre compared to WMI counterparts and as adults only the WLI males and not females, were found to continue this trait (Mehta et al., 2013).

Work from our own lab has shown that expression of depressive- and anxiety-like behaviours is mostly similar between male and female WKY rats with the few differences that were found suggesting an enhanced depressive-like phenotype in male rats (Burke et al, 2016). Behaviour in the OFT and FST were similar between males and females, with both sexes exhibiting decreased locomotor activity and increased time spent immobile respectively. However males exhibited novelty-induced hypophagia, decreased sucrose preference and decreased weight gain suggesting a more prominent depressive behavioural profile.

From the available literature, it seems that the WKY model may not be as robust as a model of depression in female animals compared to male animals. However, in order to

understand this fully, more work looking at baseline ‘depressive-like’ behaviours as well as response to antidepressant drugs would need to be carried out in both sexes.

Very little in the way of investigation into sex differences in the OB model has been carried out and results from studies we have found are often conflicting. OB female rats have been shown to have a significantly lower sucrose preference compared to OB males (Stock et al., 2000), however another study found that while the intake of sucrose was lower in female OB animals there was no difference in sucrose preference compared to males (Stepanichev et al., 2016). One consistent result reported is that OB surgery causes hyper-locomotion in both male and female operated animals in the OFT (Stock et al., 2000, Stepanichev et al., 2016, Stock et al., 2001). However reports of exploratory and emotional behaviours during the OFT are conflicting with both rearing activity and the number of faecal boli reported to be increased in OB females as is the case in male OB animals (Pandey et al., 2014, Stock et al., 2000) but also no difference between OB and sham females has also been reported (Stepanichev et al., 2016).

Measures of anxiety behaviour, mainly entries into and time spent in the open arms of the elevated plus maze are also inconsistently reported between studies. Female OB animals have been shown to enter the open arms both more than sham females (Stock et al., 2000) and no differences between OB and sham have been reported (Stepanichev et al., 2016). Also the time spent in the open arms has been reported to be significantly greater in OB animals (Stock et al., 2000) and significantly less (Stepanichev et al., 2016) compared to sham animals.

As mentioned earlier, learning and memory deficits are commonly reported in male OB operated animals. Whether this is also the case for female OB animals is unclear as there has been very little published in this area to date. One research group has suggested that OB surgery in females impairs short-term memory in a simple Y-maze test however

causes faster acquisition and better recall of two-way active avoidance (Stepanichev et al., 2016). They suggest that damage to the hippocampus caused by neurodegeneration following OB surgery improves active avoidance learning which has been shown previously (Guillazo-Blanch et al., 2002, Wang et al., 2015a).

These results may suggest that OB surgery produces only a moderate ‘depressive-like’ phenotype, however further investigations will have to take place before we are able to categorically say whether it is an appropriate model of female depression or not.

One study which looked at only female rats concluded that OB produced only moderate effects in females. This difference may be due to the different strain of rats and different test protocol used. They did observe a hyperlocomotor response in the OF however they did not see other behavioural alterations that are also associated with OF testing in the OB such as increased rearing and defecation. As regard to anxiety testing there were no differences in EPM parameters between sham and OB females which was also the case for their test for depressive-like phenotype, the FST (Stepanichev et al., 2016).

Studies investigating gender differences in the CMS model are sparse, although there are more compared to either of the previous two models mentioned earlier in this review. CMS affects both sexes with a decrease in sucrose consumption and/or preference occurring following a period of CMS treatment (Grippe et al., 2005, Duncko et al., 2001, Harden et al., 2012). However there are also many conflicting reports that have found an anhedonic effect of CMS in only males (Dalla et al., 2008, Pitychoutis et al., 2012) or only in females (Xing et al., 2013, Konkle et al., 2003). The effects of CMS on anhedonic measures appear to be more pronounced in male subjects (males differ at all time points measured whereas females don’t). A reason for the inconsistent effects of CMS on sucrose preference in females may be due to the higher baseline consumption of sucrose in female rats (Dalla et al., 2005). The CMS protocol has also been shown to affect the

oestrous cycle (Grippio et al., 2005), which may have downstream hormonal effects on taste perception. This may mean that sucrose preference is not a reliable measure of anhedonia when investigating gender differences. To attempt to avoid this confound, other measures of hedonic activity have been used. One such measure of reward behaviour that has been used in male and female rats exposed to a CMS regime is brain stimulation rewarded for lever pressing; however there was no reduction in lever pressing found in either sex (Bielajew et al., 2003).

The CMS protocol also has sexually dimorphic effects on body weight gain with male animals gaining weight slower when compared to shams but with no effect on body weight in females exposed to CMS (Duncko et al., 2001, Dalla et al., 2005, Xing et al., 2013). Effects of CMS on activity in the open field test is also sex dependent; female rats move less both compared to control females and also male animals exposed to the same CMS regime (Xing et al., 2013, Dalla et al., 2005).

### **1.11. Hypothesis**

The hypothesis under investigation in this thesis was that there would be sexual dimorphism in anxiety- and depressive-like behaviours, cognitive ability as well as responsiveness to antidepressant drug treatment in the Olfactory Bulbectomy and Wistar Kyoto models of depression.

### **1.12. Aims**

The primary objective of the work presented in this thesis is to characterise potential sex differences in two commonly used rodent models of depression and to determine if the female subjects could model the disease to the same extent as the male subjects. The goal was to first characterise baseline behaviour in a range of tests that examined depressive-like and anxiety-like behaviours as well as tests of cognitive ability. We then examined whether or not the depressive phenotype affected reproductive ability of male and female animals, maternal behaviour and whether or not there was an effect on pup development when born to a dam as a model of depression. Finally we investigated whether or not there were differences in response to treatment with different classes of antidepressant drugs in male and female models of depression.

#### **Specific Aims:**

- To characterise baseline behaviours between male and female subjects in tests of anxiety and depression and cognitive ability
- To determine whether female subjects modelled depression as well as male subjects
- To investigate if animal models of depression exhibit impaired mating abilities
- To examine if female models of depression chosen exhibited signs of altered maternal behaviour

- To investigate if offspring born to females with a depressed behavioural profile exhibited altered neonatal development
- To examine potential differences in response to antidepressant drugs between male and female models of depression

***Chapter 2:***  
***Materials and***  
***Methods***

## **Chapter 2    Materials and Methods**

This chapter describes the general materials and methods used to complete the studies that are encompassed in this thesis. More detailed information referring to individual studies is provided in the ‘Materials and Methods’ sections of subsequent results chapters.

### **2.1. Materials**

#### **2.1.1. Animal husbandry**

*Rats:* male and female Sprague-Dawley/ Wistar Kyoto, bred in-house (CNS Pharmacology Laboratory, NUI, Galway) or purchased from Envigo (formerly Harlan) (United Kingdom).

*Rat cages:* (42 cm x 25.5 cm x 13 cm), plastic bottoms with metal cage lids: North Kent Plastics (Coalville, United Kingdom)

*Water bottles:* North Kent Plastics (Coalville, United Kingdom)

*Weighing scales:* Mason Technology (Dublin, Ireland)

*Goldflakes bedding:* LBD (Serving Biotechnology) Ltd. (Surrey, United Kingdom)

*Corn cob bedding:* W.M. Lillico (Surrey, United Kingdom)

*3 Rs Paper bedding:* Fibrecycle Ltd. (North Lincolnshire, United Kingdom)

*Rat chow:* (Harlan Teklad global diets chow): ENVIGO RMS (Bicester, United Kingdom)

*Temperature/humidity monitor:* Radionics Ltd. (Dublin Ireland)

*Nesting material:* (Safe bed fluff bedding): Petworld (Galway, Ireland), Goldwool and Sizzle-Pet nesting (LBS Biotechnology, United Kingdom)

*Environmental Enrichment:* physical enrichment included nesting material, plastic tube (6" x 3.5") (constructed by Mr. Ambrose O'Halloran). Nutritional enrichment included hazelnuts, cereals, sunflower seeds, muesli

### **2.1.2. Behavioural Equipment**

*Elevated Plus Maze:* (arms 50 x 10 cm; length x width, walls of the closed arms 30 cm high): constructed by Mr. Ambrose O'Halloran (Pharmacology and Therapeutics, NUI, Galway)

*Open field:* (75 cm diameter with walls 41 cm high): constructed by Mr. Ambrose O'Halloran (Pharmacology and Therapeutics, NUI, Galway)

*Forced swim test cylinders:* (45 x 20 cm; height x diameter): apparatus constructed by Mr. Ambrose O'Halloran (Pharmacology and Therapeutics, NUI, Galway)

*Morris Water Maze pool:* (1 X 2m; height x diameter): apparatus constructed by Mr. Ambrose O'Halloran (Pharmacology and Therapeutics, NUI, Galway)

*Novel Object Recognition:* Arena used was the open field apparatus- details outlined above. Objects used consisted of Lego® blocks

*Social interaction test:* arena (0.8 x 1.8m; height x diameter): apparatus constructed by Mr. Ambrose O'Halloran (Pharmacology and Therapeutics, NUI, Galway)

### **2.1.3. Recording Equipment**

*Video cameras:* Sanyo Digital Colour LCD Camera: Radionics (Dublin Ireland)

*DVR Recorder:* 8 place inspire DVR range: Tracksys (Nottingham, United Kingdom)

*Memory storage:* SanDisk 64GB USB: Currys (Galway, Ireland)

#### 2.1.4. Surgical Equipment

*Anaesthetic Machine:* Matrx, Grays Medical (Biggar, Lanarkshire, United Kingdom)

*Isoflurane:* Chanelle Veterinary (Loughrea, Galway, Ireland)

*Oxygen:* BOC ((Galway, Ireland)

*Shaver:* Oster Golden A5 Shaver: Argos (Galway, Ireland)

*Stereotaxic frame:* (Harvard Apparatus, MA, USA)

*Eye drops:* Blink®Tears (Polythylene Glycol 400 0.25%), Boots (Galway, Ireland)

*Betadine:* 7.5% w/v, iodinated povidone, Videne®, Ecolab Ltd., (Leeds, United Kingdom)

*Surgical instruments:* Bulldog clips, forceps: Fine Science Tools (Heidelberg, Germany)

*Scalpel:* Swan Morton: Lohan's Pharmacy (Galway, Ireland)

*Drill:* RS Pro 398D PCB Drill: RS Radionics Ltd., (Dublin, Ireland)

*Drill bits:* Size 8: Transmore Ltd., (Dublin, Ireland)

*Blunted needle:* 16G x 1.5" BD Microlance (Oxford, United Kingdom)

*Vacuum pump:* Super Vega Suction Machine: Medguard (Meath, Ireland)

*Gauze:* Gauze Mediswabs: Lohan's Pharmacy (Galway, Ireland)

*Cotton buds:* Johnson & Johnson: Boots (Galway, Ireland)

*Haemostatic sponge:* Septodont: Lohan's Pharmacy (Galway, Ireland)

*Michel Clip Application Forceps and Michel Clips:* Fine Science Tools, Europe

*Recovery heating pad:* Peco Services Ltd., (Cumbria, United Kingdom)

*1ml syringes:* BD Microlance (Oxford, United Kingdom)

*Needles:* 25G x5/8" BD Microlance (Oxford, United Kingdom)

*Rapidex cleaning solution:* Fred Storey Ltd. (Comber, Co Down, Northern Ireland)

*Hot bead sterilizer:* Steri 250

### **2.1.5. Drugs and drug administration**

*Desipramine hydrochloride:* Cat # D3900 Sigma-Aldrich (Dublin, Ireland)

*Fluoxetine hydrochloride:* Cat # PHR1394, Sigma-Aldrich (Dublin, Ireland)

*1ml, 2ml and 5ml syringes:* BD Microlance (Oxford, UK)

*Needles:* (25G x 5/8") BD Microlance (Oxford, UK)

### **2.1.6. RT-PCR**

*RNase Zap:* Cat# R2020, Sigma Aldrich (Dublin, Ireland)

*RNase free water:* Cat# W45020, Sigma Aldrich (Dublin, Ireland)

*Molecular grade ethanol:* Cat# E7023, Sigma Aldrich (Dublin, Ireland)

*RNase and DNase eppendorfs:* Cat# 72.695.400, Sarstedt (Wexford, Ireland)

*RNase and DNase eppendwarfs:* Cat# 72.737.002, Sarstedt (Wexford, Ireland)

*MicroAmp® optical 96-well plate:* Cat#72.1981.202, Sarstedt (Wexford, Ireland)

*Macherey Nagel Nucleospin RNA columns:* Cat# 740955.250, Fisher Scientific (Dublin, Ireland)

*Mastero Nano drop spectrophotometer*: Medical Supply Company (Dublin, Ireland)

*Mj research thermal cycler*: Bio-rad Fannin (Dublin, Ireland)

*BDNF probe: Rn02531967\_s1 BDNF Rat (FAM MGB)*: Cat# 4331182, Bio Sciences Ltd.  
(Dublin, Ireland)

*Ntrk2 probe: Rn01441749\_m1 Ntrk2 Rat (FAM MGB)*: Cat# 4331182, Bio Sciences Ltd.  
(Dublin, Ireland)

*$\beta$ -actin probe: Rn00667869\_m1 ACTB Rat (VIC MGB)*: Cat# 4352340E, Bio Sciences  
Ltd. (Dublin, Ireland)

*High Capacity cDNA reverse transcription kit*: Cat# 4368814, Bio Sciences Ltd.  
(Dublin, Ireland)

*Taqman Universal PCR mix*: Cat# 4324018, Bio Sciences Ltd. (Dublin, Ireland)

*Optically clear plate cover*: Cat# 95.1994, Sarstedt, (Wexford, Ireland)

*StepOne plus plate reader*: Applied Biosystems, (Warrington, United Kingdom)

### **2.1.7. Computer Software**

*Microsoft Office*: Microsoft Ireland (Dublin, Ireland)

*IBM SPSS Statistics 21*: SPSS Inc. (Chicago, IL, USA)

*GraphPad Prism 5*: GraphPad Software Inc. (La Jolla, CA, USA)

*Ethovision ® XT 8.5*: Noldus (Wageningen, The Netherlands)

*StepOne software v2.3*: Applied Biosystems, (Warrington, United Kingdom)

## 2.2. Methods

### 2.2.1. Animals

All experimental procedures on animals were conducted with the approval of the Animal Care and Research Ethics Committee (ACREC) of the National University of Ireland, Galway (12/NOV/07, 15/MAR/05, 16/FEB/02), under licence from the Irish Department of Health and Children and in compliance with European Communities Council directive 86/609 guidelines. Male and female Sprague-Dawley and Wistar Kyoto rats were used for all of the experiments described and were either obtained from Envigo (U.K.) or bred in-house. Rats were individually housed in plastic-bottomed cages (42 x 25.5 x 13 cm; L x W x H), with paper bedding, or housed in 3's (1 male and 2 females) for breeding, depending on the nature of the experiment. Bedding was changed weekly. Rats were maintained on a 12-h light/dark cycle (lights on at 08:00 h) in a temperature controlled room ( $23 \pm 2$  °C) with relative humidity ranging at 35-60%. Standard rat chow pellets and water were available *ad libitum*. Animals were weighed at least once weekly, this was increased to once daily following surgery, to ensure normal growth patterns were being maintained. In all cases, animals were randomly assigned to their treatment groups.

### 2.2.2. Breeding for study animals

3-4 days before mating, males were singly housed while females remained housed in 3's. During mating males and females were housed together for 2 weeks at a ratio of 1:2 or 1:3. Bedding was changed once per week. Following the 2 week period, females were singly housed with nesting material and cardboard tubes to facilitate nest building. During the gestational period, the dams were left undisturbed except for weekly bedding changing. 3 weeks post the first night of mating was considered to be roughly GD 21 and from this point the females were closely monitored for signs of parturition. The day of

birth was considered to be postnatal day (PND) 0. Mother and pups were left undisturbed until PND 2-3 at which point the pups were counted and sexed. Any deaths or unplanned pup sacrifices were recorded. During the postnatal period the pups were counted daily and cages were changed once weekly beginning on PND 5.

### **2.2.3. Weaning**

Weaning was carried out on PND 21. Pups were housed in cages of 4/6 by sex (littermates were housed together whenever possible). Only the number of pups needed for the study were weaned, the remaining pups were either allocated to another study/research group or sacrificed if surplus to requirements. At 4 weeks old the animals were housed in cages of four if not already done so. At 7 weeks old (unless otherwise stated in study chapter) the animals were singly housed for the commencement of the study.

### **2.2.4. Behavioural testing**

#### **2.2.4.1. Elevated plus maze (EPM)**

The EPM is a test for anxiety-like behaviour (Pellow and File, 1986). This test is based on the principle of thigmotaxis, whereby the rat has a natural aversion to open areas and prefers to remain close to vertical surfaces. The EPM arena consists of a plus-shaped apparatus with two open arms, two closed arms and a central platform that are raised approximately 55 cm above the floor (Figure 2.1). All arms are 50 cm long and 13cm wide and the central platform measures 13 cm x 10 cm (width x breadth). The walls surrounding the closed arms are 30 cm high. Four 60 watt bulbs are placed above the EPM, one bulb over every arm. These bulbs are powered by dimmer switches and the light intensity was controlled so that the end of each closed arm was approximately 55-60 lux and the end of each open arm was approximately 110-120 lux. Each rat was placed

in the centre square of the EPM, facing an open arm and was allowed to freely explore the arena for 5 minutes. If the rat fell from the EPM it was discounted from the analysis. After each rat trial, the rats were placed into the open field for further analysis, after which they were returned to their home cage. The arena was cleaned with warm, soapy water in between trials.

All trials were recorded by a camera that was placed approximately 1.3 metres above the centre of the EPM. Trials were later scored by an experimenter blinded to the treatment groups. An arm entry was defined as all four of the rat's paws being on the arm. The number of entries into and the duration of time spent in the open and closed arms were manually scored using Ethovision ® XT 8.5 system using the 'mutually exclusive' setting. Total arm entries (open arm + closed arm) and total arm time (open arm + closed arm) were calculated to derive the parameters of interest – percentage open arm entries (% OAE;  $\text{open arm entries}/\text{total arm entries} \times 100$ ) and percentage open arm time (% OAT;  $\text{open arm time}/\text{total arm time} \times 100$ ). The total distance moved in the 5 minute trial was measured automatically by Ethovision ® XT 8.5.



**Figure 2. 1:** A screenshot from a recording of the EPM test.

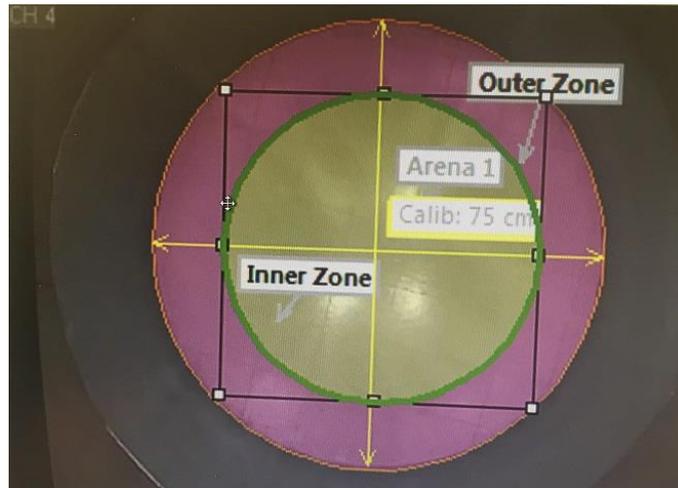
#### 2.2.4.2. Open Field

The open field test was used to measure general locomotor activity and emotionality in a novel environment (Hall *et al.* 1934). The test involves exposing a rodent to a novel environment from which they cannot escape. The apparatus consists of a white circular base (75 cm in diameter), surrounded by silver mirrored walls (41 cm high) (Figure 2.2). Four 60 watt bulbs are placed above the apparatus. These bulbs are powered by dimmer switches and the light intensity was manipulated so that it measured between 190-210 lux, measured by a lux meter at various points on the base of the arena. Each rat was placed in the centre of the open field and allowed to freely explore the arena for 5 minutes. After each trial, the rat was placed back into its home cage and the apparatus was cleaned with warm soapy water and dried thoroughly before the next rat was tested. All trials were recorded on a DVD or DVR recorder from a camera that was placed above the centre of the open field. Distance moved, measured in cm, was calculated automatically by calibrating the diameter of the arena and using the 'detection determines speed' trial setting on the Ethovision ® XT 8.5 system. An inner and outer zone were set up using Ethovision ® XT 8.5 and the amount of time spent in each zone was automatically determined. The diameter of the inner zone was determined to be 50cm and this was kept constant across all studies.

(A)



(B)



**Figure 2. 2:** Open Field apparatus. The open field apparatus illustrated from a screenshot of the video scoring image (A) and side profile of the apparatus (B).

#### 2.2.4.3. Forced Swim Test

The FST is the most widely used preclinical behavioural test for assessing antidepressant efficacy of compounds (Porsolt *et al.*, 1978). In the FST, rats are forced to swim for 15 minutes in a water-filled cylinder (45 cm x 20 cm) filled to 30cm with water (23- 25 °C) from which they cannot escape. 24 hours later, the animals swim for another 5 minutes – this 5 minute swim is the test that is scored. The rats develop an ‘immobile’ posture which is said to reflect behavioural despair. In the WKY antidepressant response study, rats received their final dose 15 minutes after the pre-swim and 24 hours prior to the 5 minute post-swim. After each swim, the rats were towel dried and placed back in their home cage. Cylinders were filled with fresh water in between each rat.

All trials were recorded by a DVD or DVR recorder from a camera that was placed at a position so that all four cylinders could be viewed together and clearly. The 5 minute trials were later scored by an experimenter blinded to the treatment groups. The

parameters of interest were immobility, climbing and swimming behaviour. Immobility was assigned when the rat exhibited only the minimum amount of activity required to keep its head above the water. Climbing behaviour consisted of forceful movements of the four limbs, with upward-directed movements of the forepaws, usually along the side of the cylinder. Swimming was defined as a movement (usually horizontal) throughout the cylinder. For all studies, these behaviours were scored using the ‘mutually exclusive’ scoring technique on Ethovision ® XT, which involved scoring the entire amount of time the rat spent undergoing each of the three behaviours over the 5 minute test.

(A)



(B)

**Figure 2. 3:** (A) Apparatus used to perform the FST and (B) a depiction of the three behaviours scored including immobility, swimming and climbing (left-right) (image adapted from (Cryan et al. (2002))).

#### **2.2.4.4. Morris Water Maze (MWM)**

The MWM is a test used to assess spatial learning and memory in rats and mice. The theory behind the test is for the animal to learn to find a hidden platform using visual cues at locations around the perimeter of the tank, (Vorhees and Williams, 2006). The tank measures 2m in diameter and is filled to a depth of 0.3m. The water was maintained at a temperature of 23-25°C. A transparent platform, 2cm below the waterline, was positioned in the South-West quadrant of the tank for the acquisition trials. Geometric shapes (printed in black on white A4 paper) were positioned around the tank to act as the visual cues. The lux was set at 25lux using dimmer switch controlled lights. Rats were brought into the MWM room for testing 4 at a time and were tested one by one 4 times, each time they were placed into the tank at a different location, as set out by Vorhees and Williams (Vorhees and Williams, 2006). Acquisition trials took place over 4 days. The rat was allowed to swim until it found the platform (time was manually recorded) or until 120 seconds had elapsed. When the rat found the platform it was left there for 10 seconds

before being removed and dried gently using a cotton towel. If the rat did not find the platform within the allotted time, it was guided there and left for 10 seconds. The rat is placed in a recovery cage and a timer is set to measure the time between trials. The probe trial took place 24 hours after the last acquisition trial. In this trial, the hidden platform is removed and the rats are placed into the pool at a new release point. All trials were recorded by a camera positioned above the centre of the pool. Ethovision ® XT software was used to measure the distance swam, the velocity of swimming and the time spent in the quadrants.



**Figure 2. 4:** Screen grab of scoring video of MWM test. Submerged platform in the south-west quadrant.

#### **2.2.4.5. Novel Object Recognition**

The NOR test assesses a rats ability to differentiate between a novel and familiar object. The test takes place over 2 days, with day 1 being a 20 minute habituation phase to the arena used for testing, in this case it was the open field arena. Day 2 is composed of 3 phases: a further 3 minute habituation period, a familiarisation period in which the rat

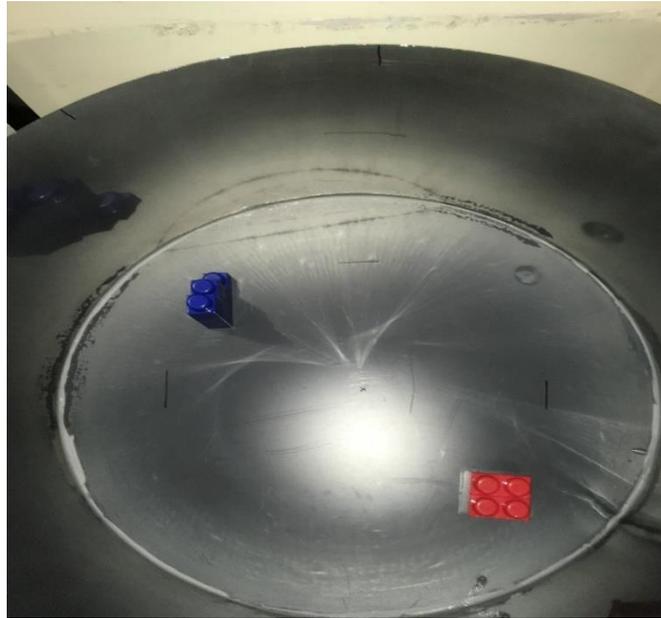
explores the two similar objects and the test phase which consists of the rat exploring familiar and a novel object. Each phase of the testing lasts 3 minutes and there is a 7 minute delay between each phase during which the rat is returned to their home cage. Lego blocks were used as the ‘familiar’ objects and a drinks can was used as the ‘novel’ object. During both the familiarisation and the testing phases, the objects were placed at opposite sides of the arena diagonally from each other and at a distance of ~ 16cm from the arena walls. The objects and the arena were washed with warm soapy water between each rat.

All trials were video recorded by a camera attached above the centre of the arena and were later scored using Ethovision ® XT software. Scoring of the time spent interacting with the objects was performed manually by an experimenter blinded to treatment groups.

(A)



(B)



**Figure 2. 5:** (A) representation of 3 minute phase with 2 similar objects and (B) 3 minute phase with one novel and one familiar object.

#### 2.2.4.6. 3 Chamber Sociability Test

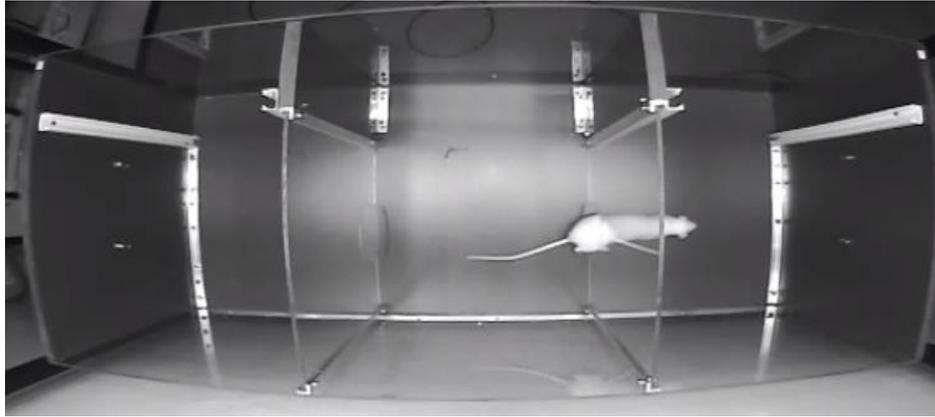
The 3 chamber sociability test is used as a measure of sociability and also social memory in rats. It is carried out in an arena made up of 3 chambers, each separated with transparent Plexiglas with a hole for access in and out of the chamber. Each chamber measures 40cm x 60cm (width x breadth). In each outer chamber a wire mesh can be inserted to block off a portion of the chamber, into which the conspecific rat is placed. The test is made up of 3 phases: habituation, sociability and novelty phase, each lasting 10 minutes. In the habituation phase, the test rat is placed into the centre chamber of the empty arena and is allowed to explore the arena. After 10 minutes, the test rat is confined to the centre chamber by blocking the holes in the partition walls and a novel conspecific rat is confined behind a wire mesh insert in one of the outer chambers. A similar wire mesh insert is placed into the opposite chamber. The test rat is allowed to explore the

arena and interact with the rat/empty chamber for a further 10 minutes. After this, the test rat is again confined to the centre chamber while a second conspecific rat is placed into the other outer chamber. The test rat is again allowed to explore and interact with the novel and familiar rats for a further 10 minutes. After this time the test rat is removed from the arena and returned to its home cage. The conspecific rats are also removed and returned to their cages and the arena is cleaned and dried ready for the next trial to begin. The conspecific rats come from a separate cohort of rats from the test rats. They can be used twice per day and can be used for the length of the testing protocol (i.e. 4 days). The placement of the first conspecific rat into the right or left outer chamber is randomly allocated for each trial. All trials were recorded using a camera attached to the centre of the roof of the arena. Trials were later scored by an experimenter blinded to the treatment groups. Time spent interacting with the rat and cage were scored in the sociability trial and the time interacting with the familiar and novel rat were scored in the novelty trial.

**(A)**



(B)



(C)



(D)



**Figure 2. 6.** Image of (A) the 3 chamber test apparatus followed by screenshots from (B) the habituation phase during which only the test rat is in the arena, (C) sociability phase which involves the addition of the first conspecific and (D) the novelty phase of the testing procedure during which the second conspecific is added to the arena.

### **2.9.1. Reproductive and neonatal parameters**

#### **2.9.1.1. Mating and determination of pregnancy**

The male: female ratio for mating in the studies was 1:2. At the beginning of the light phase the following morning, the female rats were vaginally smeared to detect for the presence of sperm cells. A cotton bud was moistened in water and inserted into the vagina. It was rotated and gently swabbed along the walls of the vagina. The cotton bud was removed and the female placed back into the cage. The cotton bud was rolled on a glass slide to transfer the cells onto the slide and the slide was examined under a light microscope to inspect for the presence of sperm. If sperm were present the female rat was transferred into a new cage with nesting material and this was considered as GD 1. If no sperm were detected the female remained in the male rat's cage until sperm was detected, if no sperm was detected after one week those females were group housed and the males were also group housed at this time.

#### **2.9.1.2. Gestation period and littering**

The females that tested positive for sperm during the mating period were monitored and their body weight, food and water intake were recorded every day. Litterings that occurred on or before 17:00 were considered to have their PND 1 on this day and litterings after 17:00 had their PND 1 the following day. On PND 1 the pups were randomly culled to 10 per litter, with a ratio of 50:50 males to females whenever possible. As only 1 male and 1 female pup were tested per litter, they were tattooed for identification purposes. A BD Micro-Fine 0.5ml needle was used to inject a small amount of tattoo ink under the top layer of skin on the rear heel.

### **2.9.1.3. Somatic development of offspring**

Somatic development of the pups was measured by recording the physical maturation of the pup. The parameters measured included pinna unfolding, fur appearance, eye-opening, ano-genital distance, body length and body weight. Pinna unfolding and fur appearance were recorded from PND 3 and eye-opening from ND 14. Recordings were taken until all pups had reached these developmental stages.

Ano-genital distance was measured to investigate for masculinising or feminising effects in the pups. Measurements were taken using digital callipers. The distance measured was from the base of the genitals to the top of the anus.

Body length was recorded using digital callipers on PND 7 and 14. Body length was regarded as the distance between the tip of the nose and the base of the tail.

Body weight was recorded frequently throughout the neonatal period.

### **2.9.1.4. Surface righting**

This test was used to examine the sensorimotor development of the pups. Pups were placed in supine position on a flat surface and were given a maximum of 30 seconds to turn themselves over to their normal prone position. If the pup did not successfully right itself in the time allowed, the test was ended and the pup was placed back into the home cage. This test was performed on PND 2, 3, 4 and 5.

### **2.9.1.5. Negative geotaxis**

The test consists of placing the pup on an inclined surface, facing downward and the time taken for the pup to turn 180° and face upward was recorded. The maximum time to complete the test was 30 seconds and if the pup did not complete the turn within this time the test was terminated. This test was performed on PND 9 and 11.

### 2.9.1.6. Forelimb grip

The forelimb grip test was used to test the grip reflex as well as the strength of the pups. The apparatus consists of a thin metal bar, ~20 cm in length and held ~25cm above the base of the platform by two poles. The pups were held at the base of the tail and lowered onto the bar in order to allow them to grip it with their forepaws. When the grip on the bar was established, the pup was lowered and released. The length of time the pup was able to hold onto the bar for before falling off was recorded. A maximum time of 30 seconds was given and if any rats were still hanging from the bar at this time they were removed and placed back into their homecage. This test was performed on PND 14 and 17.

### 2.9.2. Drug Dosing

Table 2.1 represents the route of administration, the dose volumes, and the vehicle used to make up each drug.

<b>Drug</b>	<b>Dose</b>	<b>Route(s)</b>	<b>Dose volume</b>	<b>Vehicle</b>
<b>Desipramine hydrochloride</b>	10 mg/kg	s.c., or o.g	4 ml/kg or 2 ml/kg	Saline or DH <sub>2</sub> O
<b>Fluoxetine hydrochloride</b>	10 mg/kg	s.c. or o.g	4 ml/kg or 2 ml/kg	Saline or DH <sub>2</sub> O

**Table 2. 1:** The dose, route of administration, dose volume and vehicle used for all compounds.

The specific drug dose, route, volume and vehicle used for each study are provided in the ‘Experimental protocol’ section of each results chapter. All parameters were decided based on either previous studies within our laboratory (Simpson *et al.*, 2012a, Simpson *et al.*, 2012b) or within the literature. For the olfactory bulbectomy dosing study, desipramine hydrochloride and fluoxetine hydrochloride were prepared by dissolving in saline to the required concentration and DH<sub>2</sub>O was the vehicle used in the Wistar Kyoto

dosing study. The drugs were divided into aliquots according to how much of the drug was required for each dosing day, and frozen at -20°C over the course of the study. Aliquots were thawed and brought to room temperature on the day that they were required for dosing.

### **2.9.3. Surgical Technique**

#### **2.9.3.1. Aseptic technique**

The day before surgery surgical packs were prepared. These packs consisted of surgical tools i.e. scalpel, tweezers, forceps and were wrapped in two layers of tin foil which were then autoclaved. Other materials that were also autoclaved included cotton buds, gauze, tissue, bulldog clips, blunted needles and suture clips.

Before starting the first surgery, the space was made sterile. Once anaesthetised and shaved the rat was placed into the stereotaxic frame, head held in position using ear bars. Once the rat was secure, sterile gloves were put on and from this point on the rat was not touched directly. The tin foil from the surgical packs was opened out and acted as a sterile place to put all of the surgical equipment. After each item of equipment was used, an assistant washed it in soapy water, placed it in a hot bead sterilizer and returned it back to the tin foil ready for re-use.

#### **2.9.4. Olfactory bulbectomy surgery**

OB surgery involves the bilateral removal of the olfactory bulbs which are located anterior to the frontal lobes. Removal of the olfactory bulbs in rats leads to behavioural, neurochemical and neuroendocrine changes that reflect the symptoms of depression in humans. Each rat was anaesthetised using isoflurane (5% for induction and 2% maintenance in 0.5L/min O<sub>2</sub>), and their head was shaved. The animal was then secured

using ear bars onto a stereotaxic frame (Harvard Apparatus, MA, USA) which was connected to an isoflurane pump to ensure the animal remained anaesthetised for the duration of the surgery. Eye drops were placed onto the eyeball to prevent the eyes from drying out during surgery. The head was swabbed with betadine and the initial incision was made with a sterilised scalpel along the midline of the scalp. The skin was pulled to the side and secured in place using bulldog clips. Bregma and the sagittal suture were located and the positions for drilling the holes were marked; ~5mm rostral to bregma and 2mm lateral to the midline. The drill was then used to drill two holes in the skull, through which the olfactory bulbs were aspirated. A blunted 23 gauge hypodermic needle attached to a vacuum pump was used to aspirate the bulbs through the holes. A 'suction' sound can be heard when all of the bulbs have been removed. The cavities were then plugged with haemostatic sponge to stop the bleeding and the skin was sutured using sterile Michel suture clips. For sham animals, the drill bit was manually twisted through the skull to the level of the dura. After surgery, the animals were administered 1 ml of saline intraperitoneally to promote rehydration and 5 mg/kg of an anti-inflammatory, Carprofen. Animals were dosed with the anti-inflammatory 24 hours post-surgery and their body weight and food and water intake were monitored daily to ensure there was no significant weight loss following surgery.

## **2.9.5. Animal sacrifice and tissue collection**

### **2.9.5.1. Decapitation**

For post-mortem investigations, animals were sacrificed by decapitation via guillotine; the brains were removed and immediately frozen on dry ice. Immediately following decapitation, the skin at the top of the head was removed using scissors. The optic ridge between the eyes was then broken using a rongeur. Scissors were used to make a shallow cut along the midline of the skull and the parietal and frontal bones were peeled to the

side. A spatula was used to tease the brain out of the skull, severing the optic nerve in the process. The brains were snap frozen on a bed of solid CO<sub>2</sub> pellets and stored at -80 °C until analysis.

### **2.9.5.2. Brain Dissection and tissue collection**

Brains were removed from the -80 °C freezer and placed in a Styrofoam box containing solid CO<sub>2</sub> pellets prior to dissection to allow for ease of access. Brains were taken out from the Styrofoam box and placed on an ice cold plate. The pre-frontal cortex and left hippocampus was isolated and approximately 2/3 of the tissue was used for PCR analysis.

### **2.9.6. Analysis of gene expression using quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)**

#### **2.9.6.1. RNA isolation**

Total RNA was extracted from homogenised pre-frontal cortex and hippocampus samples using NucleoSpin RNA II total RNA isolation kit (Macherey-Nagel, Fisher Scientific, Ireland). 354µl of RA1 lysis buffer containing 1% β-mercaptoethanol (M6250: Sigma-Aldrich, Ireland) was added to approximately 30mg tissue prior to homogenisation with an Ultra-Turrax Polytron tissue disrupter (Fisher Scientific, Ireland). Homogenates were then transferred to a Nucleospin filter column (purple) and centrifuged at 11,000g for 1 min. 350µl of 70% molecular grade ethanol (E7023: Sigma-Aldrich, Ireland) was added to the lysates and mixed by pipetting up and down 10 times. The samples were then transferred to another set of Nucleospin RNA II columns (blue) and centrifuged at 11,000g for 30 seconds to allow RNA bind to the column. Following centrifugation, the columns were placed in new collection tubes and 350µl of membrane desalting buffer (MDB, supplied with kit) was added prior to centrifugation at 11,000g for 1 minute. Genomic DNA was digested using a 10% v/v rDNase solution prepared in DNase reaction

buffer (supplied). 95µl of the rDNase solution was pipetted directly onto the centre of each column and allowed to stand for 15 minutes at room temperature, following which 200µl RA2 buffer was added to each column prior to centrifugation at 11,000g for 30 seconds. The columns were then placed in new collection tubes and 600µl of RA3 wash buffer was added followed by centrifugation at 11,000g for 30 seconds. The eluent was discarded and 250µl of RA3 wash buffer was added followed by centrifugation at 11,000g for 2 minutes. The columns were then placed in RNase-free collection tubes and the RNA was eluted by the addition of 60µl of RNase-free water (W4503: Sigma-Aldrich, Dublin) followed by centrifugation at 11,000g for 1 minute. The eluted RNA was stored on ice until RNA quantification was complete and then stored at -80°C until reverse transcription.

#### **2.9.6.2. RNA quantification and equalization**

The quantity, purity and quality of RNA were assessed using a Mastero Nano drop spectrophotometer (Medical Supply Co. Dublin). RNA quantity was determined by measuring optical density (OD) at 260nm (1 OD unit at 260nm corresponds to 40µg/ml RNA). RNA quality was determined by measuring the OD<sub>260</sub>/OD<sub>280</sub> ratio where a value of approximately 1.6-2.0 was deemed indicative of pure RNA. All RNA samples with a ratio >1.6 were accepted. Prior to cDNA synthesis, all samples were equalised to the same concentration of RNA (2µg/20µl) by addition of RNase free water. Equalised samples were then stored at -80°C until reverse transcribed.

#### **2.9.6.3. Reverse Transcription of mRNA to cDNA**

An adapted version of the Invitrogen protocol was used for the synthesis of complementary DNA (cDNA) via reverse transcription of mRNA (cDNA reverse transcription kit: Cat# 4368814, Bio Sciences Ltd.) 10µl of equalised RNA was added to

a PCR grade 200 $\mu$ L tube to which 2 $\mu$ L of master mix 1 was added (master mix 1 was made of 1 $\mu$ L 10X RT random primers and 1 $\mu$ L 10mM dNTP mix per sample). The tubes were heated to 65°C for 5 minutes in an 'MJ Research' thermocycler. 7 $\mu$ L of master mix 2 was then added (master mix 2 was made up of 4 $\mu$ L 5X First Strand Buffer, 2 $\mu$ L 0.1M USB Dithiothreitol (DTT) and 1 $\mu$ L RNase OUT per sample). The tubes were then incubated for 2 minutes at 37°C on the thermocycler. 1 $\mu$ L of superscript III reverse transcriptase was added and the contents were mixed by gently pipetting up and down. The tubes were incubated at room temperature for 10 minutes and then incubated for 50 minutes at 50°C following by heating to 70°C in the thermocycler. The resultant cDNA was diluted 1 in 4 with Rnase free water and stored at -80°C until quantification by qRT-PCR.

#### **2.9.6.4. Quantitative Real-time PCR (qRT-PCR) analysis of gene expression**

Gene expression of target proteins were determined using commercially available TaqMan gene expression assays (Applied Biosystems, UK) containing specific forward and reverse target primers and FAM-labelled MGB probes (Table 2.2.).  $\beta$ -actin was used as an endogenous control to normalise gene expression between samples and was quantified using a  $\beta$ -actin endogenous control assay (Cat# 4352340E, Bio Sciences Ltd.) containing specific primers and a VIC-labelled MGB probe. Assay IDs for the genes examined are given in Table 2.2. A reaction master mixture was first prepared and stored on ice for each target gene. This consisted of 0.5 $\mu$ l target primers, 0.5 $\mu$ l  $\beta$ -Actin (multiplex version) and 5 $\mu$ l TaqMan Universal PCR Master Mix (Cat # 4324018: Applied Biosystems, UK) per sample and 1.5 $\mu$ L of RNase free water. 2.5 $\mu$ l of each sample was pipetted in duplicate onto a MicroAmp® optical 96 well plate (Applied Biosystems, UK). 7.5 $\mu$ l of the relevant reaction mixture was then added to each well giving a total reaction volume of 10 $\mu$ l. Non-template controls (NTC) containing the master mix and Rnase free

water without cDNA for each target gene were also included. Plates were then covered with optical adhesive covers and spun at 1000g for 1 minute to ensure complete mixing and elimination of any bubbles. The plate was then placed in the real time PCR thermocycler (*StepOnePlus*<sup>™</sup>, Applied Biosystems, UK) pre-set to run the following Relative Quantification protocol: step 1: 95 °C for 10 minutes, step 2: 95 °C for 15 seconds followed by one minute at 60 °C. Step 2 was repeated 40 times and the fluorescence was read during the annealing and extension phase (60 °C) for the duration of the programme.

<b>Target Gene</b>	<b>Assay number</b>
BDNF	Rn02532967_s1
Ntrk2	Rn01441749_m1
<b>Endogenous Control</b>	
β-actin	Rn00667869_m1

**Table 2. 2:** List of Taqman gene expression assays used.

### 2.9.6.5. Analysis of qRT-PCR Data

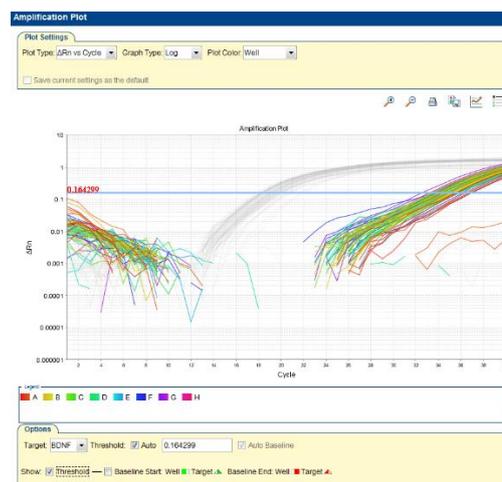
Amplification plots and copy threshold (Ct) values were examined using Applied Biosystems StepOne software V2.2.2. Ct values for each sample were analysed after setting the threshold to the linear exponential phase of the amplification plots and exporting to Microsoft Excel for final analysis (Figure 2.8). 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 control samples, allowing determination of the fold change in mRNA expression between experimental groups. This method involves 3 steps: (1) Normalisation to endogenous control (β-actin) where  $\Delta Ct$  is determined:  $\Delta Ct = Ct \text{ Target gene} - Ct \text{ Endogenous control}$ ; (2) Normalisation to control sample where  $\Delta\Delta Ct$  is determined:  $\Delta\Delta Ct = \Delta Ct \text{ Sample} - \text{average } \Delta Ct \text{ of Control group}$ ; and (3) where the fold difference is

given by  $2^{-\Delta\Delta C_t}$ . The  $2^{-\Delta\Delta C_t}$  values for each sample were then expressed as a percentage of the average of the  $2^{-\Delta\Delta C_t}$  values for the control group. In this manner the percentage increase or decrease in mRNA expression between experimental groups was determined.

A.



B.



C.



**Figure 2. 7:** Sample Amplification Plots for (A.) the endogenous control  $\beta$ -actin, (B.) BDNF and (C.) Ntrk2 receptor.

### **2.9.7. Data analyses**

All statistical analyses were carried out using IBM SPSS Statistics Version 21. A  $p$  value of less than 0.05 was considered statistically significant in all tests. Full details of statistical analyses are provided in the 'Results' section of each Results chapter (Chapters 3-5). Details on graphical and tabular representation of data are outlined in each Results chapter. Graphs were constructed in GraphPad Prism Version 5.

***Chapter 3:***  
***Sex characteristics in***  
***behavioural domains***  
***in the OB model***

### **3.1. Introduction**

As mentioned in Chapter 1, male subjects are used more in preclinical studies compared to females, particularly in the areas of neuroscience, physiology and pharmacology (Beery and Zucker, 2011). This means that most basic research of disease pathology and drug development is carried out on male subjects with the assumption that the results will be the same for females. However this thinking is flawed and it is no longer justifiable to exclude female subjects from preclinical research. In 1993, the US National Institute of Health (NIH) released an act (The NIH Revitalisation Act) requiring the enlistment of females in clinical trials which resulted in a more even use of male and female subjects in clinical research. In 2014, the NIH introduced similar guidelines for preclinical research. These guidelines state that when applying for NIH research funding, the application must describe how the researcher aims to incorporate sex as a biological variable in their experiments and whether the use of both sexes is warranted (NIH Guide Notice NOT-OD-15-102). The NIH is the world's largest public funder of biomedical research and so it is believed that these guidelines will positively impact the ratio of male to female experimental subjects in preclinical research.

As mostly male subjects were used in the development and validation of animal models of depression, it is unclear whether these models express the same validation criteria in female subjects as in males. In male rats the phenotype of the olfactory bulbectomy (OB) model of depression, which will be the focus of the following chapters, consists of hyperlocomotion due to a decreased ability to habituate to a novel environment and increased agitated behaviours as well as cognitive impairment. The OB model responds to chronic treatment with both TCAs and SSRIs meaning it has a good predictive validity. Hyperlocomotion in the OFT, the hallmark behaviour of the OB syndrome, is attenuated following chronic treatment with these drug classes. As little in the way of research using

female OB animals has been carried out, it is not known whether the behavioural manifestation of the OB syndrome is the same for females as for males.

As mentioned, hyperactivity in the OFT is the behavioural hallmark of the OB syndrome and so the OFT is one of the four behavioural tests that we will perform in this study. As well as measuring activity levels, we can also measure anxiety-like behaviour as more anxious rats will not stray from the walls of the arena, a phenomenon known as thigmotaxis which is a predisposition for rats to orient towards a touch stimulus. The EPM, which we will also perform as part of this study, also uses the thigmotaxis response to measure anxiety-like behaviours with rats that remain in the closed arms said to be more anxious than those who explore the open arms of the arena.

The FST is the most common test for measuring antidepressant response and depressive-like behaviour however even in male OB animals the published results in this test are not consistent with some reports finding increased immobility times in the OB rat compared to the sham control (Rinwa and Kumar, 2014, Jindal et al., 2015a, Wang et al., 2012), while others do not find this difference (Kelly and Leonard, 1999, Pudell et al., 2014), or only find a difference between OB and naïve rats and not when comparing OB and sham-operated subjects (Tasset et al., 2010). To the best of our knowledge, the only study that tested female OB rats in the FST found no differences between OB and sham operated rats (Stepanichev et al., 2016). The reason for these inconsistent results in the FST could be due to variability in testing procedure as it has been shown that even water temperature can affect the time that rats spend immobile as cooler water has been shown to decrease immobility time (Jefferys and Funder, 1994) and warmer (~30°C) water increasing immobility time (Drugan et al., 2005). Standardisation of testing protocols can limit this variability in the results obtained across studies and increase the reproducibility of results.

As discussed in Chapter 1, learning and memory deficits have been reported in male OB operated animals in a range of tests including passive avoidance tests which measure fear memory, the 8-arm radial maze, hole board test and Morris water maze which all assess spatial learning and memory. Again very little work examining cognitive abilities in female OB rats has been published, with one study showing impaired short-term memory (Stepanichev et al., 2016) and so we aim to increase the understanding of this domain of the depressive syndrome in female OB operated rats.

With the introduction of the NIH guidelines, it is important that we characterise the behaviour of female subjects of models of depression so that we can establish that these models can be validated in female subjects as well as to outline the similarities and differences between male and female subjects. To our knowledge, this is the first time a behavioural characterisation study has been carried out testing both male and female OB operated animals in a range of tests designed to measure anxiety- and depressive-like behaviours as well as cognitive ability.

In brief, the main aims of this chapter are to:

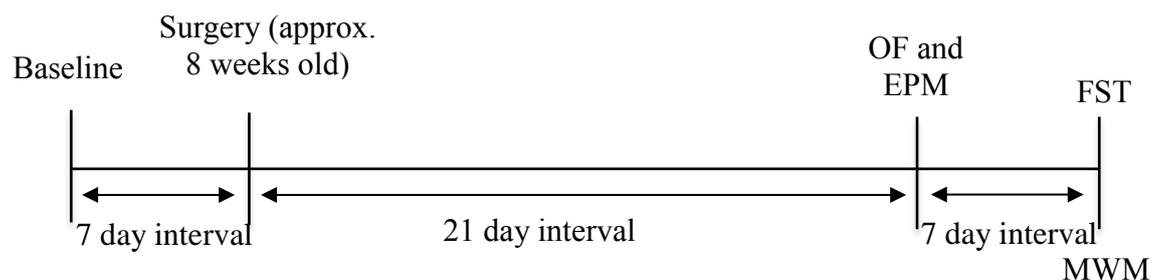
- Characterise the behaviour of female OB operated rats and determine if the OB model has face validity in female subjects
- Compare and contrast the behaviours of male and female OB rats in tests of anxiety, depression and cognitive ability

### 3.2. Experimental methods and design

A detailed description of the apparatus and test procedure used for the OFT, EPM, FST and MWM can be found in Chapter 2.

Male and female Sprague-Dawley rats were bred in-house and at 7 weeks of age were singly housed for the commencement of the study. All rats were maintained on a 12h light/dark cycle with lights on at 8am and food and water was available *ad libitum* throughout the study. At roughly 8 weeks of age OB surgery was performed on all animals across four days. The body weight for the male rats ranged between 200-250g and for the females the range was between 160-220g on day of surgery. Following surgery there were 14/15 males and 31/30 females in the OB/sham groups respectively. We used more females than males as these numbers were required for breeding purposes in the subsequent component of the study discussed in the chapter 5.

As we were not looking at any effects of drugs in this study, we tested rats in only the 15 minute swim in the FST and did not perform the 5 minute swim 24 hours later.



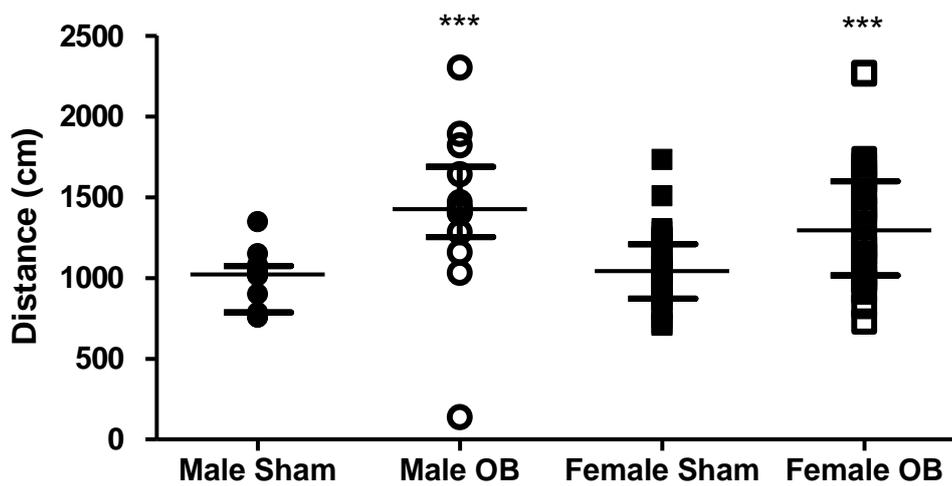
**Figure 3.1.** Experimental design for OB sex characteristics study. All subjects (n= 14/15 male OB/Sham groups and n=31/30 female OB/Sham groups) were tested in the EPM and OF which took place one directly after another on the same day. 7 subjects per group were tested in the MWM and a different 7/8 subjects per group were tested in the FST.

### 3.3. Results

#### 3.3.1. Elevated Plus Maze (EPM)

##### 3.3.1.1. Distance moved in the EPM

A Kruskal-Wallis test revealed a significant effect of surgery on the distance moved over the 5 minute trial [ $K_{(3)} = 23.141, p < 0.001$ ]. *Post-hoc* test showed that the male and female OB groups moved significantly more compared to their sham operated counterparts,  $p < 0.001$ . An outlier in the male OB group registered very little movement in the EPM as they exhibited a freezing response for the majority of the 5 minute trial.



**Figure 3.2. Distance moved in the EPM test.** Both the male and female OB groups moved significantly more compared to their sham counterparts. Data is presented as median $\pm$  interquartile range. \*\*\*  $p < 0.001$  vs sham control. Male groups  $n = 15/14$ , female groups  $n = 30/31$ .

### 3.3.1.2. Frequency and duration of open arm entries

There was a significant effect of surgery on the number of entries into the open arms during the EPM test [ $F_{(1,84)} = 8.759, p < 0.01$ ]. *Post-hoc* testing revealed that the male OB group entered the open arms significantly more compared to their sham control group.

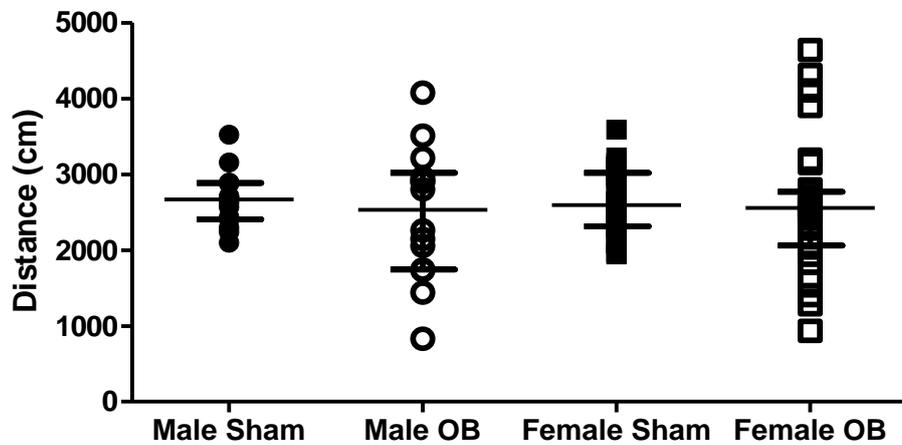
Group	Entries		Duration (s)	
	No.	%	Time	%
<b>M Sham</b>	8±5	44± 16	55± 33	26± 16
<b>M OB</b>	13± 6*	51± 12	72± 43	32± 18
<b>F Sham</b>	8± 4	49± 13	72± 36	31± 15
<b>F OB</b>	11± 7	53± 14	61± 39	25± 16

**Table 3.1. Frequency and duration of open and closed arm entries in the EPM.** Male OB rats entered the open arms more compared to the male sham group. Data presented as mean± SD. \* $p < 0.05$  vs sham control. Male groups  $n = 15/14$ , female groups  $n = 30/31$ .

### 3.3.2. Open field test (OFT)

#### 3.3.2.1. Locomotor activity in the OFT

The data did follow a normal distribution, however did not show homogeneity of variance and had unequal group numbers so was analysed by non-parametric methods (Kruskal-Wallis). The statistics confirmed that there were no differences between any of the groups, [ $K_{(3)} = 1.693, p > 0.05$ ].

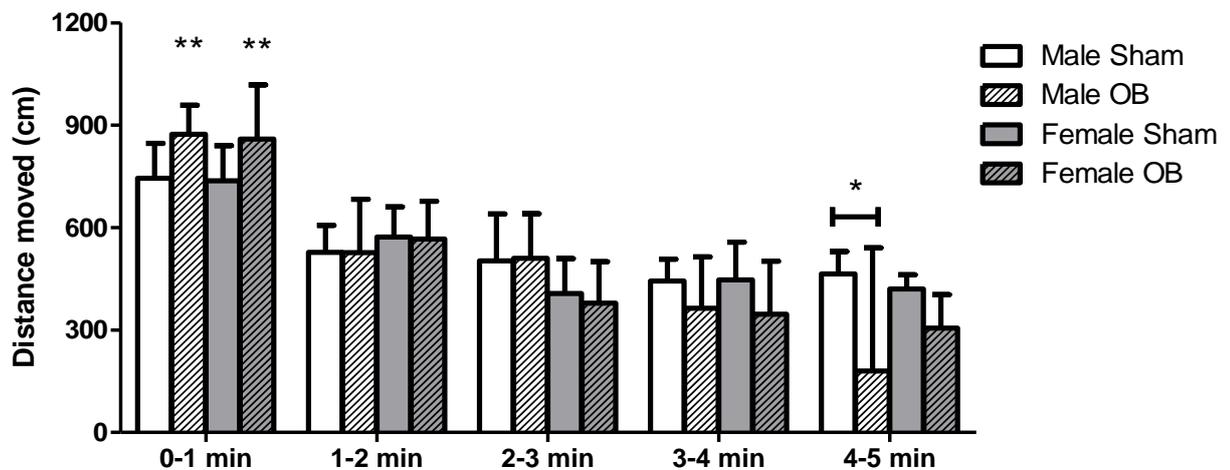


**Figure 3.3. Distance moved in the OFT 3 weeks post OB surgery.** There are no differences in the distance moved in the 5 minute open field test. Data presented as median  $\pm$  interquartile range. Male groups  $n = 15/14$ , female groups  $n = 30/31$ .

### 3.3.2.2. Distance moved in the OFT per 1 minute time bins

The 1 minute time bin data followed normal distribution and so was analysed parametrically. A Two-Way ANOVA revealed an effect of surgery between the groups [ $F_{(1,86)}= 16.061, p<0.001$ ]. *Post-hoc* Student-Newman-Keuls revealed that both male and female OB groups moved significantly more than their sham operated counterparts ( $p=0.001$ ).

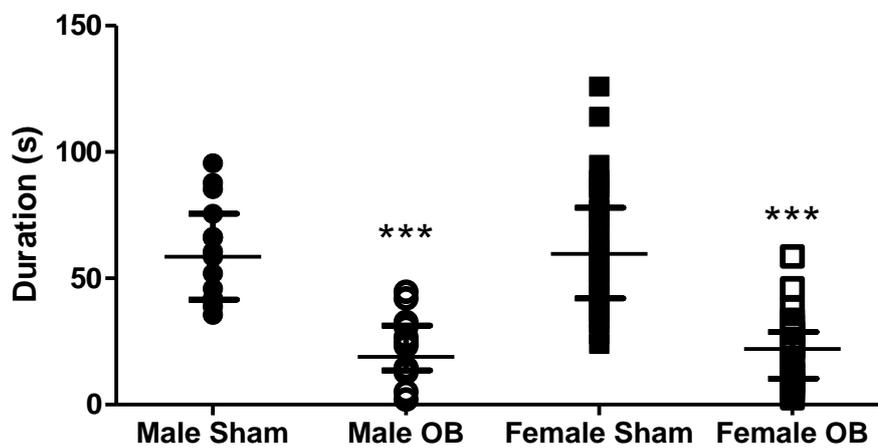
The other time bins did not meet requirements for normality and so were analysed using non-parametric methods. The 4-5 minute time bin was analysed with Kruskal-Wallis which revealed a significant difference between the groups [ $K_{(3)}= 12.885, p<0.01$ ]. *Post hoc* tests revealed that the male OB group moved significantly less compared to the male sham group ( $p<0.05$ ).



**Figure 3.4. Distance moved in the OFT per 1 minute time bins.** In the first minute of the OFT both male and female OB groups moved significantly more compared to their sham operated counterparts. In the fifth minute the male OB group moved significantly less than the male sham group. Data presented as mean + SD. \*\* $p<0.01$  and \* $p<0.05$  vs. same sex control. Male groups  $n= 15/14$ , female groups  $n=30/31$ .

### 3.3.2.3. Duration spent in the centre of the OFT arena

This data was analysed by non-parametric methods (Kruskal-Wallis). Analysis revealed a significant effect of surgery, [ $K_{(3)}= 55.393, p<0.001$ ] on inner zone duration. *Post-hoc* testing revealed a significant difference between the male sham and OB groups and between the female sham and OB groups. Both the male and female OB groups spent significantly less time in the centre of the arena compared to their sham operated counterparts.



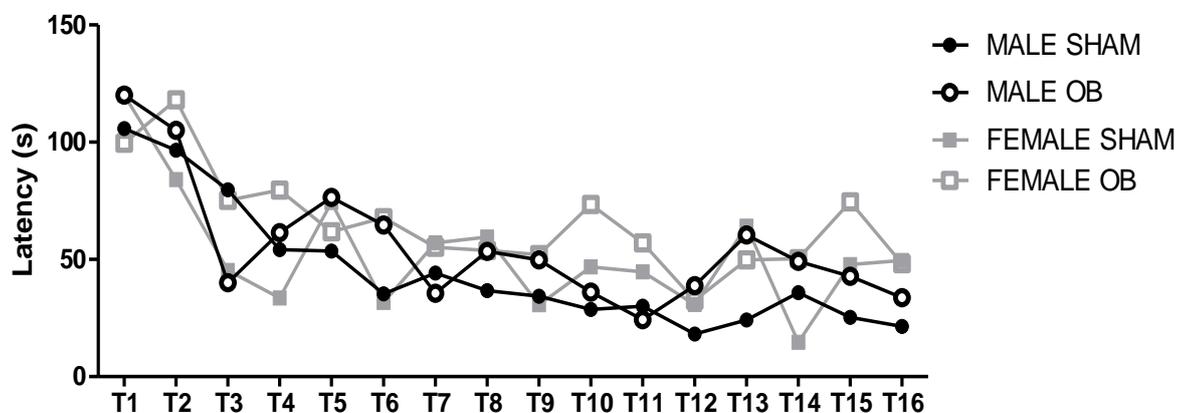
**Figure 3.5. Duration of time spent in the centre of the arena.** Both male and female OB operated animals spent significantly less time in the centre of the arena. Data presented as median  $\pm$  interquartile range. Male groups n= 15/14, female groups n=30/31. \*\*\* $p<0.001$  compared to SD control.

### 3.3.3. Morris Water Maze (MWM)

#### 3.3.3.1. Training data from the MWM

Each rat underwent 16 trials in which they were trained to find the hidden platform. The time taken to find the platform was measured and if it was not found in 2 minutes or less the rat was guided to the platform.

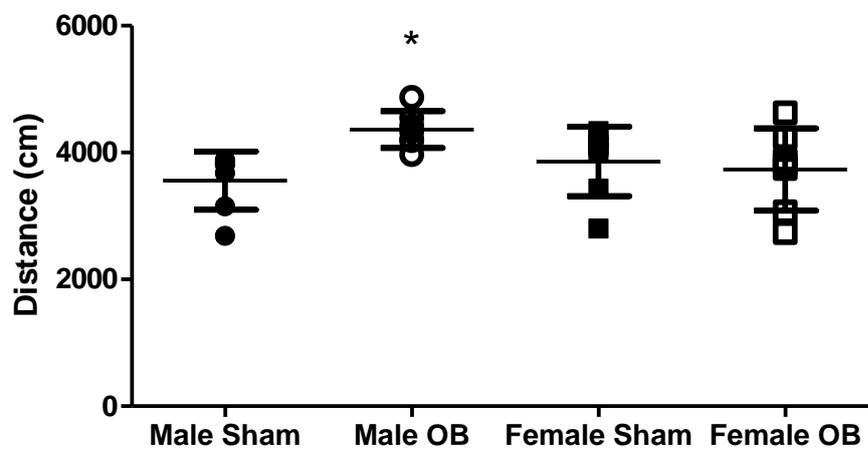
A two- way ANOVA revealed a significant interaction effect of sex\* surgery on the amount of time taken to find the hidden platform in trial 3 [ $F_{(1,24)}=4.968, p<0.05$ ]; a significant effect of surgery on the amount of time taken to find the hidden platform in trial 6 [ $F_{(1,24)}=5.193, p<0.05$ ] and a significant effect of sex on the time taken to find the hidden platform in trial 10 [ $F_{(1,24)}=4.863, p<0.05$ ] however, *post-hoc* testing revealed no differences between the groups.



**Figure 3.6. Graph of training trials for the MWM.** There were no differences found between any of the groups in the time taken to find the hidden platform during the training trials. Data expressed as mean. N= 7 per group.

### 3.3.3.2. Distance moved in the probe trial of the MWM

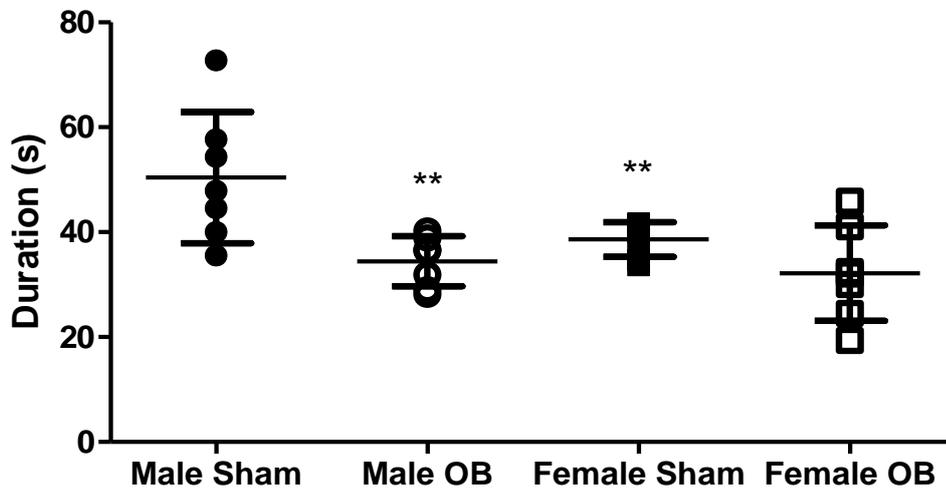
Data followed a normal distribution and so was analysed parametrically. A Two-way ANOVA revealed a significant interaction effect of sex and surgery on the distance swam in the MWM,  $F_{(1)} = 6.001$ ,  $p < 0.05$ . *Post-hoc* Student-Newman-Keuls test revealed that the male sham and male OB groups are significantly different, with the male OBs swimming more than their sham counterparts.



**Figure 3.7. Distance moved in the MWM probe trial.** The male OB group moved significantly more compared to their sham operated counterparts. Data is presented as mean  $\pm$  SD. N= 7 per group. \*  $p < 0.05$  vs control.

### 3.3.3.3. Duration in the target quadrant during probe trial

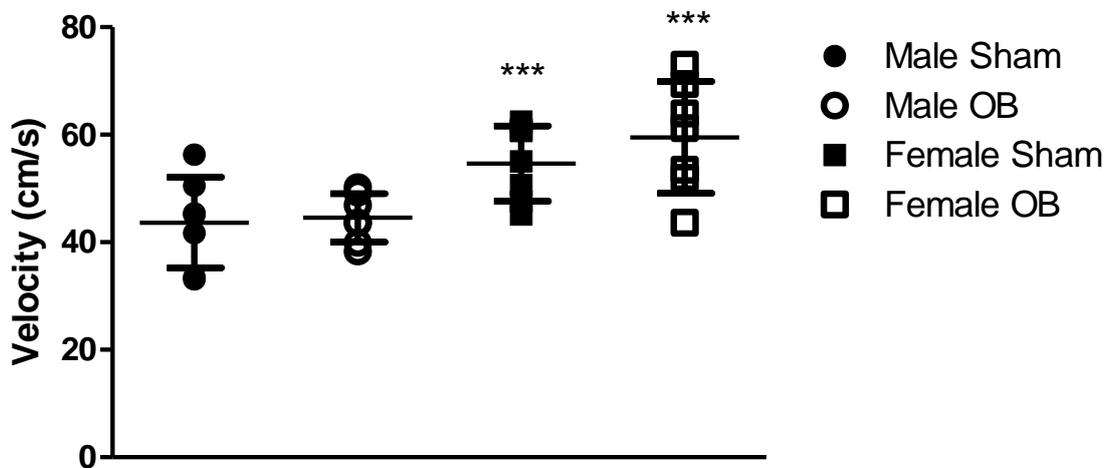
Data followed a normal distribution and so was analysed parametrically. A Two-way ANOVA revealed a significant effect of both sex [ $F_{(1,24)}=5.083$ ,  $p<0.05$ ], and surgery [ $F_{(1,24)}=12.848$ ,  $p<0.01$ ], on the time spent in the target quadrant during the test trial. *Post-hoc* Student-Newman-Keuls test revealed that the male sham group spent significantly more time in this quadrant compared to both the male OB and female sham groups.



**Figure 3.8. Duration spent in the target quadrant.** Male OB group and female sham group spent less time in the target quadrant compared to the male sham group. Data presented as mean  $\pm$  SD. N=7 per group. \*\* $p<0.01$  vs male sham group.

### 3.3.3.4. Maximum velocity in the MWM probe trial

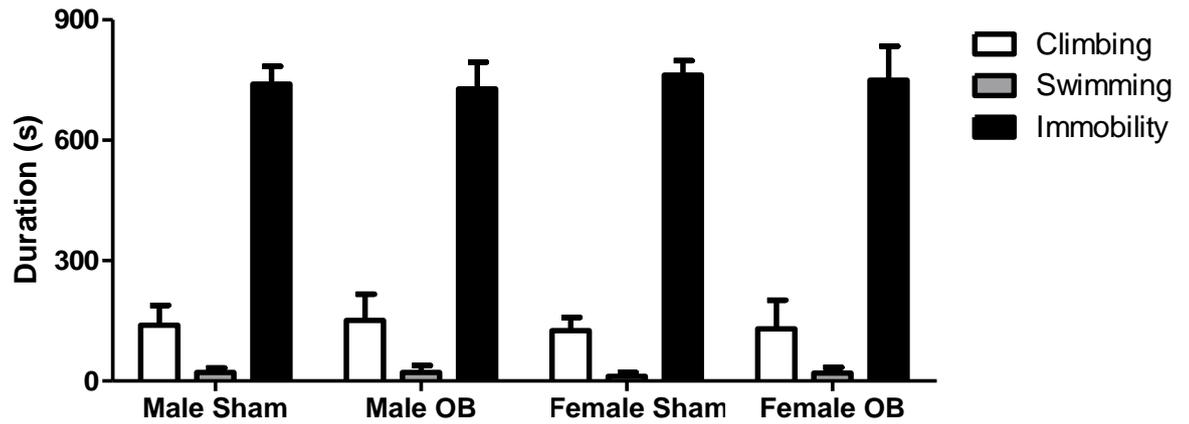
A two-way ANOVA revealed a significant effect of sex [ $F(1,24)=5.083$ ,  $p<0.05$ ] on the maximum swimming velocity during the MWM probe trial. *Post-hoc* testing revealed that the female sham and OB groups had a significantly higher maximal swimming velocity compared to their respective male group.



**Figure 3.9. Maximum swimming velocity in the MWM probe trial.** Female sham and OB groups had a greater maximum swimming velocity compared to their relative male group. Data presented as mean  $\pm$  SD. N=7 per group. \*\*\* $p<0.001$  vs male group.

### 3.3.4. Forced Swim Test

Data was normally distributed and so was analysed by parametric measures. A two-way ANOVA was carried out between the groups for each behaviour, however no differences were found.



**Figure 3.10. Analysis of behaviours during 15 minute FST.** There were no differences between the groups in any of the behaviours scored. Data presented as mean+ SD. N= 7/8 per group.

### **3.4. Discussion**

The main aim of this chapter was to assess whether female OB operated rats exhibited the same behavioural phenotype as male OB animals which have been validated for use as a model of depression. To do this we employed the use of a range of behavioural tests to measure various domains of the depressive phenotype including anxiety- and depressive-like behaviours as well as cognitive ability.

As mentioned previously, hyperactivity is the main behaviour measured in the OB model of depression and the test most commonly used to assess this is the open field test, however, OB-induced hyperactivity in the open field test was not detected in this study. We were unsure as to the reason why hyperactivity was not detected because, as mentioned previously, this behaviour is the hallmark of the OB syndrome and we allowed for a 3 week recovery period post-surgery in order to ensure enough time had elapsed to allow the development of the syndrome, reflecting the standard practice of having at least a 2 week recovery period between surgery and prior to the initiation of behavioural testing (Stepanichev et al., 2016, Pudell et al., 2014, Machado et al., 2012, Stock et al., 2001). We believe the reason for the lack of hyperactivity of OB animals in the open field was due to their first being tested in the elevated plus maze directly before open field testing. Previous unpublished work in our lab has shown that testing in the EPM directly followed by OF testing has no impact on the behaviour of non-OB operated animals in the OFT, however this was the first time this testing protocol had been performed on OB animals in our lab and to the best of our knowledge in any other lab. Other studies identified that used both the OFT and EPM and reported the order of behavioural testing, first tested the OB rats in the OFT then in the EPM later the same day (Holubova et al., 2016), the following day (Jindal et al., 2015b) or 3 days later (Stepanichev et al., 2016).

When we analysed the 5 minute OFT in 1 minute time bins, we found that OB rats did exhibit increased locomotion in the first minute of the test. This was not apparent in the later minutes of the test, thought to be due to a habituation effect. There is evidence to suggest that multiple or prolonged exposures to new environments results in habituation and a loss of the hyperlocomotion seen in OB animals (Gigliucci et al., 2014, Holubova et al., 2016). Holubova *et al* reported a normalisation of the increased locomotor activity in OB rats in the last 2 minutes of a 10 minute OFT.

Another possible cause of the lack of difference in locomotor activity between sham and OB operated animals is the observation that the locomotor scores for the sham groups appear to be higher than those found previously in both published (Burke et al., 2014, Burke et al., 2015) and unpublished work from our laboratory using the same OF testing protocol and arenas. The reason for this difference in behaviour of sham operated animals is unknown. It is the first time in our lab that we housed both male and female OB/sham animals in the same room which may have affected the animals' behaviour. Only two other studies could be found that involved male and female OB animals being housed and tested together, both of these studies came from the same lab (Stock et al., 2000, Stock et al., 2001) group however their results for locomotor activity in sham rats were similar to other findings.

As mentioned previously, rats have a natural tendency to remain close to the perimeters of their surroundings, particularly in a novel environment, which is an inherent response called thigmotaxis. In our results for the OFT, we can see that both male and female OB rats spent less time in the centre of the arena and so more time in the perimeter, indicating an enhanced thigmotaxic response which is associated with increased anxiety. It has previously been shown that male OB rats spend more time in the perimeter of the OFT arena (Roche et al., 2008, Pandey et al., 2014) and that female OB rats leave the centre

of the OFT arena faster than sham animals (Stepanichev et al., 2016). However it has not been reported before whether or not female OB rats spend significantly less time in the centre of the arena over the duration of the trial, and so we believe that this study is the first to report this.

We found hyperlocomotor activity in the EPM in both male and female OB animals. As this is also a novel anxiogenic environment it could be equated to the hyperlocomotor activity usually found in the OFT. The EPM also uses bright lights in order to create an anxiogenic environment, similar to the OF, and it has been previously shown that a bright lux is necessary in order to elicit the hyperlocomotor response, (Kelly and Leonard, 1995). For the purposes of this study, we are going to assume that the hyperactivity in the EPM as observed here is the same as that found in the OFT in other studies.

In line with previous studies which have reported increased entries into the open arms of the EPM arena (Stock et al., 2001, Jindal et al., 2015b, McGrath and Norman, 1999, Holubova et al., 2016), we found increased entries into the open arms by male OB animals compared to their sham counterparts, however female OB rats fail to exhibit this characteristic of the OB syndrome.

Impaired learning and memory has been widely reported in male OB animals. Different aspects of learning and memory have been investigated in OB animals including spatial memory which has been measured by various tests including the holeboard test (Borre et al., 2012a); the T-maze test which was also used by Borre et al., who reported deficits in short term hippocampus dependent spatial memory in OBs (Borre et al., 2012b). Fear memory has also previously been shown to be impaired in male OB rats, tested by passive avoidance tests (Douma et al., 2011). Our findings in the MWM reveal no effects of OB on the learning/acquisition phase of the test, i.e. the 16 acquisition trials performed over 4 days. However in the probe trial we did see an effect of OB surgery in male animals,

with the OB group spending significantly less time in the target quadrant compared to their sham counterparts. Our results agree with the above mentioned studies which also found deficits in spatial memory in OB male animals. A literature search revealed only one study that has examined the cognitive abilities of female OB rats which reported impaired short-term memory, as measured by the Y-maze test, however they also reported ‘more rapid acquisition and efficient recall’ in a two-way active avoidance test (Stepanichev et al., 2016). This conflicting report coupled with our lack of observation of impairments in spatial learning and memory in female OB rats suggest that further investigation should be carried out examining both spatial and non-spatial aspects of cognition.

The preclinical literature generally supports a male advantage in cognitive abilities, in particular when it comes to spatial learning and memory, confirmed by a rodent meta-analysis study (Jonasson, 2005). There is reported to be a male advantage in the water maze task however this advantage was affected by variation in testing protocols and strain. Testing protocols that utilised pre-training/learning phases, dampened the male advantage. Strain has also been shown to have an effect on the magnitude of sex differences in spatial memory with Sprague-Dawley rats showing a greater male advantage than any other strain and Wistar rats showing the smallest male advantages (Jonasson, 2005). Possible causes of this superiority of males in regards to spatial memory include anatomical and physiological differences in the hippocampus (Roof and Havens, 1992) Several research groups have looked into the effects of hormonal fluctuations caused by the oestrous cycle as well as testosterone on spatial learning, however the results are inconsistent and so it is difficult to draw conclusions from them (Roof, 1993, Warren and Juraska, 1997, Daniel et al., 1999). Our findings agree with the literature in that the male shams outperformed the female shams in the probe trial of the MWM, suggesting better spatial memory in the male animals.

There is evidence in the literature for increased immobility time in the FST in OB rats, (Rinwa and Kumar, 2014, Tasset et al., 2010, Wang et al., 2012) as well as reports of no differences between sham and OB animals (Kelly and Leonard, 1999) and even those of decreased immobility time in OB animals (Kalshetti et al., 2015). Procedural differences between studies may account for some of the inconsistent reports of immobility time in the FST, for example in the three studies reporting increased immobility time in OB rats the subjects were injected once daily including on testing days whereas in the study carried out by Kelly and Leonard (1999) which found no differences between OB and sham rats, the subjects were injected three times between the pre-swim and the test swim and finally rats were only tested in the FST for 5 minutes in the study which reported decreased immobility time in OB animals (Kalshetti et al., 2015). A lack of consistency in the protocols for behavioural tests across studies and laboratories results in a decrease of reproducible results. As will be discussed in more detail in later chapters, many consider there to be a problem in preclinical research with the ability to reproduce results and one option to improve reproducibility would be to standardise behavioural tests so that the same or very similar protocols are used across laboratories.

Only one study could be identified which investigated immobility time in female OB rats in the FST (Stepanichev et al., 2016) which reported no differences between the sham and OB groups (Stepanichev et al., 2016). Our findings show no differences in either the male or female OB and sham groups in any of the FST behaviours that were measured. However we must keep in mind that we tested the animals in the 15 minute swim only and did not perform the 5 minute swim 24 hours later and so this increases the difficulty in comparing our results to what has previously been reported. Even when the data was broken down in to 5 minute time bins and each time bin analysed separately no differences were found between the groups.

To summarise our main findings in this chapter, we found hyperactivity in both male and female OB animals which is the hallmark of the OB syndrome, however this was found in the EPM and not in the OF. The reasons for this lack of hyperactivity in the OF is thought to be a combination of both habituation to anxiogenic environments as well as unusually high sham locomotor activity scores. To the best of our knowledge, this is the first time hyperactivity has been reported in OB rats in a test other than the OFT. Our findings support previous studies which report hyperactivity in female OB animals compared to their sham counterparts, suggesting that OB is a suitable model to use for studying depressive-like behaviours in both male and female subjects. As we did not detect OB-induced deficits in spatial memory in female subjects, further work should be carried out to determine if other aspects of cognition are affected in female OB animals.

***Chapter 4:***  
***Response to chronic***  
***antidepressant***  
***treatment in male and***  
***female OB rats***

#### 4.1. Introduction

Women were first used as subjects in clinical trials in 1993 with the introduction of the NIH Revitalisation Act, meaning that testing of the efficacy and safety of pharmaceuticals had taken place on males only until that point. This would imply that information regarding the efficacy and safety of drugs in females is inferred from the results obtained in males and may not accurately represent the true clinical scenario. There are reports that suggest a sex difference in efficacy of treatment with different classes of antidepressant drugs, specifically, that females respond better to SSRIs and males to TCAs; however there are also many contradictory findings and so is it difficult to draw definitive conclusions regarding the effect of sex on response to antidepressant treatment.

In 2000 it was reported by Kornstein *et al*, that women responded better to sertraline than to imipramine and that the opposite was true for males, i.e. they responded better to imipramine than to sertraline (Kornstein *et al.*, 2000). Other reports in agreement with these findings include reports of females responding better to citalopram, an SSRI, than to reboxetine, an SNRI (Berlanga and Flores-Ramos, 2006), females are more likely to respond and reach remission with citalopram than men (Young *et al.*, 2009) and premenopausal females respond more favourably to SSRIs than to TCAs (Martenyi *et al.*, 2001).

As mentioned above there are also studies that did not find any sex differences in efficacy of treatment with different classes of antidepressants with no differences between males and females in remission rates or time to remission when treated with MAOIs, SNRIs, SSRIs or TCAs (Entsuah *et al.*, 2001, Parker *et al.*, 2003, Quitkin *et al.*, 2002, Thiels *et al.*, 2005).

Meta-analysis studies have been carried out, combining data from multiple clinical trials, in order to draw better conclusions from the inconsistent individual observations. Two

large meta-analysis studies found no effect of sex on the efficacy of treatment with antidepressant drugs (Cuijpers et al., 2014, Kornstein et al., 2014). One of these studies found that sex was not a factor in response to either pharmacotherapy or CBT in the treatment of depression (Cuijpers et al., 2014). The second study found no effect of sex on efficacy of response to venlafaxine or fluoxetine, an SNRI and SSRI respectively (Kornstein et al., 2014). Limitations are apparent in both of these meta-analyses. The first study mentioned above carried out by Cuijpers et al (2014), grouped all classes of antidepressant drugs together as ‘pharmacotherapy’ comparing it to CBT and so it is possible that effects of sex on responsiveness to individual drugs is being missed (Cuijpers et al., 2014) and the second study mentioned is limited to looking at only the SNRI and SSRI drug classes (Kornstein et al., 2014).

It is suggested that menopausal status may impact the efficacy of different classes of antidepressant drug treatment. Premenopausal women prescribed TCAs report more adverse effects compared to postmenopausal women also taking TCAs and premenopausal women responded better to SSRI treatment compared to treatment with TCAs, whereas there was no difference in response rate between the two drug classes in postmenopausal women (Kornstein et al., 2000, Martenyi et al., 2001, Pinto-Meza et al., 2006). These findings suggest a role of ovarian hormones in the action and efficacy of antidepressant treatment. Oestrogen is known to interact with the serotonergic system which may affect the action of antidepressant drugs, in particular SSRIs. In support of this, studies have found that depressed postmenopausal women treated with supplemental oestrogen in the form of hormonal replacement therapy (HRT), either alone or in combination with an SSRI exhibited improved responses compared to those receiving the SSRI alone (Schneider et al., 1997, Liu et al., 2004, Gordon and Girdler, 2014).

Reasons for the inconsistent findings may be down to differences in therapeutic agents used as well as different dosages, dosing regimes and duration of treatment. Another important factor which may account for some of the variability in results is the level of adherence to treatment by the subjects (Sramek et al., 2016). The criteria for determining a significant response to treatment also varied across studies making comparisons difficult. For example, one study used paired t-test to analyse the difference in HAM-D-17 score from baseline to assessment (Martenyi et al., 2001), whereas another study classed response as at least a 50% decrease in HAM-D-17 score (Entsuah et al., 2001).

Rodents are routinely used in laboratories for drug discovery and safety testing. The use of male animals vastly outnumbers that of females in preclinical research meaning that it is possible that drugs found to be efficacious in males may not be in females or may have more adverse side-effects. Recently the NIH which is the largest funder of basic research in the world, has brought in guidelines to ensure the appropriate use of female subjects in NIH funded preclinical research. As depression affects almost twice as many more women than men, it is prudent that we ensure the animal models used to study depression appropriately model the disease as well in female subjects as in males in order to allow for the use of female subjects as models of depression in future research.

The olfactory bulbectomy (OB) model of depression was developed based on the theory that lesions to areas in the limbic system or brain areas that project to or from the limbic system may result in a depressive phenotype due to the high involvement of this brain area in emotional processing. Olfactory bulbectomy results in anterograde and retrograde degeneration of neurons that connect the bulbs with other brain regions including the amygdala, hypothalamus, ventral hippocampus and ventral tegmental area. Chronic administration of both TCAs and SSRIs reverse the behavioural profile of the OB model

which does not occur with acute dosing regimes (Jancaš and Leonard, 1981) which gives the model a high predictive validity.

Few investigations into possible sex differences in the OB model have been carried out thus far. Importantly, the main behavioural endpoint measured in the OB rat, OFT hyperactivity, is maintained in female OB subjects (Stock et al., 2000, Stock et al., 2001). There are inconsistent findings regarding other behaviours in the OFT with female OB subjects reported to have both increased rearing activity and faecal boli counts compared to shams (Pandey et al., 2014) as well as no differences between female OB and sham groups (Stepanichev et al., 2016). A literature search found no studies that have investigated the possibility of sex differences in responsiveness to antidepressant drugs in the OB model. As mentioned, male OB animals respond to chronic but not acute treatment with two common antidepressant drug classes, SSRIs and TCAs, and so it is important that we characterise the response of female OB animals to these drugs in order to determine if sex differences exist and also to conclude if the OB model is an appropriate method of modelling depression in female subjects.

Neurotrophic factors (NTFs) are biomolecules that promote the survival, growth and differentiation of both mature and developing neurons. Brain-derived neurotrophic factor (BDNF) is the most studied of these molecules with regards to links with depression. It is thought that decreased levels of BDNF leads to neuronal atrophy in the hippocampus, a key limbic region implicated in depression. The hippocampus has important roles in learning and memory and regulation of the HPA axis. It also has connections to the amygdala and prefrontal which are involved emotion and cognition and are linked to major symptoms in depression. Supporting this theory is the finding of decreased hippocampal volume in imaging studies of patients with depression (Gerritsen et al., 2015, McKinnon et al., 2009, Videbech and Ravnkilde, 2004).

Both physical activity and antidepressant treatment have been shown to increase levels of BDNF mRNA in the hippocampus of rats (Russo-Neustadt et al., 2000, Van Hoomissen et al., 2003). There is also evidence for antidepressants to increase hippocampal BDNF in humans with higher levels of BDNF found in post-mortem hippocampal samples taken from patients diagnosed with depression who were being treated with an antidepressant medication at the time of death (Chen et al., 2001).

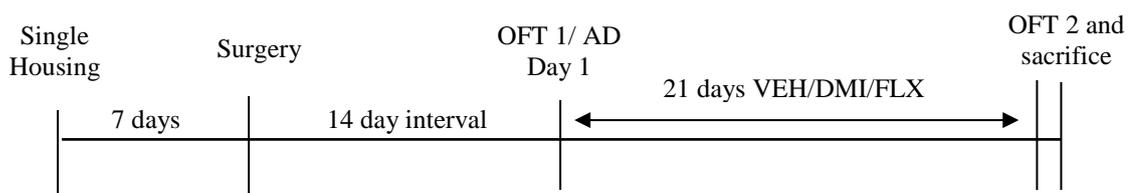
OB males have been shown to exhibit lower hippocampal levels of BDNF (Pudell et al., 2014, Maturana et al., 2015) and so we plan on measuring BDNF mRNA to determine if this effect of OB is also observed in females and to examine whether BDNF can be used as a biomarker for detecting depression or response to antidepressant treatment. In order to do this we will measure BDNF and its receptor NTRK2 mRNA levels in both the hippocampus and pre-frontal cortex of sham and OB operated male and female rats.

Aims for this chapter:

- Assess the effect of chronic antidepressant treatment on the main behavioural endpoint of OB male and female rats
- Investigate possible sex differences in responsiveness to two classes of antidepressant drugs
- Investigate if chronic treatment with an SSRI or TCA affects BDNF mRNA levels in two discrete brain regions of OB rats
- Investigate if chronic treatment with an SSRI or TCA affects TrkB mRNA levels in two discrete brain regions of OB rats

## 4.2. Experimental design

Male and female Sprague- Dawley rats were bred in-house and at ~7 weeks of age were singly housed for commencement of the study. Animals were maintained under a 12h light cycle (lights on at 08:00h) with food and water provided *ad libitum*. Desipramine (DMI) and fluoxetine (FLX) were dissolved in sterile distilled water which was used for vehicle (VEH) injections. Drugs were administered s.c. at a volume of 4ml/kg at a dose of 10mg/kg daily for 21 days at approx. 2-4pm. The OFT was performed 14 days post-surgery and the scores for locomotor activity were used to determine drug groups. To do this, the score for distance moved was sorted from highest to lowest for the sham and OB groups and then drugs were randomly allocated so that the mean distance moved for each drug group was as similar as possible to the overall distance moved for the group as a whole. The OFT was performed for a second time 18-23 hrs after the last dose was administered. All rats were killed immediately following the second OFT by decapitation and brains were removed and snap frozen on dry ice for later post-mortem analysis which consisted of rt-qPCR analysis of relative BDNF and NTRK2 mRNA levels.



**Figure 4.1.** Experimental design for OB antidepressant study

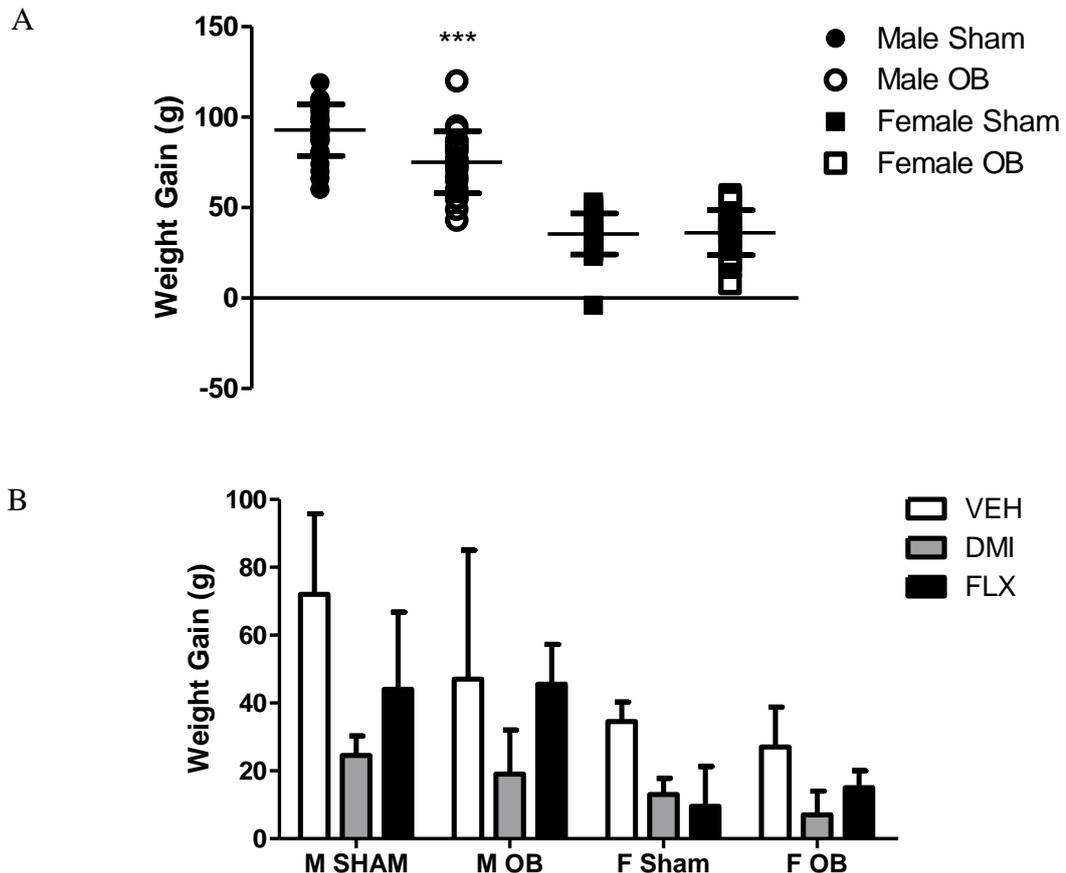
Upon post-mortem examination, the brains were visually examined and any deemed to have olfactory bulb remaining or cortical damage were removed from all behavioural and neurochemical analysis so that the final group numbers were as follows:

N= 10 for all male and female sham groups; n=7 male OB Veh; n=7 male OB DMI, n=8 male OB FLX; n= 8 female OB Veh; n= 7 female OB DMI; n=7 female OB FLX.

### 4.3. Results

#### 4.3.1. Weight gain following surgery and antidepressant treatment

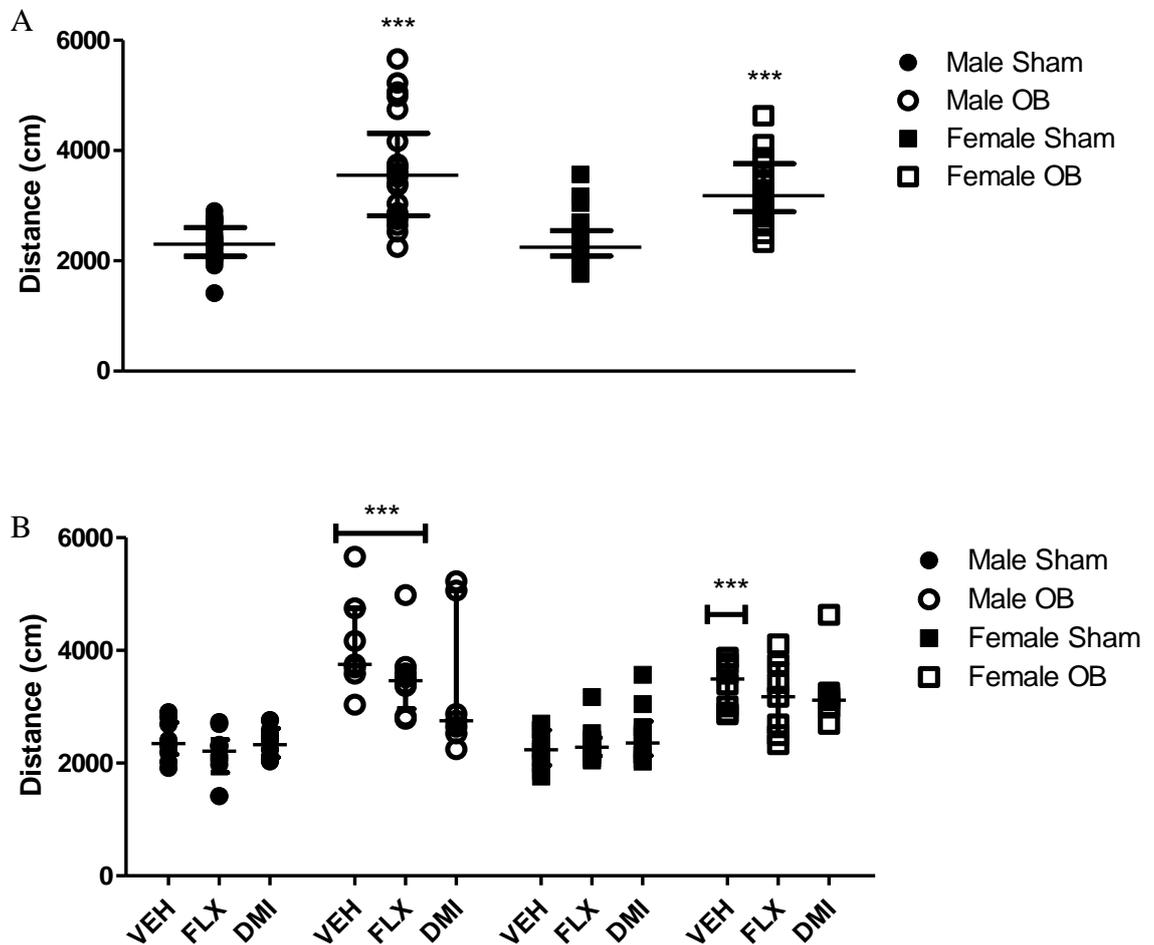
A two-way ANOVA revealed a significant effect of sex, surgery and an interaction effect between sex and surgery [ $F_{(1,100)}= 309.192, p<0.001$ ;  $F_{(1,100)}= 10.929, p<0.01$ ;  $F_{(1,100)}= 10.168, p<0.01$ ]. *Post-hoc* test revealed that the male OB group gained significantly less weight compared to their sham counterparts in the 14 day recovery period following surgery. Weight gain following 21 days of drug dosing was analysed with a Kruskal-Wallis test which revealed a significant difference between the groups [ $K_{(11)}= 65.410, p<0.001$ ] however *post-hoc* testing revealed no relevant differences.



**Figure 4.2.** Male OB group gained significantly less weight compared to their control group in the recovery period following surgery. There were no differences in weight gain following drug treatment. Data presented as (A) mean  $\pm$  SD and (B) median  $\pm$  interquartile range. (A)  $n= 22-30$ ; (B)  $n= 7-10$ . \*\*\*  $p<0.001$ .

## 4.3.2. Distance moved in the OFT 2 weeks post OB

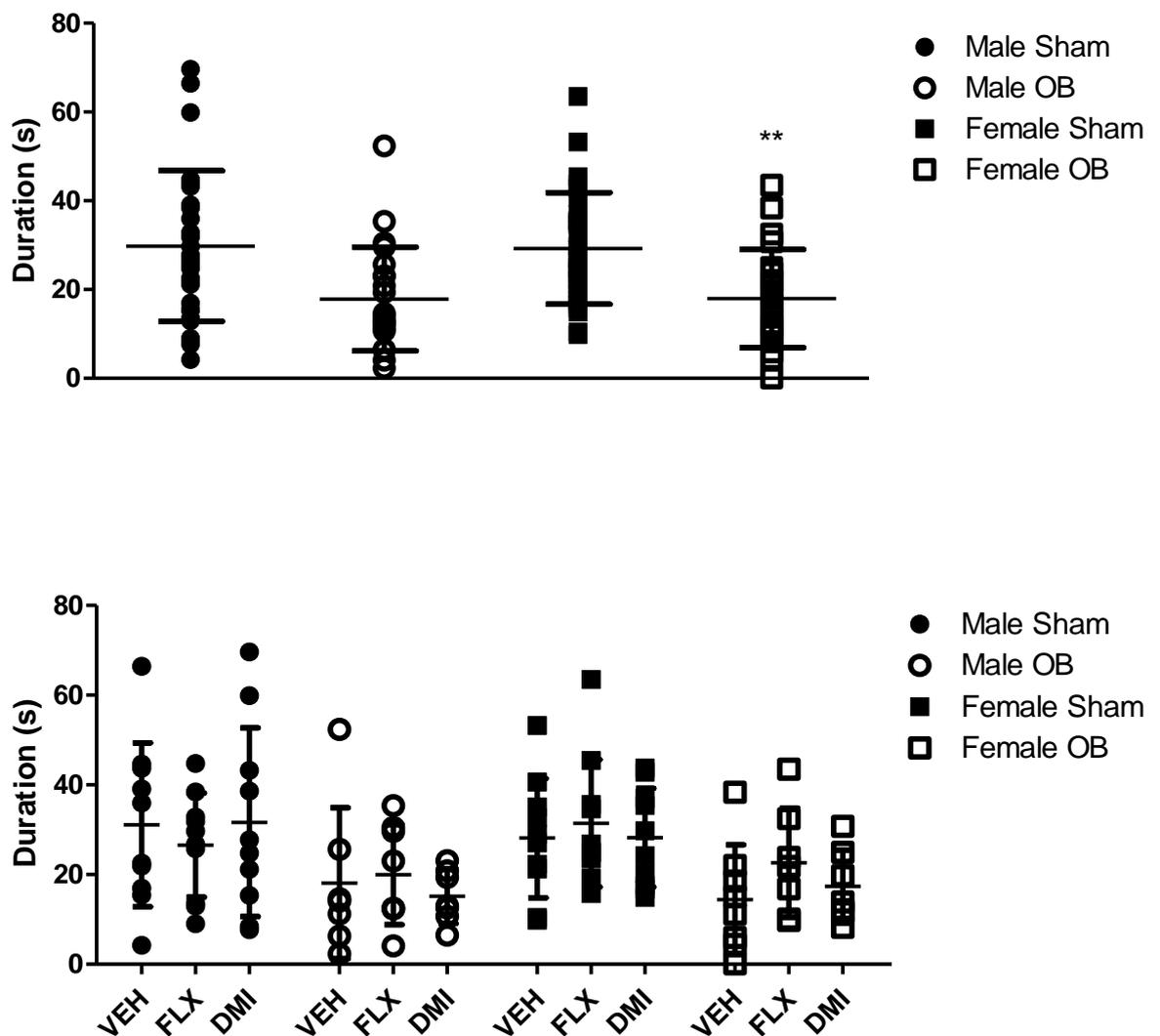
A Kruskal-Wallis test revealed a significant effect of surgery [ $K=37.320, p<0.001$ ]. *Post-hoc* testing revealed that both male and female OB groups moved significantly more compared to their sham counterparts,  $p<0.001$ . Distance moved was re-analysed following assignation of rats to dosing groups which revealed a significant difference between the groups [ $K=62.210, p<0.001$ ]. *Post-hoc* test showed that the Male OB vehicle and fluoxetine groups moved significantly more compared to their sham counterparts and the Female OB vehicle group moved significantly more than their sham counterparts.



**Figure 4.3. OFT 2 weeks post OB surgery.** Graph A shows the distance moved in the OFT between the sham and OB groups 2 weeks post-surgery. Graph B shows the same results but with the sham and OB groups further split into their drug groups. Data presented as median  $\pm$  interquartile range. (A)  $n=22-30$ ; (B)  $n=7-10$  per group. \*\*\* $p<0.001$  vs. sham group.

## 4.3.3. Duration in Inner Zone of OFT 2 weeks post OB

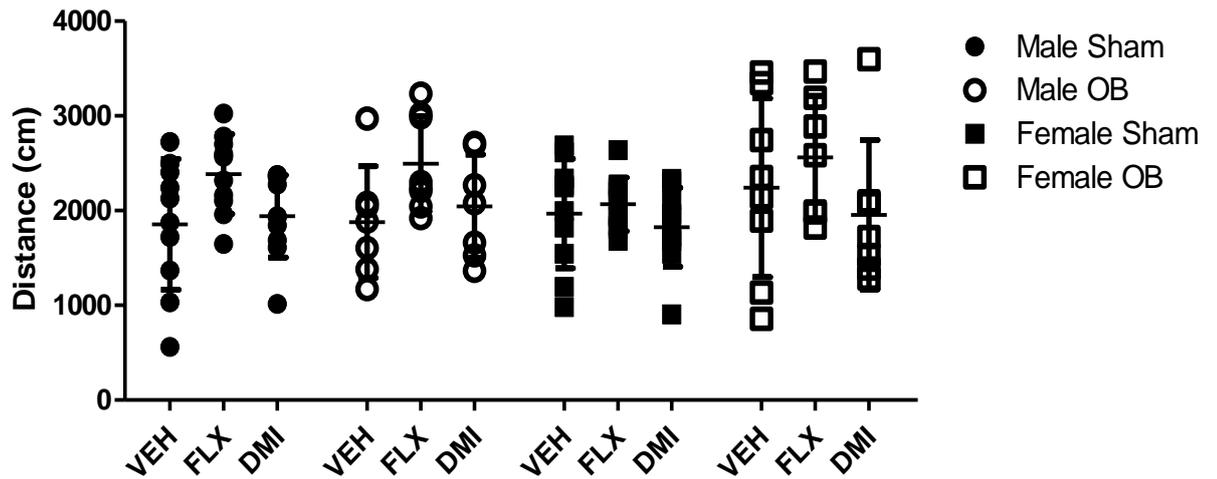
This data passed tests of normality and homogeneity of variance and so a two-way ANOVA was used to analyse it which revealed a significant effect of surgery on the duration of time spent in the inner zone of the OFT arena [ $F_{(1,100)}= 18.614, p<0.001$ ] [ $F_{(1,92)}= 17.531, p<0.001$ ]. *Post-hoc* analysis revealed that the female OB group spent significantly less time in the centre compared to the sham group however when assigned to dosing groups no differences between the groups,  $p=0.053$ .



**Figure 4.4. Duration spent in the inner zone of the OFT.** There was a trend for a surgery effect in the time spent in the centre of the OFT arena however post-hoc testing failed to detect differences between the groups. Data presented as mean $\pm$ SD. N= 7-10 per group. \*\*\* $p<0.001$  vs. sham group.

**4.3.4. Distance moved in the OFT post 21 days dosing**

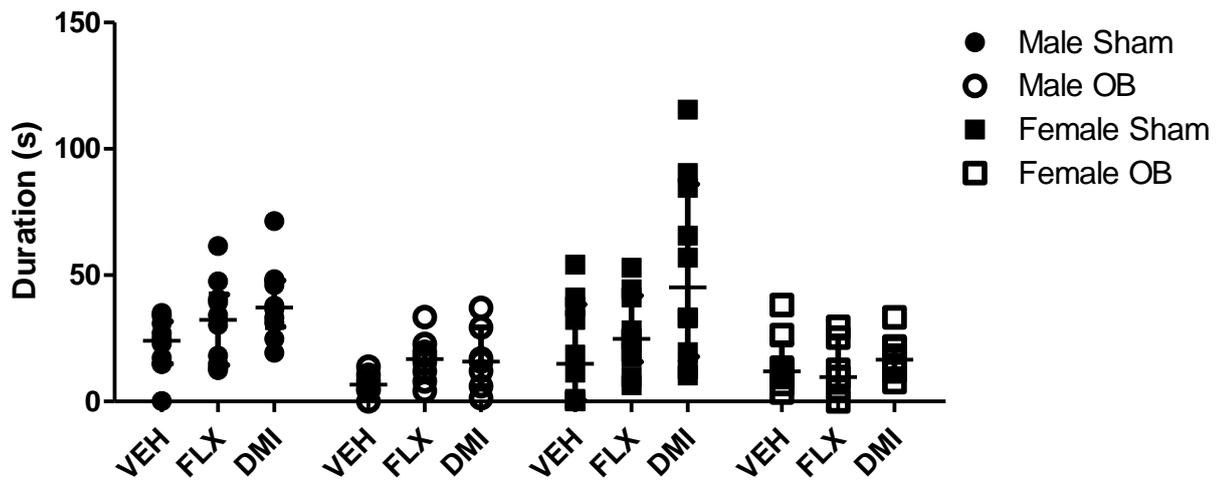
A Two-Way ANOVA revealed a significant effect of drug on the distance moved in the OFT following 21 days of dosing [ $F_{(2,92)}= 5.778, p<0.01$ ]. *Post-hoc* analysis revealed no differences between the groups.



**Figure 4.5. Distance moved in the OFT following 21 days drug dosing.** No differences were found between the groups for distance moved in the OFT following dosing with either DMI or FLX. Data presented as mean± SD. N= 7-10 per group.

#### 4.3.5. Duration in Inner Zone of OFT post dosing

As the data did not meet the standards to be analysed parametrically, a Kruskal-Wallis non-parametric test was used which revealed a significant difference between the groups [K= 35.994,  $p < 0.001$ ]. *Post-hoc* testing revealed no meaningful differences between the groups.



**Figure 4.6. Duration in the inner zone of the OFT post 21 days drug dosing.** No differences were found between the groups. Data presented as median  $\pm$  interquartile range. N= 7-10 per group.

#### 4.3.6. Relative levels of BDNF and NTRK2 mRNA in the hippocampus and frontal cortex following antidepressant treatment

BDNF and NTRK2 mRNA levels in the hippocampus were analysed non-parametrically with a Kruskal-Wallis test which revealed no differences between the groups.

A Kruskal-Wallis test was used to analyse data for the levels of BDNF and NTRK2 mRNA in the frontal cortex of rats following 21 days of antidepressant treatment. The analysis revealed no differences between the groups.

Group	Hippocampus		Frontal Cortex	
	BDNF	Ntrk2	BDNF	Ntrk2
<b>M Sham VEH</b>	1± 1.18	1± 1.28	1± 0.75	1± 1.48
<b>M Sham DMI</b>	1.26± 1.3	0.58± 1.04	1.79± 2.06	1.09± 1.66
<b>M Sham FLX</b>	1.67± 1.51	0.63± 1.15	1.82± 1.9	0.91± 0.69
<b>M OBX VEH</b>	0.82± 0.72	0.88± 1.47	1.3± 1.7	0.91± 1.12
<b>M OBX DMI</b>	1.09± 1.67	0.58± 0.86	0.29± 0.36	0.72± 1.02
<b>M OBX FLX</b>	0.83± 0.67	1.11± 1.19	0.46± 0.49	0.6± 0.89
<b>F Sham VEH</b>	1± 0.56	1± 1.06	1± 0.63	1± 1.95
<b>F Sham DMI</b>	1.56± 1.49	0.51± 0.82	1.13± 1.64	0.83± 1.22
<b>F Sham FLX</b>	0.95± 0.79	0.53± 0.78	1.74± 1.73	0.94± 1.54
<b>F OBX VEH</b>	1.07± 0.74	0.8± 0.99	0.99± 0.93	0.79± 1.01
<b>F OBX DMI</b>	1.54± 1.16	0.61± 0.92	2.73± 2.57	0.91± 1.22
<b>F OBX FLX</b>	1.23± 0.89	0.39± 0.65	0.59± 0.37	0.76± 1.12

**Table 4.1. Relative levels of BDNF and NTRK2 mRNA in the hippocampus and frontal cortex.** The male and female sham VEH groups were used as comparator groups, i.e. relative mRNA levels were calculated for other groups based on values from control groups. There were no effects of drug treatment on the level of BDNF or NTRK2 mRNA in the FC or hippocampus of male and female OB and sham operated rats. N= 6-10 per group. Data mean± SD.

### **4.3. Discussion**

In this chapter we aimed to investigate potential sex differences in response to chronic antidepressant drug treatment in the OB model of depression. Hyperactivity in the OFT is the main behavioural output measured in the OB model of depression as so we chose to assess the behaviour of male and female OB and sham operated animals in this test to determine if sex differences occurred in this model in regards to responsiveness to treatment with different classes of antidepressants.

We observed a reduced weight gain in male OB operated animals in the 2 week recovery period following surgery when compared to the male sham group which has previously been reported (Kelly et al., 1997). This effect of OB surgery was not observed in female animals. In the 21 day drug dosing period there were no differences in the amount of body weight gained in male or female groups suggesting that the surgery effect observed in males had disappeared at this stage and that there were no effects of DMI or FLX on body weight gain. Although there was a trend for decreased weight gain in both the male and female sham DMI treated groups compared to their respective vehicle dosed control. Chronic desipramine treatment has been previously shown to cause a reduction in body weight gain in rats (Lucki and Frazer, 1985, Nobrega and Coscina, 1987) which is opposite of the clinical scenario which often reports that chronic treatment with antidepressants including desipramine results in increased weight gain (Gobshtis et al., 2007).

When all subjects were grouped together, hyperactivity was observed in the male and female OB groups 14 days post-surgery, however when the subjects were allocated into their treatment groups and the scores for distance moved in the OFT were re-analysed using these groups, there were no longer differences in the distance moved between some of the sham and OB groups, namely the male DMI groups and the female FLX and DMI

groups. A possible reason for this lack in difference in locomotor activity between the sham and OB groups may be due to the higher than usual scores for distance moved in the sham groups. In previous studies carried out in our lab the average score for distance moved in sham groups was lower than the scores reported in this chapter. In order to overcome this obstacle we decided to compare each group pre- and post-dosing scores so that each group acts as its own control. These results show that all of the male and female groups exhibited decreased locomotor activity following chronic dosing, however as the vehicle treated groups also exhibited decreased locomotor activity compared to their pre-dosing scores, we cannot definitively say that the reduction in distance moved in the groups dosed with desipramine and fluoxetine was due to antidepressant activity. This would suggest that the decrease in locomotor activity observed here is due to habituation to the testing arena which has been shown to occur in OB rats previously (Holubova et al., 2016). This level of habituation appears to be the same in both male and female rats.

We observed a decreased time spent in the centre of the arena in female OB rats compared to their sham operated counterparts 14 days post-surgery which was not evident in the male group. The centre of the open field arena is considered to be more anxiogenic compared to the outer edges as rats have a natural proclivity to be in contact with the walls of their environment, called thigmotaxis. This result would suggest increased anxiety-like behaviour in the female OB group compared to their sham control. There were no differences between the male OB and sham groups similar to reports published previously from our lab (Burke et al., 2010). While there was a trend for a decreased duration spent in the inner zone in male OB rats compared to their sham controls ( $p=0.07$ ) it did not reach significance and so this result failed to replicate that in our previous chapter in which we did find decreased time in the inner zone of the OFT in both male and female OB rats.

Another aim of this chapter was to determine if chronic antidepressant treatment had an effect on the levels of BDNF or NTRK2 mRNA in male or female OB animals. Brain derived neurotrophic factor (BDNF) is a member of the neurotrophin family of peptides which have neuroprotective qualities. BDNF has been found to play a role in neuronal cell differentiation, neuronal survival and synaptogenesis. Clinically, decreased levels of BDNF in the serum of depressed patients is a common finding (de Azevedo Cardoso et al., 2014, Fornaro et al., 2015) however the relevance of serum BDNF in regards to depression is not yet fully understood. It has also been found that there is reduced levels of both BDNF and its receptor *trkB* mRNA has been found in the hippocampus of victims of suicide (Banerjee et al., 2013). Treatment with antidepressant medications has been shown to increase brain BDNF levels in MDD patients compared to non-treated patients (Sheldrick et al., 2017).

BDNF has an important role in dendritic plasticity and growth of neurons and it is believed that decreased BDNF levels contribute to the decreased hippocampal volume previously reported in depressed patients (Gerritsen et al., 2015, McKinnon et al., 2009). Decreased BDNF and *TrkB* mRNA levels have previously been reported in post-mortem hippocampal samples of depressed patients (Dwivedi et al., 2003) as well as in stress models of depression (Rasmusson et al., 2002). As the frontal cortex is involved in cognitive processing together with the hippocampus, it is suggested that this brain area may be involved in mediating memory impairments and cognitive disruption observed in depressed patients (Nestler et al., 2002). Similar to the hippocampus, post-mortem studies have reported decreased BDNF and *TrkB* mRNA levels in the pre-frontal cortex and antidepressant treatment increases both hippocampal and PFC BDNF levels to within normal ranges (Castren, 2004). The clinical evidence for the implication of altered BDNF levels in the hippocampus and pre-frontal cortex contributed to our decision to investigate these brain regions in male and female OB rats.

Neither hippocampal nor frontal cortex levels of BDNF or NTRK2 mRNA were altered in the OB rats nor were they altered following chronic antidepressant treatment. There are varying reports as to the effect of OB on BDNF levels with some finding a reduction in BDNF following OB (Rinwa et al., 2013, Hendriksen et al., 2012) while it has also been reported that OB has no effect on brain levels of BDNF (Van Hooymissen et al., 2003). Evidence in the literature suggests that BDNF levels are increased in a manner that is associated with antidepressant drug efficacy, for example, chronic administration of the traditional antidepressants DMI at a dose of 10mg/kg (Liu et al., 2014) and FLX at doses of 10mg/kg and 20mg/kg (Wang et al., 2016, Xie et al., 2015), have decreased depressive-like behaviour with an associated increase in hippocampal BDNF. As we did not observe any antidepressant induced reduction in hyperactivity in the open field test, our finding of no treatment effect on brain BDNF mRNA levels did not come as a surprise.

Similar to our results for BDNF, NTRK2 mRNA levels were not altered either by OB or following chronic antidepressant treatment. Although the study of the role of the TrkB receptor in depression and antidepressant response is not as common as that of BDNF, some studies assessing chronic effects of DMI (Nibuya et al., 1995) and FLX (Wang et al., 2015b) on TrkB levels have shown alterations in the hippocampus, however, the association of these alterations with a decrease in depressive-like behaviour has not been demonstrated.

In conclusion, we did not observe any sex differences in the OB model of depression in regards to responsiveness to chronic antidepressant treatment with both a TCA and an SSRI drug. We did find that female OB rats exhibited an anxiety-like behaviour 14 days following OB surgery which was not evident in male animals which may suggest the presence of an anxiety-like phenotype in female OB rats that has not been characterised

in males. We observed neither behavioural nor neurochemical responses to chronic treatment with antidepressant drugs in this study, however, a drug effect on hyperactivity in the open field may have been obscured due to habituation of the animals to the arena following multiple exposures. To prevent this confounding effect of habituation on hyperactivity in future studies, we suggest to limit the exposure of rats to the OFT arena to once where possible.

***Chapter 5:***  
***Olfactory Bulbectomy***  
***Reproductive Ability***  
***and Neonatal***  
***Development Study***

## **5.1. Introduction**

As previously discussed, women are almost twice as likely to be diagnosed with depression during their lifetime compared to men. The increased rate of depression is true for women of all age groups from the age of 16+ (Angst et al., 2002a). It has recently been reported that in the U.S., 1 in 20 women of childbearing age (20-44 years) experience major depression (Guo et al., 2018) and because of this, the rates of depression during or shortly after pregnancy are relatively high, with the prevalence of perinatal depression estimated to be 11.9% (Woody et al., 2017).

Depression occurring around pregnancy was not classified as being any different from regular depression until the release of the DSM-IV in 1994 which outlined a ‘postpartum onset specifier’ which was defined as onset of a depressive episode within 4 weeks of childbirth. In the most recent version, DSM-5, the postpartum onset specifier was changed to ‘peripartum onset’ defined as the most recent episode which occurred during pregnancy or within the 4 weeks following childbirth.

Perinatal, particularly postpartum, depression has a strong genetic component. Perinatal depression is more common in women who have exhibited depressive episodes at other times in life or who have a family member diagnosed with depression. Evidence of a genetic component is described in a study which found that out of 31 new mothers who had a sister with previously diagnosed postpartum depression, 29% of these were themselves diagnosed with postpartum depression. This is significantly higher than the 12% of new mothers who were diagnosed with PPD who did not have a sister previously diagnosed with the disorder (Forty et al., 2006).

Perinatal depression has been linked to multiple negative effects in pregnancy as well as in the neonate, including preeclampsia (Kurki et al., 2000), preterm birth (Jesse et al., 2003, Orr et al., 2002), low birth weight (Greene et al., 2015), as well as impaired mother-

child bond and adverse effects later in childhood. A possible cause of growth delays and low birth weight seen in neonates born to depressed mothers may be due to elevated gestational cortisol. Elevated cortisol is a common feature of depression and cortisol has the ability to cross the placental barrier and so it is believed that maternal cortisol crosses the placenta, entering the foetus which results in foetal HPA hyperactivation, which then affects foetal growth (Diego et al., 2006). Foetuses with mothers exhibiting high levels of cortisol were more likely to display growth delays at mid-gestation, including reduced head and abdominal circumference and weight (Field et al., 2006).

A meta-analysis of studies reporting on the early interactions between postpartum depressed mothers and their infants found that these mothers were more likely to show irritability and hostility, to have lower rates of play interaction and show less loving emotions towards their infants (Lovejoy et al., 2000). Data also shows that children whose mothers had depression, had increased number of Accident and Emergency hospital visits in the first year of life and these children had decreased receipt of preventative services, such as age-appropriate GP visits and up-to-date vaccinations (Minkovitz et al., 2005).

Animal models of disease are used in order to better study disease aetiology and pathophysiology. Animal models of neuropsychiatric diseases, including depression, have advanced our knowledge of these diseases as well as therapeutic options. However, due to the underuse of female subjects in pre-clinical research, little investigation has been carried out on perinatal depression using animal models.

One model of postnatal depression used in pre-clinical research is the hormone withdrawal model. It is based on the rapid decrease in levels of circulating ovarian hormones, from the high levels occurring during pregnancy to pre-pregnancy levels that occurs shortly after birth. Clinical evidence for the implication of ovarian hormone

fluctuations in the onset of postpartum depression has been provided by way of withdrawal from 8 weeks of supraphysiological doses of oestradiol and progesterone inducing depressive symptoms in women with a history of PPD (Bloch et al., 2000). In the rodent model, the female subjects are ovariectomised and then dosed with oestradiol and progesterone to mimic the levels observed during gestation. After 21-24 days (depending on the protocol used) administration of ovarian hormones ceased to mimic the postpartum period and behavioural tests are carried out in order to measure anxiety- and depressive-like behaviours. It has been shown that rats exposed to oestradiol withdrawal exhibit increased immobility and decreased struggling and swimming behaviours in the FST (Galea et al., 2001, Schiller et al., 2013) and anhedonia in the form of reduced sucrose preference (Navarre et al., 2010) and reduced responding to electrical stimulation of the reward pathway (Schiller et al., 2013).

Gestational stress models are another form of perinatal depression rodent model. This approach uses chronic stress during pregnancy to elicit a stress-induced postpartum depression. Different variations of stressors are used with one model subjecting dams to restraint stress and overcrowding on alternate days from gestational day (GD) 4-16 (Hillner et al., 2011). A similar protocol uses daily restraint stress from GD 10-20 and on PND 3-4 the females were tested in the FST. Gestational stress was found to increase immobility scores compared to control as well as decrease maternal behaviours such as arched back nursing (Smith et al., 2004).

As mentioned previously, the OB model of depression was validated in male subjects and so its effectiveness at modelling depression in females has not been fully investigated. In this chapter we investigate whether OB affects the ability to reproduce, littering parameters including neonatal deaths and maternal care via observation of pup development.

Previous reports on the effect of OB on sexual behaviour and reproductive ability have shown a decrease in sexual behaviour in male rats with these animals showing no preference for sexually receptive versus non-receptive females and a neutral compartment (Edwards et al., 1990, Lumia et al., 1987). However it has been shown that males exposed to sexual behaviours prior to OB surgery did not lose sexual motivation following surgery (Larsson, 1975). A reason for this decrease in copulatory behaviour in male OB rats is thought to be due to a reduction in androgen receptor binding in the amygdala which occurs as early as 2 days post-surgery (Lumia et al., 1992). This would suggest that input from the olfactory bulbs to the amygdala is necessary for the initiation of sexual behaviours in male rats but only of minor importance for the maintenance of these behaviours throughout the reproductive period of a male rat's life.

There are seemingly opposite effects of OB surgery on sexual behaviour in female rats with reports of increased levels of sexual proceptivity, measured as ear wiggling and darting, and sexual receptivity, lordosis (Lumia et al., 1981). It is thought that OB increases female sexual behaviour by increasing neural sensitivity to oestrogen (Williams et al., 1991, Williams et al., 1992).

As mentioned, we sought to investigate whether the OB-induced depressive phenotype had effects on maternal instincts via observation of pup development. We hypothesised that this OB-induced depressive-like phenotype would negatively impact maternal instincts and caring for pups which would in turn result in a negative impact on pup development. To the best of our knowledge, no previous studies have investigated the effects of maternal depression or postnatal depression on pup neonatal development; instead opting to examine the effects on behaviour of offspring once they have reached adolescence or adulthood (Brummelte et al., 2012, Gobinath et al., 2016, Brummelte et al., 2006). Here two aspects of pup development were investigated, namely somatic

development and behavioural parameters. Somatic development refers to the physical development of the offspring and the age at which they reach certain developmental milestones, i.e. eye opening, pinna unfolding and fur appearance. Neonatal behavioural testing involves the use of tests that measure pup co-ordination, balance and strength. The age at which these behavioural tests are performed is important as different behavioural mechanisms develop at different stages in development, i.e., the righting from a supine to prone position in the surface righting test is an evaluation of vestibular efficiency and is a basic motor pattern that pups should display from PND 1 (Jamon, 2014).

For each neonatal parameter that we will measure, one male and one female pup will be tested from each litter. This is to avoid effects of litter and also to test for differences in development between male and female pups. Sex differences have been found in offspring of models of PPD in adolescence and adulthood (Brummelte et al., 2006, Brummelte et al., 2012).

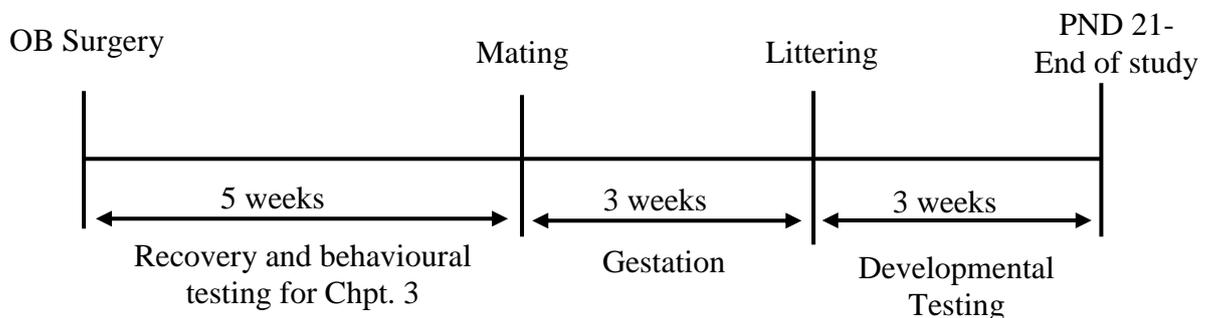
The aims of this chapter are to investigate the effects of OB-induced depressive-like phenotype on reproductive ability, littering parameters and early maternal instincts and the effect of having a ‘depressed’ mother on pup somatic and behavioural development.

## 5.2. Experimental protocol

At approximately 8 weeks old, male and female Sprague-Dawley rats underwent OB/Sham surgery (surgery protocol described in detail in Chapter 2). Approx. 5 weeks post-surgery the male and female sham and OB animals were mated in the following combinations:

- Male Sham X Female Sham
- Male OB X Female Sham
- Male Sham X Female OB
- Male OB X Female OB

The rats mated were the same rats tested in the study describe in Chapter 3 with the addition of 30 more female rats who were operated on but were not included in behavioural testing.



**Figure 5.1.** 15/14 male Sham and OB and 30/31 female Sham and OB rats were mated in the above combinations. From these pairings, 13 Sham/Sham litters were born and 11 litters were born in the remaining groups. Due to neonatal death, the number of litters per group decreased throughout the neonatal period, see legends of graphs for numbers per group for each measurement.

### 5.3. Results

#### 5.3.1. The effect of OB surgery on mating

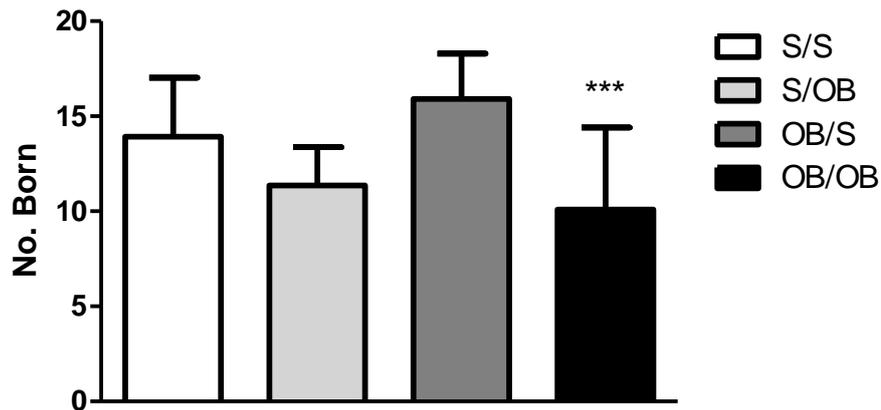
Chi square test revealed there was no difference in the number of pairs of animals that failed to successfully mate, ( $X_1^2 = 3.934$ ,  $p > 0.05$ ).

Group	Mated	%	Pregnant	%
M Sham X F Sham	14/15	93	13/14	93
M Sham X F OB	14/15	93	11/14	79**
M OB X F Sham	13/15	87	11/13	85
M OB X F OB	14/16	87	11/14	79**

**Table 5.1. No effect of OB surgery on reproductive ability.** There were no differences in the percentage of females to test positive for sperm following overnight housing with males however the percentage of female OB rats to become pregnant was significantly lower than sham females.

### 5.3.2. Litter sizes and mortalities

A two-Way ANOVA revealed a significant effect of OB surgery on the number of pups born per litter [ $F_{(1,42)} = 21.1, p < 0.001$ ]. *Post-hoc* testing showed that female OB operated animals that were mated with OB operated males had smaller litters compared to their sham operated controls.

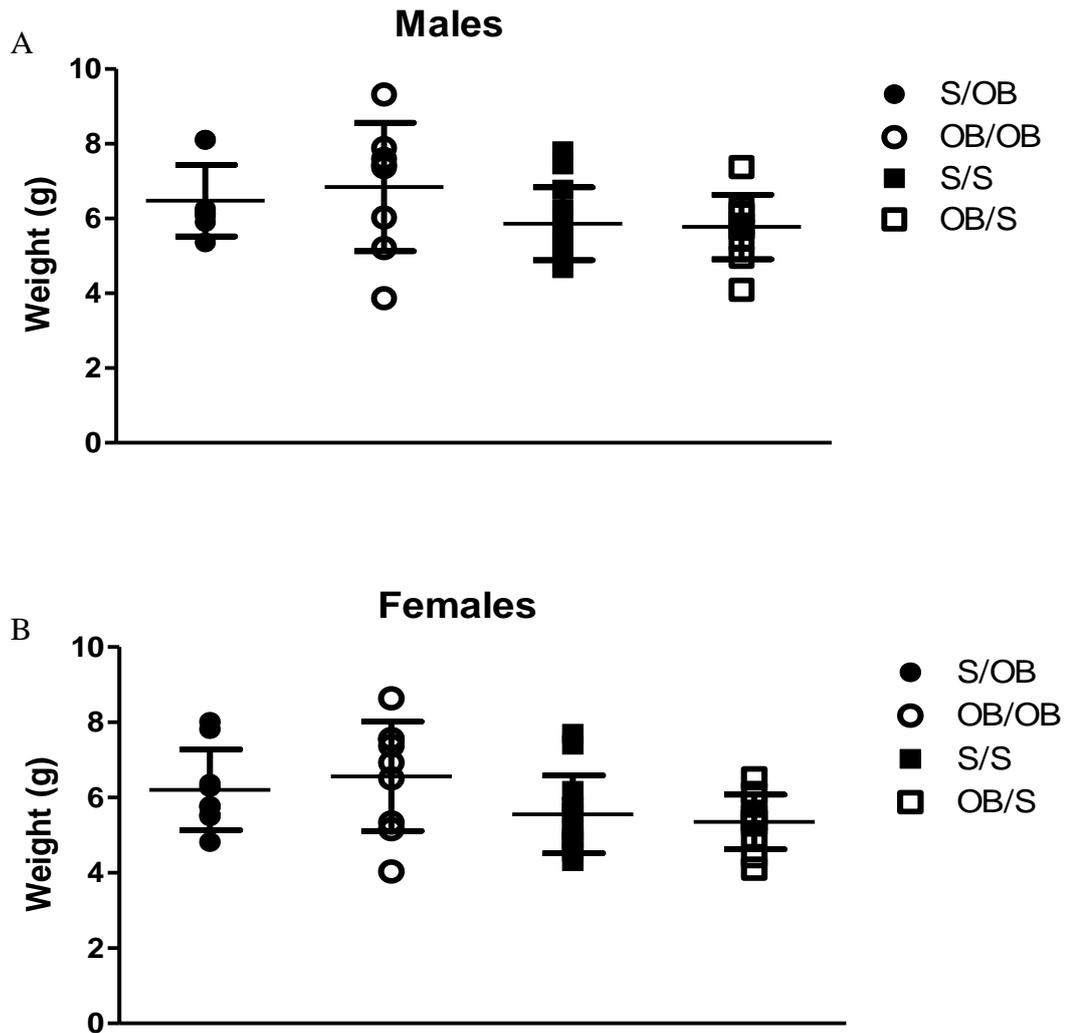


**Figure 5.2. The number of pups born in each group.** The litters in the OB/OB mated group had a significantly smaller number of pups compared to that Sham/Sham mated group. Data presented as mean+ SD. N=11-13 per group. \*\*\* $p < 0.001$  vs. Sham/Sham group.

There was no difference in the number of stillborn pups in the groups [ $K_{(3)} = 1.689, p > 0.05$ ], or in the number of pups that died or had been eaten in the neonatal period [ $K_{(3)} = 1.002, p > 0.05$ ] and [ $K_{(3)} = 1.822, p > 0.05$ ].

### 5.3.3. Birth Weights

A Two-Way ANOVA revealed a significant effect of maternal treatment on the birth weights of the pups [ $F_{(1)}=12.618$ ,  $p<0.001$ ], however this difference failed to reach significance in the *post-hoc* test.

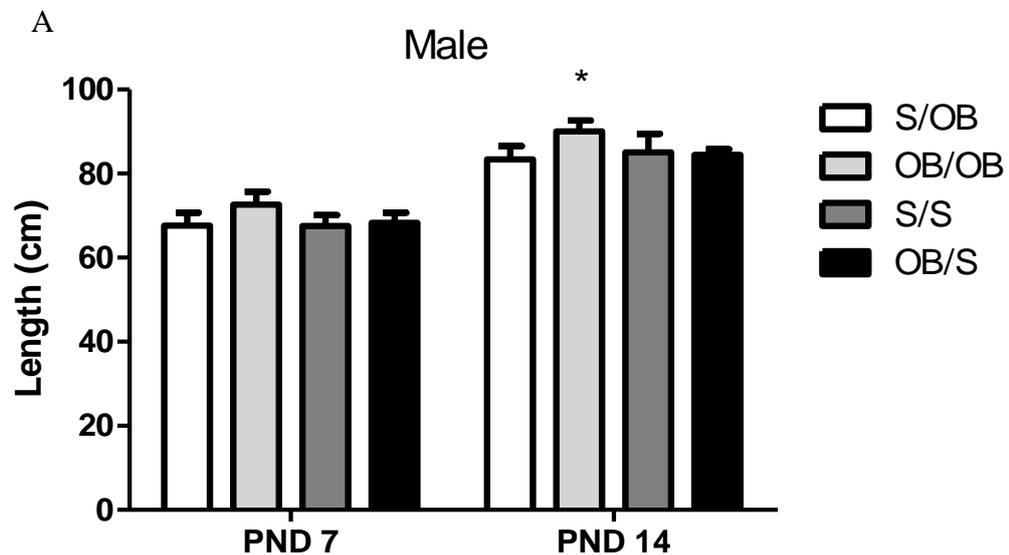


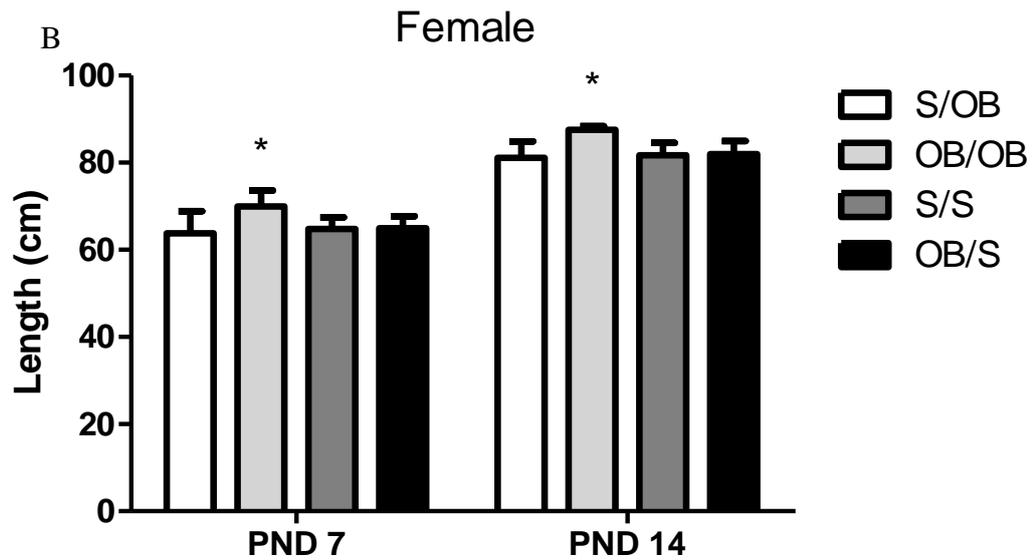
**Figure 5.3.** The effect of parental treatment on birth weights for (A) male and (B) female pups. Statistical testing revealed no significant differences in birth weights between the groups however there was a trend for pups born to OB operated mothers to be heavier at birth. Data presented as mean $\pm$  SD. N= 8-12 per group.

### 5.3.4. Offspring Parameters

#### 5.3.4.1. Body Length

A two-way ANOVA revealed a significant effect of paternal treatment [ $F_{(1,48)}=12.311$ ,  $p<0.001$ ], maternal treatment [ $F_{(1,48)}=5.858$ ,  $p<0.05$ ] and an interaction effect [ $F_{(1,48)}=8.768$ ,  $p<0.01$ ] on the body length of pups at PND 7. *Post-hoc* test found that the female pups in the OB/OB group were significantly longer than the female pups in the Sham/OB group. On PND 14 again a two-way ANOVA found an effect of paternal treatment [ $F_{(1,48)}=14.461$ ,  $p<0.001$ ], maternal treatment [ $F_{(1,48)}=7.142$ ,  $p<0.05$ ] and an interaction effect [ $F_{(1,48)}=16.315$ ,  $p<0.001$ ] on the body length of pups. *Post-hoc* analysis revealed that male pups in the OB/OB group had a significantly longer body length than the male pups in the Sham/OB and the OB/Sham groups. *Post-hoc* testing also showed that the female pups in the OB/OB group had a significantly longer body than the female pups born in the other groups.





**Figure 5.4. The effect of parental treatment on pup body length.** On PND 7 female pups in the OB/OB group had a greater body length than the female pups in the Sham/OB group. On PND 14 the male pups in the OB/OB group had a greater body length than the pups in the Sham/OB and the OB/Sham groups. Female pups in the OB/OB group had a significantly longer body than the female pups born in the other groups. Data is expressed mean+ SD. N= 5-9 per group. \* $p < 0.05$  vs relevant control.

### 5.3.4.2. Eye Opening

On PND 14 there was no difference between the groups in the number of pups that had both eyes open. On PND 15 Chi Square test revealed there was a significant effect of parental treatment on eye opening [ $X_1^2= 15.659, p<0.05$ ]. *Post-hoc* analysis revealed this difference was in the male pup groups and more specifically between the sham/sham mated group and the sham/OB mated group,  $p<0.001$ .

There was no effect of parental treatment on the rate of development of fur on the pups as there was no difference in the number of pups with fur on PND 3, ( $X_1^2= 5.630 p>0.05$ ).

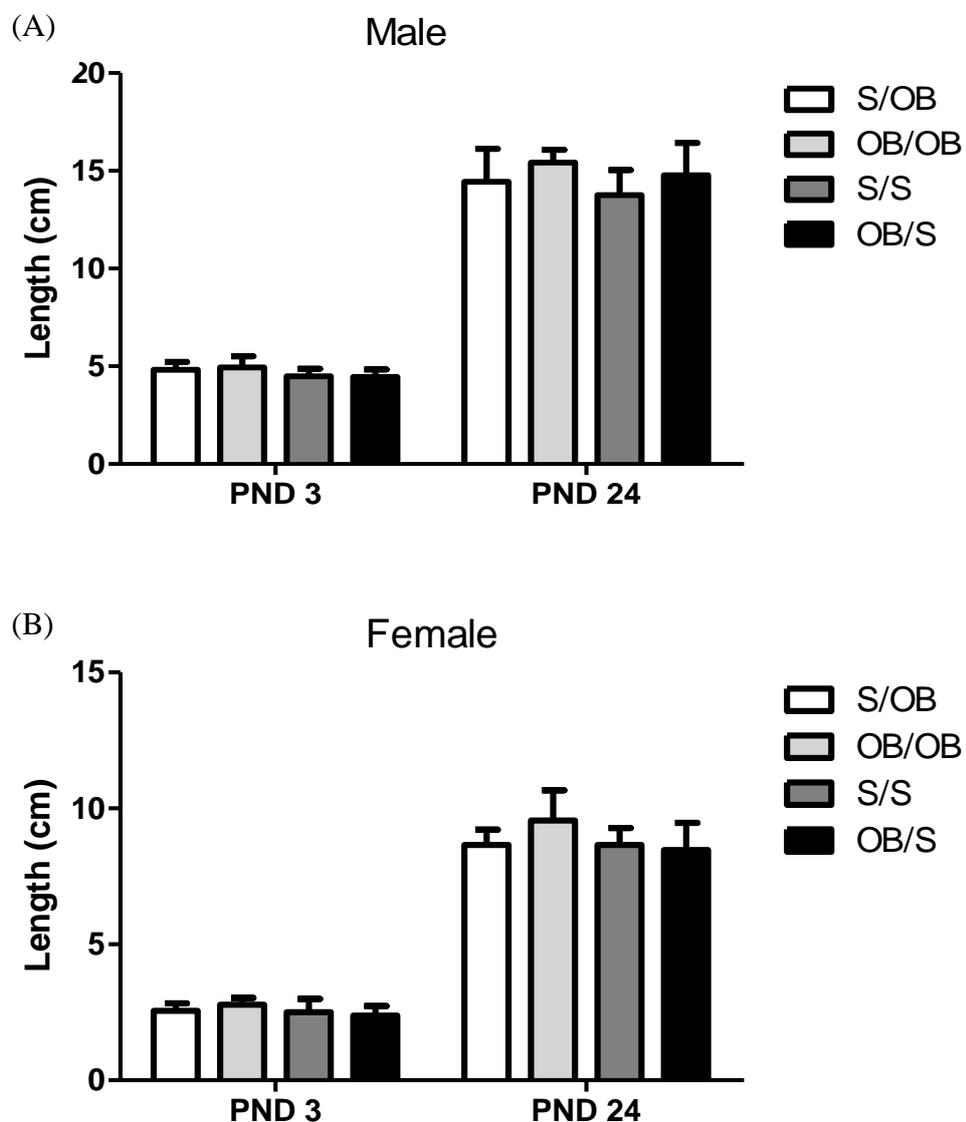
There was no effect of parental treatment on the rate of pinna unfolding for the pups on PND 3 ( $X_1^2= 6.314 p>0.05$ ).

Group	Eye Opening		Fur Appearance	Pinna Unfolding
	PND14	PND15	PND 3	PND 3
<b>Male Pups</b>				
<b>M Sham X F Sham</b>	1/9	3/9	7/9	4/9
<b>M Sham X F OB</b>	1/6	3/6***	4/6	2/6
<b>M OB X F Sham</b>	1/8	8/8	6/8	3/8
<b>M OB X F OB</b>	1/5	4/5	4/5	4/5
<b>Female Pups</b>				
<b>M Sham X F Sham</b>	2/9	3/9	5/9	6/9
<b>M Sham X F OB</b>	1/6	5/6	4/6	3/6
<b>M OB X F Sham</b>	1/8	7/8	6/8	3/8
<b>M OB X F OB</b>	3/5	5/5	4/5	4/5

**Table 5.2. The effect of parental treatment on eye opening.** Significantly more male pups in the sham/OB mated group had both eyes open on PND 15 compared to male pups in the sham/sham mated group. Data presented as number of pups successfully meeting criteria out of total pups tested. \* $p<0.05$  vs relevant control.

### 5.3.4.3. Ano-genital Distance

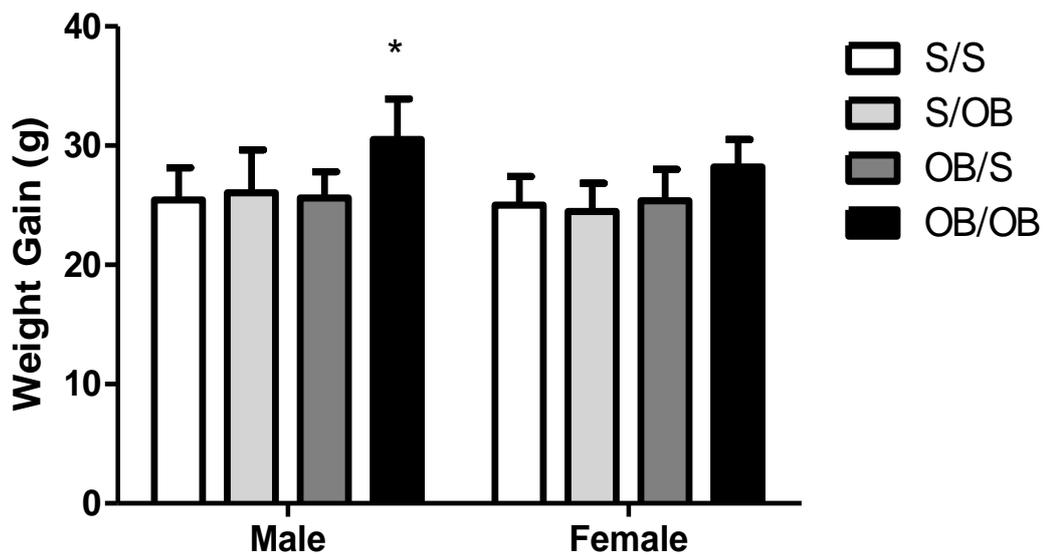
A two-way ANOVA revealed a significant effect of sex [ $F_{(1,46)}=337.803$ ,  $p<0.001$ ] and maternal treatment ( $[_{(1,46)}=7.565$ ,  $p<0.01$ ] on ano-genital distance on PND 3. *Post-hoc* testing revealed the difference lay between the male and female groups. On PND 24 again there was a significant effect of sex [ $F_{(1,46)}=297.901$ ,  $p<0.001$ ] and also an effect of paternal treatment [ $F_{(1,46)}=4.098$ ,  $p<0.05$ ]. *Post-hoc* testing revealed the difference lay between the male and female groups.



**Figure 5.5. The effect of parental treatment on pinna unfolding.** The only difference found between the groups was a sex difference on both PND 3 and 24. Data presented as mean+ SD. N=4-9 per group.

#### 5.3.4.4. Total body weight gain PND 1-21

The body weight of the pups was measured at frequent intervals during the neonatal period. A two-Way ANOVA revealed a significant effect of paternal [ $F_{(1,46)}=8.298$ ,  $p<0.01$ ], maternal [ $F_{(1,46)}=6.630$ ,  $p<0.05$ ] treatment as well as an interaction effect [ $F_{(1,46)}=6.526$ ,  $p<0.05$ ] on the total amount of body weight gained by the pups over the 21 day period. *Post-hoc* testing showed that the only difference between the groups was in the male pups, with the pups born in the OB/OB group putting on more weight than the pups in the Sham/Sham and OB/Sham groups.



**Figure 5.6. The effect of parental treatment on body weight gain in pups during the neonatal period.** The male pups in the OB/OB group put on significantly more body weight than the male pups in the Sham/Sham and OB/Sham groups. Data presented as mean+ SD. N= 4-9 per group. \* $p<0.05$  vs. relevant control.

### 5.3.5. Behavioural Tests

#### 5.3.5.1. Surface Righting

The parental treatment had no effect on the ability of the pups to turn themselves from a supine to a prone position when placed on a flat surface on any day of testing, PND 2 ( $X_1^2= 6.913, p>0.05$ ), PND 3 ( $X_1^2= 4.207, p>0.05$ ), PND 4 ( $X_1^2= 9.036, p>0.05$ ) and PND 5 ( $X_1^2= 3.321, p>0.05$ ).

Group	PND2	PND3	PND4	PND5
<b>Male Pups</b>				
<b>Sham X Sham</b>	3/9	6/9	7/9	8/9
<b>Sham X OB</b>	5/6	5/6	6/6	5/6
<b>OB X Sham</b>	5/8	5/8	8/8	7/8
<b>OB X OB</b>	4/5	4/5	3/5	4/5
<b>Female Pups</b>				
<b>Sham X Sham</b>	6/9	5/9	6/9	6/9
<b>Sham X OB</b>	3/6	2/6	4/6	5/6
<b>OB X Sham</b>	6/8	5/8	8/8	7/8
<b>OB X OB</b>	2/5	3/5	4/5	5/5

**Table 5.3. No effects of parental treatment on pup surface righting.** Data expressed as the number of pups able to successfully right themselves in 15 seconds or less. N=5-9 per group.

### 5.3.5.2. Negative Geotaxis

There was no effect of parental treatment on the ability of the pups to perform the negative geotaxis test on PND 9 ( $X_1^2 = 13.939, p = 0.052$ ) or PND 11 ( $X_1^2 = 5.124, p > 0.05$ ).

Group	PND 9	PND 11
<b>Male Pups</b>		
Sham X Sham	5/9	6/9
Sham X OB	1/6	4/6
OB X Sham	3/8	7/8
OB X OB	2/5	4/5
<b>Female Pups</b>		
Sham X Sham	7/9	8/9
Sham X OB	5/6	4/6
OB X Sham	3/8	8/8
OB X OB	5/5	4/5

**Table 5.4. No effect of parental treatment on negative geotaxis testing.** Data expressed as the number of pups able to successfully complete negative geotaxis test in 15 seconds or less.

### 5.3.5.3. Forelimb Grip

There was no effect of parental treatment on the ability of the pups to perform the forelimb grip test on PND 14 ( $X_1^2 = 3.567, p > 0.052$ ) or PND 17 ( $X_1^2 = 5.111, p > 0.05$ ).

Group	PND 14	PND 17
<b>Male Pups</b>		
Sham X Sham	3/9	9/9
Sham X OB	2/6	4/6
OB X Sham	2/8	5/8
OB X OB	2/5	3/5
<b>Female Pups</b>		
Sham X Sham	4/9	6/9
Sham X OB	4/6	5/6
OB X Sham	2/8	6/8
OB X OB	2/5	4/5

**Table 5.5. No effect of parental treatment on forelimb grip testing.** Data expressed as the number of pups able to perform forelimb grip test for 20 seconds or more.

#### **5.4. Discussion**

This chapter has revealed that the OB syndrome did not affect reproductive ability in male rats however in female OB rats it did effect the rate of pregnancy following mating. There was also a significant effect of OB induced syndrome on littering parameters, with OB mothers giving birth to fewer pups compared to the controls. We also found differences in the somatic development of pups born to OB mothers however the cause of this may be related more to smaller litter sizes in these groups.

The first aim of this chapter was to investigate the reproductive ability in male and female OB induced model of depression. We measured reproductive ability by testing for the presence of sperm in a vaginal swab following male and female subjects being housed together for a dark phase of the light/dark cycle. Contrary to previous reports, we did not observe a reduction in sexual behaviours in male OB animals as there was no difference in the number of females per group to test positive for the presence of sperm. As mentioned previously, it has been shown that sexual impairment occurs in male rats following OB surgery (Larsson, 1975, Lumia et al., 1987, Edwards et al., 1990), which is thought to be caused by a reduction in androgen receptor binding in the amygdala (Lumia et al., 1987). However, one research group also show that male rats housed with other intact males prior to OB surgery exhibited only minor deficits in sexual behaviours following surgery (Larsson, 1975), which could explain why we didn't observe any deficits in sexual performance in the current study.

In contrast to the effects in male rats, OB females do not exhibit impaired sexual behaviour according to the literature. As mentioned previously, OB surgery has even been found to facilitate sexual proceptivity behaviours, including darting and ear wiggling, as well as sexual receptivity, lordosis, in response to oestrogen administration (Williams et al., 1991, Lumia et al., 1981). The mechanism by way this occurs is believed

to be an increased neural sensitivity to oestrogen caused by OB surgery (Williams et al., 1992, McGinnis et al., 1985). In the current study we found no impairment of sexual behaviour in female OB rats however as we did not examine sexual behaviours we are unable to remark on whether olfactory bulbectomy had an effect in this regard.

We did find that a significantly lower number of OB females that were shown to have successfully mated, went on to actually become pregnant. The reason for this is unclear however it may be due to effects of OB surgery on the female oestrous cycle. Previous studies have shown that OB surgery can disrupt the rat oestrous cycle, with one study carried out in rats reporting irregular oestrous cycles characterized by prolonged diestrous stages. However these changes were transient and rectified after 4-5 cycles (Larsson, 1977). A later study also reported that OB caused a significant number of female rats to exhibit a continuous diestrous smear and these animals also had lighter reproductive organs including ovary, oviduct and uterus (Gala et al., 1984). This may have also occurred in the subjects in this study and be the cause of the high percentage of OB females that failed to become pregnant.

In regards to the effects of OB on birth and littering parameters, we found that female OB rats had smaller litters compared to their sham controls. Little research has been carried out in this area and so making comparisons to other findings is difficult. One study found that did examine the development of pups born to OB mothers did not report any differences in litter sizes (Slamberova et al., 2017). They reported that pups from OB mothers had lower birthweights, however as litters from OB mothers had fewer pups in our study, there was a trend for these pups to be heavier compared to those born to sham mothers. We found no effect of sex on birth weights between the male and female pups in the sham groups, this effect was reported by Slamberova *et al.*, (Slamberova et al., 2017), however our results are in line with previous studies from our lab which also

reported no differences (McDonnell-Dowling and Kelly, 2015, McDonnell-Dowling and Kelly, 2016, McDonnell-Dowling et al., 2017).

Our results show a significant effect of both maternal and paternal treatment on the total amount of weight gained by the pups over the neonatal period. Male pups with both OB mother and father gained more weight compared to the male pups in the sham counterpart group. Again this is in contrast to the study by Slamberova et al, who reported that pups born to OB mothers gained significantly less weight compared to pups born to sham mothers (Slamberova et al., 2017). We believe the reason for this increase in weight gain is due to small litter sizes in the litters with OB mothers which allows pups to feed more easily and often and so causes more rapid weight gain, (Romero et al., 1992). Small litter sizes have also been linked to accelerated development of physical landmarks and development of reflexes, (Carvalho et al., 2016).

With regards to somatic development of the offspring, we found no deficits in those born to OB mothers; in fact we found that both the male and female pups born to OB mothers were bigger in weight and length at different time points. We also found that more male pups from the sham/OB mated groups had both eyes open on PND 15 compared to the male pups in the sham/sham mated groups. There was also a trend for more male pups in the OB/OB group to have both their eyes open at this time point. This again contradicts with the results reported by Slamberova who suggested that OB in mother rats prolonged the time until eye opening in pups (Slamberova et al., 2017). As mentioned, little work has been published in this area and so it is difficult to conclude the true effects of OB maternal care on somatic development based on the contrasting results from our work and the study mentioned above.

Our results show no effect of OB maternal care on the functional development of offspring with no differences in development of co-ordination or strength found between

pups born to OB or sham mothers. Again this is in contrast to the results published by Slamberova as they found that pups born to OB mothers exhibited impaired strength in the forelimb grip test (Slamberova et al., 2017). However as the pups born to OB mothers in our study were larger than those born to sham mothers, we believe this to be the reason for the lack of impairment in these pups. A possible reason for the increase in weight and lack of impaired development in pups born to OB mothers could be due to the smaller litter sizes in the OB groups. Pups born in smaller litters have been previously shown to exhibit more rapid somatic and cognitive development due to easier access to food and attention from the dam (Carvalho et al., 2016, Romero et al., 1992).

The results presented in this chapter lead to the conclusion that the OB model of depression may not be the most suitable model for investigating the effects of maternal depression on offspring development. As mentioned, there is little published work examining the effects of maternal depression on the neonatal development of offspring with most studies examining the effects of maternal stress and depression on anxiety- and depressive-like behaviours in the offspring in adolescence and adulthood. Pre-clinically, gestational stress and depression models have been shown to cause increased depressive-like behaviour in male and female offspring (Smith et al., 2004, Brummelte et al., 2012). These studies are clinically relevant as it has been shown that children with depressive mothers are more likely to suffer from anxiety and depression in later life (Pilowsky et al., 2006, Gluschkoff et al., 2017).

***Chapter 6:***  
***Sex characteristics in***  
***behavioural domains***  
***in the WKY model***

## **6.1. Introduction**

Genetics are involved in the aetiology of many psychiatric disorders, including depression. Meta-analysis studies have estimated the heritability of depression to be ~37% (Sullivan et al., 2000). However, despite this evidence for a role of genetics in depression, so far no specific gene variants have been identified as having a significant role in the onset of the disorder. Genome-wide association studies (GWAS) of depression have been carried out but due to lack of consistency and underpowered studies, no genetic variants of significance have been identified (Cohen-Woods et al., 2013). Genome-wide genotyping and sequencing studies are providing evidence that depression is polygenic (Wray et al., 2014), meaning that many genetic variants, each with small effect sizes, combine to cause a genetic susceptibility.

The Wistar Kyoto (WKY) rat strain was originally bred from outbred Wistar rats as a normotensive control line to the spontaneously hypertensive rat (SHR), but is now widely used as a genetic model of anxiety and depression. Paré et al first describe the depressive-like behaviours of the WKY rat in 1989. They described a ‘behavioural despair’ exhibited by this strain as well as a susceptibility to the development of stress ulcers (Pare, 1989). This lab group were the first to describe an increased immobility time in the WKY rat in the FST; a finding which has been commonly replicated, with reports of increased immobility time in WKY vs. both Sprague-Dawley controls (Lopez-Rubalcava and Lucki, 2000, Carr et al., 2010, Burke et al., 2016) and Wistar controls (Tejani-Butt et al., 2003, Nagasawa et al., 2015). Increased anxiety-like behaviours are also evident in the WKY rat, with decreased locomotor activity in the open field (Ferguson and Gray, 2005, Luo et al., 2015, Chen et al., 2014) and decreased entries into and time spent in the open arms of the EPM test (Langen and Dost, 2011, Shepard and Myers, 2008) common findings.

Memory dysfunction has been previously reported in male WKY rats compared to male SD rats in the MWM (Grauer and Kapon, 1993, Wyss et al., 2000) and the water radial arm maze (Clements and Wainwright, 2006). However, when comparing the WKY to the SHR strain, there are contradicting reports with some finding deficits in the WKY strain (Sontag et al., 2013), and others finding the SHR strain to perform worse than the WKY in tests of memory (Meneses et al., 2011, Leffa et al., 2016). These results highlight the importance of choosing an appropriate control strain when using the WKY model.

As mentioned previously, the genetics underlying the anxiety- and depressive-like phenotype of the WKY model have not been fully elucidated, however several QTL have been mapped in WKY rats for the FST behaviours which share overlapping candidate regions with human loci for depression (Solberg et al., 2004).

Another genetic component of the WKY model is that genes encoding for the synthesis and metabolism of NE were more highly expressed in WKY compared to SD rats. As NE is one of the neurotransmitter systems thought to be involved in human depression, these findings may suggest that a dysfunction in NE turnover leads to the phenotype observed in this rat strain (Pearson et al., 2006). As well as this, WKY rats show a 4-7 fold increase in levels of catechol-O-methyltransferase (COMT) mRNA, COMT being the enzyme involved in the degradation of catecholamines, in the cerebral and frontal cortices compared to SD rats (Walker et al., 2004). This would suggest a lowered synaptic level of catecholamines which may be a factor in the models depressive-like phenotype.

Although classed as an inbred rat strain, there is evidence of genetic heterogeneity between sources of commercially available WKY rats. This is important as it results in biological variability in WKY rats from different suppliers. The most likely cause of this genetic variability is that WKY rat breeding animals were distributed to supplier

companies before the rats were fully inbred with evidence to suggest the breeding stock may have been sent to suppliers as early as the F 10 generation (Kurtz et al., 1989).

Variability in depressive-like behaviours have been reported in WKY rats obtained from different suppliers (Pare and Kluczynski, 1997), however rats from these suppliers are used equally and interchangeably. In the last 5 years an equal number of studies used rats from Harlan and Charles River to investigate the depressive-like phenotype of the WKY strain (n= 17 studies each) (Zhang-James et al., 2013).

It has been shown that WKY rats obtained from Harlan, WKY/NHsd, displays a three times greater behavioural variability compared to other in- and out-bred rat strains (Will et al., 2003) with genetic variability observed even within supplier populations. The WKY strain has been bred at Harlan for over 67 generations which may suggest that genetic mutations (such as base pair substitutions or indels) within the population may account for some of the genetic and behavioural variability.

As mentioned previously, a majority of pre-clinical research, particularly in the fields of neuroscience and pharmacology, are carried out on male subjects (Beery and Zucker, 2011), and so most work on investigating the WKY strain as a model of depression has been carried out on male rats. However, a select few studies have investigated potential sex differences in the WKY model with differences between male and female WKY rats observed as far back as by the group who first described the WKY strain as a model of depression (Pare and Redei, 1993). They reported that WKY females were more active in the OFT and more immobile in the FST compared to male animals and when they took the oestrous cycle stage into account they found that females in proestrous-estrous stage were less active in the OFT and more immobile in the FST compared to diestrous females (Pare and Redei, 1993). The same group also reported that female WKY rats were more vulnerable to chronic stress which caused decreased activity in the OFT and poor passive-

avoidance responses, suggesting female WKYs were more vulnerable to chronic stress induced depressive-like behaviours (Pare et al., 1999).

Results from our own lab also suggest the presence of sex differences in anxiety- and depressive-like behaviours in the WKY model compared to SD controls. However these sex differences were not found in the classical tests of these behaviours, i.e. the OFT and FST, as was reported by Paré et al (1999) but in tests of anhedonia and novelty-suppressed feeding (Burke et al., 2016). In this study, males exhibited enhanced novelty-induced hypophagia and anhedonia, as measured by sucrose consumption, compared to their SD controls, neither of which were observed in female WKY rats.

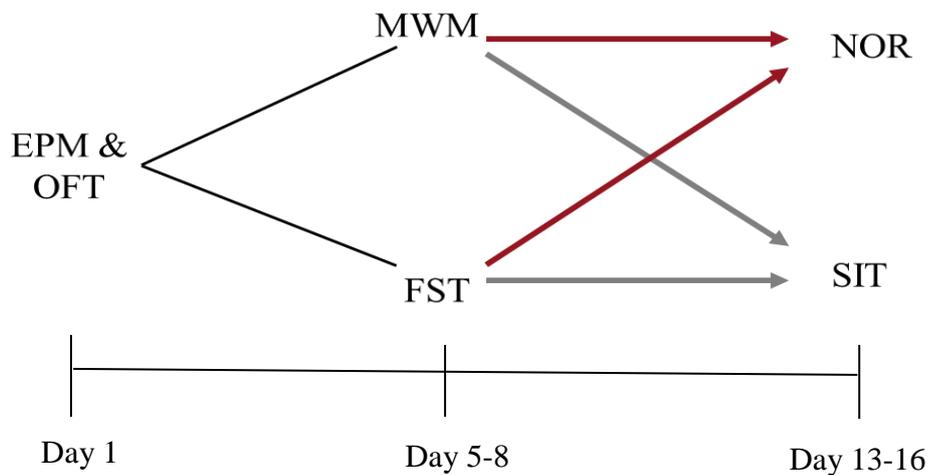
In regards to whether sex differences are present in cognitive ability in the WKY model of depression only one study could be found which investigated recognition, learning and memory in both male and female WKY rats; it reported no sex differences in cognitive abilities in the Novel Object Recognition test, (Gonzales et al., 2015). This test is now considered to be the hallmark method for examining non-spatial object memory in rodents (Cohen and Stackman, 2015).

Similar to the work we presented in Chapter 3, in this chapter we aim to characterise and compare anxiety- and depressive-like behaviours in male and female WKY rats compared to SD controls. We have put an emphasis on attempting to elucidate potential sex differences in learning and memory abilities in the WKY rat as we believe this is the most unclear aspect of the model when comparing the sexes from the currently available literature.

## 6.2. Experimental methods

Male and female WKY/NHsd and Sprague-Dawley rats were received from Harlan UK (Envigo UK) aged approx. 7 weeks upon arrival. Due to low weight of some female rats (some were below 100g) testing had to be delayed for 4 weeks so animals were approx. 11 weeks at the start of testing. They were housed with a 12h light/dark cycle (lights on at 08:00h). Food and water was available *ad libitum*.

The rats used in this study were then mated for use in the study described in Chapter 8.



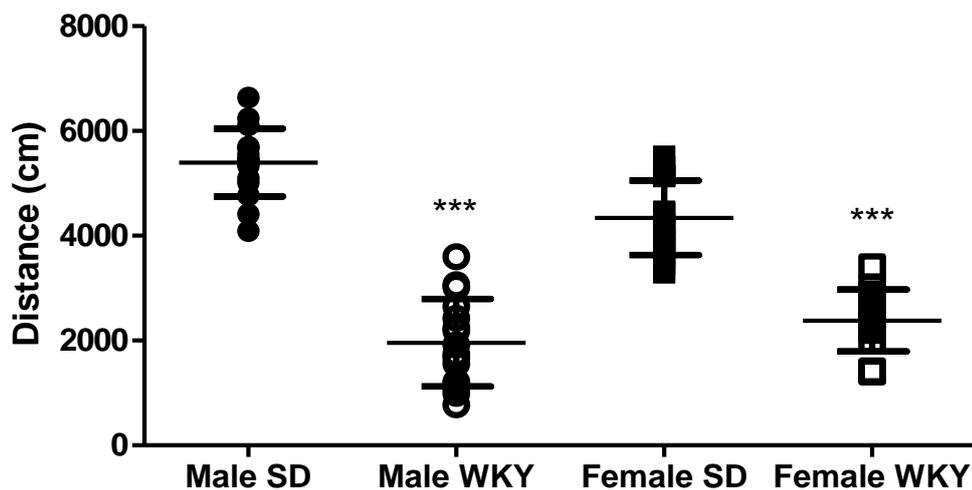
**Figure 6.1.** Diagram outlining the order of behavioural testing. MWM was carried out days 5-8 and the FST was performed on day 8. NOR test took place on days 13 and 14 and the SIT was performed on days 15 and 16. For EPM and OFT  $n=16$  per group, half of these rats,  $n=8$ , were then tested in the MWM and  $n=8$  were tested in the FST. 4 rats per group tested in the MWM (represented by red arrow) were tested in the NOR and the other 4 rats per group (represented by grey arrow) were tested in the SIT and the same allocation of rats was used for those tested in the FST, meaning  $n=8$  per group in the NOR test and SIT.

### 6.3. Results

#### 6.3.1. Open field test (OFT)

##### 6.3.1.1. Distance moved in the OFT

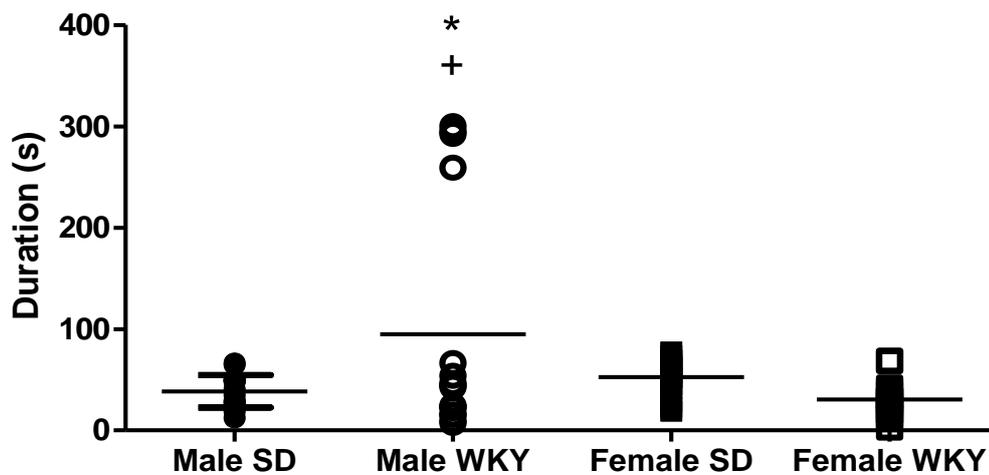
The data met the criteria to be analysed parametrically. A two-way ANOVA was used to determine if sex or strain affected the distance moved. A significant effect of strain, as well as a sex\*strain interaction was found, [ $F_{(1,60)}=237.68$ ,  $p<0.001$ ;  $F_{(1,60)}=17.836$ ,  $p<0.001$ ]. *Post-hoc* Student Newman-Keuls test revealed that the male and female WKY groups displayed a significantly reduced locomotor activity compared to their respective SD counterpart.



**Figure 6.2. Effect of sex and strain on distance moved in the OFT.** Both male and female WKY rats moved less compared to their SD counterparts. Data are expressed as mean $\pm$  SD. n=16 per group. \*\*\* $p<0.001$  vs same sex control.

### 6.3.1.2. Duration spent in the inner zone of the OFT

A two-way ANOVA revealed a significant strain\* sex interaction on the duration spent in the centre of the OFT arena [ $F_{(1,60)} = 6.899, p < 0.05$ ]. *Post-hoc* Student Newman-Keuls test revealed that the male WKY group displayed a significantly increased time in the inner zone compared to both the male SD group and the female WKY group.



**Figure 6.3. Effect of sex and strain on inner zone duration in the OFT.** Male WKY rats moved more compared to their SD counterparts and compared to female WKY rats. Data are expressed as mean  $\pm$  SD.  $n = 16$  per group. \* $p < 0.05$  vs same sex control, + $p < 0.05$  vs same strain.

### 6.3.2. Elevated Plus Maze (EPM)

#### 6.3.2.1. Distance moved in the EPM

The data met the criteria to be analysed parametrically. A Two-Way ANOVA was used to determine if sex or strain affected the distance moved. A significant effect of sex [ $F_{(1,60)}= 21.54, p<0.001$ ], and strain [ $F_{(1,60)}=208.269, p<0.001$ ]. *Post-hoc* Student Newman-Keuls test revealed that all groups were significantly different to each other.



**Figure 6.4. Effect of sex and strain on distance moved in the EPM.** Both male and female WKY rats moved less compared to their SD counterparts, females of both strains moved more than their male strain counterparts. Data are expressed as mean $\pm$  SD. n=16 per group. \*\*\* $p<0.001$  vs SD control, +++ $p<0.001$  vs. male.

**6.3.2.2. Frequency and duration of open arm entries in the EPM**

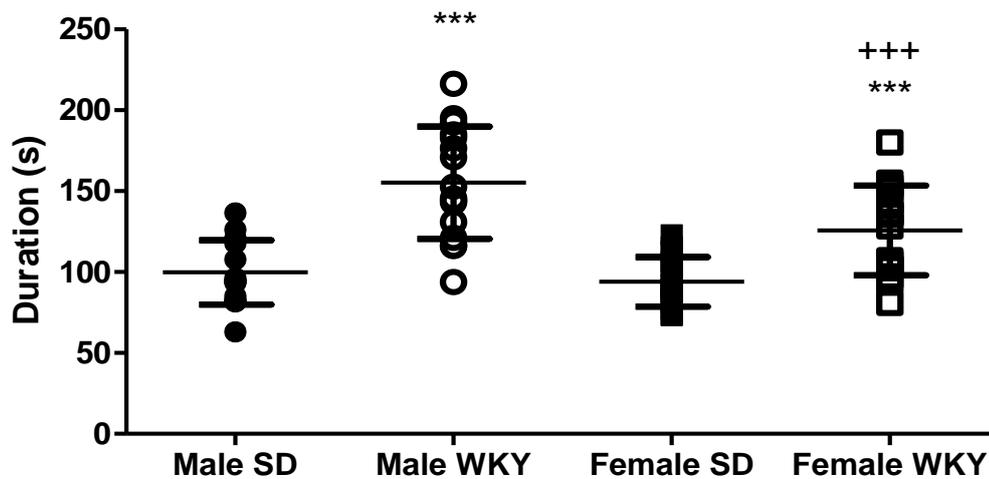
A two-way ANOVA revealed a significant effect of strain on the number of entries into the open arms as well as the duration spent on the open arms [ $F_{(1,60)}=13.345$   $p<0.01$ ;  $F_{(1,60)}=5.265$   $p<0.05$ ]. *Post-hoc* analysis revealed that both male and female WKY rats entered the open arms less than their SD counterparts but no differences were found between the groups for the duration of time on the open arms.

Open Arms				
Group	Entries		Duration(s)	
	No.	%	Time	%
<b>M SD</b>	4±3	22±14	37±27	19±13
<b>M WKY</b>	2±1**	22±15	26±20	19±16
<b>F SD</b>	5±3	25±13	40±26	20±13
<b>F WKY</b>	3±1**	23±11	26±15	15±8

**Table 6.1. Frequency and duration of open arm entries in the EPM.** Male and female WKY rats entered the open arms less compared to their SD counterparts. Data expressed as mean± SD. \*\*  $p<0.01$  vs SD control. N= 16 per group.

### 6.3.2.3. Duration of time spent in the centre of the EPM

A Two-Way ANOVA revealed a significant effect of sex [ $F_{(1,60)}=7.691, p<0.01$ ], and strain [ $F_{(1,60)}=46.852, p<0.001$ ]. *Post-hoc* Student Newman-Keuls test revealed that male and female WKY rats were significantly different to their SD controls and female WKY rats were significantly different to male WKY rats.

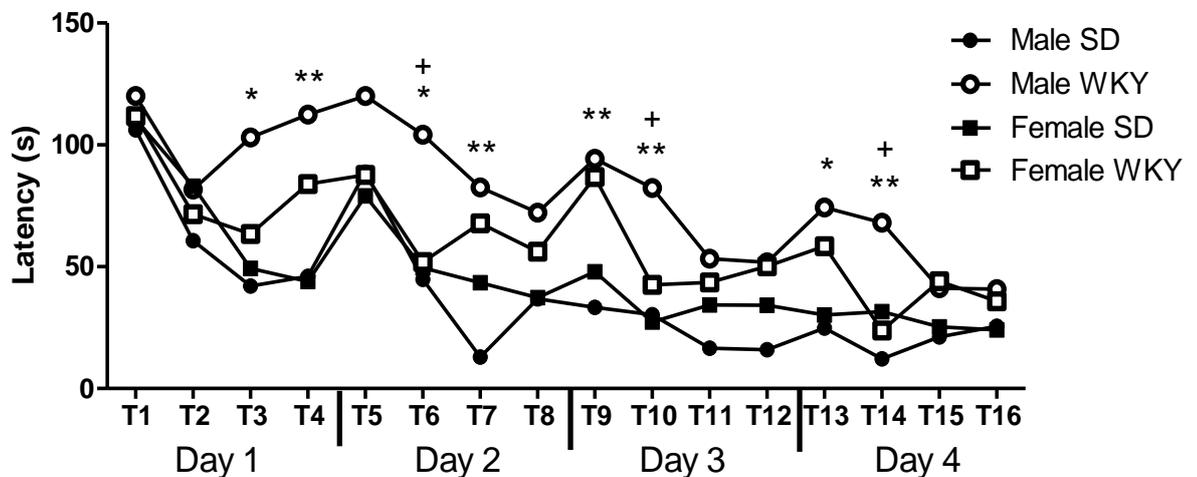


**Figure 6.5. Effect of sex and strain on duration spent in centre of the EPM.** Both male and female WKY rats spent significantly more time in the centre of the arena compared to their SD counterparts and the female WKY group spent less time here than the male WKY group. Data are expressed as mean $\pm$  SD. n=16 per group. \*\*\* $p<0.001$  vs SD control, +++ $p<0.001$  vs. male WKY.

### 6.3.3. Morris Water Maze (MWM)

#### 6.3.3.1. Training graph from the MWM test

This data represents amount of time taken for each group to find the submerged platform in the 16 training trials of the MWM test. The data met the criteria to be analysed parametrically. A Repeated Measures ANOVA was used to determine if sex or strain affected the time taken to find the platform. Tests of within-subject effects revealed a significant effect of trial [ $F_{(7, 214)}=19.194, p<0.001$ ]. Tests of between subject effects revealed a significant effect of strain [ $F_{(1,28)}=17.148, p<0.001$ ] and a significant sex\*strain interaction [ $F_{(1,28)}=4.226, p<0.05$ ]. *Post-hoc* Student Newman-Keuls test revealed that the male WKY group was significantly different the male SD group in Trial 3 (T3), T4, T6, T7, T9, T10, T13 and T14. Female WKY rats were significantly different to male WKY rats in T6, T10 and T14.

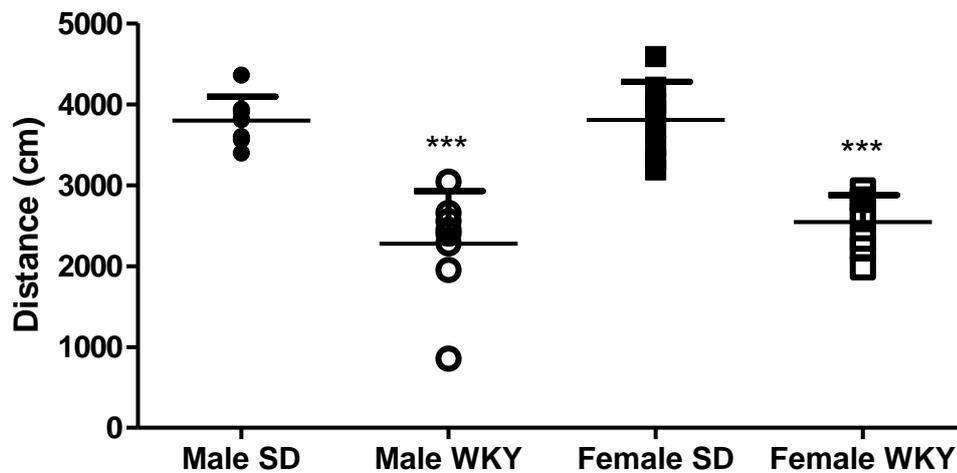


**Figure 6.6. Effect of sex and strain on the latency to find platform on training days.**

There were significant differences in the time taken to find the platform between the male WKY and male SD groups, the female WKY and female SD groups and also between the male and female WKY groups. Data are expressed as mean.  $n=8$  per group. \* $p<0.05$ , \*\* $p<0.01$  vs. SD control, + $p<0.05$  vs. female WKY.

### 6.3.3.2. Distance moved in the MWM

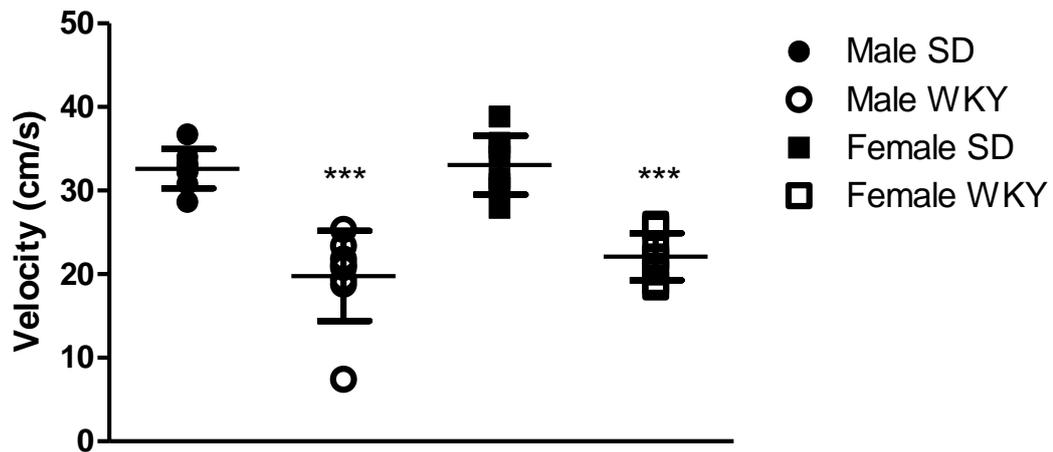
The data met the criteria to be analysed parametrically. A Two-Way ANOVA was used to determine if sex or strain affected the distance moved. A significant effect of strain [ $F_{(1,28)}=73.29, p<0.001$ ], was found. *Post-hoc* Student Newman-Keuls test revealed that the WKY groups were significantly different their SD counterpart group.



**Figure 6.7. Effect of sex and strain on distance moved in the MWM.** Both male and female WKY rats moved less compared to their SD counterparts. Data are expressed as mean $\pm$  SD. n=8 per group. \*\*\* $p<0.001$  vs SD control.

**6.3.3.3. Swimming velocity in the MWM probe trial**

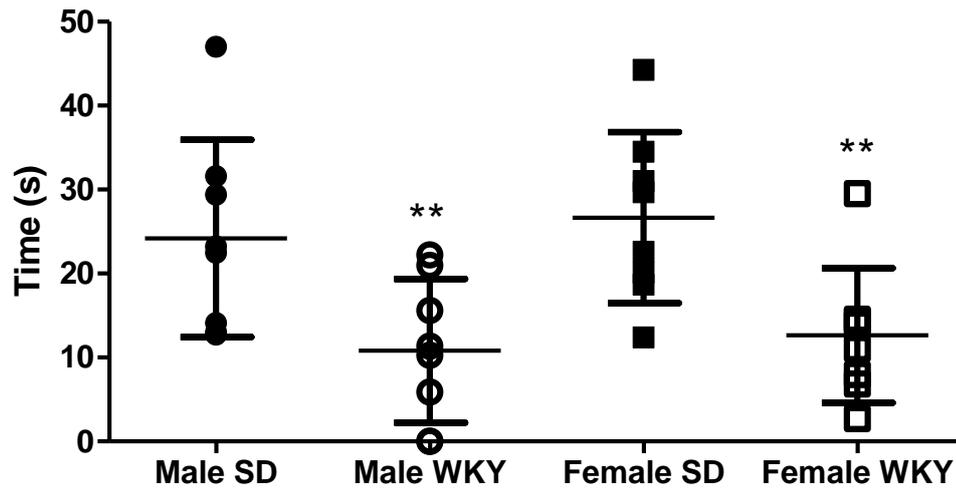
A two-way ANOVA revealed an effect of strain in the maximum velocity swam, [ $F_{(1,28)}=82.281, p<0.001$ ]. Post-hoc analysis revealed that both the male and female WKY groups achieved a significantly smaller maximum velocity compared to their relative SD group.



**Figure 6.8. Maximum swimming velocity in the MWM.** Both male and female WKY rats had a lower maximal swimming velocity compared to their SD counterparts. Data are expressed as mean±SD. n=8 per group. \*\*\* $p<0.001$  vs SD control.

### 6.3.3.4. Time spent in the target quadrant in MWM probe trial (1<sup>st</sup> minute)

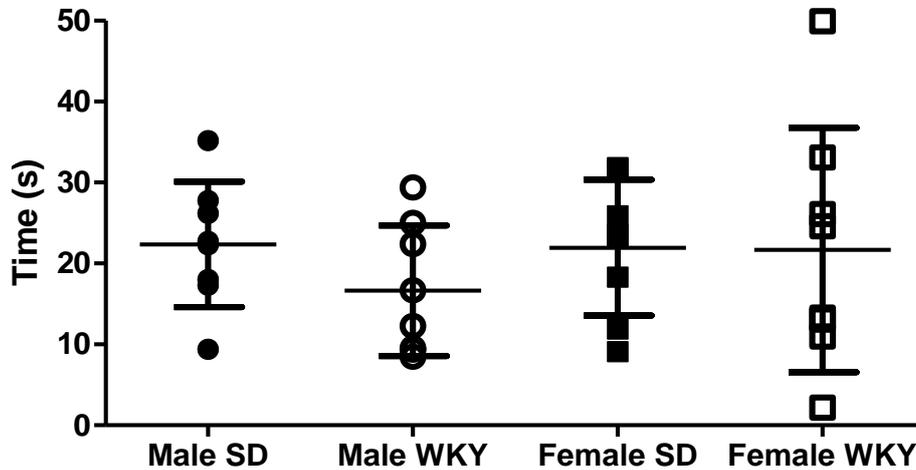
A Two-Way ANOVA revealed a significant effect of strain [ $F_{(1,28)}=1505.468$ ,  $p<0.001$ ], on the duration spent in the target quadrant. *Post-hoc* Student Newman-Keuls test revealed that the male and female WKY groups were significantly different to their SD counterpart groups.



**Figure 6.9.** Effect of sex and strain on time spent in the target quadrant of the MWM (1<sup>st</sup> minute). Both male and female WKY rats spent less time in the target quadrant during the 1<sup>st</sup> minute of the trial compared to their SD counterparts. Data are expressed as mean $\pm$ SD. n=8 per group. \*\* $p<0.01$  vs SD control

6.3.3.5. Time spent in the target quadrant in MWM probe trial (2<sup>nd</sup> minute)

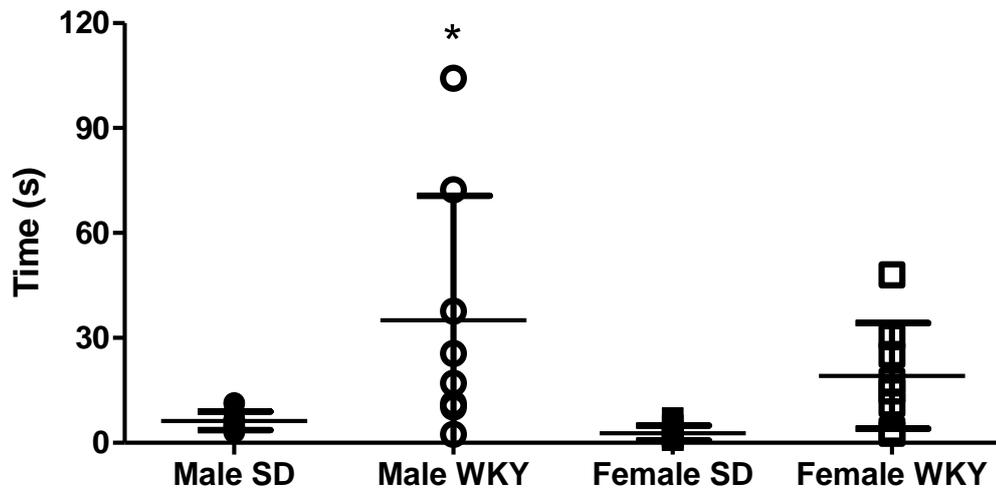
No differences were found in the time spent in the target quadrant in the 2<sup>nd</sup> minute of the MWM probe trial.



**Figure 6.10.** Effect of sex and strain on time spent in the target quadrant of the MWM (2<sup>nd</sup> minute). No differences were found between the groups for the time spent in the target quadrant during the second minute of the trial. Data presented as mean± SD. N=8 per group.

### 6.3.3.6. Latency to enter target quadrant in MWM probe trial

This data represents the amount of time taken for each group to enter the target quadrant of the MWM probe trial. The data met the criteria to be analysed parametrically. A Two-Way ANOVA was used to determine if sex or strain affected the time. A significant effect of strain [ $F_{(1,28)}=10.866, p<0.01$ ], was found. *Post-hoc* Student Newman-Keuls test revealed that the male WKY group was significantly different the male SD group.

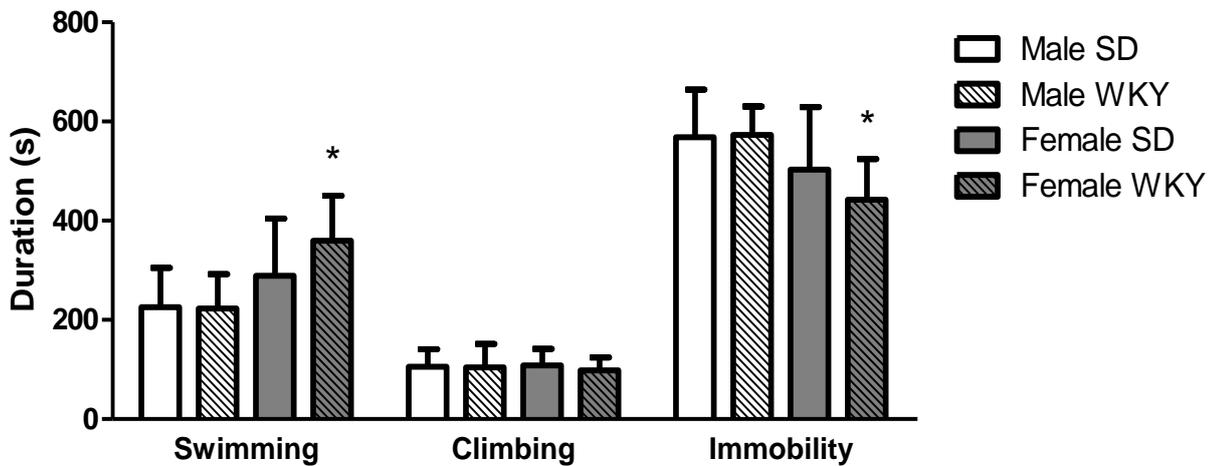


**Figure 6.11. Effect of sex and strain on the latency to enter the target quadrant.** Male WKY rats took significantly longer to enter the target quadrant compared to their SD counterparts. Data are expressed as mean $\pm$  SD.  $n=8$  per group.  $*p<0.05$  vs same sex control.

### 6.3.4. Forced Swim Test (FST)

#### 6.3.4.1. Analysis of behaviours in FST

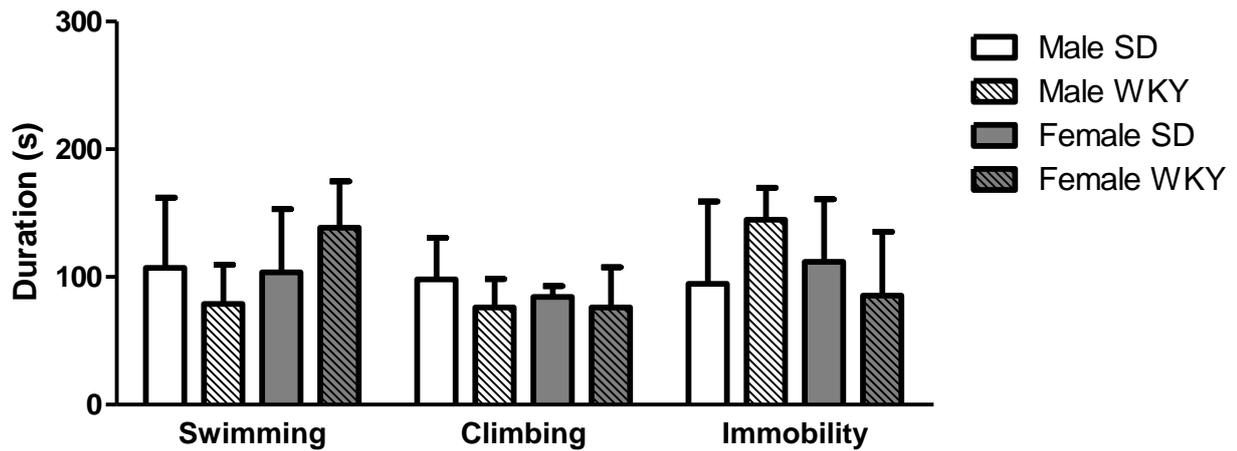
There was an effect of sex on both the time spent swimming [ $F_{(1,28)} = 9.784, p < 0.01$ ] and the time spent immobile [ $F_{(1,28)} = 8.756, p < 0.01$ ] in the FST. *Post-hoc* analysis revealed that the female WKY group spent more time swimming than the male WKY group and they also spent less time immobile compared to the male WKY group.



**Figure 6.12.** FST analysis. Female WKY rats spent more time swimming and less time immobile compared to male WKY rats. Data presented as mean+ SD. \* $p < 0.05$  compared to male WKY group. N= 8 per group.

### 6.3.4.2. FST behaviours broken into 5 minute time bins

There was no effect of sex or strain on the time spent swimming or climbing in the first 5 minutes of the FST. There was a significant interaction effect of sex\*strain on immobility behaviour [ $F_{(1,28)}=4.897$ ,  $p<0.05$ ], however *post-hoc* analysis revealed no differences between the groups.

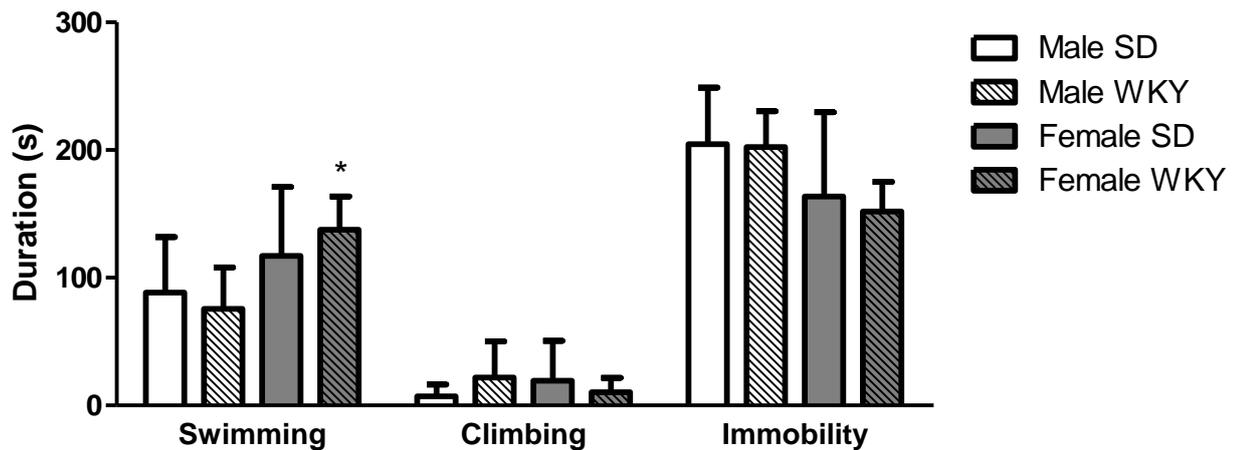


**Figure 6.13. FST behaviours displayed in the first 5 minutes.** No differences were found between the groups. Data presented as mean+ SD. N= 8 per group.

### Analysis of FST behaviours 5-10 minutes

A two-way ANOVA revealed a significant effect of sex on the time spent swimming in the second 5 minute time bin of the FST [ $F_{(1,28)}=10.092$ ,  $p<0.01$ ]. A *post-hoc* analysis revealed that the female WKY group spent more time swimming compared to the male WKY group.

There was no effect of sex or strain on the time spent climbing in the second 5 minute time bin of the FST. A two-way ANOVA revealed a significant effect of sex on the time spent immobile in the second 5 minute time bin of the FST [ $F_{(1,28)}=8.754$ ,  $p<0.01$ ]. *Post-hoc* analysis revealed no differences between the groups.

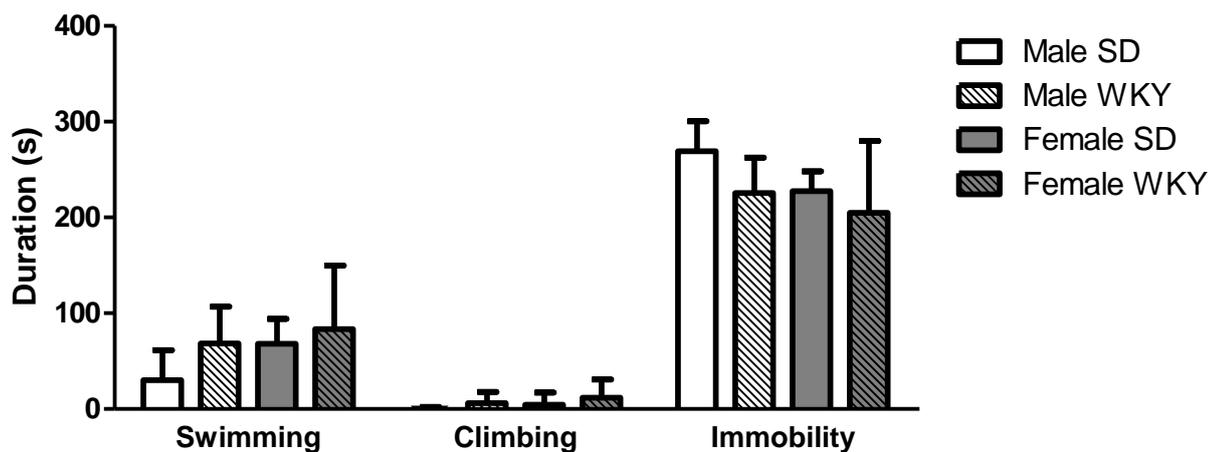


**Figure 6.14. Effect of sex and strain on FST behaviours in second 5 minute time bin.**

Female WKY group spent significantly more time swimming compared to the male WKY group and there was a trend for the female WKY group to spend significantly less time immobile than the male WKY group. Data presented as mean+ SD. N=8 per group.

**Analysis of FST behaviours 10-15 minutes**

There was no effect of sex or strain on the time swimming or climbing in the last 5 minutes of the FST. There was a significant effect of strain on immobility behaviour [ $F_{(1,28)}=4.179, p<0.05$ ]. *Post-hoc* test did not find any differences between the groups.



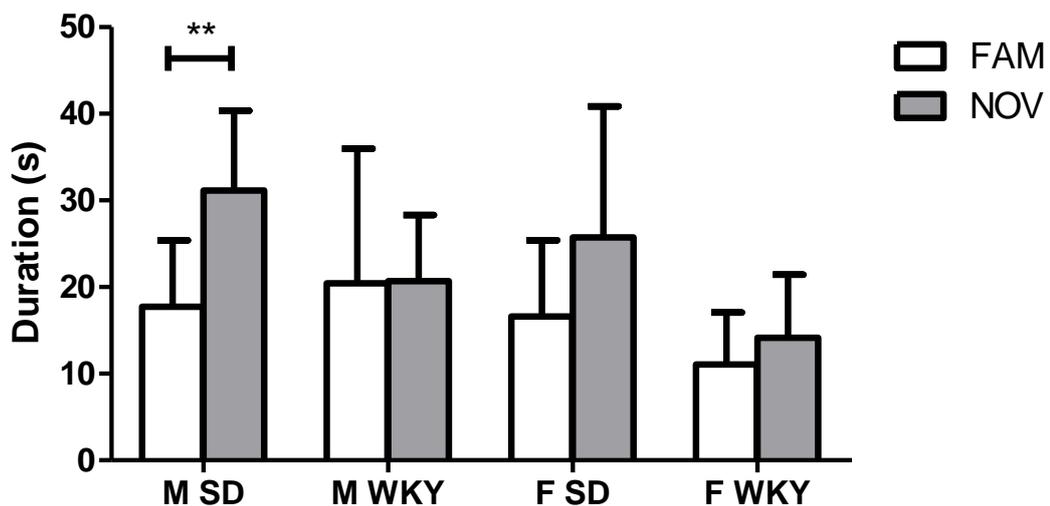
**Figure 6.15. No differences in FST behaviours in last 5 minutes.** Data presented as mean+ SD. N= 8 per group.

### 6.3.5. Novel object recognition test

#### 6.3.5.1. Time sniffing familiar vs. novel object

There was no effect of sex or strain on the time spent sniffing the familiar object in the 3 minute trial. A Two-Way ANOVA revealed a significant effect of strain on the total time spent sniffing the novel object [ $F_{(1,28)}=9.185$ ,  $p<0.01$ ]. *Post-hoc* test revealed no meaningful differences between the groups.

When comparing the time spent interacting with the familiar vs. the novel object within the same group, T-Tests revealed that the male SD group spent significantly longer interacting with the novel object ( $t=3.652$   $df=7$ ,  $p<0.001$ ).

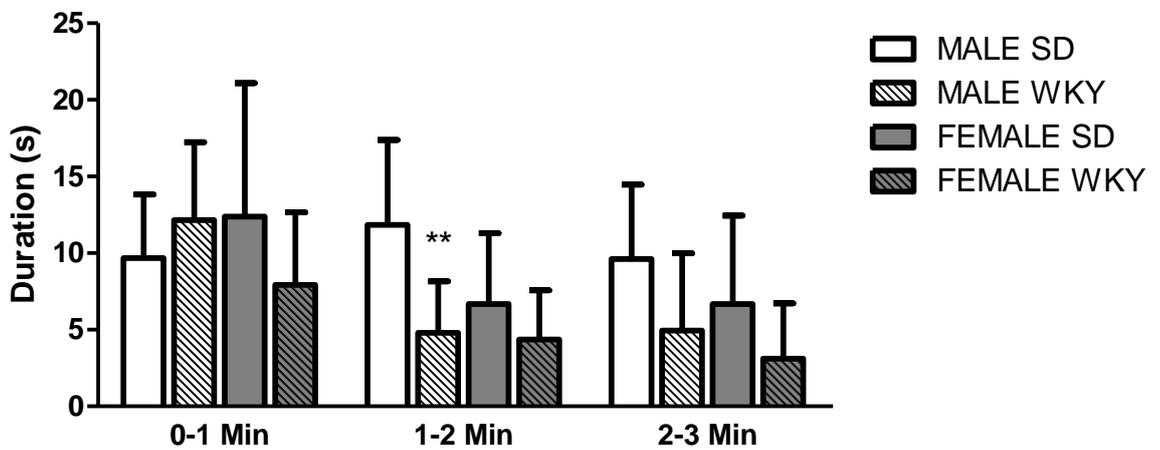


**Figure 6.16. Comparison of time spent sniffing the familiar vs. the novel object.**

Total time spent sniffing the familiar vs. the novel object over the 3 minute trial. The only difference between the groups was between the male SD and female WKY groups; not marked on graph as this comparison is not relevant to our experiment. Data presented as mean+ SD. N= 8 per group.

### 6.3.5.2. Time sniffing novel object in 1 minute time bins

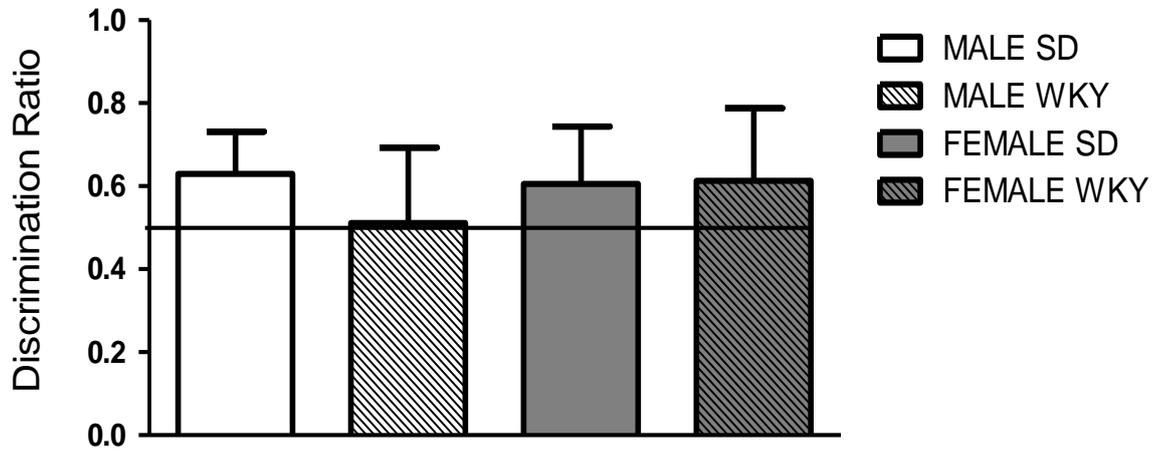
A two-Way ANOVA revealed no effects of sex or strain on the time spent sniffing the novel object in the first minute. In the second minute, a significant effect of strain was observed [ $F_{(1,28)}=9.556, p<0.01$ ]. *Post-hoc* analysis revealed that the male WKY group sniffed the novel object significantly less than the male SD,  $p<0.01$ . In the third minute, again a two-Way ANOVA revealed a significant effect of strain [ $F_{(1,28)}=5.700, p<0.05$ ], however *post-hoc* analysis failed to show any differences between the groups.



**Figure 6.17. Time sniffing novel object in 1 minute time bins.** In the 2<sup>nd</sup> minute male WKY rats spent significantly less time sniffing the novel object compared to the male SD group. Data presented as mean+ SD. N=8 per group. \*\* $p<0.01$  vs. male SD.

### 6.3.5.3. Discrimination ratio

A two-way ANOVA revealed no differences between the groups in the discrimination ratio of time spent investigating the novel compared to the familiar object.



**Figure 6.18. Discrimination ratio.** There were no differences found between the WKY and SD groups in their preference for exploring the novel compared to the familiar object. Data presented as mean+ SD. N=8 per group.

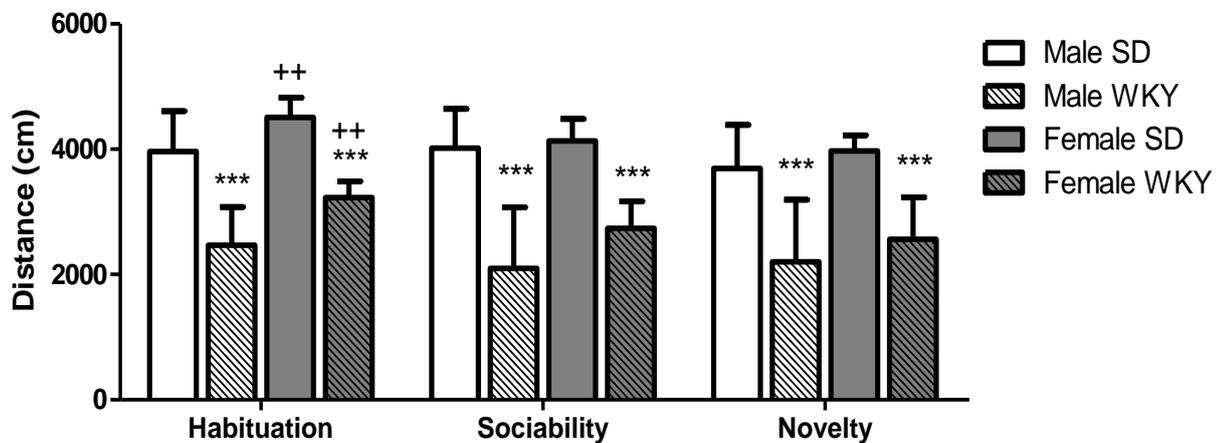
### 6.3.6. 3 Chamber sociability test

#### 6.3.6.1. Distance moved in the 3 chamber sociability test

The distance moved in each of the 3 x 10 minute trials was measured and analysed. In the habituation trial, a Two-Way ANOVA found significant effects of both sex [ $F_{(1,27)}=13.494$ ,  $p<0.01$ ], and strain [ $F_{(1,27)}=60.877$   $p<0.001$ ], on the distance moved. *Post-hoc* analysis revealed that all groups were significantly different to each other.

In the sociability trial, a Two-Way ANOVA found a significant effect of strain [ $F_{(1,27)}=50.212$ ,  $p<0.001$ ]. *Post-hoc* analysis revealed that the male and female WKY groups moved significantly less compared to their SD counterparts.

In the preference trial, again a Two-Way ANOVA found a significant effect of strain [ $F_{(1,27)}=31.794$ ,  $p<0.001$ ]. *Post-hoc* analysis showed that the male and female WKY groups moved significantly less compared to their SD counterparts.

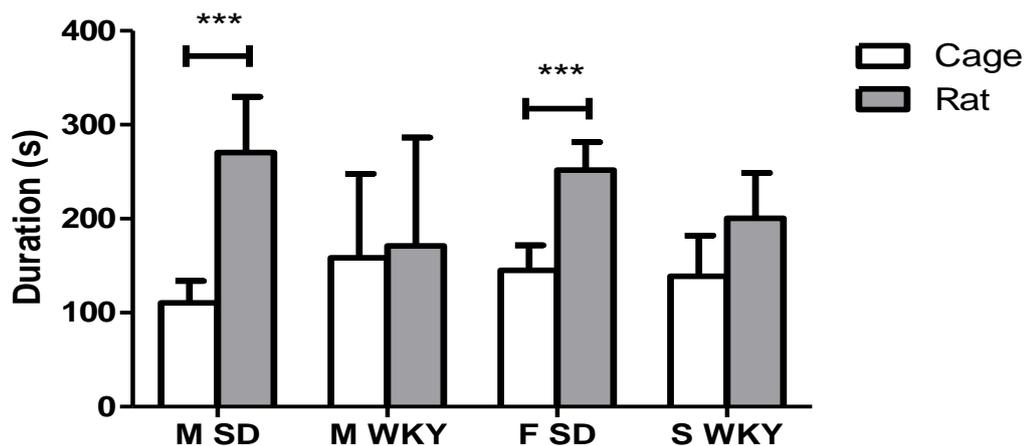


**Figure 6.19. Distance moved in the 3 trials of the 3 chamber sociability test.** In the habituation trial the male and female WKY groups moved less compared to their SD counterparts. The females of both strains moved more compared to their male counterparts. Data represented as mean+ SD. N= 8 per group. \*\*\* $p<0.001$  vs. SD counterpart. ++ $p<0.01$  vs. male counterpart.

### 6.3.6.2. Time interacting with novel rat and empty cage in sociability trial

A Two-Way ANOVA showed a significant effect of strain on the time spent interacting with the novel rat [ $F_{(1,27)}=8.504, p<0.01$ ]. *Post-hoc* testing revealed a trend for the male WKY group to spend less time investigating the novel rat however this failed to reach statistical significance,  $p=0.052$ . There were no effects of sex or strain on the time spent investigating the empty cage.

When comparing the time spent interacting with the rat vs. the cage within the same group, T-Tests revealed that the male and female SD groups spent significantly longer interacting with the rat, ( $t=6.466$   $df=7, p<0.001$ ;  $t=7.620$   $df=6, p<0.001$  respectively).

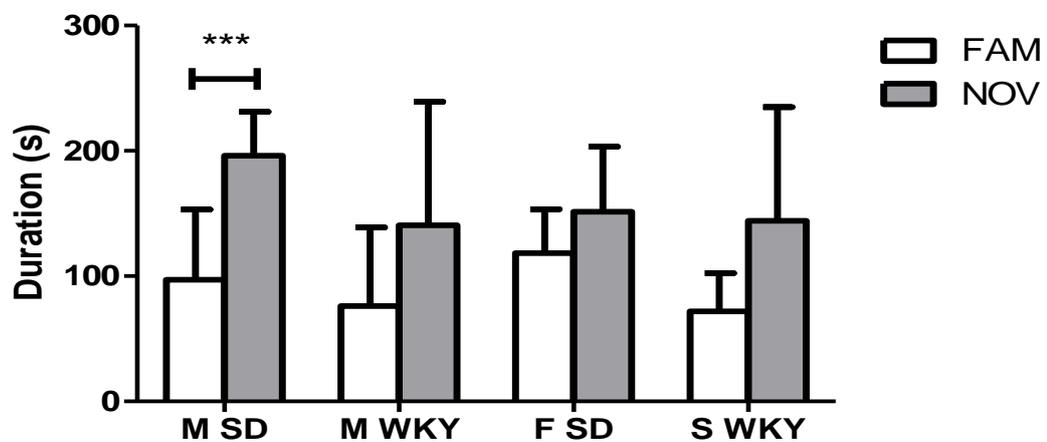


**Figure 6.20. Time spent investigating the novel rat vs. the empty cage in the sociability trial.** There were no differences between the groups in the time spent interacting with either the rat or the cage. Male and female SD groups spent more time with the rat than with the cage. Data represented as mean+ SD. N= 8 per group. \*\*\* $p<0.001$  cage vs. rat.

### 6.3.6.3. Time interacting with familiar vs. novel rat in novelty trial

There were no significant differences between the groups in either the time spent with the familiar rat or the novel rat.

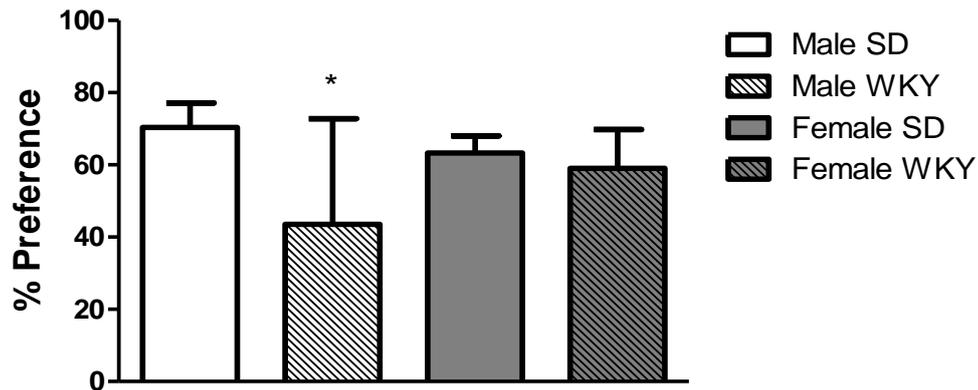
When comparing the time spent interacting with the familiar rat vs. the novel rat within the same group, T-Test revealed that only the male SD group spent significantly longer interacting with the novel rat ( $t=3.432$   $df=7$ ,  $p<0.05$ ).



**Figure 6.21. Time spent investigating the familiar rat vs. the novel rat in the novelty trial.** There were no differences between the groups in the time spent interacting with either rat. Data represented as mean+ SD. N= 8 per group. \*\*\* $p<0.001$  familiar vs. novel rat.

**6.3.6.4. Percentage preference for rat vs. cage in sociability trial**

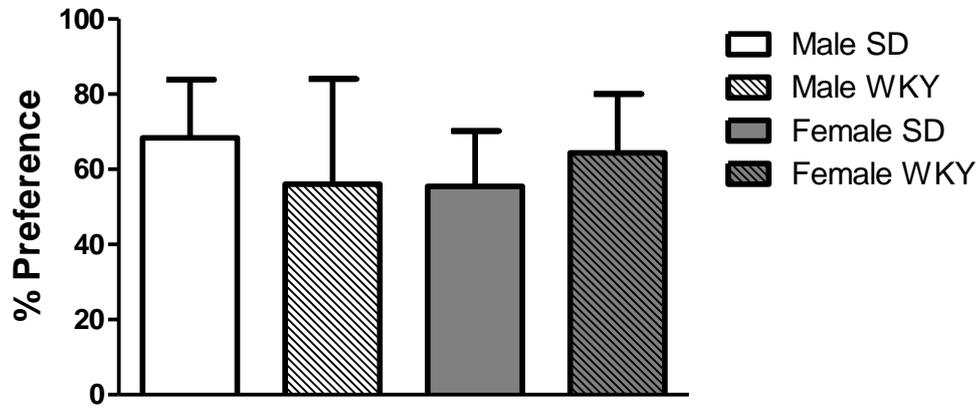
A Two-Way ANOVA revealed a significant effect of strain [ $F_{(1,27)}=6.970, p<0.05$ ], on the percentage preference for the novel rat. *Post-hoc* test revealed the male WKY group had a significantly lower percentage preference for the novel rat compared to the male SD group.



**Figure 6.22. Percentage preference for rat vs. cage.** The male WKY group exhibited a significantly reduced percentage preference for the novel rat compared to the male SD group. Data represented as mean+ SD. N= 8 per group. \* $p<0.05$  vs. male SD group.

### 6.3.6.5. Percentage preference for novel vs. familiar rat in novelty trial

There were no differences between the groups for the percentage preference for the novel vs. familiar rat.



**Figure 6.23. Percentage preference for novel vs. familiar rat in novelty trial.** Data represented as mean+ SD. N= 8 per group.

#### **6.4. Discussion**

The aims of this chapter were to investigate potential sex differences in the WKY model across a range of behaviours including anxiety- and depressive-like behaviours as well as in a range of tests of cognitive ability. Our findings show that both male and female WKY animals display increased anxiety-like behaviours, the degree of which are mainly equal between the sexes, in comparison to the SD counterparts. However we failed to show increased “depressive-like” activity in the FST in either male or female WKY animals. In regards to cognitive abilities, both male and female WKY groups exhibited deficits compared to their SD counterparts, with male WKYs showing more cognitive deficiencies compared to the female WKY groups.

Contrary to the behaviour expected from the OB model of depression, which is a hyperactive, agitated phenotype, the WKY model displays a hypoactive, timid phenotype. This can be observed in the OF results with both male and female WKYs displaying a significantly smaller distance moved during the test compared to their SD counterparts which is a commonly reported behaviour in the WKY model (Pare, 1994b, Luo et al., 2015, Burke et al., 2016, Tejani-Butt et al., 2003). We found an increased time in the centre of the OF arena in the male WKYs compared to both the male SD group as well as the female WKY group. We believe this is due to freezing behaviour exhibited by male WKYs upon exposure to an anxiogenic environment. This behaviour has been previously reported in fear conditioning tests (DaSilva et al., 2011), and in the conditioned defensive burying test (Pare, 1992).

In the EPM, again the WKY groups exhibited a hypoactive response with a decreased distance moved for both male and female WKYs compared to their SD counterpart. We also found that females of both strains moved significantly more compared to their counterpart male group. Sexual dimorphism in rats in regards to locomotor activity has

previously been studied with results showing increased locomotor activity in females by 8 weeks of age (Hyde and Jerussi, 1983). This finding has also been reported in WKY females (Pare and Redei, 1993, Chelaru et al., 2012).

Increased anxiety levels were observed in WKY animals in the EPM regardless of sex. WKY groups entered the open arms significantly less times and there was also a trend for the WKY groups to spend less time in the open arms compared to their corresponding SD control group. Decreased entries into and time spent in the open arms of the EPM test have been reported previously and are a feature of the anxiety-like phenotype of the WKY strain (Shepard and Myers, 2008, Langen and Dost, 2011). Our findings also show significantly less entries into the closed arms of the EPM test in both WKY groups compared to their SD controls. This was more pronounced in male WKYs as the females entered the closed arms more times than them. The male WKYs also spent less time in the closed arms, which was not observed in female WKYs. This smaller number of entries into the arms and time spent in the arms of the EPM may be due to the hypo-locomotor activity observed in the WKY groups. As the EPM test possesses a clear locomotor component, this feature of the WKY may skew the interpretation of the results.

Both the male and female WKY groups exhibited more time in the centre of the EPM arena compared to their SD control which again demonstrates the freezing behaviour synonymous with this model. This behaviour is suggestive of a novelty-induced freezing which alludes to an anxiety-like phenotype. Fear or extreme dislike for the new and unfamiliar is called neophobia and has been measured in the male WKY rat previously, in tests such as hyponeophagia (Pare, 1994a), and latency to approach and duration of exploration of a novel object placed in an unfamiliar arena (Delini-Stula and Hunn, 1985). Novelty-induced hypophagia (NIH) has also been investigated in female WKYs, however

unlike in male WKYs, females did not exhibit hypophagia when presented with food in a novel environment (Burke et al., 2016).

Previous investigations into the behaviour of the WKY rat in the FST have found an increased immobility time in male WKYs compared to SD controls (Lopez-Rubalcava and Lucki, 2000, Carr et al., 2010, Pare and Tejani-Butt, 1996) as well as in female WKYs compared to SD controls (Burke et al., 2016), however we failed to detect any differences in immobility time between the WKY and SD groups. This may be because we tested the animals in the 15 minute pre-swim only and did not carry out the 5 minute swim 24 hours later which is the result presented in the above cited studies. One study which did analyse the 15 minute pre-swim data and compared the results across 3 WKY substrains, showed that the WKY/NHsd, which is the substrain used in our study obtained from Harlan Laboratories, did not show increased immobility scores whereas the WKY/NCrl substrain from Charles River Laboratories displayed significantly higher immobility times compared to the other 2 strains (the third strain being the WKY/NTac which are supplied by Taconic) (Browne et al., 2015). This finding has major implications on the decision of where to source WKY animals for behavioural studies as it implies a variation in behavioural profile between WKY animals obtained from different vendors.

Cognitive impairment is one of the main symptoms listed in the DSM diagnostic criteria for depression (“Diminished ability to think or concentrate”), and so it is important that we reflect this in animal models of depression. Most work carried assessing the cognitive ability of the WKY rat has been with the use of the WKY strain as a control to the SHR strain which is used as a rodent model of ADHD (King et al., 2000, Gattu et al., 1997, Bayless et al., 2015) and so the cognitive ability of the WKY rat as a model of depression compared to a standard outbred rat strain is still not fully clear, especially in female subjects as these are seldom used in pre-clinical research.

From the studies that were identified, it is proposed that the WKY strain exhibits impaired working and reference memory compared to the SD strain in male subjects (Grauer and Kapon, 1993, Clements and Wainwright, 2006) and learning and memory compared to Wistar rats also (Luo et al., 2015). To our knowledge, our work is the first time cognitive ability has been studied in both male and female WKY rats, comparing them against a commonly used ‘control’ strain. Our results suggest that male WKY rats display a more pronounced cognitive impairment compared to female WKY rats. In the MWM training period, WKY males were slower to find the platform compared to SD males in half of the trials performed and were slower than the WKY females in 3 out of the 16 trials performed. The female WKY rats did not show any impairment in learning across the 16 trials compared to their SD counterpart.

In the probe trial of the MWM, both male and female WKY rats exhibited a similar level of impairment in the time spent in the target quadrant, with both the sexes spending less time here compared to their SD controls which is indicative of an impaired ability to remember where the platform is located. Again male WKY rats showed greater levels of memory deficits as they took significantly longer to enter the target quadrant in the probe trial compared to their SD controls whereas female WKY rats did not show this memory deficit.

In a test of object learning and recognition memory, we did not find any deficits in the short-term memory of the WKY model. There are mixed reports regarding the cognitive ability of the WKY strain in the NOR test. One study has found that male WKY rats perform better in this task compared to the SHR strain which was being studied as a model of ADHD (Leffa et al., 2016). However when this test was used to measure long-term memory in the WKY rat, it was found that male WKYs performed worse compared to the SHR rat (Langen and Dost, 2011). A study using female rats which also takes two WKY

substrains into account (WKY NCrl from Charles River Laboratories and WKY NHsd from Harlan Laboratories), found that the WKY NHsd, the same strain used in our study, showed no difference in preference for the novel vs. familiar object compared to SD counterparts (Zhang-James et al., 2014). This study was again looking at long term memory, as it had an inter-trial delay of 24 hours, but described opposite results to the study mentioned above that looked at long-term memory in male rats. This would suggest a sex difference in long-term memory abilities in the WKY strain which will need to be explored in further detail before definitive conclusions can be made. Differences in study protocol as well as the reporting of results make comparisons across lab groups difficult for the NOR test.

We found no differences in social recognition memory in WKY rats compared to the control SD groups as there was no difference in the percentage preference for the novel rat compared to the familiar rat in the novelty trial. Female WKY rats have been previously shown to spend less time with a novel rat compared to female SD rats (Zhang-James et al., 2014), however it was not stated whether or not female WKYs spent more time with the novel rat than the familiar rat (judging by the graph in the paper it seems this may be the case) and so we cannot fully compare the results of this study to our results.

The main findings from our three tests of cognition are that WKY rats exhibit deficits in spatial learning and memory compared to SD controls; which are more pronounced in males compared to females. Little to no effects of strain on cognition were found in the NOR test and SIT, with the only strain differences being an increased preference for social interaction in SD animals compared to WKYs.

Overall our findings support the use of both male and female WKY rats as a model of anxiety, however we failed to detect depressive-like behaviours in the FST. In future

experiments, the inclusion of another test of depressive-like behaviour would be appropriate in order to allow a more well-rounded measure of the depressive-like phenotype such as a measure of anhedonia analysed with the sucrose preference test as an example.

We show that the extent of the cognitive deficits in the WKY model are primarily in spatial learning and memory, with little impairments found in object or social recognition memory. We also show that these cognitive deficits are sexually dimorphic, with male WKY rats exhibiting more pronounced learning impairments.

***Chapter 7:***  
***Response to chronic***  
***antidepressant***  
***treatment in male and***  
***female WKY rats***

## **7.1. Introduction**

Variability in response to treatment with antidepressant drugs in the WKY model of depression is commonly reported. Previous studies have shown that neither acute nor chronic treatment with SSRI drugs, which are the first line of treatment for depression, result in anti-depressive effects in this model of depression. Fluoxetine and paroxetine have been shown to have no effect on the time spent immobile in the FST even at high doses in male WKY rats<sup>1</sup> (Lopez-Rubalcava and Lucki, 2000, Griebel et al., 1998, Tejani-Butt et al., 2003).

Some success has been achieved using the TCAs desipramine and imipramine which both reduce immobility time in the FST (Lopez-Rubalcava and Lucki, 2000, Lahmame et al., 1997), however these effects are observed when the drugs are administered both acutely as well as chronically which is not representative of the clinical scenario in which antidepressant effects are only observed following chronic administration.

Due to the seemingly resistant nature of the WKY model to SSRI drugs, it has been proposed that it could be used to detect novel antidepressant compounds that are not reliant on altering serotonin levels. Supporting this theory, ketamine, which has been proven to have clinical benefit for depression, causes a rapid and lasting antidepressant effects in the WKY rat (Tizabi et al., 2012, Akinfiresoye and Tizabi, 2013).

Very little work has been previously carried out comparing the response to antidepressant drugs between male and female WKY rats. It is believed that there may be a sex difference in the response to different classes of antidepressants in the clinical scenario and so it is important that we establish if a sex difference exists in our animal models of depression. Clinically it is thought that females respond more favourably to SSRIs than the classical TCAs (Kornstein et al., 2000, Martenyi et al., 2001) as oestrogen is thought to play a role in modulating the serotonergic system in females. A literature search found no previous

reports investigating the female WKY response to SSRIs and whether they do indeed respond to this class of drugs, although as mentioned above, it has been shown that male WKY rats do not.

Clinically there are also reported sex differences in response to TCAs, with less favourable responses in females (Martenyi et al., 2001). Whether this is also true for the WKY model is unknown as a literature search showed no previous studies researching this avenue of investigation.

In this chapter we aim to explore whether sex differences do occur in the WKY model of depression in the response to the two most common classes of antidepressants, an SSRI and a TCA. We have chosen to use a chronic dosing regime of 21 days as this is more clinically relevant compared to acute drug administration. The FST was used as a measure of antidepressant response as this is the most well-established test of antidepressant drug action currently used in animals. We also chose to perform a test of memory and cognition in order to determine if antidepressant treatment causes improvement in cognitive ability in a model of depression.

The neurotrophic theory of depression suggests that a decrease in neurotrophic factors (NTFs) may play a role in the onset of depression. NTFs are biomolecules that promote the survival, growth and differentiation of both mature and developing neurons. Brain-derived neurotrophic factor (BDNF) is the most studied NTF with regards to links with depression. It is thought that decreased levels of BDNF caused by genetic mutation or stress leads to suboptimal function in the hippocampus, a key limbic region implicated in depression. One of the most widely studied genetic mutations in the BDNF gene is the single nucleotide polymorphism (SNP) Val66Met; rs6265 which results in the substitution of valine (Val) for methionine (Met). This Met allele has been linked with reduced BDNF activity and is associated with deficits in short-term memory and

abnormal hippocampal activation (Hosang et al., 2014, Roy et al., 2014). A meta-analysis study showed a significant interaction between life stresses and a role for BDNF in depression when this Met allele was present (Hosang et al., 2014). This may suggest that this mutation in the BDNF gene conveys a predisposition to depression which then may turn into clinical depression following exposure to life stressors.

BDNF binds to the neurotrophic tropomyosin receptor kinase B (TrkB) receptor, coded for by the NTRK2 gene, which is in the tyrosine kinase receptor family. Binding with TrkB mediates the effects of BDNF including neuronal growth and survival. Little is known about the role (if any) TrkB has in depression however a meta-analysis study did find a link between genetic polymorphisms in the NTRK2 gene and increased treatment resistant depression in MDD patients (Li et al., 2013). NTRK2 polymorphisms have also been linked to reduced white matter structural integrity (Murphy et al., 2012).

There are mixed reports as to whether BDNF levels are decreased in the WKY model of depression with some studies showing decreased levels in both the frontal cortex and hippocampus compared to Wistar controls (Vinod et al., 2012, Hauser et al., 2011) while others have found no differences in BDNF levels compared to either Wistar or Sprague-Dawley rats (O'Mahony et al., 2011, Kyeremanteng et al., 2014). According to a literature search no sex comparisons of basal BDNF levels and changes in levels following treatment with antidepressants could be found and so the final aspect of this chapter is to investigate whether there are sex differences in basal BDNF and Ntrk2 mRNA levels and to examine if treatment with two classes of antidepressant drugs alters these levels in either sex.

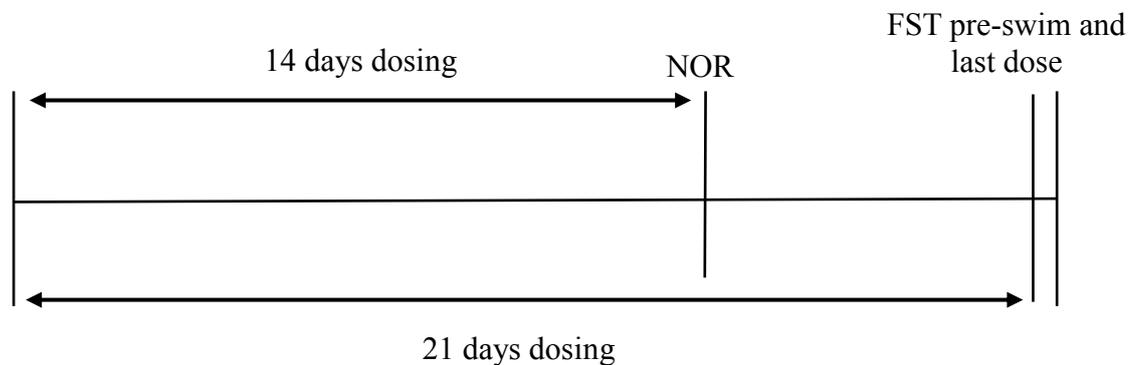
## Aims

The aims of this chapter are as follows:

- To ascertain whether sex differences exist in behavioural responses to chronic treatment with an SSRI, fluoxetine, or a TCA, desipramine
- To determine if treatment with antidepressant improves cognitive ability in the WKY model
- To determine if sex differences exist in levels of BDNF and Ntrk2 mRNA in the hippocampus and frontal cortex of WKY rats
- To determine if antidepressant treatment elevates levels on BDNF mRNA in male or female WKY rats

## 7.2. Experimental methods

Male and female Sprague- Dawley (SD) and Wistar Kyoto (WKY) rats were bred in-house and at ~7 weeks of age were singly housed for commencement of the study. Animals were maintained under a 12h light cycle (lights on at 08:00h) with food and water provided *ad libitum*. Desipramine and fluoxetine were dissolved in a sterile distilled water which was used for the vehicle group. Drugs were administered via oral gavage at a volume of 2ml/kg at a dose of 10mg/kg daily for 21 days between 2-4pm each day. Animals were randomly assigned to their drug group. The novel object recognition test (NOR) was performed 18-23 hours following the 14<sup>th</sup> dose. Rats were given their final dose 15 minutes following their FST pre-swim and put into the test swim 24 hours after the final dose, followed immediately by sacrifice.

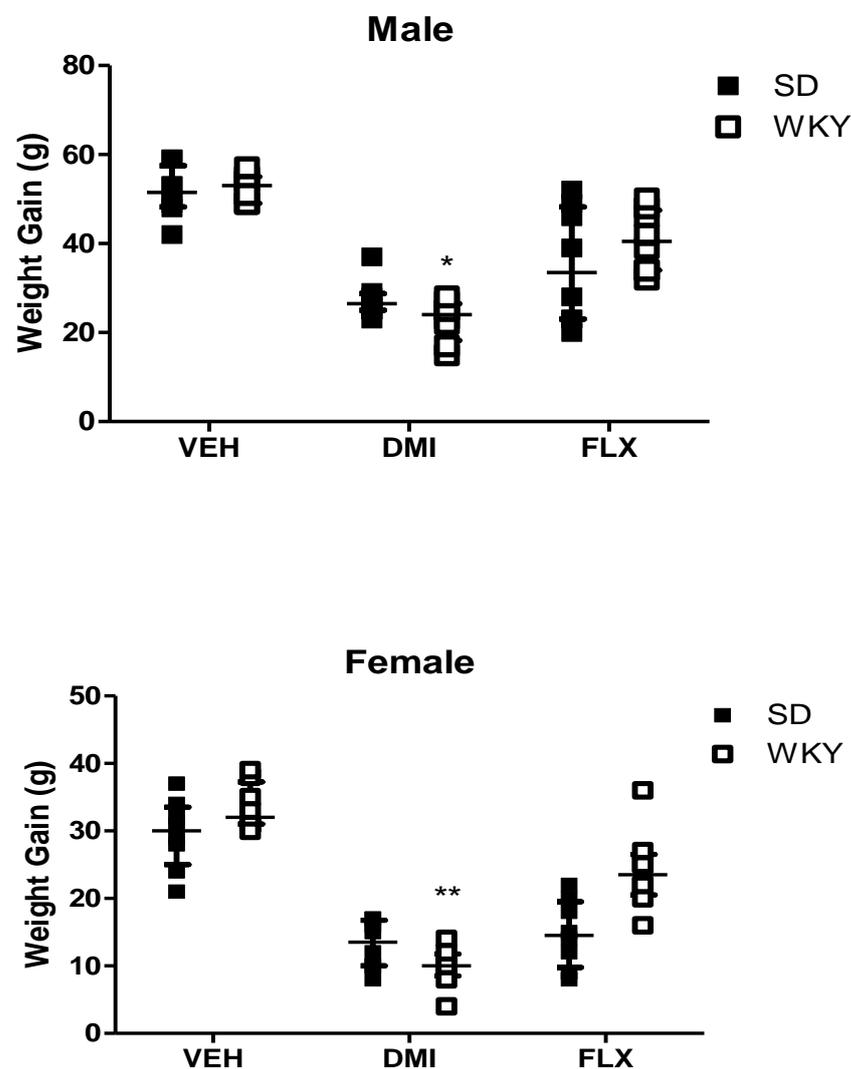


**Figure 7.1.** Experimental design for WKY antidepressant response study. N= 8 per group were tested in both the NOR test and the FST.

### 7.3. Results

#### 7.3.1. Body Weight

The effect of antidepressant treatment on body weight gain was analysed with a Kruskal-Wallis test [ $K_{(11)} = 79.684$   $p < 0.001$ ]. *Post-hoc* analysis with Dunn-Bonferroni pairwise comparison test revealed that the male and female WKY desipramine groups gained significantly less weight compared to their vehicle dosed controls.

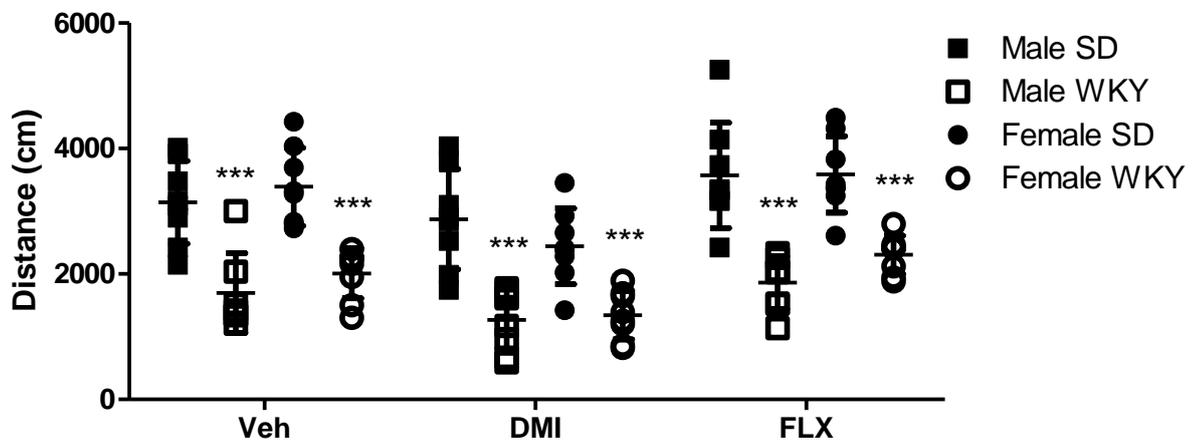


**Figure 7.2. The effect of antidepressant dosing on body weight gain.** Male and female WKY DMI dosed groups gained less weight compared to the WKY VEH dosed groups. \* $p < 0.05$  vs MWKY VEH, \*\* $p < 0.01$  vs FWKY VEH.  $N = 7-8$  per group.

### 7.3.2. Open field

#### 7.3.2.1. Distance moved in open field

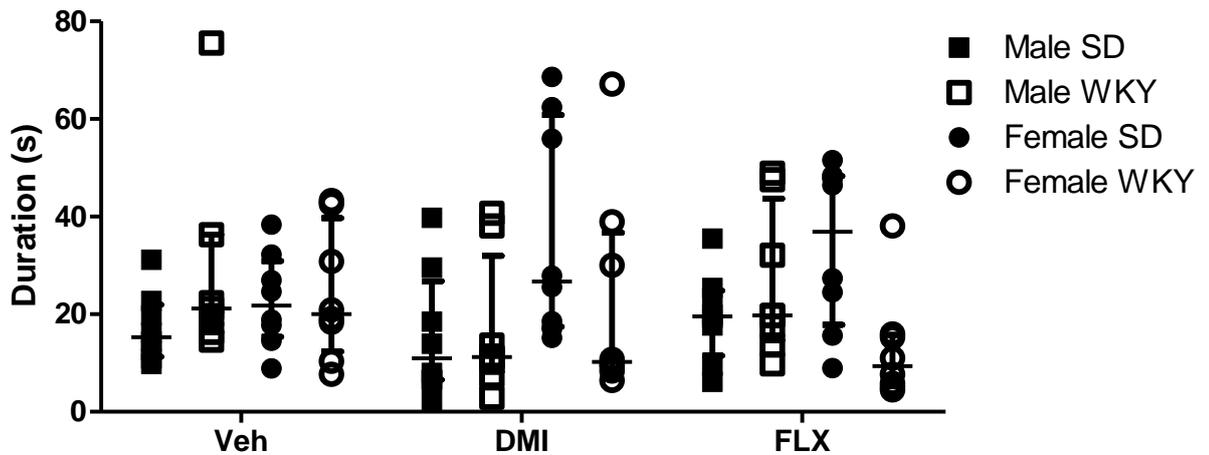
A Three-Way ANOVA revealed a significant effect of drug [ $F_{(2,83)}=17.559, p<0.001$ ], and strain [ $F_{(1,83)}=139.359, p<0.001$ ]. *Post-hoc* Student-Newman-Keuls test revealed that the male and female WKY drug groups were significantly different compared to their SD counterpart groups. It also revealed that the female SD and WKY FLX groups were significantly different to their VEH counterpart groups.



**Figure 7.3.** The effect of sex, strain and drug on distance moved in the OFT. All male and female WKY groups moved significantly less compared to their SD counterparts. The female SD and WKY groups treated with FLX moved significantly more compared to their counterpart groups that were treated with DMI. Data presented as mean  $\pm$  SD. N= 7-8 per group. \*\*\* $p<0.001$  vs. SD group.

## 7.3.2.2. Inner Zone duration in the OFT

Data was analysed via the non-parametric Kruskal-Wallis test which revealed a significant difference between the groups,  $K_{(11)} = 20.595$ ,  $p < 0.05$ . Dunn-Bonferroni *post-hoc* procedure revealed no differences between the groups.

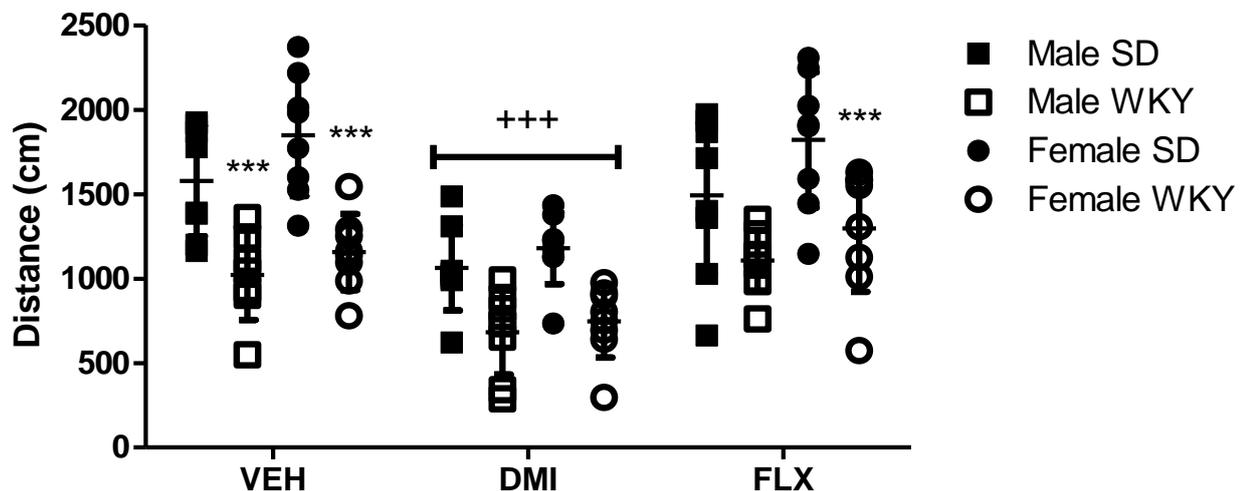


**Figure 7.4.** The effects of sex, strain and drug treatment on IZD in the OFT. The female WKY FLX treated group spent significantly less time in the centre of the OF arena compared to the female SD WKY group, the male WKY FLX group and the female WKY VEH group. Data presented as median  $\pm$  interquartile range. N= 7-8 per group.

### 7.3.3. Novel Object Recognition

#### 7.3.3.1. Distance Moved

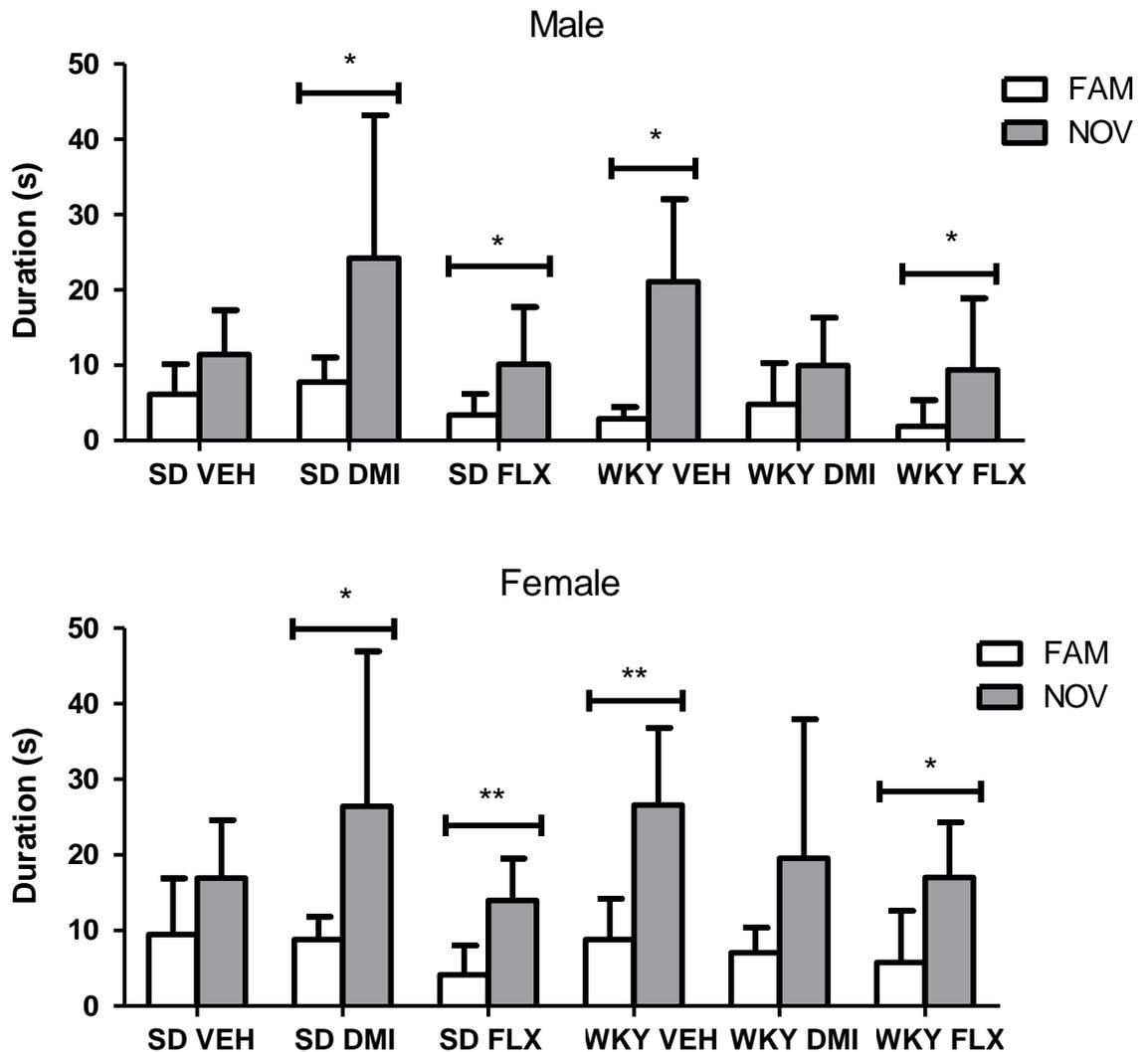
Distance moved in the NOR test was analysed by a three way AVOVA test which revealed effects of sex, strain and drug [ $F_{(1,83)}= 8.566, p<0.01$ ;  $F_{(1,83)}= 61.957, p<0.001$ ;  $F_{(2,83)}= 27.927, p<0.001$ ]. *Post-hoc* analysis revealed that both the male and female WKY VEH groups moved less compared to their SD control, all groups treated with DMI moved less compared to their VEH treated control and the female WKY FLX group moved significantly less compared to the female SD FLX group.



**Figure 7.5. Distance moved in the NOR habituation trial.** Male and female WKY VEH groups moved significantly less than their SD controls. All DMI treated groups moved significantly less compared to their VEH treated control. The female WKY FLX group moved significantly less compared to the female SD FLX group. +++ $p<0.001$  vs relevant VEH group, \*\*\* $p<0.001$  vs SD group. N= 7-8 per group.

## 7.3.3.2. Time sniffing familiar vs novel object

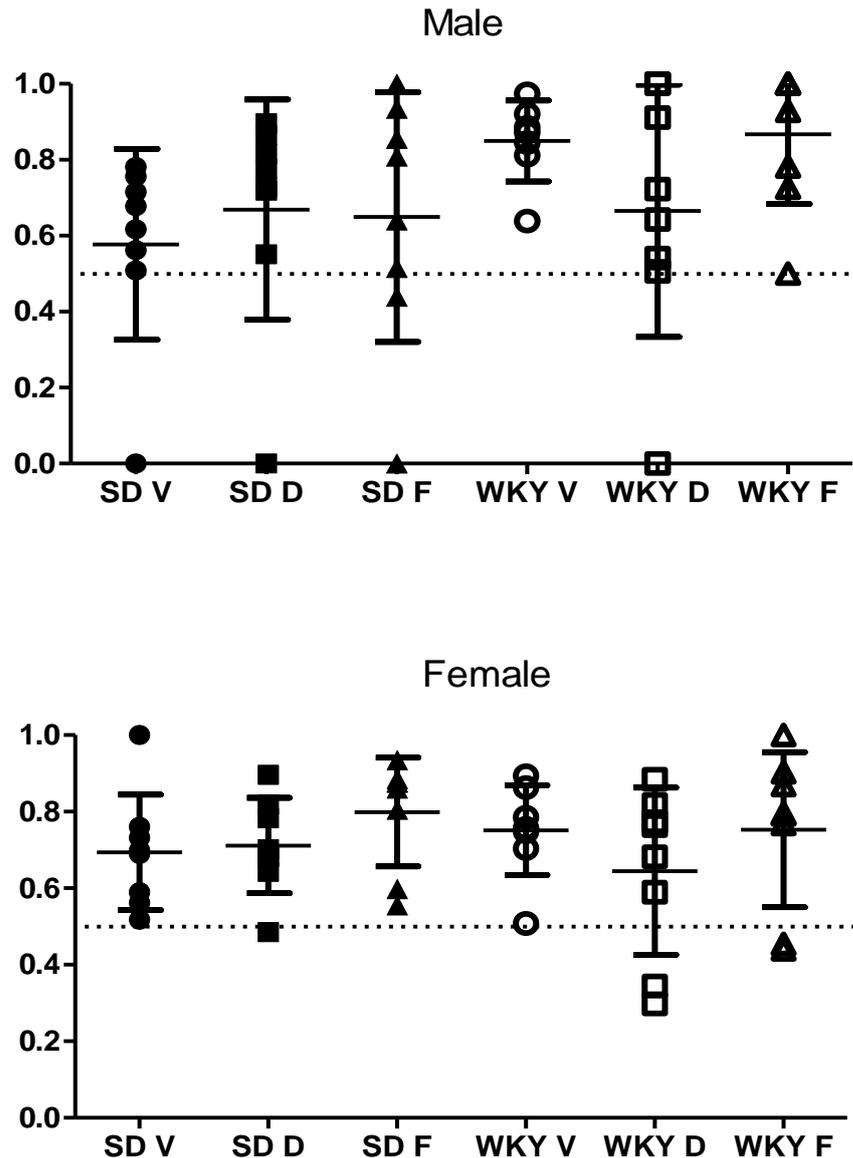
Three way ANOVA revealed significant effects of sex and drug treatment on the time spent sniffing the familiar and novel objects in the discrimination trial [ $F_{(1,83)}= 9.440$ ,  $p<0.01$ ;  $F_{(1,83)}= 5.254$ ,  $p<0.01$ ] and [ $F_{(1,83)}= 5.436$ ,  $p<0.05$ ;  $F_{(1,83)}= 3.609$ ,  $p<0.05$ ] respectively. Analysis with *post-hoc* Student Newman Keuls test found no relevant differences between the groups.



**Figure 7.6. Time spent interacting with familiar and novel objects.** Male and female WKY VEH groups moved significantly less than their SD controls. All DMI treated groups moved significantly less compared to their VEH treated control. The female WKY FLX group moved significantly less compared to the female SD FLX group. \* $p<0.05$  familiar vs. novel object. \*\* $p<0.001$  familiar vs. novel object. N= 7-8 per group.

## 7.3.3.3. Discrimination Ratio

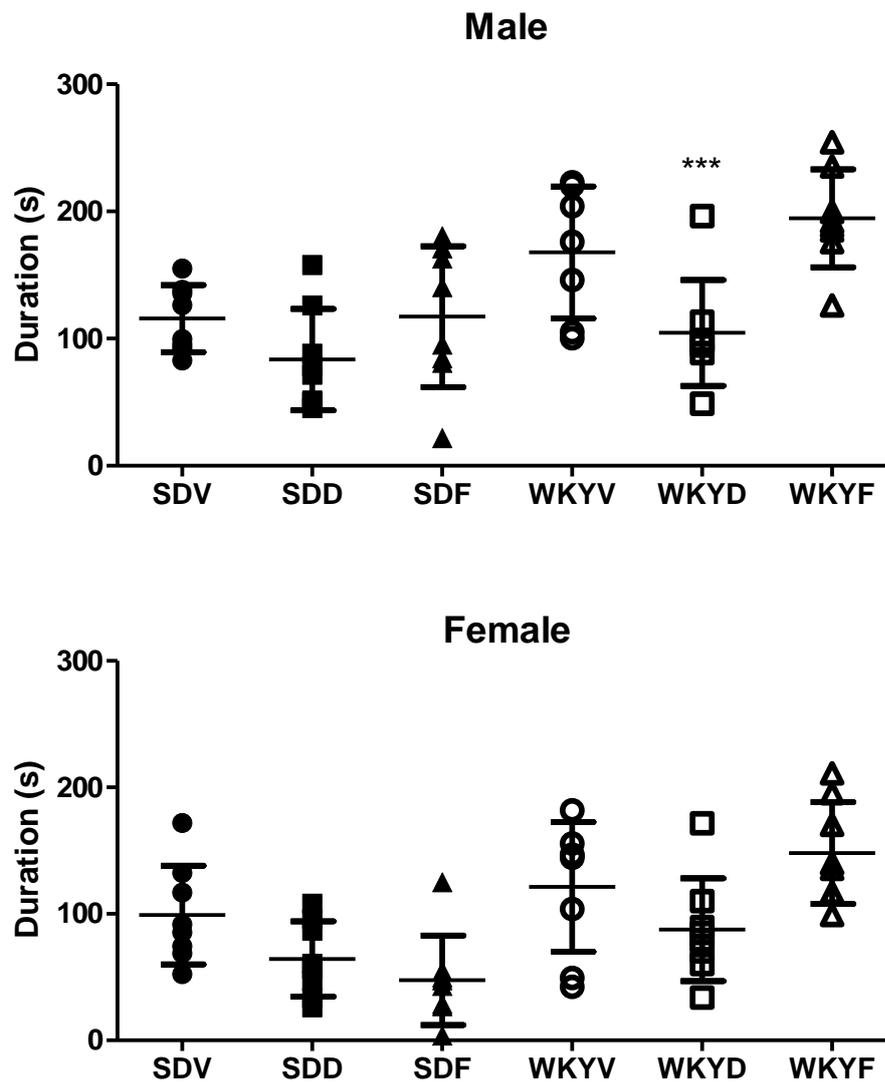
The discrimination ratio is calculated as the time spent exploring the novel object divided by the total time spent exploring both objects. A 3-way ANOVA revealed an interaction effect of sex\*strain [ $F_{(1,83)} = 4.036$ ,  $p < 0.05$ ] however *post-hoc* analysis revealed no differences between the groups.



**Figure 7.7. Discrimination ratio for time interacting with the novel vs. familiar objects.** No differences were found between the groups in the ability to discriminate between the novel and familiar objects following 14 days of antidepressant drug dosing. Data presented as mean  $\pm$  SD. N= 7-8 per group.

### 7.3.4. Forced Swim Test

A three-way ANOVA revealed significant effects of sex, strain and drug on immobility time in the FST [ $F_{(1,83)}= 17.785, p<0.001$ ;  $F_{(1,83)}= 33.555, p<0.001$ ;  $F_{(2,83)}= 10.595, p<0.001$ ]. *Post-hoc* analysis revealed that the male WKY DMI group spent less time immobile compared to the VEH group. The female WKY FLX group spent significantly more time immobile compared to the SD FLX group.



**Figure 7.8. Immobility time in the FST.** The male WKY DMI group spent less time immobile compared to the WKY VEH group. The male and female WKY FLX groups were more immobile compared to the SD FLX group. \*\*\* $p<0.001$  compared to WKY VEH group. Data expressed as mean $\pm$  SD. N= 7-8 per group.

### 7.3.5. Relative levels of BDNF and NTRK2 mRNA in the hippocampus and frontal cortex of male and female WKY rats following 21 days of antidepressant treatment

Statistical analysis of the data for the levels of BDNF and NTRK2 mRNA in the hippocampus was carried out using a Kruskal-Wallis test which revealed no differences between the groups.

Group	Hippocampus		Frontal Cortex	
	BDNF	Ntrk2	BDNF	Ntrk2
<b>M SD VEH</b>	1± 0.83	1± 0.84	1± 0.82	1± 0.43
<b>M SD DMI</b>	0.53± 0.8	0.27± 0.27	2.22± 1.79	0.95± 0.26
<b>M SD FLX</b>	1± 0.99	0.78± 0.61	1.11± 0.7	0.86± 0.27
<b>M WKY VEH</b>	1.08± 1.28	0.58± 0.48	2.15± 1.64	0.85± 0.27
<b>M WKY DMI</b>	0.33± 0.2	1.58± 1.38	1.15± 0.63	1± 0.31
<b>M WKY FLX</b>	0.88± 1.06	0.61± 0.35	1.26± 0.7	0.94± 0.34
<b>F SD VEH</b>	1± 0.98	1± 1.24	1± 1.4	1± 0.64
<b>F SD DMI</b>	0.93± 1.06	0.86± 0.78	1.33± 0.43	0.76± 0.2
<b>F SD FLX</b>	0.55± 0.57	1.01± 0.67	1.54± 1.72	0.57± 0.16
<b>F WKY VEH</b>	0.18± 0.16	0.87± 0.64	1.38± 1.22	0.57± 0.15
<b>F WKY DMI</b>	0.23± 0.19	1.09± 0.86	1.22± 0.85	0.74± 0.2
<b>F WKY FLX</b>	0.26± 0.09	0.72± 0.71	1.19± 0.77	0.7± 0.29

**Table 7.1. Relative levels of BDNF and NTRK2 in the hippocampus and frontal cortex of male and female WKY rat.** There were no differences found in the levels of BDNF or NTRK2 mRNA in the hippocampus or frontal cortex of male and female WKY rats following 21 days of antidepressant treatment. Data is expressed as mean± SD. N= 4-8 per group.

#### **7.4. Discussion**

The aim of this study was to determine if there are sex differences in responsivity to two classes of antidepressant drugs in the WKY model of depression. In addition to this we also examined potential involvement of neurotrophin signalling in mediating antidepressant responses in the WKY model and whether there are sex differences present.

Body weight was measured each day throughout the 21 day dosing period and when the amount of weight gained for this period was analysed we found that both male and female rats dosed with DMI gained significantly less weight compared to their SD DMI dosed counterparts. This effect on weight gain was apparent in both sexes and was not observed in either the VEH or FLX groups. Clinically, antidepressants often cause increased weight gain thought to be caused by various physiological mechanisms including antihistaminergic effects, modulation of hormonal signalling of leptin and ghrelin and impact on genes (Himmerich et al., 2015). The reason for the opposite effect of DMI on weight in rats is not fully understood and so further investigation could be warranted.

We found a decrease in locomotor activity in the OFT which is a common finding in this model due to its hypoactive phenotype which is present in both male and females (Pare, 1994b, Tejani-Butt et al., 2003, Burke et al., 2016). Unlike in Chapter 5 we did not see increased freezing behaviour in the male WKY groups in the OFT as there was no increased time spent in the centre of the arena in these or any other groups. It has previously been found in our lab that male and female WKY rats spend less time in the centre of the OFT arena (Burke et al., 2016) which is indicative of increased anxiety, however we did not find this in the current study.

In the novel object recognition (NOR) test that was performed following 14 days of dosing, we again observed hypoactivity in the habituation period in both the male and

female WKY vehicle treated groups compared to their SD controls. We also observed a drug effect of DMI on locomotor activity in all 4 groups as rats treated with this drug moved significantly less compared to their vehicle treated controls. Previous unpublished work from our lab found no effects of chronic DMI administration on locomotor activity in the home cage while other groups have reported a depression of locomotor activity in the OFT following chronic DMI administration similar to the results presented in this chapter (Hadweh et al., 2010, Wrona et al., 2013). The NOR test was performed in the OFT arena and so the reason why we see this effect of DMI on locomotor activity here and not in the OFT is unknown and may warrant further exploration.

In the SD groups both the male and female groups that were dosed with DMI and FLX spent more time interacting with the novel object compared to the familiar object. This differentiation between the objects was not observed in the vehicle dosed groups. The ability to remember the previously encountered object is necessary in order for the rats to discriminate between the novel and familiar objects and so this increase in time spent with the novel object in the DMI and FLX SD groups indicates improved memory in these groups compared to the SD groups that received vehicle treatment. Cognitive impairment can be a debilitating symptom of MDD and so it is important that medications used in the treatment of this disease can rectify these deficits often observed in patients. Our finding of improved object recognition following chronic FLX administration supports previous work showing improved spatial memory following a similar drug dosing regime (Cui et al., 2016). As for the literature regarding the effects of DMI administration on memory, the results are less clear with reports that DMI improves memory in rats with reduced noradrenaline levels but impairs cognitive ability in controls (Clinton et al., 2006) which is contradictory to the results reported in this chapter. However results reported by Walling *et al*, support our finding of enhanced object recognition in SD rats following chronic DMI treatment (Walling et al., 2016). Novel

spatial learning as well as memory consolidation and reconsolidation have also been shown to be impaired by DMI (Watts et al., 2012) however this was following acute administration and so we cannot assume that these results would be the same following chronic DMI administration.

Both the male and female WKY VEH treated groups interacted more with the novel object compared to the familiar object which would indicate that this model does not exhibit deficits in object recognition memory. Previous reports have found impaired spatial memory in male WKY rats in the MWM (Wyss et al., 2000) and the water radial arm maze (Clements and Wainwright, 2006) which contradict the findings here however as these tests assess spatial memory and the NOR test measures non-spatial memory there may be little benefit to comparing these results.

The DMI treated WKY groups spent a similar amount of time with the novel and familiar objects suggesting an impairment of memory compared to the VEH groups. Chronic DMI administration with a low dose of 3mg/kg has previously been shown to impair short-term object recognition memory (Walling et al., 2016) however, as mentioned above, the same study found that a dose of 7.5mg/kg improved memory and so this suggests that chronic DMI administration exerts different effects between SD rats and WKY rats. DMI has been shown to exert antidepressant effects in behavioural parameters in the WKY model of depression, discussed further below, however may not be a useful compound for investigating learning and memory in this model.

In the FST we found that chronic DMI administration caused a decrease in the time spent immobile in the male WKY group compared to the WKY vehicle treated group. This is in agreement with previous studies that reported TCA-induced decreases in immobility following sub-chronic dosing with DMI (Lopez-Rubalcava and Lucki, 2000) as well as chronic dosing with imipramine (Lahmame et al., 1997). This effect of DMI was not

observed in the female WKY group suggesting a sex difference in responsivity to TCAs in the WKY model. This finding is particularly interesting as males are reported to respond better to TCAs compared to females in the clinical scenario also (Kornstein et al., 2000, Martenyi et al., 2001).

There is general agreement in the literature that WKY males, are resistant to the antidepressant effects of SSRIs (Griebel et al., 1999, Willner and Belzung, 2015). Our findings support this as we did not observe any effects of fluoxetine on immobility time in male or female WKY rats. These findings suggest that the depressive-like phenotype of the WKY model is not caused by depleted levels of serotonin or dysregulation of the serotonergic system but perhaps by alterations in the noradrenergic system because chronic treatment with DMI, which acts as a noradrenaline transporter blocker, decreased immobility in the FST. As DMI caused reduced immobility in both males and females, it may be assumed that the noradrenaline system has a similar role in both male and females in depression pathophysiology.

We found no differences between the groups in the levels of BDNF mRNA in the hippocampus and prefrontal cortex following antidepressant treatment. Both the male and female WKY VEH groups exhibited similar levels of BDNF mRNA in both brain regions which is similar to previous reports (O'Mahony et al., 2011, Kyeremanteng et al., 2014). The WKY model has been found to exhibit lower BDNF hippocampal protein levels compared to Wistar controls (Vinod et al., 2012, Hauser et al., 2011) which may suggest that the Wistar rat strain is a more appropriate strain for neurochemical comparisons as it is more closely related to the WKY strain.

The role of the BDNF receptor TrkB, encoded by the NTRK2 gene, in depression pathogenesis is unclear and little research on this particular receptor has been carried out in the WKY model of depression. Our results showed that there are no differences in the

expression of NTRK2 mRNA in the hippocampus or prefrontal cortex between male and female WKY rats and the SD control strain. We also found no differences in mRNA expression following chronic dosing with either desipramine or fluoxetine.

To summarise our findings in this chapter, chronic DMI treatment caused reduced weight gain in both male and female WKY rats, male and female WKY rats exhibited hypolocomotion in the OFT and this is not affected by either chronic DMI nor FLX treatment; in the NOR test DMI reduced locomotor activity in male and female SD and WKY rats in the habituation phase and both DMI and FLX treatment increased the time spent with the novel object in both male and female SD rats. DMI treatment had the opposite effect in the WKY groups in that it reduced the time spent interacting with the novel object compared to the familiar object. In the FST, no antidepressant-like effects of FLX treatment were found however a sexually dimorphic effect of DMI treatment was observed with a reduction in immobility time found in male WKY rats only. Finally, we did not find any differences in brain levels of BDNF mRNA between the WKY rats and their SD controls either in the vehicle groups nor did we find any effects of antidepressant treatment on mRNA levels of BDNF or NTRK2.

In conclusion, we found sex differences in the WKY model of depression in response to chronic administration of the antidepressant desipramine. In the most widely validated behavioural test of antidepressant activity, the FST, desipramine reduced immobility time (behavioural despair) in male WKY rats only. This suggests that female WKY rats could be used as a model of treatment resistant depression and may be valuable in the search for antidepressant medications that do not rely on manipulation of neurotransmitter systems.

***Chapter 8:***  
***Wistar Kyoto***  
***Reproductive ability***  
***and neonatal***  
***development study***

## **8.1. Introduction**

Animal models of depression are used in order to aid in the investigation of underlying neurobiological mechanisms of the disease as well as in the development of new pharmacotherapies. Clinically it is widely reported that females are roughly twice as likely to suffer from depression than males and so an effort must now be made to examine the reliability of the animals models of depression that were validated in male animals for use in female subjects.

Genetic models of depression have been developed which exhibit hereditary vulnerability to depressive-like behaviours, similar to clinical observations that have shown depression to have up to 31-42% genetic heritability (Sullivan et al., 2000). As previously discussed, the WKY model of depression was originally developed as a normotensive control strain to the SHR strain and was later observed to exhibit hyper-responsiveness to stress and a 'behavioural despair' (Pare, 1989). Issues regarding genetic and behavioural variability of the WKY strain have been described however it is still one of the most commonly used genetic models of depression (Overstreet, 2012). Previous work, including studies from our own lab, have demonstrated anxiety- and depressive-like behaviours in both male and female WKYs but have also described some sex differences. When compared to SD controls, female WKYs did not exhibit novelty-induced hypophagia or anhedonia as male WKYs did (Burke et al., 2016); male WKYs have also been reported to exhibit more robust immobility scores in the FST compared to female animals (Will et al., 2003), however the opposite has also been found, with females exhibiting greater immobility scores (Pare and Redei, 1993). While it can be assumed that female WKYs model depression at least as well as males, it is fair to accept that sex differences exist, however the precise nature of these differences is still inconclusive.

Due to the high prevalence rates of depression, the risk for an occurrence of a depressive episode in the perinatal period are high with roughly 19.2% of new mothers experiencing a depressive episode within the first 3 months postpartum (Gavin et al., 2005). An important consideration to take into account in female rodent models of depression is whether they can model perinatal depression because, as mentioned, it affects a large proportion of women.

As described in a previous chapter, specific models of perinatal depression have been developed that involve either hormonal manipulation to imitate hormonal fluctuations observed clinically during pregnancy, i.e. the hormone withdrawal model, or application of stress during the gestational period such as restraint stress (Li and Chou, 2016), in order to elicit a depressive-like behaviour in the dam. These models have been shown to exhibit increased immobility scores in the FST with the gestational stress model also exhibiting reduced maternal behaviours (Smith et al., 2004).

The Flinders Sensitive-Line (FSL) genetic model of depression exhibited deficits in maternal behaviour as well as physiological and behavioural impairments in pups. FSL dams displayed increased immobility times in the FST as well as decreased non-nutritive contact and licking of pups (Lavi-Avnon et al., 2005). Maternal-offspring interactions have been shown to play an important role in pups' emotional, social, sexual and cognitive development (Caldji et al., 2000). When pre-pubertal FSL rats were tested in the EPM and OF, they were found to exhibit significantly lowered patterns of anxiety-like behaviours (Braw et al., 2006a), and so it was suggested that as FSL rats mature they move from lower anxiety levels to normal level as adults.

WKY dams have been shown to spend less time nursing and licking their pups and were also out of their nest more compared to SHR dams, however WKY rats rearing cross-fostered pups displayed reversed behaviour towards these pups of the opposite strain, i.e.

WKY dams spent more time with the SHR pups and SHR dams spent less time with WKY pups (Cierpial et al., 1990). Similarly when comparing maternal behaviour of WKY dams compared to Fischer 344 dams, another inbred rat strain, a study found that WKYs spent less time in contact with their pups (Ahmadiyeh et al., 2004). WKY dams were found to produce more milk at PND 6 compared to SHR dams and cross-fostering of SHR pups to WKY dams resulted in an antihypertensive effect thought to be produced by this increase in milk intake (Gouldsbrough et al., 1998). When undisturbed maternal observations were carried out investigating differences in behaviour of WKY and Wistar strains, it was found that WKY dams spent more time nursing pups with less self-directed activity compared to Wistar dams (Braw et al., 2009). Both positive and negative aspects of WKY maternal behaviours have been described and we aim to increase the body of work investigating this aspect of the WKY model of depression by comparing the behaviour of WKY dams to that of another commonly used, outbred rat strain, i.e. the Sprague-Dawley strain.

Most reports found examining neonatal development in WKYs is with the use of the WKY strain as a control for the SHR strain as a model of ADHD, this makes interpretation of the results difficult as they haven't been compared to a standard rat strain. One study was found that did use both WKY and SD strains as controls to the SHR strain, found that WKY pups exhibited delayed eye opening and impaired balance compared to SD pups (Ferguson et al., 2003).

Exposure to early-life stress impacts neurodevelopment and stress susceptibility in adulthood. When early-life stress in the form of maternal separation is applied to WKY pups, which are genetically hyper-responsive to stress, results in decreased anxiety- and depressive-like behaviours in adulthood in with increased exploration in OF, decreased immobility time in the FST and increased social interaction times. Maternal separation

had opposite effect in Wistar rats causing increased anxiety- and depressive-like behaviours in adulthood. This response in WKYs is thought to be caused by early-life stress leading to an adaptive response and stress resilience later in life (Rana et al., 2015).

To the best of our knowledge, an in depth examination of the effects of having one or more WKY rat as a parent on neonatal developmental endpoints has not been carried out previously. The use of an outbred commonly used laboratory rat strain as a control in this study, allows us to compare neonatal development in WKYs against a standard as well as allowing us to obtain a basic insight into whether any developmental alterations found are inherited or are due to maternal care, via the use of the cross-bred groups.

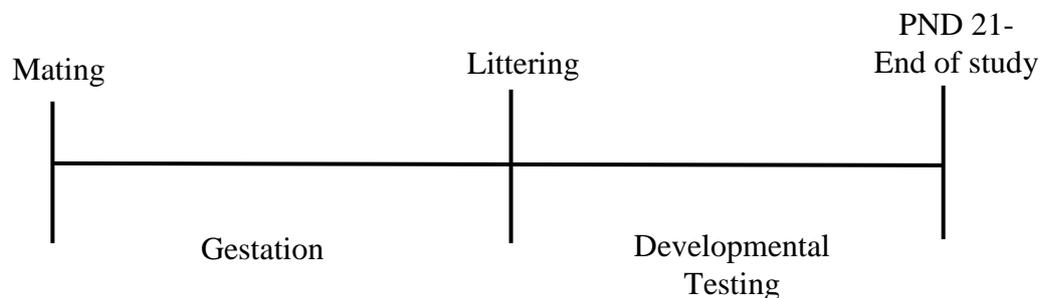
The main aims of this chapter are to investigate whether WKY rats, a genetic model of depression and anxiety, exhibit deficits in reproductive parameters; to examine if female WKY rats exhibit altered maternal behaviours and also to investigate the effect of having one or more WKY rat as a parent on neonatal developmental parameters. All compared to a commonly used outbred rat strain, the Sprague-Dawley.

## 8.2. Experimental methods

Male and female SD and WKY rats were obtained from Envigo LTD. Following behavioural testing carried out for the study described in Chapter 6, when the rats were approximately 14 weeks old they were mated in the following combinations:

- Male SD X Female SD
- Male SD X Female WKY
- Male WKY X Female SD
- Male WKY X Female WKY

Littering characteristics including litter size, pup sex, neonatal death were recorded and pups' development was measured from PND 1-21 and behavioural tests were carried out at set time points during the neonatal period. Male and female WKY and SD rats, including pups, were culled post-weaning (PND 21).



**Figure 8.1.** 16 male SD and WKY (32 total) rats were mated with 32 female SD and WKY rats (64 total) in the combinations mentioned above. Following mating, 13 litters were born in the SD/SD and WKY/SD groups, 14 litters were born in the SD/WKY group and 15 in the WKY/WKY group.

### 8.3. Results

#### 8.3.1. Reproductive ability

Chi square test revealed a difference between the groups in the number of pairings that failed to mate, determined by failure to observe sperm in vaginal smear following overnight housing together ( $X_1^2 = 14.250, p < 0.01$ ). More pairings in the WKY x WKY mated group successfully mated compared to the other groups.

Chi square test revealed there was no difference in the number of females that became pregnant following successful mating ( $X_1^2 = 7.158, p > 0.05$ ).

Group	Mated	%	Pregnant	%
M SD X F SD	14/16	87.5	13/14	92.8
M SD X F WKY	14/16	87.8	14/14	100
M WKY X F SD	14/16	87.5	13/14	92.8
M WKY X F WKY	16/16**	100	15/16	93.7

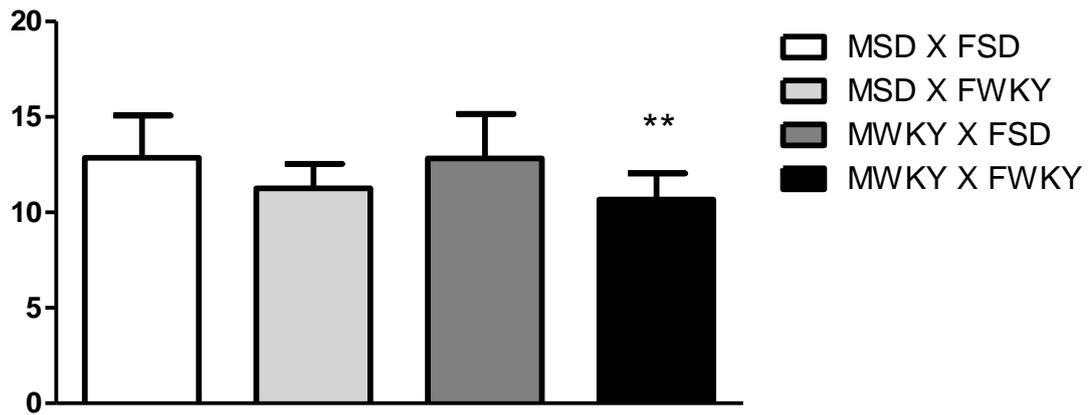
**Table 8.1.** The number of successfully mated pairs and successful pregnancies.

\*\* $p < 0.01$  vs SD X SD group.

#### 8.3.2. Littering Parameters

##### 8.3.2.1. Litter Sizes and Mortalities

A Two-Way ANOVA revealed a significant effect of maternal strain on the number of pups born [ $F_{(1,50)} = 14.013, p < 0.001$ ]. Post-hoc SNK test showed that this difference lay between the WKY/WKY group and the SD/SD group with the WKY group having smaller litter sizes,  $p < 0.01$ .



**Figure 8.2. The effect of parental strain on litter size.** Litters in the WKY/WKY bred group were significantly smaller compared to those in the SD/SD control group. Data presented as mean+ SD. N = 12-16 per group.

There were no effects of parental strain on the number of male pups born. A Two-Way ANOVA revealed an effect of maternal strain on the number of female pups born [ $F_{(1,50)}=4.548$ ,  $p<0.05$ ], however this failed to reach statistical significance in *post-hoc* testing.

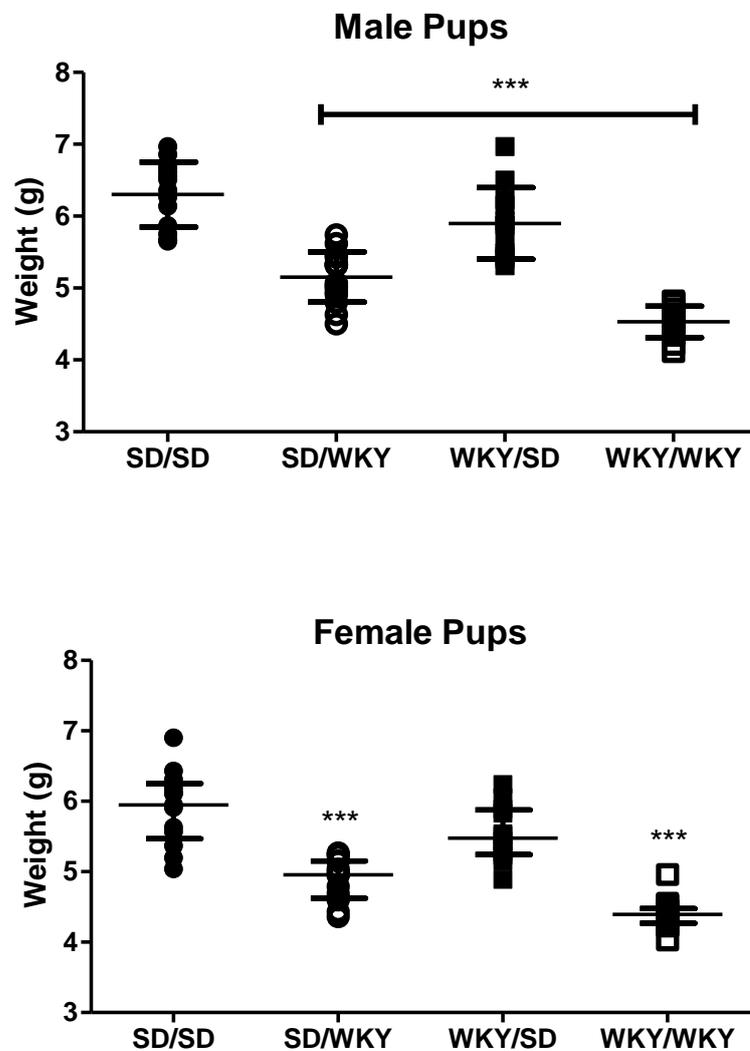
Chi square test revealed a difference between the groups for the number of stillborn pups ( $X_1^2=16.379$ ,  $p<0.001$ ). Following correction of the *p*-value, *post-hoc* tests revealed there were significantly more stillborn pups in the SD/SD group compared to the SD/WKY group.

Chi square test revealed a difference between the groups for the number of pups who died in the first postnatal week ( $X_1^2=15.301$ ,  $p<0.01$ ). Following correction of the *p*-value, *post-hoc* tests revealed significantly more pups died in the WKY/WKY group compared to the WKY/SD group.

No differences between the groups were found in the number of pups eaten during the first postnatal week.

### 8.3.2.2. Birth Weights

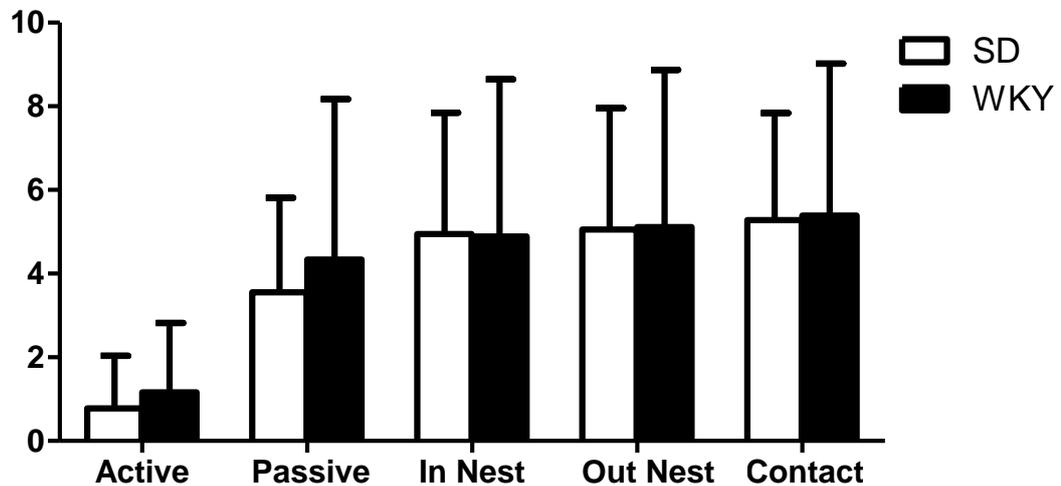
A Two- Way ANOVA revealed a significant effect of both maternal and paternal strain on the birthweight of male pups [ $F_{(1,51)}=23.815, p<0.001$ ] and [ $F_{(1,51)}=143.776, p<0.001$ ] respectively. *Post-hoc* analysis revealed that male pups in all groups with either WKY mother or father or both had a significantly lower birth weight compared to male pups in the SD/SD group. Data for the birth weights of female pups was analysed with Kruskal-Wallis test which revealed a significant difference between the groups [ $K=41.427, p<0.001$ ]. *Post-hoc* testing revealed that female pups born in the maternal WKY groups had a significantly lower birth weight compared to those born in the SD/SD group.



**Figure 8.3. The effect of parental strain on birth weight.** Data presented as median± interquartile range. N = 12-16 per group.

### 8.3.3. Maternal Behaviour

No differences between WKY and control mothers were found in a range of behaviours including active and passive nursing and pup contact during the observation test.



**Figure 8.4. The effect of strain on maternal behaviour.** No differences were found between SD and WKY dams in the number of times they performed a range of behaviours including active and passive nursing, pup contact as well as the number of times they were in and out of the nest. N= 9 per group. Data presented as mean+ SD.

### 8.3.4. Offspring parameters

#### 8.3.4.1. Eye opening

On PND 16, Chi Square test revealed a difference between the male groups ( $X_1^2= 17.383$ ,  $p<0.01$ ) and female groups ( $X_1^2= 16.161$ ,  $p<0.01$ ). *Post-hoc* test revealed that less male and female pups in the maternal WKY groups had open eyes compared to the male and female SD control groups.

On PND 17, Chi Square test revealed a difference between the male groups ( $X_1^2= 18.076$ ,  $p<0.001$ ) and the female groups ( $X_1^2= 24.947$ ,  $p<0.01$ ). *Post-hoc* test revealed that less male and female pups in the maternal WKY groups had open eyes compared to the SD control groups.

#### 8.3.4.2. Fur appearance

There were no differences between the groups in the number of pups with fur on PND 3 ( $X_1^2= 12.814, p>0.05$ ).

#### 8.3.4.3. Pinna unfolding

On PND 3, Chi Square test revealed a difference between the male pups ( $X_1^2= 29.670, p<0.001$ ) and the female pups ( $X_1^2= 30.726, p<0.001$ ). *Post-hoc* tests showed that the male and female pups in the groups with a WKY mother were less likely to have both pinnae unfolded compared to male and female SD groups.

On PND 4, again a difference was found between the male pups ( $X_1^2= 28.670, p<0.001$ ) and the female pups ( $X_1^2= 32.249, p<0.001$ ). Significantly fewer male and female pups with a WKY mother had both pinna unfolded.

On PND 5, Chi Square test revealed a significant difference between the male ( $X_1^2= 19.998, p<0.001$ ), and female pups ( $X_1^2= 19.998, p<0.001$ ). *Post- hoc* testing revealed that less pups in the WKY/WKY group had both pinna unfolded compared to pups in the sham/sham group.

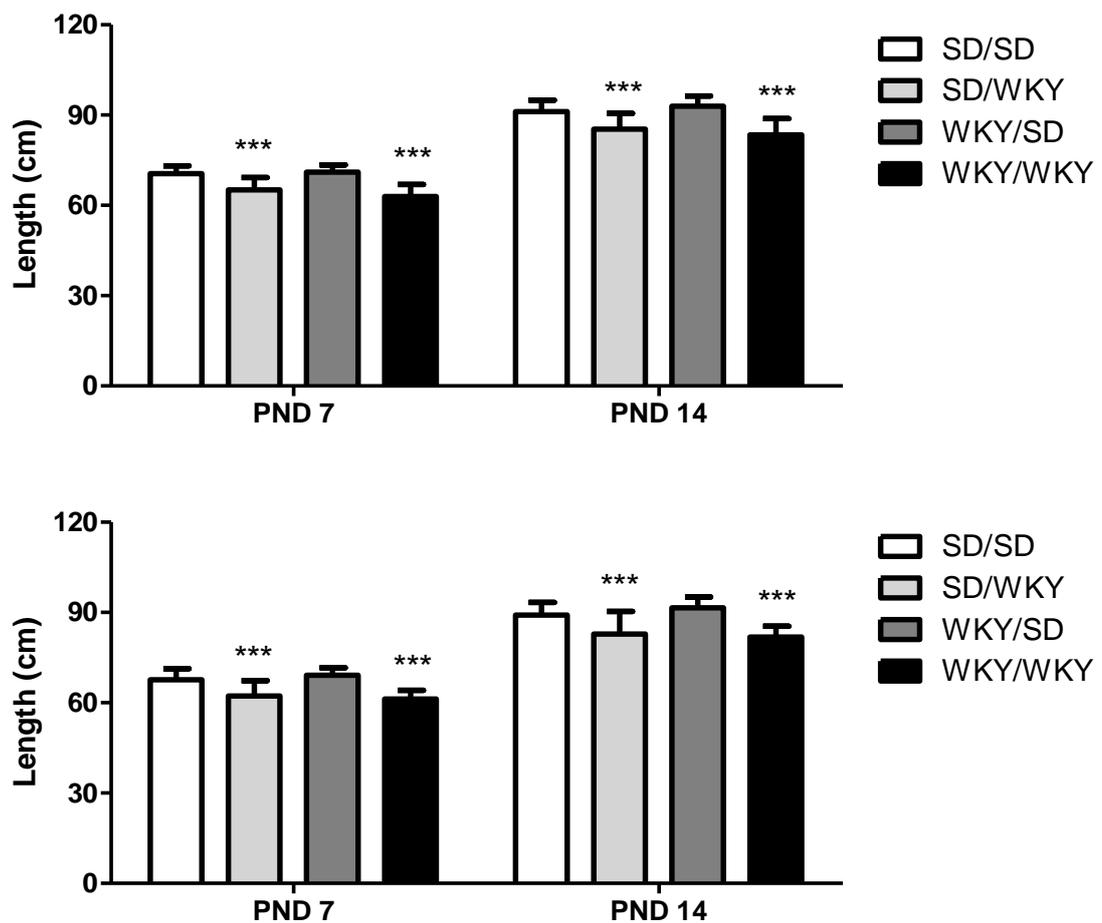
Group	Eye Opening		Fur Appearance	Pinna Unfolding		
	PND16	PND17	PND 3	PND 3	PND 4	PND 5
<b>Male Pups</b>						
<b>M SD X F SD</b>	7/14	11/14	14/14	10/14	13/14	15/14
<b>M SD X F WKY</b>	0/16**	2/16**	10/16	0/16***	2/16***	11/16
<b>M WKY X F SD</b>	5/12	8/12	11/12	1/12**	7/12	13/12
<b>M WKY X F WKY</b>	0/13**	3/13**	10/13	0/13***	0/13***	5/13***
<b>Female Pups</b>						
<b>M SD X F SD</b>	5/14	12/14	13/14	11/15	14/15	15/15
<b>M SD X F WKY</b>	0/16**	3/16**	10/16	0/16***	3/16***	11/16
<b>M WKY X F SD</b>	6/12	10/12	11/12	2/13**	9/13	13/13
<b>M WKY X F WKY</b>	0/13**	2/13**	8/13	0/13***	0/13***	5/13***

**Table 8.2. Effect of parental strain on pinna unfolding.** Maternal strain affected pinna unfolding in that pups born to WKY mothers were slower to reach this stage of development. N=12-16 per group. \*\* $p < 0.01$  vs SD/SD group; \*\*\* $p < 0.001$  vs SD/SD group.

#### 8.3.4.4. Body length

On PND 7, a Two-Way ANOVA revealed a significant effect of maternal strain on the body length of pups [ $F_{(1,102)}=93.718$ ,  $p<0.001$ ]. *Post-hoc* analysis revealed a difference between the male and female pups in the WKY maternal groups compared to the pups in the SD/SD group.

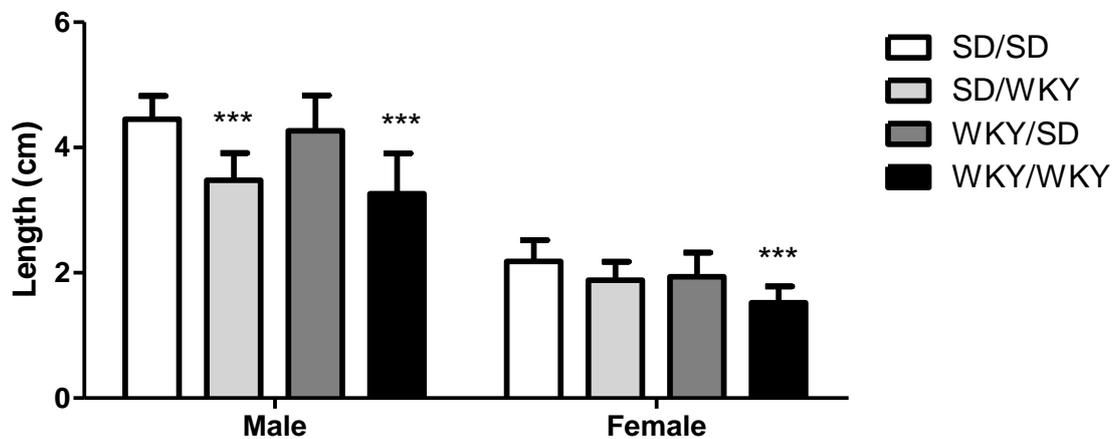
On PND 14, a Two-Way ANOVA revealed a significant effect of maternal strain [ $F_{(1,102)}=70.205$ ,  $p<0.001$ ]. *Post-hoc* analysis revealed that the male and female pups in the groups with a WKY mothers had significantly lower body weights compared to pups born in the SD/SD group.



**Figure 8.5. Effect of parental strain on pup body length.** Pups born in groups with a WKY mother had significantly smaller body lengths compared to pups in the SD/SD group on both PND 7 and PND 14. Data presented as mean+ SD. N= 12-16 per group. \*\*\* $p<0.01$  vs. SD/SD group.

### 8.3.4.5. Ano-genital distance

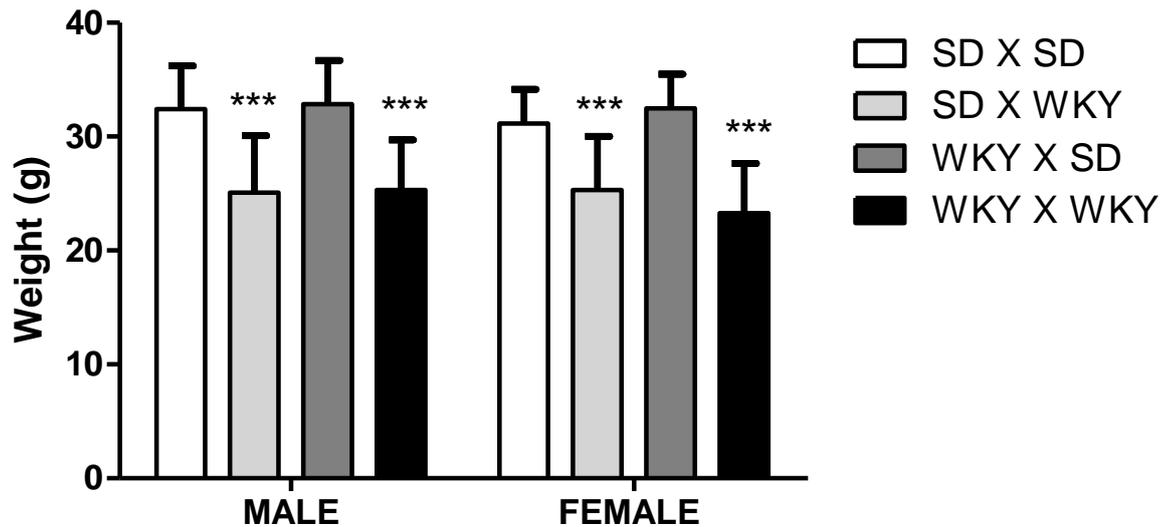
A Three- Way ANOVA revealed a significant effect of paternal strain,  $F_{(1,102)}=5.031$ ,  $p<0.05$ , maternal strain,  $F_{(1,102)}=44.405$ ,  $p<0.001$ , and sex,  $F_{(1,102)}=409.872$ ,  $p<0.001$ . *Post-hoc* analysis revealed that the male pups born in the groups with WKY mothers had significantly smaller ano-genital distances compared to pups in the SD/SD group. It also showed that female pups in the WKY/WKY group had significantly smaller ano-genital distances compared to the female pups in the SD/SD group.



**Figure 8.6. Effect of paternal strain on ano-genital distance.** Maternal strain had a significant effect on ano-genital distance. Male pups born in groups with a WKY mother had significantly smaller ano-genital distance compared to pups in SD/SD group. Female pup sin the WKY/WKY group had a smaller ano-genital distance compared to pups in the SD/SD group. Data presented as mean+ SD. N= 12-16 per group. \*\*\* $p<0.01$  vs. SD/SD group.

### 8.3.4.6. Total body weight gain

A Two- Way ANOVA found a significant effect of maternal strain on the total body weight gained by the pups over the neonatal period [ $F_{(1,102)}=76.097, p<0.001$ ]. *Post-hoc* testing revealed that the male and female pups in the groups with WKY mothers put on significantly less weight compared to the male and female pups in the SD/SD group.



**Figure 8.7. The effect of parental strain on the total body weight gained by pups over the neonatal period.** Male and female pups born to WKY mothers gained significantly less weight over the neonatal period compared to pups in the SD/SD group. Data presented as mean+ SD. N= 12-16 per group. \*\*\* $p<0.01$  vs. SD/SD group.

### 8.3.5. Offspring behaviour test

#### 8.3.5.1. Surface righting

Chi Square test revealed no differences between the groups in the surface righting test on PND 2, 3 or 4. On PND 5, Chi Square test revealed a difference between the male pups ( $X_1^2= 9.257, p<0.05$ ). *Post-hoc* analysis revealed that following p-value adjustment, the only significant difference was between the SD/WKY group and the SD/SD group. Differences were also revealed between the female pups ( $X_1^2= 8.578, p<0.05$ ). *Post-hoc* analysis revealed that following p-value adjustment, the only significant difference was between the WKY/WKY group and the SD/SD group.

Group	PND 2	PND 3	PND 4	PND 5
<b>Male Pups</b>				
<b>SD X SD</b>	4/14	13/14	11/14	14/14
<b>SD X WKY</b>	11/16	10/16	14/16	8/16*
<b>WKY X SD</b>	7/12	8/12	9/12	8/12
<b>WKY X WKY</b>	7/13	10/13	8/13	9/13
<b>Female Pups</b>				
<b>SD X SD</b>	2/14	7/14	7/14	13/14
<b>SD X WKY</b>	8/16	10/16	10/16	10/16
<b>WKY X SD</b>	7/12	8/12	7/12	10/12
<b>WKY X WKY</b>	6/13	7/13	8/13	6/13*

**Table 8.3. The effect of parental strain on the ability of pups to perform surface righting test.** Maternal strain had an effect on pups' ability to perform the surface righting test in 15 seconds or less. Data presented as number of pups successfully performing task out of total pups tested. N= 12-16 per group. \* $p<0.05$  vs. SD/SD group.

**8.3.5.2. Negative geotaxis**

On PND 9, Chi Square test revealed a difference between the male pups ( $X_1^2= 16.959$ ,  $p<0.05$ ). *Post-hoc* analysis revealed that following p-value adjustment, the only significant difference was between the WKY/WKY group and the SD/SD group. There was also a difference found between the female groups ( $X_1^2= 8.300$ ,  $p<0.05$ ), however post-hoc testing did not find any statistical difference between the groups.

On PND 11, Chi Square test revealed a significant difference between the female groups ( $X_1^2= 11.615$ ,  $p<0.001$ ). *Post-hoc* analysis revealed that the pups in the groups with WKY mothers were less likely to be able to perform the test compared to the SD/SD group.

<b>Group</b>	<b>PND 9</b>	<b>PND 11</b>
<b>Male Pups</b>		
<b>SD X SD</b>	10/14	12/14
<b>SD X WKY</b>	6/16	9/16
<b>WKY X SD</b>	8/12	7/12
<b>WKY X WKY</b>	3/13*	8/13
<b>Female Pups</b>		
<b>SD X SD</b>	9/14	11/14
<b>SD X WKY</b>	5/16	5/16*
<b>WKY X SD</b>	9/12	9/12
<b>WKY X WKY</b>	4/13	4/13*

**Table 8.4. The effect of paternal strain on the ability of pups to complete the negative geotaxis test.** Maternal strain effected the ability of the pups to perform the negative geotaxis test in 15 seconds or less. Data presented as number of pups successfully performing task out of total pups tested. N= 12-16 per group. \* $p<0.05$  vs. SD/SD group.

**8.3.5.3. Forelimb grip**

On PND 14 there was no difference between the groups in the ability to perform the forelimb grip test for 20 seconds or more. On PND 17 there was a significant difference between the male groups [ $\chi^2 = 15.804$ ,  $p < 0.01$ ]. *Post-hoc* analysis revealed that the groups with a WKY mother were less likely to be able to perform the test compared to the pups in the SD/SD group. There were no differences found in the female pups.

<b>Group</b>	<b>PND 14</b>	<b>PND 17</b>
<b>Male Pups</b>		
<b>SD X SD</b>	4/14	7/14
<b>SD X WKY</b>	1/16	1/16**
<b>WKY X SD</b>	5/12	6/12
<b>WKY X WKY</b>	0/13	0/13**
<b>Female Pups</b>		
<b>SD X SD</b>	4/14	7/14
<b>SD X WKY</b>	1/16	5/16
<b>WKY X SD</b>	2/12	7/12
<b>WKY X WKY</b>	3/13	2/13

**Table 8.5. The effect of paternal strain on the ability of pups to complete the forelimb grip test.** Maternal strain effected the ability of the pups to perform the forelimb grip test for 20 seconds or more. Data presented as number of pups successfully performing task out of total pups tested. N= 12-16 per group. \* $p < 0.05$  vs. SD/SD group.

#### **8.4. Discussion**

Our results show that while the WKY rat has been validated as a valid model of depression and anxiety, they exhibit no impairments in reproductive parameters and WKY dams show no deficits in maternal behaviour compared to a standard laboratory rat strain. We did observe negative effects of parental strain on the somatic and behavioural development of pups with maternal WKY having the most effects on neonatal parameters, however mating a male SD with a female WKY appeared to negate at least some of these effects.

The first aim of this chapter was to investigate the reproductive ability in the male and female WKY model of depression. We measured reproductive ability by testing for the presence of sperm in a vaginal swab following male and female subjects being housed together for a dark phase of the light/dark cycle. We did not observe a reduction in sexual behaviours in male or female WKY rats, in fact all WKY/WKY pairings successfully mated which was significantly more compared to the SD/SD mated group. We hypothesised that due to the timid nature of the WKY rat that less male WKYs would mate with SD females however this was not the case. There were also no differences in the rate of pregnancies following successful mating.

No evidence of differential litter sizes between WKYs and other strains could be found in the literature however we found that litters in the WKY/WKY mated group were significantly smaller compared to the SD/SD mated group. Mating female WKYs with male SDs appeared to negate this effect on litter size as the number of pups in these litters was not different to controls.

Clinically, preterm birth and low birth weight of neonates born to depressed mothers have been commonly reported (Szegda et al., 2014, Grote et al., 2010a). Our results show an effect of maternal depressive phenotype on the birth weight of both male and female pups,

with pups born to WKY mothers having significantly lower birth weight compared to those born to a standard rat strain. We also found decreased body length, at both 1 and 2 weeks of age, and decreased amount of total body weight gain during the neonatal period in the pups born to WKY mothers suggesting a delay in development.

As mentioned, little work has been carried out investigating differences in neonatal development between pups born to WKY rats and those born to standard outbred rats. Delayed eye opening and impaired balancing ability have previously been reported in WKY neonates compared to SD pups (Ferguson et al., 2003). We found similar results in our study as both male and female pups born to a WKY mother, irrespective of paternal strain, exhibited delayed eye opening compared to the pups born in the SD/SD mated groups. Another delay in somatic development found in our study was the delayed unfolding of pinna in both male and female pups in all groups with WKY mother until PND 5 on which day only male and female pups in the WKY/WKY mated group still exhibited this delay in development. There was also a smaller effect of paternal strain on the rate of pinna unfolding as less male and female pups in the WKY/SD mated groups had both pinna unfolded on PND 3 compared to pups in the SD/SD groups. This effect was not seen in PND 4 or 5. Reports from clinical studies carried out in high income countries have not found any link between maternal depression and measures of growth in offspring (Grote et al., 2010b, Wisner et al., 2013). However an association between maternal depression and stunting of growth rates in children has been reported in the developing country Ghana (Wemakor and Mensah, 2016).

Mother-infant attachment and interaction has been found to be negatively affected by maternal depression. Disturbances in caregiving activities such as feeding and sleep routines are commonly disturbed (Field, 2010), which have been related to poorer language abilities at 26 months (Stein et al., 2008). Most previous work investigating the

maternal behaviour of WKY rats have done so with the use of the WKY strain as a control strain against which to compare the SHR strain as a model of hypertension or ADHD. It was found that WKY dams spend less time nursing and licking their pups compared to SHR dams (Cierpial et al., 1990, Myers et al., 1989). One other study was found which compared WKY maternal behaviour to that of the outbred Wistar strain, which reported that WKY dams spent more time nursing their pups and performed less self-directed behaviours. Such as grooming, compared to Wistar dams. These results are in contrast to those mentioned above which found deficits in WKY maternal behaviour compared to the SHR strain. In our study we compared the maternal behaviour of the WKY strain against the outbred SD which acted as the control. We found no differences in a range of behaviours measured over a period of 1 hour during the light cycle on PND 19. This is a later time point than what is normally studied, and at this age pups are becoming less reliant on the mother which may be why we failed to detect any differences in the behaviour of the dams towards their pups. Analysis of maternal behaviour at an earlier time point should be carried out in order to determine if there are differences in behaviour between WKY and SD dams towards their offspring at a time when the pups are more dependent of the mother for feeding and interaction.

Neonatal behavioural testing involves the use of tests that measure pup co-ordination, balance and strength. In our study we found a general negative effect of maternal WKY strain on the development of pups, however the deficits in the parameters of development found were not as robust as those observed in the measures of somatic development. The surface righting test is a measure of the ability of a pup to right themselves from a supine to prone position and is taken as an evaluation of vestibular efficiency and is a basic motor pattern that pups should display from PND 1 (Jamon, 2014). Our results found a small deficit in the ability of male pups in the SD/WKY group and female pups in the WKY/WKY groups to perform this task on PND 5 only. In a test of grip strength, again

maternal strain had a negative effect on the performance of pups, however only male pups were affected. Our data shows no improvement in grip strength in male pups born in either of the maternal WKY groups.

In the negative geotaxis test, there was a negative effect of maternal strain which differentially affected male and female pups. When tested on PND 9 male pups in the WKY/WKY group performed worse compared to their controls and on PND 11 only the female pups performed worse. This suggests that the maternal depressive phenotype has a more pronounced effect the sensorimotor development in females. As mentioned previously, clinical reports show that maternal depression is linked to impaired cognitive development in the neonatal period. Less than optimal orientation has been previously described in neonates born to depressed mothers (Suri et al., 2014, Davalos et al., 2012), which is similar to our findings of impaired performance in the surface righting and negative geotaxis test. There are also reports of habituation and inferior response to stimuli, hypotonicity, as well as lower scores on cognitive scales (Field, 2011, Marcus et al., 2011).

In conclusion, while we did not find any deficits in maternal behaviour of WKY females, we did observe various developmental delays in pups born to these mothers which are similar to those observed in the clinical scenario. Further investigation of maternal behaviour at an earlier time point may reveal disturbances in behaviours of WKY dams that were not found in this study. Further investigation in this area, perhaps with the inclusion of cross-fostering pups in the study design may help to elucidate the true effects of maternal behaviour on the developmental deficits found.

***Chapter 9:***  
***General Discussion***

## 9.1. Discussion

The work presented in this thesis aims to develop our knowledge of sex differences in distinct behavioural domains in two animal models of depression as well as their response to antidepressant drugs. The OB and WKY models were chosen as they represent two different classes of animal model of depression. There are three main classes of rodent models of depression that are used in preclinical research which are genetic/ selectively bred models, stress models and the pharmacologically or surgically-induced group (O'Leary and Cryan, 2013). The WKY model of depression is a selectively bred model of depression and the OB model is a surgical model that is classed with pharmacological models such as the reserpine model. The two models in question also have a history of use in our laboratory in numerous published and unpublished studies (Burke et al., 2010, Burke et al., 2015, Burke et al., 2016), and so this factor also influenced our choice of models as we were able to draw on the wealth of past experience concerning their utility.

Following the decision of which animal models to use in our experiments we then had to decide on the appropriate control groups. For the OB studies the choice for control group was simple as sham surgery could be performed on the same strain rats, i.e. Sprague-Dawley rats, which would act as the control. For the WKY studies the choice of control group was less straightforward. Many reports published on the WKY rat use the WKY as a control strain for the SHR (Langen and Dost, 2011, McDougall et al., 2000, Gentsch et al., 1987) or compare the WKY with other inbred rat strains such as the Fischer-344 and Flinders Sensitive Line (FSL) rats (Pare, 1993, Malkesman and Weller, 2009), however we wanted to characterise the behavioural phenotype of male and female WKY rats against a commonly used laboratory rat strain whose behavioural phenotype has been extensively studied which led to our use of the Sprague-Dawley strain which has a history of use in our own laboratory.

The three behavioural domains we aimed to evaluate were anxiety- and depressive-like behaviours and cognitive abilities. The decision to study these domains was based on their clinical relevance to depression as well as the ability to conduct tests to measure these behaviours in our laboratory with equipment previously shown to elicit the behaviours in question.

The open field test and elevated plus maze were chosen to measure anxiety related behaviour. These are commonly used tests that have been used previously in male subjects of both models however there is little information regarding the behaviour of female subjects in these tests. The open field test is also used to elicit the hallmark behaviour of the OB model, hyperactivity, and so the open field test was also performed in the OB antidepressant study as it has been previously shown in male subjects that chronic treatment with antidepressant drugs attenuates this behaviour (Leonard, 1984) however there is little evidence to suggest whether females respond similarly to drug treatment. The forced swim test was chosen as a measure of depressive-like behaviour in the characterisation studies and also as a measure of antidepressant response in WKY rats. The Morris water maze was performed on both models in order to measure spatial learning and memory and further tests of cognitive ability were performed on the WKY model only, namely the novel object recognition test and the social interaction test.

Our results in the OB characterisation study indicate that the female OB animals exhibit a similar behavioural phenotype as the males in regards to the hallmark behaviours associated with the OB model but some sex differences were found. We also found that the behaviour of male and female WKY rats was mostly similar in the tasks performed with the main sex differences evident in the tests of cognitive ability.

While we failed to detect hyperactivity in the open field test in male and female OB rats, we did find that OB rats exhibited increased locomotor activity in the elevated plus maze

compared to their sham counterparts. As we tested rats in the elevated plus maze first followed by the open field test, we believe this to be the cause for the lack of open field hyperactivity, i.e. the OB rats were habituated to the stressful environment. This is supported by the finding that over the course of a 10-minute open field test, OB rats are found to be hyperactive in the first 2 minutes but not in the last 2 minutes which suggests that they habituated to the anxiogenic environment (Holubova et al., 2016). We believe that the hyperactivity observed in the elevated plus maze in this study can be equated to the hyperactivity usually exhibited in the open field test as the same behaviour was found but it was just in response to a different but still anxiogenic environment.

Contrary to the hyperactive behaviour expected from the OB model of depression, the WKY model displays a hypoactive, timid behavioural phenotype. This was observed in the open field results with both male and female WKY groups displaying significantly smaller distance moved scores compared to their SD counterparts which is a commonly reported behaviour in the WKY model (Pare, 1994b, Luo et al., 2015, Burke et al., 2016, Tejani-Butt et al., 2003). Both male and female WKY groups again exhibited a hypoactive response in the elevated plus maze, with a smaller distance moved compared to their SD counterparts. We also found that both SD and WKY females moved significantly more compared to their counterpart male group in the elevated plus maze. Sexual dimorphism in rats in regards to locomotor activity has previously been studied with results showing increased locomotor activity in females by 8 weeks of age (Hyde and Jerussi, 1983).

Rats exhibit a thigmotaxic response upon exposure to a novel environment meaning they remain close to surrounding walls however this response competes with rats' innate curiosity and willingness to explore and so it is believed that rats who explore more of their environment are displaying less anxiety-like behaviour. OB rats spent significantly

less time in the centre of the open field arena compared to their sham counterparts, suggesting heightened anxiety. This response is not always observed in OB animals (Burke et al., 2010) as the hyperactivity usually displayed in the open field test means they cross the centre zone of the arena, but it was present in this study and to a similar degree in both males and females.

A sex difference in WKY rats was observed in the time spent in the centre of the open field with the male WKYs exhibiting increased time in the centre zone compared to female WKYs. We believe this is due to freezing behaviour exhibited by male WKYs upon exposure to an anxiogenic environment. This behaviour has been previously reported in WKY males in fear conditioning tests (DaSilva et al., 2011) and in the conditioned defensive burying test (Pare, 1992).

The first sexually dimorphic result we discovered in OB rats was that only male OB rats have an increase in open arm entries in the elevated plus maze compared to their sham counterparts. Increased open arm entries has been previously observed in male OB animals and is regarded as one of the principal behavioural changes that follows OB surgery (Song et al., 1996). There are contradictory reports as to whether female OB rats exhibit this behaviour with previous findings showing female OB rats behaving similar to male OBs in the elevated plus maze by displaying increased open arm entries and duration (Stock et al., 2001) and also findings supporting the results presented in this thesis which show no difference between female OB and sham animals (Stepanichev et al., 2016). This variability in reports may be caused by differences in testing protocol which highlights the need for authors to report all relevant information regarding testing protocol, e.g. lux settings, which phase of the light/dark cycle testing was carried out etc., in order to improve reproducibility of findings.

Both male and female WKYs entered the open arms of the elevated plus maze less compared to their control groups. This is indicative of the anxiety-like behaviours displayed by this model. Previous work examining the behaviour of WKY rats in the elevated plus maze have used different control strains, such as the Wistar or SHR strains, and so comparisons to our results are difficult to make. One study that did use male and female WKY and SD rats did not analyse the results by strain and sex separately (Ferguson and Gray, 2005), again making it hard to compare their findings to the results presented here.

To summarise our findings regarding anxiety-like behaviours, we found that female OB rats displayed more anxiety-like behaviour compared to males whereas in the WKY model it seems that male rats display increased levels of anxiety in the form of freezing behaviour. As mentioned, the female OB rats did not exhibit the increase in arm entries in the elevated plus maze which has been described as a hallmark of OB behaviour in male rats. This may suggest that the OB syndrome involves a more anxiety-like phenotype in female rats compared to males. Male and female WKY rats also exhibit a differential responses to stress with males more likely to exhibit freezing behaviour upon entering a novel environment. This may suggest that female WKY rats are not as susceptible to short-term stressors as males.

We failed to detect “depressive-like” behaviour (as evidenced by increased immobility time in the forced swim test) in either model in our studies. Increased immobility time has been reported in male OB rats previously (Yang et al., 2014, Jindal et al., 2015a), however it was not observed in females (Stepanichev et al., 2016) and is not the main behaviour typically studied in the OB model. There is debate as to the relevance of using immobility time in the forced swim test as a marker for depression in rodents. Factors related to depression such as early life stress as well as stress during adulthood, have been

shown to increase immobility in the forced swim test (Veenema et al., 2006, Johnson et al., 2006) and as a result, increased immobility time in the forced swim test is now regularly classed as a 'depressive-like' behaviour, which some consider to be an over simplification (Commons et al., 2017). The description of immobility in the FST as a move from an active to a passive coping strategy is thought to be a more appropriate definition of the behaviour. Despite this debate as to the applicability of forced swim test results to depression research, it is estimated that one paper is published per day that involves the use of the forced swim test (Molendijk and de Kloet, 2015).

Increased immobility in the male WKY model is a common finding with less robust findings in female WKYs. The reason for the lack of this depressive-like behaviour in our findings is thought to be due to differences in behavioural phenotypes between sub-strains of the WKY rat (Browne et al., 2015). Browne (2015) found that the WKY/NHsd rats obtained from Harlan Laboratories failed to exhibit increased levels of immobility compared to the rats obtained from Charles River Laboratories (WKY/NCrl). One reason for this difference in behaviour of WKY rats from different suppliers is thought to be caused by the distribution of breeding animals to vendor companies before the strain was genetically stable (Pare and Kluczynski, 1997). This has resulted in behavioural and genetic variability between supplier strains with three times greater variability reported to be displayed by the WKY/NHsd sub-strain compared to other inbred and outbred strains of rat (Will et al., 2003). As well as this it has been found that both behavioural and genetic variability exist within the same supplier populations (Kurtz et al., 1989). Another possible explanation for the variability in the WKY phenotype across suppliers could be due to mutations which could have accumulated over the course of the ~70 generations that have been bred in the supplier companies (Will et al., 2003). Despite the knowledge of behavioural and genetic differences, WKY rats from different suppliers are used equally in depression research with an equal number of studies published between

2009-2013 using Harlan or Charles River WKY rats (n=17 studies each) (Zhang-James et al., 2013).

Impaired cognitive abilities including diminished ability to think or concentrate are one of the main symptoms of depression and so it is important that we are able to investigate the underlying mechanisms causing this impairment. Animal models are one method by which we can attempt to elucidate the neural circuitry involved in this aspect of the depressive disorder and they can also be used to examine whether antidepressant drugs have an affect alleviating this symptom. OB surgery is associated with deficits in cognitive abilities in male rats, particularly in spatial learning and memory, however less is known about the effects of OB surgery on females in regard to this aspect of depression symptomology. Our findings support previous research in that male OB rats exhibit deficits in a spatial learning and memory, as we observed in the MWM. Only one previous study examining spatial working memory in female OB rats could be found and they reported impaired short-term memory in the Y-maze task but they also found no deficits in long-term declarative memory (Stepanichev et al., 2016).

Similar to the OB study, we observed more cognitive impairments in the male WKY rats compared to the female rats in the various tests of learning and memory employed over the course of these studies. Previous reports of the spatial learning and memory abilities of male WKY rats is conflicting with deficits found between WKY and SD rats in a spatial maze task (Wyss et al., 2000) whereas others found no difference in the MWM between WKYs and SDs (Ferguson and Cada, 2004) or between WKY and Wistar rats (Kyeremanteng et al., 2014). Our findings support a previous report of no deficits in spatial learning and memory in female WKY rats compared to their SD controls (Ferguson and Cada, 2004).

In regards to non-spatial learning and memory, we found no deficits in object or social recognition in the WKY rats over the course of experimentation. There has been little previous work carried out comparing the cognitive ability of the WKY rat to a SD control in non-spatial tasks. In female rats it has been reported that there are no differences between WKYs and SDs in long term memory as no difference were found between them for preference of a novel object in the NOR test (Zhang-James et al., 2014).

Overall it appears that male models of depression, at least the two models examined in this thesis, are more likely to exhibit deficits in cognitive ability compared to females. This decreases the potential usefulness of females in modelling this aspect of the depressive phenotype.

Below is a summary table listing the sexually dimorphic behaviours in both models compared to their respective control groups.

	<b>OB</b>	<b>WKY</b>
<b>OFT</b>	---	♂ ↑ IZD
<b>EPM</b>	♂ ↑ OAE; ♀ ↑ CAE	♀ ↑ DM; ♂ ↑ IZD
<b>FST</b>	---	♀ ↑ Climbing
<b>MWM</b>	♂ ↑ DM; ♂ ↓ Time in target zone	♂ ↑ Time to find platform; ♂ ↑ latency to enter target zone
<b>NOR</b>		♂ ↓ NOR
<b>SIT</b>		♂ ↓ Sociability

**Table 9.1.** Summary table comparing sex differences found in the OB and WKY models. OB- Olfactory bulbectomy, WKY- Wistar Kyoto, OFT- open field test- EPM- elevated plus maze, FST- forced swim test, MWM- Morris water maze, NOR- Novel object recognition, SIT- social interaction test, IZD- inner zone duration, OAE- open arm entries, CAE- closed arm entries, DM- distance moved, ♂- male, ♀- female.

Our aim for the antidepressant dosing studies was to examine the response of male and female rats to distinct classes of antidepressant drugs. We opted to use an SSRI, fluoxetine and a TCA, desipramine, as these are two commonly prescribed classes of antidepressants and they are also subject to discussion regarding sex differences in treatment outcome in the clinical situation. The dose and route of administration of drugs in the OB study was chosen based on previous unpublished research from our laboratory. Both fluoxetine and desipramine were dissolved in distilled water and administered at a dose of 10mg/kg via subcutaneous injection. As previous experiments in our laboratory showed that chronic administration of TCAs by subcutaneous injection leads to irritation of the skin, we increased the dilution volume of the drugs in the hope that the less concentrated dose would lead to less irritation. However this was not the case and upon recommendation from the designated veterinarian we chose to administer the drugs by oral gavage for the WKY study.

In these studies we repeated one or two selected tests from the characterisation study in order to determine the effects of antidepressant drugs on these behaviours as well as to attempt to reproduce the findings from the characterisation study. As mentioned briefly in a previous chapter, there is a perceived crisis regarding the lack of reproducible findings, particularly in preclinical behavioural research (Baker, 2016). As basic and preclinical research provide the foundation on which clinical research is conducted, a lack of reproducible findings are thought to contribute to the high clinical failure rate and associated high costs in pharmaceutical research design (Peers et al., 2012). As biological measures are inherently prone to variability, especially behavioural measures, it is accepted that every detail in experiments will not be exactly replicated but the main results and conclusions should agree. The NIH is attempting to tackle this problem by promoting the publishing of all relevant information needed to replicate the experiment as well as organising training modules on experimental design which will include

information on how to properly control experiments and limit biases that may occur (Collins and Tabak, 2014). While many researchers avoid replication of studies for fear of obtaining differing results, we purposefully repeated a select number of tests in order to determine if the behaviours described in females are replicable and robust.

OB males have been repeatedly shown to exhibit good predictive validity in that treatment with antidepressant drugs reverses the hyperactivity associated with the OB phenotype (Breuer et al., 2007, Jarosik et al., 2007, Pandey et al., 2014). This responsiveness to antidepressant drugs is not observed following acute treatment but only chronic treatment (Wang et al., 2012) which is an advantage of the OB model as it accurately replicates the clinical scenario. In this study we were unable to definitively report antidepressant activity with either desipramine or fluoxetine in male or female OB rats. Although the drug treated OB groups displayed locomotor activity levels similar to their sham control groups following 21 days of treatment, the vehicle treated OB groups also exhibited similar scores for distance moved as their sham control group, meaning we are unable to report that the normalisation of behaviour in the open field is due to antidepressant activity. The reason for the normalisation of behaviour in the vehicle treated OB groups is unclear but one possibility is that the rats became habituated to the open field arena as this was now their second exposure to the test. Previous studies from our laboratory have also reported habituation and cessation of hyperactive behaviour following multiple exposures to the open field arena which has also been reported in the literature (Gigliucci et al., 2014).

In our OB characterisation study, we reported that male and female OB rats displayed hyperactivity in the elevated plus maze but then failed to display this behaviour in the open field test which was performed directly afterwards. In the dosing study we report similar results in that we found hyperactivity in both male and female OB rats which was

then no longer present upon re-testing. These two studies shed light on what may be considered a disadvantage of the OB model in that the behavioural phenotype becomes attenuated following repeated behavioural testing.

The WKY model of depression has been previously reported to be unresponsive to both acute and chronic treatment with SSRI drugs (Griebel et al., 1999, Willner and Belzung, 2015) whereas treatment with TCA drugs was found to exert antidepressant effects even at doses that had no effects in other strains (Lopez-Rubalcava and Lucki, 2000). Our findings are in agreement with the literature as neither male nor female WKY rats exhibited an antidepressant response to treatment with an SSRI drug. This finding may suggest that the depressive-like phenotype of the WKY model is not caused by depleted levels of serotonin or dysregulation of the serotonergic system but other monoamine systems may be involved as TCA treatment does result in an antidepressant response in male WKYs as described below.

A differential response to treatment with desipramine was found between male and female WKY rats. Male WKY rats exhibited an antidepressant response in the FST, i.e. reduced immobility time compared to the vehicle treated WKY group, whereas the female WKYs treated with desipramine failed to exhibit any differences in behaviour in the test. To the best of our knowledge this is the first time that a sexually dimorphic response to chronic desipramine has been reported in WKY rats. This finding is important as it agrees with clinical findings that there is a differential treatment outcome in males and females in response to treatment with TCA drugs (Kornstein et al., 2000). This suggests that female WKY rats could be used as a model of treatment resistant depression and may be valuable in the search for antidepressant medications that do not rely on manipulation of neurotransmitter systems.

The novel object recognition test was performed following 14 days of drug dosing in order to determine if antidepressant treatment exerted any effects on the cognitive ability of the WKY model. Both the male and female WKY vehicle treated groups interacted more with the novel object compared to the familiar object which would indicate that this model does not exhibit deficits in object recognition memory, as also concluded in the WKY characterisation study. The desipramine-treated WKY groups spent an equal amount of time with the novel and familiar objects which may suggest an impairment of recall ability in this group; however we also found that desipramine decreased the distance moved in all groups compared to their vehicle control groups and so the effect of desipramine we see on time spent interacting with the objects may solely be due to the effects on the locomotor activity of the animals. As there were no differences in the discrimination ratios between the groups, we must conclude that treatment with desipramine or fluoxetine had no effect on short-term memory in WKY rats.

The results for the vehicle treated groups in both the forced swim test and novel object recognition test are comparable to those from the characterisation study in which we reported no differences in immobility time and that there are no apparent deficits in non-spatial memory in WKY males and females compared to the SD control strain. This suggests that these are robust findings that can be replicated with repeated testing.

We hypothesised that BDNF mRNA and possibly its receptor gene NTRK2 mRNA could be used as biomarkers for antidepressant response and so to investigate this we analysed the levels of BDNF and NTRK2 mRNA in the hippocampus and frontal cortex of the rats that had undergone chronic antidepressant/vehicle treatment. We failed to observe any effects of drug treatment on mRNA levels of either BDNF or NTRK2 in the OB and WKY rats. The identification of a biomarker that could accurately predict whether a patient will respond to a particular treatment either before commencement of the

treatment or in the early stages of the trial period would have a significant impact in alleviating the problems associated with depression treatment. BDNF is of interest in both clinical and preclinical biomarker research as it has been previously shown to be increased following antidepressant treatment in both humans and rats. Decreased protein levels of BDNF in the brain of OB and WKY rats has been previously reported (Jindal et al., 2015b, Vinod et al., 2012) however our findings have failed to replicate this.

As part of this project we examined the reproductive abilities of these models as well as the neonatal development of offspring. Rodent models of postpartum depression are utilised to investigate the effect of depression in the early neonatal period in both offspring and dams (Aguggia et al., 2013, Fernandez et al., 2014). As the OB and WKY rats are recognised as models of depression, we wanted to explore the possibility that female OB and WKY rats exhibited maternal deficits that could be measured by delays or impairments in neonatal development.

Reduced sexual activity has previously been reported in male OB rats (Edwards et al., 1990, Lumia et al., 1987) which has been suggested to be caused by a reduction in androgen receptor binding in the amygdala following OB surgery (Lumia et al., 1992). Opposite effects have been reported in female OB rats as increased levels of sexual proceptivity, measured as ear wiggling and darting, and sexual receptivity, exhibited by lordosis behaviour, have been reported following OB surgery (Lumia et al., 1981). It is thought that OB increases female sexual behaviour by increasing neural sensitivity to oestrogen (Williams et al., 1991, Williams et al., 1992). In our study we measured sexual activity by looking for the presence of sperm in vaginal smear samples following coupled housing. We found no differences in the number of smears that tested positive for the presence of sperm, suggesting there were no differences in sexual activity between OB and sham rats. An interesting effect of OB surgery we did find was that a smaller

percentage of female OB rats who tested positive for sperm went on to actually give birth. The reason for this is unknown and was beyond the scope of our investigation but may be a result of altered neural sensitivity to changes in levels of ovarian hormones which occur in pregnancy. As for the WKY rats, we found that the WKY groups were more likely to mate compared to the SD groups and that there were no differences in pregnancy rates following successful mating.

As the OB dams had smaller litter sizes in our study we believe this may have masked potential impairments in the development of the pups. It has been shown that pups from smaller litters exhibit more rapid somatic and cognitive development due to easier access to milk (Romero et al., 1992, Carvalho et al., 2016) and so this effect may have obscured negative effects of OB maternal care in this study. In the WKY experiment, again we saw smaller litter sizes in the ‘depressive-like’ dam strain. As well as this we found that more pups in the WKY/WKY mated group died in the neonatal period compared to the number who dies in the WKY/SD mated group, which would suggest a deficit in maternal care in this group, however when maternal behaviour was analysed on PND 19 we found no differences between the WKY and SD dams. Pups born to WKY dams exhibited a number of developmental delays compared to their SD controls including delayed eye opening, pinna unfolding and development of strength and reflexes. These results suggests that the WKY model may represent a model of detrimental maternal care however more investigation into this will have to be carried out.

Throughout the course of this project we learned about the Research Domain Criteria (RDoC) and how this approach could be used to expedite the development of new pharmacological approaches for depression treatment. The RDoC is a framework set up by the NIMH for the investigation of mental disorders whose ultimate goal is to “Develop, for research purposes, new ways of classifying mental disorders”. The framework aims

to support the move away from the use of classical models of disease which aim to reproduce the symptoms that are described in DSM-5, and instead to focus research on defined measures of neurobiology and behaviour that are present across multiple categories of mental disorders. This means that RDoC inspired animal models will attempt to recreate a single clinical symptom and focus on elucidating the underlying biology relating to that symptom or behaviour e.g. cognitive impairments or anxiety related behaviours. The information obtained from these types of studies can then be used as a foundation on which a new system of mental disease classification and diagnosis can be built as well as revealing new approaches for treatment.

The results in this thesis have highlighted the difficulty of modelling a multifaceted rodent model of depression in males and females; while the OB rats did exhibit their characteristic hyperactive response, the relevance of this behaviour to clinical depression has been questioned and females failed to display other behaviours synonymous with this model. We failed to demonstrate the “depressive-like” behaviour that is characteristic of the WKY model, i.e. immobility in the forced swim test and again females only displayed anxiety-like behaviour and no other aspects that have been previously described in males. These results lend credence to the method of preclinical behavioural research set out by the RDoC which would involve one aspect of the depressive phenotype being modelled accurately in both male and female rodents which can then be thoroughly investigated in regards to pathophysiology. This will also mean that research would not be limited to one mental disorder as a number of disorders share some common symptoms such as cognitive deficits and anxiety.

## 9.2. Conclusions

The work described in this thesis was carried out with the main aim of characterising sex differences in behaviour and antidepressant responsivity in two commonly used rat models of depression. In terms of the characterisation of behaviours in the models we found that female OB rats displayed increased anxiety-like behaviour as they failed to exhibit increased open arm entries in the EPM which is commonly found in male OB rats. The opposite was true for the WKY rats with the males exhibiting an increased freezing response upon introduction to the anxiogenic open field and elevated plus maze arenas. Sex differences in cognitive deficits were also found in the models with both the male OB and WKY rats exhibiting impairments in spatial learning and memory.

We were unable to detect antidepressant activity of drugs in the OB model however possibly the most interesting results came from the WKY rats which exhibited a sexually dimorphic response to chronic TCA treatment. Male WKY rats treated with desipramine displayed decreased immobility time in the forced swim test whereas females showed no responses to treatment.

From our results we can see that female OB rats exhibit the main behavioural characteristic of the OB syndrome, hyperactivity in an anxiogenic situation, however we failed to observe other behavioural aspects that have previously been reported in male OB rats including altered behaviour in the EPM and cognitive deficits. Similar observations were found in the WKY females as they did exhibit the hypoactive, anxious phenotype associated with this model but failed to exhibit cognitive deficits or a depressive-like phenotype. To conclude, these findings highlight the need for the evaluation of sex differences in rodent models of psychiatric disease as sex-related differences could direct towards new areas of research into novel therapeutic approaches.

### 9.3. Limitations and Future Work

- As mentioned previously, there are three classes of rodent models of depression; genetic/selectively bred models, stress models and those that fall in the "other category". At the start of this project our aim was to investigate sex differences in one model from each class, however it became clear that time constraints would limit the number of models we could investigate from three to two. As stress is believed to affect males and females differently, a future experiment could be to investigate if male and female stress-induced models of depression exhibit sexually dimorphic behavioural characteristics or response to antidepressant treatment.
- Upon careful consideration of the studies incorporated within this thesis, several limitations and possibilities for future investigation can be identified. In the OB characterisation study the OFT should have been performed first as hyperactivity in this test is the main behavioural alteration observed in OB rats. While it has been previously shown in our lab that testing in the elevated plus maze first followed by the OFT does not affect the behaviour of naïve rats in either test, our results in chapter 3 show that the behaviour of OB rats in the OFT was affected by prior testing in the EPM as seen by a lack of hyperactivity in the OFT. Future studies should carefully consider the order in which behavioural tests are carried out in OB animals to prevent this unwanted effect.
- As there is debate as to the relevance of immobility behaviour in the forced swim test in modelling "depressive-like" behaviour in rats, another measure relating to the depressive phenotype of models should be performed in future studies such as the sucrose preference test which is commonly used for measuring anhedonia in rats. As well as this, other tests of anxiety-like behaviour and cognitive ability that do not rely on a locomotor component, such as the novelty-induced

hypophagia, could be used as the WKY model exhibits reduced movement which could have confounding effects on tests that contain a locomotor aspect.

- A limitation of the investigation into the maternal care in the OB study was the small litter sizes in the OB groups which may have obscured effects of OB surgery on maternal behaviour. A method of preventing this in future work would be to cross-foster the pups in all of the groups and ensure that an equal number of pups were then given to each dam. In the WKY study our observation of maternal behaviour should have been carried out at an earlier time point in order to examine dams' behaviour towards her pups at a time when they are more reliant on her. Tests such as the pup retrieval test could also have been carried out to get a better understanding of whether deficits in maternal care are present. Also further testing of the offspring could be carried out in adolescence and adulthood in order to determine if having a mother with depressive-like features affects the long-term well-being of the pups.

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