Investigation of the central serotonergic system in the olfactory bulbectomized rat model of depression

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Contents

Abstract

Depression is a devastating heterogeneous disease that has a high lifetime prevalence. Despite over 50 years of research, the aetiology of depression is still largely undetermined but the improper functioning of the central serotonergic system is believed to play a considerable role. The olfactory bulbectomized (OB) rat model of depression is well validated, exhibiting behavioural, physiological and neurochemical alterations that are reflective of the clinical condition; open field hyperactivity is the primary behavioural hallmark, which is attenuated by chronic and not acute antidepressant treatment, reflective of the clinical condition. To date there is an absence of a comprehensive evaluation of the central serotonergic system in this model, encompassing multiple parameters of central serotonergic transmission as well as an assessment of its functionality. The work presented aims to address these limitations, assessing a time course of changes and effect of chronic antidepressant treatment (fluoxetine, venlafaxine and imipramine) on the following parameters: tryptophan hydroxylase expression (DRN, striatum, substantia nigra and amygdala), 5-HT_{1A} and 5-HT_{2A} functionality (via an acute agonist challenge of 8-OH-DPAT and DOI respectively) and mRNA expression (prefrontal cortex, hippocampus and amygdala) measurement of 5-HT, 5-HIAA and 5-HIAA/5-HT (amygdala) and finally expression of SERT (DRN, prefrontal cortex, hippocampus and amygdala). Initially a time course characterisation of the serotonergic system was carried out, determining that neither lesion nor time following lesion had an impact on the serotonergic parameters assessed. Functionality of the 5-HT_{1A} and 5-HT_{2A} revealed an enhanced dose dependent increase in locomotor activity in OB rats, with altered OB responsiveness to components of the panel of behaviours. Assessment of chronic fluoxetine, venlafaxine or imipramine treatment resulted in limited effects on the serotonergic parameters investigated. Functionality of the receptors, exhibited by increased locomotor activity, was unaffected by antidepressant treatment, whilst imipramine increased rearing in assessment of 8-OH-DPAT in stereotyped behaviours, whilst fluoxetine reduced flat body posture and increased forepaw treading but had no effect on DOI-induced stereotyped behaviours. Fluoxetine increased PFC 5-HT_{1A} mRNA in sham-lesion rats, whilst also reducing 5-HIAA levels in the amygdala regardless of lesion type and further potentiating the reduction in OB rats. Venlafaxine increased 5-HIAA/5-HT ratio in sham lesioned rats. Alteration of behaviour in the open field was not observed with the OB model due habituation occurring upon re-exposure. Nocturnal homecage hyperactivity in the OB rat was evident from 1 week following lesion and remained significantly elevated for 2 weeks before waning thereafter. However this raises the possibility of a window of opportunity for detecting antidepressant responses. In conclusion, this work demonstrates that although limited central serotonergic structural changes were found, there is evidence of altered functionality, an area worthy of further investigation to help complete the picture.

Author's Declaration

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

Chapter 3 Time course investigation of central serotonergic parameters in the OB rat

- Assisted by Prof. John Kelly or Dr. Nikita Burke, Aoife Thornton, Katherine Obudzinski
- HPLC was carried out by Eoin O'Neill and Prof. Andrew Harkin, Trinity College, Dublin

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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:_____

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List of Figures

List of Tables

List of abbreviations

5-HT	5-Hydroxytryptamine (Serotonin)
8-OH-DPAT	8-Hyroxy-2-(dipropylamino)tetraline hydrobromide
ACTH	Adrenocorticotropic hormone
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BDI	Beck Depression Inventory
BDNF	Brain derived neurotrophic factor
CLI	Clomipramine
CMS	Chronic mild stress
CNS	Central nervous system
CRF	Corticotrophin releasing factor
CRH	Corticotrophin releasing hormone
CSF	Cerebrospinal Fluid
Ct	Copy threshold
CUMS	Chronic unpredictable mild stress
DA	Dopamine required
DAG	diacylglycerol
DALYs	Disability-adjusted life years
DAT	Dopamine transport
5,7-DHT	5,7-dihydroxytryptamine
DOI	1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane
DRD	Dorsal DRN
DRC	Cauadal DRN
DRI	Interfasicular DRN
DRN	Dorsal Raphe Nucleus
DRV	Ventral DRN
DRVL	Ventro-lateral DRN
DSM	Diagnostic and Statistical Manual of Mental Disorders
ELISA	Enzyme-linked immunosorbent assay
FDA	Food and Drug Administration
Flx	Fluoxetine
fRMI	Functional Magnetic resonance imagining
FST	Forced swim test

GABA	Gamma-amino butyric acid
GPCR	G-Protein coupled receptor
GR	Glucocorticoid
GD	Gestational day
HAM-D	Hamilton rating scale for depression
HCA	Homecage activity
HPA	Hypothalamic-pituitary-adrenal axis
HPLC	High Performance Liquid Chromatography
i.p.	intraperitoneal
ICD	International Classification of Diseases
IHC	Immunohistochemistry
Imi	Imipramine
IL	Interleukin
LPS	Lipopolysaccharide
MAO	Monoamine oxidase
MAOI	Monamine oxidase inhibitor
MADRS	Montgomery-Asberg Depression Rating Scale
MASSA	Melatonin agonist and specific serotonin antagonist
MDD	Major depressive disorder
MR	Mineralocorticoid
MRI	Magnetic resonance imagining
MRN	Median raphe nucleus
NA	Noradrenaline
NaSSA	Noradrenergic and specific serotonergic antidepressant
NDRI	Noradrenaline and dopamine reuptake inhibitor
NET	Noradrenaline reuptake transporter
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NSAID	Non steroidal anti-inflammatory drug
OB	Olfactory Bulbectomy
OF	Open field
PCK	Protein kinsase C
PET	Positron Emission Tomography

PFC	Prefrontal cortex
PND	Postnatal day
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
RNA	Ribonucleic acid
s.c.	subcutaneous
SDRI	Selective dopamine reuptake inhibitor
SERT	Serotonin transporter
SNRI	Selective noradrenaline reuptake inhibitor
SSRI	Selective serotonin reuptake inhibitor
TCA	Tricyclic antidepressant
TNF	Tumour necrosis factor
TPH	Tryptophan hydroxylase
TrkB	Tyrosine receptor kinase B
Veh	Vehicle
Ven	Venlafaxine
VMAT 2	Vesicular monoamine transporter
WHO	World Health Organisation
WKY	Wistar-Kyoto

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Chapter 1:

General Introduction

Depression is a common and devastating neuropsychiatric disorder, which as of February 2017, the World Health Organisation (WHO) reports that 300 million people worldwide suffer from the disease (www.who.int). It is a highly comorbid disease (Carney *et al.,* 2002), considered the most common comorbidity in psychiatry (Strakowski *et al.,* 2013) with a life time prevalence of 20% (Breuer *et al.,* 2009). Depression, together with pain and cardiovascular diseases, are the most commonly reported disorders across low, middle and high income countries (Alonso *et al.,* 2011). In the Global Burden of Disease Study of 2010, major depressive disorder ranked 5th in non-communicable diseases in the global disability-adjusted life years (DALYs), reflecting an increase of 37% from 1990-2010 (Murray *et al.,* 2012). The burden of this disease is only set to continue, as by 2020 it is predicted it will be the second leading cause of DALYs, second only to ischemic heart disease (Murray and Lopez, 1997). It has been proposed that the drivers for this increase are a combination of population growth, shift in age structure towards those groups at highest risk, as well as stable age-specific prevalence rates (Murray *et al.,* 2012). Other reasons cited include changes to the employment landscape due to deteriorating economic conditions (Greenberg *et al.,* 2015), increasing incidence of co-morbid diseases such as obesity (Hidaka, 2012) and cardiovascular disease (Carney *et al.,* 2002).

Reflecting the global pattern, in Western Europe major depressive disorder ranks 4th in DALYs in 2010. Moreover, the collective term affective disorders (including major depressive disorder, bipolar disorder, etc.) is ranked 3rd in the total number of brain disorders reported in Europe in 2004 (Figure 1.1) (Andlin-Soboki, 2005). The cost of affective disorders in Europe was estimated at €106 billion in 2004, with costs in Ireland amounting to €4 billion and indirect costs (such as lost work days and reduced productivity) accounting for a further €1.7 billion (Andlin-Soboki, 2005). In high income North America, major depressive disorder also ranked 4th in DALYs in 2010 (Murray *et al.,* 2012), with the incidence increasing by nearly 2 million to 15.4 million in 2005-2010 (Greenberg *et al.,* 2015). In the USA, of employees reporting chronic health conditions, those reporting depression had the highest work impairment (36.4% reduction in productivity) and second greatest risk of absence from work, accounting for the highest cost per worker per year (Collins *et al.,* 2005). The substantial rise in cases in 2005-2010, was associated with a 21.5% increase in economic burden, with

workplace, direct and suicide related causes, accounting for 37% of the overall economic burden (Greenberg *et al.*, 2015)

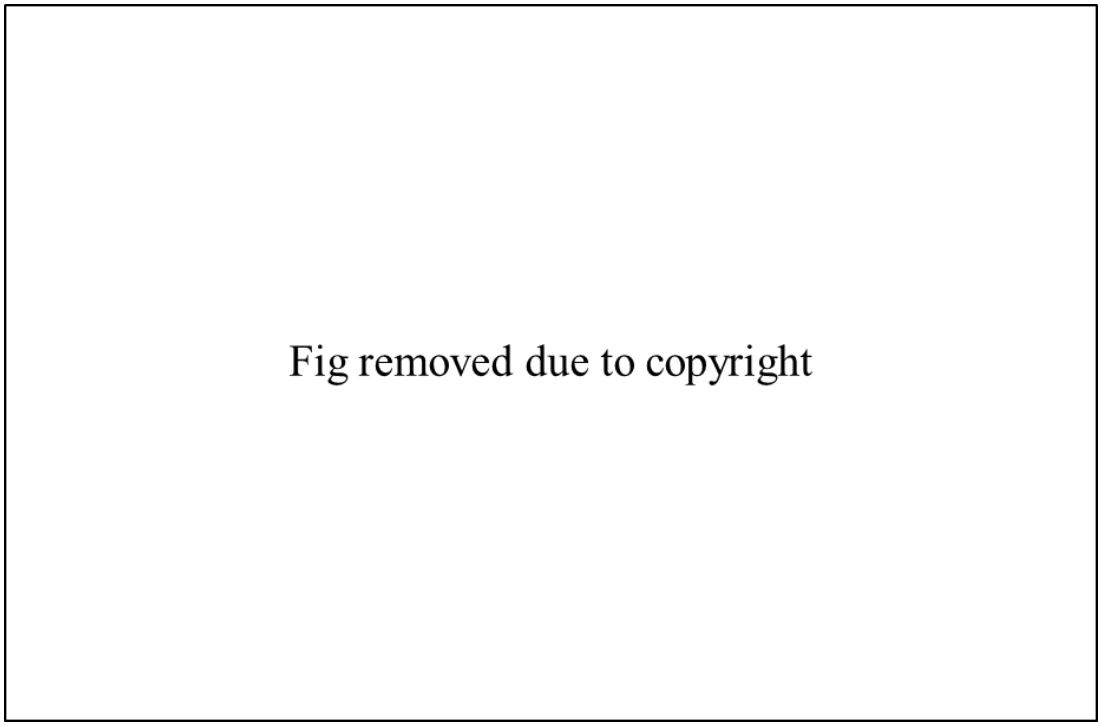


Figure 1.1: The frequency of different brain disorders in Europe (Andlin-Sobocki, 2005)

1.1 Diagnostic criteria for psychiatric disease

The term melancholia (black bile) first appeared in the *Corpus Hippocraticum* (460-370 BC) but it was only in the early 19th century that the term "depression" was initially used, being listed as an illness that included melancholia (Ban, 2014). Throughout the years classification and elaborations of the disease were made, with European and American psychiatry remaining largely in independent of each other until the late 1960s (Ban, 2014) Today there are two major consensus classifications for psychiatric diseases that are accepted across the world. These are the International Classifications of Disease (ICD) of the WHO and the Diagnostic and Statistical Manual (DSM) of the American Psychiatric Association.

The origins of the ICD can be traced back to the end of the 19th century and by the 1980s, was in its 9th revision (ICD-9) at which point it had been adopted by numerous countries worldwide. ICD-10-CM emerged in 2013, with significantly more codes and categories (Topaz *et al.*, 2013). In the USA, DSM-1 was released in the early 1950s, as a variation of ICD-6, which was the most up-to-date classification at the time. This

objective of trying to keep the two systems as similar as possible continued with the publication of DSM-III to coincide with ICD-9. DSM-III was the first widely accepted consensus-based classification system and introduced a multitude of innovations including "explicit diagnostic criteria, a multiaxial diagnostic assessment system, and an approach that attempted to be neutral with respect to the causes of mental disorders" (www.psychiatry.org). The DSM-V development process began in 1999 and was published in 2013, the outcome of which was the creation of criteria with greater specificity, the addition of dimensional measures of symptoms and severity (e.g. including new depressive disorders such as grief) and better alignment with the WHO's forthcoming ICD-11 (Blashfield *et al.*, 2014; Kronish *et al.*, 2016). However, although both classifications are primary diagnostic tools, both rely on patient reported symptomology. Thus the diagnosis of many psychiatric disorders are still reliant on self-reporting and as of yet, do not rely on the understandings of the underlying biological systems.

1.1.1 Diagnostic criteria for depression

There are three broad categories of depression in terms of its impact on the patient:

- Major/severe
- Moderate
- Mild

Whilst there are some differences between the two diagnostic systems they both rely on the presence of a number of similar symptoms to determine the relevant severity of the disease. According to DSM-V/DSM-5, diagnosis of a major depressive episode is based on the presence of 5 or more of the symptoms listed below. Critically these must include depressed mood and reduced interest in pleasure in all or nearly all activities for 2 weeks or more:

1. **Depressed mood**
2. **Reduced interest in pleasure, in all or in nearly all activities**
3. Weight loss or change in appetite
4. Changes in sleeping patterns – insomnia or hypersomnia
5. Psychomotor agitation or retardation
6. Fatigue or loss of energy

7. Diminished ability to think or concentrate
8. Recurrent thoughts of death or suicidal ideation
9. Feelings of worthlessness or guilt

In addition to experiencing these symptoms for 2 weeks, they must also be present every day, have a substantial impact on important areas of functioning, such as work and social life and should not be due to an additional medical condition or substance abuse. The diagnosis can then be further classified on the basis of severity, presence of psychotic features, as well as whether the episode is a new, singular occurrence or if it is a recurrent feature. In the case of recurrence, the patient may have been in remission and has relapsed. DSM-V/DMS-5 defines remission as a period of 2 or more months where the patient experiences no symptoms or only 1-2 symptoms mildly.

According to ICD-10, diagnosis of a mild depressive episode is based on the presence of 2 out of 3 'typical symptoms':

1. Depressed mood
2. Loss of interest and enjoyment
3. Increased fatiguability

Which must be accompanied by 2 of the following symptoms:

1. Reduced concentration or attention
2. Reduced self-esteem or self confidence
3. Feelings of guilt or worthlessness
4. Pessimistic views of the future
5. Suicidal ideation or self-harm
6. Disturbed sleep
7. Reduced appetite

As with DSM-V/DSM-5, they must be present for 2 weeks but the symptoms will not be felt intensely by the patients. With regards to a moderate depressive episode, patients must again experience 2 out of 3 'typical symptoms', in addition to 3-4 of the additional symptoms for a period of 2 weeks. A severe depressive episode, without psychotic symptoms, will generally have all 3 of the 'typical symptoms' and at least 4 of the others, generally at a severe intensity. Like a mild and moderate depressive

episode, these symptoms will generally be present for 2 weeks. Given nature of the disease, at times it can be possible to diagnose a severe depressive episode if been present for less than 2 weeks.

1.1.2 Subtypes of depression

Given the heterogeneity of the disease, two patients, each presenting with different symptomology can both be classified with the same conditions. This has led to a theory that the heterogeneous nature of the disease is due to different subtypes of depression (Nestler *et al.*, 2002) (Table 1.1). Although these subtypes are based on symptomology as opposed to biological differences, they do go some way in attempting to explain the heterogeneity of the disease. Melancholic depression is similar to the traditional 'endogenous depression' which is caused by innate factors, whereas reactive depression is similar to the traditional 'exogenous depression' which is caused by external factors (Nestler *et al.*, 2002).

Table 1.1 Examples of Proposed Subtypes of Depression

Depression Subtype	Main Features
Melancholic depression	Severe symptoms; prominent neurovegetative abnormalities
Reactive depression	Moderate symptoms; apparently in response to external factors
Psychotic depression	Severe symptoms; associated with psychosis e.g. believing depression is a punishment for past errors or hearing voices that depression is deserved
Atypical depression	Associated with labile mood, hypersomnia, increased appetite, weight gain
Dysthymia	Milder symptoms, but with more protracted course

Adapted from Nestler *et al.*, 2002

A number of rating scales have been developed to allow assessment of the severity and progression of the treatment of depression. The Hamilton-D rating scale (HAM-D) and the Montgomery-Asberg Depression Rating Scale (MADRS) are two of the most commonly used observer rating scales used in depression research (Leentjens *et al.*, 2000). These tools enable researchers to attach a categorical score to the severity of the disease, allowing both disease and treatment progression to be assessed. The HAM-D scale was developed in the 1950s and later published in 1960 (Hamilton, 1960). It was devised with the aim of overcoming some the disadvantages of the scales at the time, for example differentiating certain symptoms whilst also weighting the importance of certain symptoms depending on relevance to the disease in question e.g. anxiety in a patient with schizophrenia had equal weighting in terms of impact on condition as a patient suffering only with anxiety (Hamilton, 1960). The original 17-item questionnaire has developed into a 21-item questionnaire, with each item being scored on a 3 or 5 point scale. MADRS was developed in 1979 and was devised with the aim of detecting change in response to antidepressant treatment, the basis being looking for changes in the 17 most commonly occurring symptoms in primary depressive illness in a combined cohort of depressed patients (Montgomery and Asberg, 1979). These symptoms were then used to create a scale of 10 symptoms showing the greatest changes with treatment and the highest correlation to overall change. While the HAM-D score correlates to the severity of the depressive episode, the MADRS score correlates to the effectiveness of the treatment of the depressive episode.

The Beck Depression Inventory (BDI) is the most widely used self-rating measure of depressive symptoms (Demyttenaere and De Fruyt, 2003), was developed in 1961 (Beck, 1961). The original scale has gone through multiple revisions with the most recent augmented to minimise the possibility of reporting high estimates of depression in those patients with comorbid or additional medical conditions.

In a review of all 3 scales, Demyttenaere and De Fruyt (2003) highlight some limitations of each of the scales. For example the HAM-D scale does not differentiate between subtypes of depression, thus impeding the search for distinctive depressive subtypes, which may have preferential responses to different classes of antidepressants. This inability to differentiate between different subtypes of depression is also reflected in the MADRS scale, in addition it is also not sensitive in

detecting changes in symptomology in response to different classes of antidepressants. In relation to the BDI, one of the main criticisms is the over estimation of the severity of depression in patients with somatic conditions (e.g. irritable bowel syndrome), as well as the reactivity of the scale with the measurement itself.

1.2 Pathophysiology of depression

1.2.1 Methodological approaches

The prevalence and debilitating nature of depression is clear, however there still remains a lack of understanding of its aetiology. Attempts to summarise findings regarding the neurobiology and aetiology have been reviewed many times (Coppen, 1967; Nestler *et al.,* 2002; Duman, 2002; Krishnan and Nestler, 2008; Drevets *et al.,* 2008; Krishnan and Nestler, 2010; Oakes *et al.,* 2017). The most common ways in which neuropathological or neuroanatomical changes have been assessed in depression have been via neuroimaging techniques, post-mortem analysis and peripheral measurements of specific analytes in cerebrospinal fluid (CSF) and in blood (serum/plasma). Whilst very different in approach, each method provides insights into the pathophysiology of depression. Non-invasive neuroimaging techniques can report on functional and volumetric abnormalities that may exist in numerous brain regions. Post-mortem analysis can provide the resolution needed for identification of specific neuronal populations, as well as other components such as axonal systems and synapses, related molecules and genes that may be altered in depression (Rajkowska, 2003). Analysis of peripheral markers in CSF can also be used for monoaminergic and immunological assessments. Thus whilst one methodological approach assessing one parameter in isolation will provide a limited amount of information, collating information from all 3 approaches can only further our knowledge. Notwithstanding this they are not without limitations and deficits, for example, selection of a region(s) or neurocircuitry of interest.

In regards to neuroimaging techniques, several approaches can be used. MRI is used for anatomical imaging; fMRI detects increased activity in particular brain regions. It is non-invasive as it does not require external contrast agents. PET is used to assess metabolic changes (Kherlopian *et al.,* 2008). In brief, as summarised by Kherlopian *et al.,* (2008) all methodologies have advantages and limitations. MRI provides clear tissue contrast but often it can lead to nausea and visual problems. fMRI and PET

imaging are non-invasive, however PET imaging relies on external tracers which must be injected immediately after production.

To date, no single brain region or neurotransmitter has been identified as the primary pathophysiological feature of this disease. It has been proposed that this deficit is due to the difficulty in observing pathological changes in the brain compared to other conditions (Krishnan and Nestler, 2008). Certain disorders of the brain, such as Parkinson's disease have a clear neuropathological diagnosis (degeneration of the dopaminergic neurons in the substantia nigra), which is lacking in depression. Another confounding factor is the heterogeneity of the disease. This is highlighted in the limitations of the rating scales used to assess both severity of the disease and the effectiveness of treatment, which cannot differentiate between depressive subtypes and the specificity of different classes of antidepressants to alleviate different symptoms. This therefore results in it being very difficult to stratify patients with regard to the subtype of depression.

1.2.2 Neuroanatomical changes

As depression is a heterogeneous disorder, it is likely that numerous brain structures play a role. Regions most commonly implicated or investigated include the prefrontal cortex (PFC), amygdala, hippocampus and striatum (Maletic *et al.,* 2007; Palazidou, 2012). The hippocampus may mediate the cognitive aspects of depression such as memory impairment and feelings of worthlessness and guilt, whereas the amygdala, PFC and striatum may mediate the emotional deficits and the loss of interest in pleasure (Nestler *et al.,* 2002).

In vivo imaging suggests that in depressed patients amygdala volume is altered. Meta-analyses reveals decreased volume in patients, who are drug naïve and those who are currently taking medication (Zhang *et al.,* 2016; Sacher *et al.,* 2012). Functional imaging has demonstrated that depressed patient's exhibit increased responses to negative stimuli compared to healthy controls, suggesting that the amygdala is involved in the processing of negative emotion (Hamilton *et al.,* 2012). Assessment of individual studies also implicated that the reduction and increase in the amygdala volume is correlated with the severity of the symptomology (Kronenberg *et al.,* 2009; van Eijndhoven *et al.,* 2009). However, meta-analysis, acknowledge the discrepancies in findings regarding amygdala volume (Sacher *et al.,* 2012). The disparities in the

results however could be methodological as determining the distinct boundaries for this particular region are often challenging (Koolschijn *et al.,* 2009). Taken together these results suggest that the amygdala is both structurally and functionally altered in depression but that methodological limitations may make it difficult to definitively reach a consensus.

Hippocampal volume is one of the most widely investigated in depression (Koolschijn *et al.,* 2009). Assessment of individual studies suggest that hippocampal volume is reduced in depression, across a range of ages (Taylor *et al.,* 2014; Arnone *et al.,*2013; Chan *et al.,* 2016). Others have found no change in volume (Vakili *et al.,* 2000; van Eijndhoven *et al.,* 2009). A search for meta-analyses was carried out to obtain a more comprehensive evaluation. These primarily support the findings of hippocampal volume reduction in unipolar depressed patients, with a wide demographic of characteristics (Videbech and Ravnkilde, 2004). Hippocampal volume reduction in depressed patients (Campbell *et al.,* 2004; Kempton *et al.,* 2011) is evident in those experiencing their first episode of depression (Cole *et al.,* 2011). Interestingly, there was no impact irrespective of whether the patient was experiencing their first episode or recurrent episode on hippocampal volume (Kempton *et al.,* 2011). One of the ethical limitations of clinical assessments of depression is the inability to leave patients without therapy. As a result, many of the meta-analyses included cohorts who are drug naïve, are drug free at the time of assessment or who are currently undergoing therapy (Cole *et al.,* 2011; Kempton *et al.,* 2011). Although antidepressant treatment does not have an effect on the on hippocampal volume in depressed patients (Kempton *et al.,* 2011), patients in remission (who had previously been on antidepressant therapy) have increased hippocampal volumes compared to those currently depressed (Kempton *et al.,* 2011). Taken together, this could suggest the changes elicited by antidepressants are effective in the long term and as such normalise the reduction in hippocampal volume.

Assessment of individual studies find conflicting results with regards to the PFC. A reduction in grey matter volume in drug free patients compared to unmedicated remitted and healthy patients (Salvadore *et al.,* 2011) has been reported. Others have found no significant change in volume (Smith *et al.,* 2013). Although the majority of meta-analyses deals with the amygdala and the hippocampus, many that investigate the PFC do so in terms of functionality. Resting state activity in depressed patients is

increased in the ventromedial PFC compared to healthy controls (Kuhn and Gallinat, 2013). Whilst in suicidal patient's victims, there is a general consensus that in metabolism studies, there is a decrease in prefrontal metabolism with a reduction in basal functioning (Desmyter *et al.,* 2011).

In addition to imaging studies, post-mortem meta-analyses give insight into the reasons why there may be changes in volume or functioning of particular regions. As with some cases presented in the imaging studies, no change in amygdala volume has been found in post-mortem analysis (Bowley *et al.,* 2002). In some, this corresponds to no change in cell counts (Bowley *et al.,* 2002) or glia in the basolateral amygdala (Rubinow *et al.,* 2016). Others have found decreases in glial cell numbers of depressed patients (Bowley *et al.,* 2002). This disparity could be due to the severity of the disease, as while depressed patients show no alteration in volume of the basolateral amygdala, suicide victims have increased numbers of neurons compared to those who died of natural causes (Bowley *et al.,* 2002).

Findings in post-mortem hippocampal tissue of depressed patients have been reviewed by Malykhin and Coupland (2015), as well as a meta-analysis carried out by Stockmeier *et al.,* (2004). One of the proposed mechanisms of hippocampal reduction is due to neuronal cell death, with genetic analysis of post-mortem brains revealing pathways involved in apoptosis and cell survival are some of the most dysregulated in the hippocampus (Duric *et al.,* 2013). However, there appears to be insufficient evidence that the reduction in volume is due to neuronal death and alternatively may be due to loss of dendritic branches (Malykhin and Coupland, 2015). Analysis into the molecular basis of volume reduction appears to support this latter theory, with a decrease in neuropil (lattice of glial cells, their processes and dendrites) found per cell, which the authors postulated could contribute to the reduction in volume (Stockmeier *et al.,* 2004).

With regards to the PFC, the majority of studies investigating the role in depression do so in regards to functionality and thus there is limited data relating to post-mortem investigations. Although meta-analysis literature is sparse, Dwivedi et al, (2013) demonstrated that there was reduction in neurotrophic factor PFC in post-mortem tissue of suicide patients.

In an attempt to integrate the anatomical data, 'depression circuits' have been proposed (Krishnan and Nestler, 2010). There are two proposed circuits, the first being amygdala-centric, which postulates that the emotional symptoms could be brought about by a functional impairment in the striatum or the PFC leading to a disinhibition of the amygdala and downstream structures. The second circuit, the anatomical cluster centric circuit, compartmentalises depressive endophenotypes based on clusters of brain regions that have a strong anatomical linkage (Figure 1.2).

Therefore taken together, this then demonstrates that while imaging may show a gross change in the structure or function in the brains of depressed patients, the addition of post-mortem analysis allows further insight into the cellular changes that may be underlying those gross changes observed through imaging.

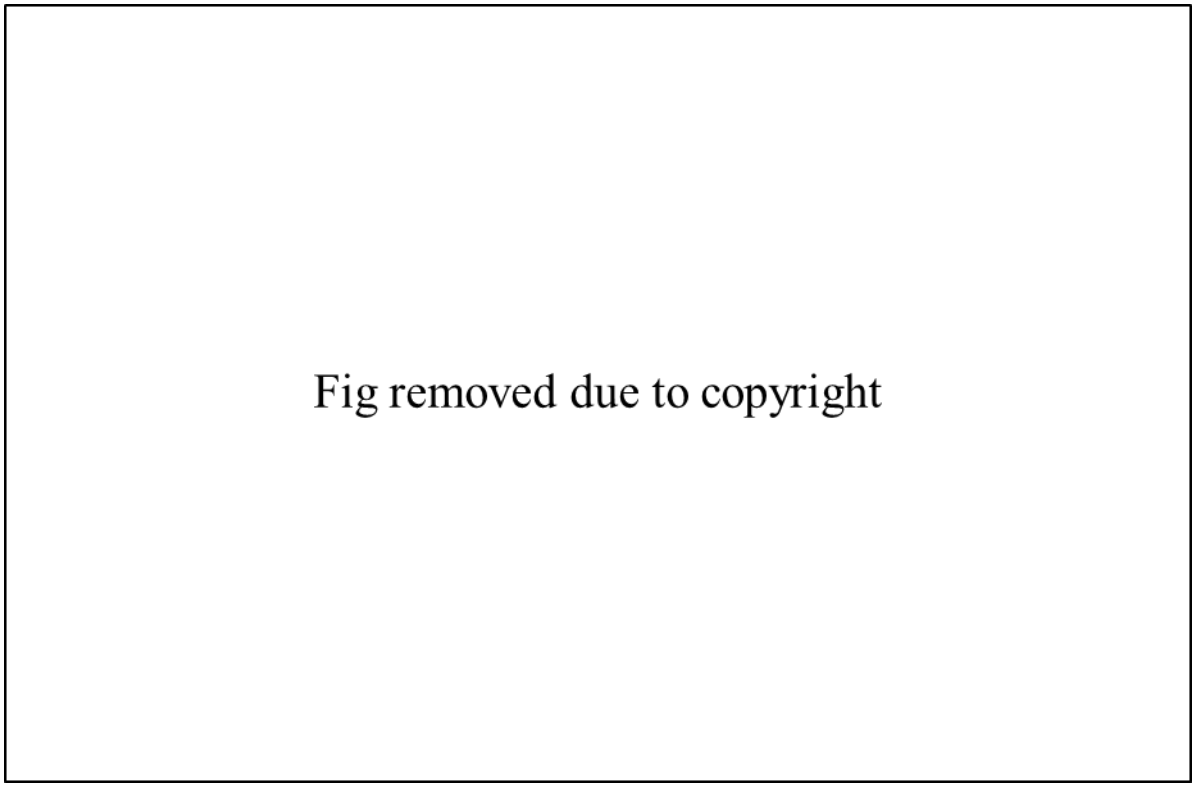


Figure 1.2 Depression circuits (A) Amygdala-centric circuit (B) Anatomical-cluster centric circuit (Adapted from Krishnan and Nestler, 2010)

Neuroimaging and post-mortem studies provide a wealth of evidence regarding the changes in the central nervous system, in terms of tissue volume and functionality. Peripheral measurements, such as blood and saliva, are relatively low-invasive procedures that can be used to assess several parameters using quantitative techniques (Hepgul *et al.*, 2013). Peripheral measurements have the potential to act as biomarkers, allowing objective information regarding diagnosis of the disease, whilst, also allowing further investigations into the pathophysiology.

The variety of methodologies employed in the attempt to elucidate the neuroanatomy and pathophysiology of the disease, demonstrates that they should not be thought of as separate entities but rather as complementary techniques whereby the limitation of one is redeemed by the advantages of other. It is these investigations that have allowed numerous theories as to the pathophysiology of depression to be developed and elucidated over the years. The main theories will be discussed below. It also highlights the importance in selection of the most appropriate methodological technique for the parameter to be assessed. In so much as, if cellular assessment is required, post-mortem analysis may be the best technique, whereas peripheral measurements may be best to assess potential biomarkers.

1.3 Theories of depression

It is clear that depression is a multifaceted disease, with numerous interconnecting neuroanatomical components. However much of the understanding as to the underlying neurobiology has evolved from serendipitous discoveries, with the first being that compounds that modulate the monoaminergic system improve the symptoms of depression. This led to the formulation of the monoamine theory of depression, which remains the basis of many investigations and is the mechanism by which current antidepressants exert their effects. Given the diagnostic challenges of identifying depressive subtypes and the responsiveness of antidepressants to specific symptomologies, in conjunction with the need for novel antidepressant therapies, several other theories have emerged, including the Neurotrophic Theory, HPA- axis Dysregulation Theory, the Neuroinflammatory Theory and the Glutamate Theory. Each of these theories will be discussed in more detail below.

1.3.1 Monoamine theory of depression

The monoamine theory was first postulated by Schildkraut in 1965 when he proposed that "some, if not all, depressions are associated with an absolute or relative decrease in catecholamines, particularly norepinephrine" (Schildkraut, 1965). It is based on the principle that depression is due to reduced levels of brain monoaminergic signalling, encompassing noradrenaline (NA), dopamine (DA) and serotonin (5-HT). The theory evolved from the serendipitous observations of the effects of compounds on monoaminergic function. During the 1960s the role of serotonin was greatly developed, with the observation of that tryptophan the precursor to serotonin, increased the effects of compounds that inhibited the enzyme responsible for the breakdown of monoamines (Coppen *et al.,* 1963) For example reserpine, a vesicular transport blocker, was shown to cause depression in a subset of hypertensive patients, even though these patients were later shown to have a pre-existing mental condition, thus increasing the likelihood for the development of depression (Baumeister *et al.,* 2003). Although the depressive effects of reserpine occurred in a subset of patients who had an underlying condition, it was found to cause prolonged central depletion of not only catecholamines but also of serotonin in the brain (Shore *et al.,* 1955, Brodie *et al.,* 1956 and Carlsson *et al.,* 1957). Such blockade of the vesicular transporter was thought to lead to the breakdown of the neurotransmitter with reduced availability for release into the synapse (Henry and Scherman, 1989).

Iproniazid, the anti-tuberculosis drug, reduced apathy and increased well-being in tuberculous patients (Robitzek *et al.,* 1952). Later it was found that this drug was capable of rapidly increasing brain levels of serotonin, through the inhibition of monoamine oxidase (Zeller *et al.,* 1952; Undenfriend *et al.,* 1957). Subsequently, clinical trials demonstrated its antidepressant effect in non-tuberculous depressed patients (Loomer *et al.,* 1957). The second serendipitous antidepressant discovery was made in the late 1950s by Roland Kuhn (Kuhn, 1958) who discovered that imipramine, the first tricyclic antidepressant, improved the mood of his schizophrenic patients. Once again, modulation of the monoaminergic system was implicated as imipramine had been shown to reduce platelet uptake of serotonin (Marshall *et al.,* 1960), whilst also blocking the reuptake of noradrenaline in presynaptic terminals (Axelrod *et al.,* 1961 in Baumeister *et al.,* 2003). These two discoveries led to the development of the first two classes of antidepressants, namely the tricyclic antidepressants (TCAs) and

monoamine oxidase inhibitors (MAOIs). A series of TCAs were developed, during the 1960s (Lopez-Munoz and Alamo, 2009).

The discovery that tryptophan increased the effects of MAOIs in animals (Coppen *et al.*, 1963), led the way for the development of serotonin reuptake inhibitors (SSRIs). These were the first family of psychoactive drugs developed with rational and directed drug design (Lopez-Munoz and Alamo, 2009). Fluoxetine, the first SSRI, was initially reported in 1974 (Wong *et al.*, 1974) and almost a decade later clinical trials demonstrated that it was as effective as TCA but with fewer side effects (Lopez-Munoz and Alamo, 2009). It was approved by the FDA in 1987 and to this day remains one of most commonly prescribed antidepressants. Given that the monoamine theory of depression gained credence from the mechanism of action of antidepressants, it is no surprise that the majority of antidepressants, including the newer generations, exert their effects through modulation of the monoaminergic system. This is still one the strongest factors in the support of monoamines having a substantial role in the pathophysiology of depression, nevertheless there is clinical and pre-clinical evidence for alterations in monoaminergic signalling in depression.

1.3.1.1 Evidence for the involvement of monoamines in clinical depression

As described previously, traditional antidepressants increase noradrenaline and/or serotonin by inhibiting their reuptake into the pre-synaptic neuron or by preventing their degradation (Brunello *et al.*, 2002). Along with the mechanism of action of antidepressants supporting the role of the monoaminergic system in depression, a meta-analysis investigating the depletion of serotonin, noradrenaline or dopamine does not impact the mood of healthy patients (Ruhe *et al.*, 2007). Rather, it was consistently found that remitted patients who were still taking antidepressant therapy, were more likely to relapse into a depressive state. Further support for the role of the noradrenergic dysfunction in depression is the demonstration α_2 receptor, mRNA levels are also increased in the frontal cortex of suicide victims (Escriba *et al.*, 2004) and dysfunctional α2 receptors in suicide victims (reviewed by Cottingham and Wang, 2012). This dysfunction is believed to be due to an upregulation of the receptors, either in absolute expression level or overall receptor activity. However, Cottingham and Wang (2012), acknowledge that the changes observed are unlikely to be the sole causative factor in depression, given that the neurobiology of the disease is complex

and multifactorial and even postulate that the changes summarised in the review could be causative or a consequence of aberrant noradrenergic signalling.

In addition the noradrenaline reuptake transporter (NET) is decreased at the cell body level in the locus coeruleus (Klimek *et al.,* 1997). However, two separate meta-analyses into the polymorphisms in the gene encoding for the noradrenaline reuptake transporter suggest that there is no link between genetic alterations in the transporter and MDD (Zhou *et al.,* 2014; Zhao *et al.,* 2013). However, it has been hypothesised that perhaps the genetic modification may not be due to a single polymorphism, rather it may be made up of multiple ones, which singly contribute a small effect but together may exert a larger collective impact on the disease (Zhou *et al.,* 2014). A recent meta-analysis assessing functional and structural neuroimaging studies in suicidal patients found that no alterations in the dopaminergic transporter was found in between suicide victims and controls (Desmyter *et al.,* 2011)

Given that the focus of this thesis will be on the serotonergic system, alterations in the serotonergic system will be discussed briefly here but will be covered in greater detail in subsequent sections. Genetic alterations in the rate limiting synthetic enzyme, tryptophan hydroxylase 2 (TPH2) has also been found in depressed patients (Zhang *et al.,* 2005), with increased protein levels found at the cell body level, in the dorsal raphe nucleus (DRN) of depressed suicide victims (Boldrini *et al.,* 2008). Serotonin, its metabolite 5-HIAA and serotonergic turnover is altered in cases of depression (Hou *et al.,* 2006; Mitani *et al.,* 2006) though this appears to depend on the methodologies used in the assessment. Depressed suicide victims show a reduced number of neurons in the dorsal raphe nucleus (DRN) expressing serotonin reuptake transporter (SERT) mRNA (Arango *et al.,* 2001).

1.3.2 Neurotrophic theory of depression

A family of neurotrophic factors, known as neurotrophins, are endogenous proteins that are structurally and functionally similar. They are responsible for the survival, growth, morphological plasticity and synthesis of proteins, as well as the differentiation of numerous neuronal populations in the central and peripheral nervous system (Boyd and Gordon, 2003). Neural plasticity is the term used to describe this ability of the central nervous system to modify connections, in response to

environmental conditions, intrinsic or extrinsic, creating new neurons that are capable of integrating into the brain's existing neural circuitry (Emsley *et al.,* 2005; Sharma *et al.,* 2013). This flexibility allows humans and animals alike, to adapt to new information and new challenges and can take the form of new neural outgrowths, strengthening of existing connections or recruitment of existing neural networks. The neurotrophin family consists of nerve growth factor (NGF), the first to be discovered (Aloe, 2004), brain derived neurotrophic factor (BDNF), which was found to have unique antigenic and functional properties compared to NGF (Barde *et al.,* 1982) and finally neurotrophins 3 and 4 (Hohn *et al.,* 1990).

In terms of depression, BDNF and its high affinity tyrosine receptor kinase B (TrkB) receptor (Autry and Monteggia, 2012) have been the most widely studied. BDNF is synthesised from a precursor protein, pro-BDNF, and is cleaved into mature BDNF, with both being localised to the superficial layers of nerve terminals (Zhou *et al.,* 2004). Depressed patients often exhibit cognitive imairments (Rock *et al.,* 2014) and it is hypothesised that this is due to a reduced ability of the brain to make adaptive changes to environment stimuli, potentiated by a decrease in the synaptic plasticity (Dwivedi *et al.,* 2013). BDNF is highly expressed in the hippocampus and as depression is associated with reduced hippocampal volume (Neumeister *et al.,* 2005), increasing BDNF signalling should help reverse the reduction in synaptic plasticity. Adding further credence to the neurotrophic theory of depression, BDNF is a down-stream target of many monoaminergic systems modulated by antidepressants and changes in its expression is postulated to contribute to the therapeutic lag exhibited by antidepressants (Racagni and Popoli, 2008).

1.3.2.1 Evidence for the involvement of BDNF in clinical depression

Clinical evidence for the role of BDNF in depression has been shown in numerous studies. Drug naïve suicide patients have reduced BDNF levels in the PFC and hippocampus (Karege *et al.,* 2005; Hayley *et al.,* 2015), with peripheral serum, plasma and whole blood BDNF levels also reduced in the depressed patients (Piccinni *et al.,* 2008; Kreinin *et al.,* 2015; Hasselbalch *et al.,* 2012). Moreover the reduced BDNF levels exhibited a significant correlation with depression severity in female patients (Kreinin *et al.,* 2015). These levels are normalised after antidepressant treatment (Kreinin *et al.,* 2015; Serra-Millas *et al.,* 2011; Shimizu *et al.,* 2003). Polyakova *et al.,*

(2015) conducted a recent meta-analysis of a commonly investigated peripheral marker in depression, BDNF. Reduced levels of BDNF found in the serum and plasma of depressed patients, was normalised in response to antidepressant treatment. This led the authors to conclude that serum BDNF is a successful biomarker for the treatment of depression, with serum levels being more reliable than plasma (Polyakova *et al.,* 2015).

1.3.3 HPA-axis dysregulation

Stress is an element of many depressive episodes and the body has an inbuilt system that is designed to help and cope with stress. The principal components of the stress response are localised to the hypothalamus, pituitary gland and the adrenal gland, collectively known as the HPA-axis (Smith *et al.,* 2006). The paraventricular nucleus in the hypothalamus releases corticotrophin releasing factor (CRF). Upon subsequent binding to its receptors on the pituitary corticotropes, adrenocorticotropic hormone (ACTH) is released, which in turn results in the release of glucocorticoids (cortisol in humans and corticosterone in rodents) from the adrenal glands (Smith *et al.,* 2006). Glucocorticoids exhibit self-regulation, with feedback inhibition through the glucocorticoid (GR) and mineralocorticoid (MR) receptors (Pariante and Lightman, 2008). Glucocorticoids have a variety of physiological effects, mediated through activation of the GRs (Zhou *et al.,* 2013). In addition to the stress response, glucocorticoids also have a role in electrolyte control, immunity, systemic fuel metabolism and fluid homeostasis (Zhou *et al.,* 2013) as well as immune modulation (Carvalho *et al.,* 2014).

1.3.3.1 Evidence for the involvement of HPA-axis dysregulation in clinical depression

In general it is considered that depressed patients will have peripheral alterations such as, increased salivary cortisol (Grynderup *et al.,* 2013), also evident in older depressed patients compared to healthy controls (O'Brien *et al.,* 2004). In addition, depressed patients have reduced levels of GR in peripheral circulating monocytes (Carvalho *et al.,* 2014). In adolescents (Goodyer *et al.,* 2000) and women (Harris *et al.,* 2000) high peak morning cortisol levels are indicative of an increased risk of developing depression. Depressed female suicide attempters also had significantly higher CSF and

plasma cortisol levels, with concomitant significant reduction in the 5-HIAA levels (Chatzittofis *et al.*, 2013). Post-mortem analysis demonstrates GR is also significantly reduced in the prefrontal cortex and amygdala of suicide victims (Pandey *et al.*, 2013) but not in the hippocampus (Pandey *et al.*, 2013, Medina *et al.*, 2013).

1.3.4 Neuroinflammatory theory of depression

The mammalian body has two defence mechanisms – the adaptive and innate immune system. The adaptive immune system is activated upon the detection of specific pathogens, whilst the innate system is not as specific and responds to all infections and insults to the body (McGeer and McGeer, 1995). Microglia and astrocytes are the two main immune cells found in the central nervous system; microglia control the brain's endogenous immune response and astrocytes are believed to play a central role in the chaperoning of the neurons to their synaptic sites (McGeer and McGeer, 1995).

Sickness, the body's response to an infection, is comprised of endocrine, autonomic and behavioural changes, mediated by soluble mediators known as pro-inflammatory cytokines (Dantzer *et al.*, 2008). These include interleukin 1-α and 1-β (IL-1 α and IL-1β), tumour necrosis factor- α (TNF- α) and interleukin-6 (IL-6) and it has been shown that these pro-inflammatory cytokines induce major depression disorders in patients that are physically ill but have no previous history of mental illness (Dantzer *et al.*, 2008).

1.3.4.1 Evidence for the role of neuroinflammation in clinical depression

Smith (1991) first proposed 'The macrophage theory of depression'. The inflammatory theory of depression is reviewed by (Maes *et al.*, 1995b) which postulated that the overproduction of the IL-1 and interferons resulted in symptoms that were considered to be at the core of depressive episodes (Maes *et al.*, 1995b). In fact it was Maes that carried out thorough investigations into the role of cytokines in depressed patients, showing that IL-6 was significantly raised in depressed patients (Maes *et al.*, 1995a). Meta-analysis of the role of cytokines in clinical depression has shown that there is a positive correlation between c-reactive protein (CRP), IL-6 and IL-1 in depression (Howren *et al.*, 2009). Meta-analysis revealed significantly higher serum levels of TNF-α, IL-6 were found in depressed patients, with no significant differences found between IL-1β, IL-4, IFN-ϒ, IL-2, IL-8 and IL-10 (Dowlati *et al.*,

2010). TNF- α and IL-6 were found to be significantly increased in depressed patients compared to healthy controls but there was no significant difference in IL-1β, IL-4, IFN-ϒ, IL-2, IL-8 and IL-10 (Liu *et al.,* 2012).

1.3.5 Glutamate theory of depression

As previously stated, neuroplasticity is a key mechanism in how the brain adapts to new information, forming new neural connections and adapting to environmental changes or lesions. However in depressed patients, this ability is known to be diminished, with alterations in the glutamatergic system implicated in the reduced neuroplasticity capabilities. Glutamate is the main excitatory neurotransmitter in the brain and as well as being implicated in many neurodegenerative diseases (Maeng *et al.,* 2007), glutamate excitotoxicity is thought to cause neuronal death in epilepsy (Cho *et al.,* 2013) The main receptor targets for glutamate are the NMDA, AMPA and kainite receptors, which mediate is rapid postsynaptic actions (Barygin *et al.,* 2017).

1.3.5.1 Evidence for the role of glutamate in clinical depression

Reduced levels of glutamate are found in severely depressed patients in the anterior cingulate gyrus (Auer *et al.,* 2000). Depressed bipolar patients and those with refractory affective disorder also have significantly reduced CSF glutamate, which was higher in those with non-melancholic depression (Frye *et al.,* 2007a; 2007b). Although glutamatergic alterations are evident in clinical depression, the interest in this system stems from the therapeutic lag, which is the main drawback of current antidepressants, and whether targeting the glutamatergic system may produce faster onset effects. Ketamine, an NMDA receptor antagonist has recently shown to have antidepressant properties, with patients showing symptomatic improvement 3 days after treatment (Berman *et al.,* 2000). This was further supported in a study in treatment resistant depressed bipolar patients, where they experienced improvement from 40 min to 2 hr post injection, peaking at approximately day 2 (Diazgranados *et al.,* 2010) but lasting for up to 7 days in drug free patients with major depressive disorder (Zarate *et al.,* 2006), with some reporting a continued effect for 4 weeks (Ibrahim *et al.,* 2012). Patients treated with ketamine also demonstrated a reduction in suicidal ideation (Ballard *et al.,* 2014). Although effective, the above studies administered ketamine via the intravenous route and therefore raises both the issue of

relevance to the clinical scenario and patient compliance. In an attempt to address this, patients in hospice care treated with ketamine orally for 28 days, reported significant improvement in symptoms at 14 and 28 days of dosing (Irwin *et al.,* 2013).

1.3.5.2 Comparison of current theories of depression

Table 1.2 Theories of depression

Theory	Associated regions	Neurotransmitters/ Chemical mediators
Monoamine	Hippocampus, amygdala, prefrontal cortex, frontal cortex, hypothalamus, ventral tegmental area, substantia nigra, striatum, midbrain, brainstem	Primarily: Noradrenaline and Serotonin Additionally: Dopamine
Neurotrophic	Hippocampus	BDNF
HPA-axis dysregulation	Hypothalamus, Pituitary and Adrenal Glands	Cortisol
Neuroinflammatory (Cytokines and pathogen associated molecular patterns thought to activate primary afferent neurons, such as the vagal nerve)	Peripheral (blood); nucleus tractus solitaries (NTS); parabrachial nucleus (PB); ventrolateral medulla (VLM), hypothalamic paraventricular and supraoptic nuclei, central amygdala and bed nucleus of the stria terminalis	Interleukins (IL), interferons (IF), Tumour Necrosis Factor (TNF)
Glutamatergic	Hippocampus, amygdala and prefrontal cortex	Glutamate

Blomstedt *et al.,* 2008; Dantzer *et al.,* 2008; Sanacora *et al.,* 2012

1.3.5.3 Towards a combined theory of depression

Given the wide range of abnormalities found in depression, an attempt has been made to formulate a unified theory of depression. Maletic *et al.,* (2007) and Dean and Keshavan (2017) have formulated an integrated view of depression based on the neurobiology of depression. Encompassing all the theories proposed above, Maletic *et al.,* (2007) propose that numerous molecular processes are disrupted by stress and depression leading to cascade of downstream events. This encompasses 4 main stages (Figure 1.3)

1. Stress increases the release of glucorticoids and pro-inflammatory cytokines
2. Disruption of serotonin, noradrenaline and dopamine in depression damage the feedback loops responsible for turning off the stress response
3. Over activity in the sympathetic system leads to the release of inflammatory cytokines, further impacting monoaminergic and neurotrophic signalling
4. Increased inflammatory cytokines may reduce corticosteroid receptor sensitivity, leading to disruption of the feedback control

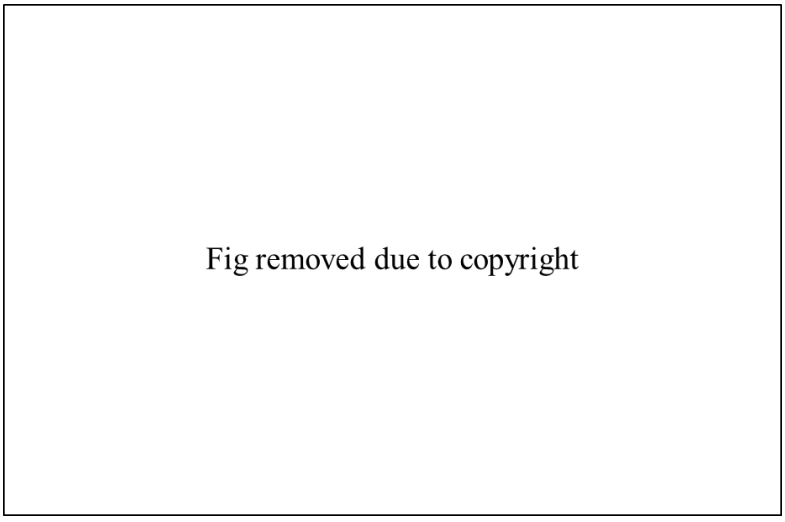


Figure 1.3 Molecular processes impacted by stress and depression (adapted from Maletic *et al.,* 2007)

Dean and Keshaven (2017) have proposed that the common end pathway in the neurobiology of depression is reduced neurogenesis, whilst acknowledging that how reduced neurogenesis causes depression is still largely unknown. They suggest, that targeting this common pathway through further investigation into how HPA-axis

dysregulation, inflammation and alterations in neurotransmitter may hold substantial promise for worthy clinical outcomes (Dean and Keshaven, 2017)

1.4 Antidepressants

As mentioned previously in section 1.3.1, the mechanism of action of antidepressants is one of the strongest features of the monoamine theory of depression and intervention via such a mechanism remains the first line pharmacological option. The period following on from the discovery of the first antidepressants in the 1950s, iproniazid and imipramine, led to the development of numerous other pharmacological compounds.

As already mentioned MAOIs inhibit the enzyme responsible for the catabolism of monoamines, however the use of these drugs declined in the 1960s as it was shown that they were less effective compared to TCAs (Lopez-Munoz and Alamo, 2009). Therefore over time, TCAs replaced them as the primary therapy. This class of antidepressants block the reuptake of both serotonin and NA, to varying specificity - imipramine and amitriptyline block serotonin and NA reuptake, clomipramine is more selective towards serotonin, whereas desipramine is more selective towards NA (Elhwuegi *et al.*, 2004). Side effect profile of TCAs are often considered unfavourable, with sedation and anti-cholinergic effects, together with a high toxicity in overdose being characteristic (Bleakley, 2009). These issues prompted the development of a new class of antidepressant, SSRIs (e.g. fluoxetine) and as the name suggests, these drugs act by blocking the reuptake of serotonin, thus increasing the level of the monoamine at the synapse. In the years since the discovery and development of the original antidepressants, several newer distinct pharmacological compounds have been developed although they still have as their mechanism of action some modulation of the central monoaminergic system (timeline of development is given in Table 1.3).

Table 1.3 Main classes of antidepressants and key development dates

Family/ Mechanism of action	Prototype	Period
TCA	Imipramine	1957-1980
MAOI	Phenelzine, Maprotiline, Mianserine	1960-1980
SDRI	Bupropion	1980-1990
SSRI	Fluoxetine	1980-1990
RIMA	Moclobemide, Nefazodone	1980-1995
NaSSA	Mirtazapine	1975-2000
NSRI	Venlafaxine	1975-2000
SNRI	Reboxetine	1980-2000
Melatonin & 5-HT2C	Agomelatine	2003
SERT & 5-HT$_{1A}$	Vortioxetine	2010

Adapted from: Lopez-Munoz and Alamo, 2009; Bang-Anderson *et al.*, 2011; San and Arranz, 2008. TCA = Tricyclic antidepressant; MAOI = Monoamine oxidase inhibitor; SDRI = Selective dopamine reuptake inhibitor; SSRI = selective serotonin reuptake inhibitory; RIMA = Reversible MAOI; NaSSA - Antagonists of α_2 auto- and hetero-receptors, 5-HT$_2$ and 5-HT$_3$ receptors; NSRI = Noradrenaline reuptake inhibitor; SNRI = noradrenaline and serotonin reuptake inhibitor; SERT = serotonin reuptake inhibitor

In recent times an attempt has been made to improve the current nomenclature in psychopharmacology, leading to the development of the Neuroscience-based Nomenclature (NbN) (Nutt and Blier, 2016). This system is a pharmacologically driven system, that the authors believe will benefit translational neuroscience and the discovery of new treatments (Nutt and Blier, 2016). In practice this nomenclature will divide the traditional classes of antidepressants based on pharmacological properties of the drug. For example, desipramine and imipramine traditionally belong to the TCA family. Under the NbN system, based on their pharmacology they target noradrenaline

and serotonin/noradrenaline respectively, with a mode of action being reuptake inhibitors. Thus providing a system that is based on pharmacological knowledge of the drug.

In a recent survey, the number of prescriptions of antidepressants in Ireland has increased considerably over a 10 year period, with 1.7 million written in 2004 compared to almost 3 million in 2013 (Garvey and Kelly, 2014). Within this analysis, it was also revealed that the majority of antidepressants had some form of serotonergic component to their mechanism of action, specifically 2,900,503 out of a total of 2,912,955. More strikingly perhaps is that of the top 10 antidepressants prescribed in 2013, all of them exert their therapeutic effect through the modulation of the serotonergic system (Figure 1.4). Thus further highlighting the central role of this neurotransmitter plays in the treatment of depression. One of the major criticism of the role of serotonin in depression comes from the 'placebo effect' whereby SSRIs have not been found to be superior compared to SSRIs in treating depression. A recent analysis aimed to address this, by taking into account how the efficacy of these trials are assessed (Hieronymus *et al.,* 2016). It was reported that when the studies analysed were re-evaluated with depressed mood being the only affect parameter the incidence of negative results decreased from 56% to 9%. This therefore suggests that the scales used to assess antidepressant activity may be a barrier to finding efficacious therapeutic drugs.

Figure 1.4 Number of prescriptions written for the top 10 antidepressants in Ireland in 2013 (Garvey and Kelly, 2014)

Other components of the serotonergic system known to have a therapeutic role are the receptors, particularly the 5-HT_{1A} and 5-HT_{2A} receptor subtypes. Reviews by Celada *et al.,* (2004) and Argitas (2013) has summarised the therapeutic role of these two receptors in the treatment of depression. Briefly, the 5-HT_{1A} receptors are expressed at the level of the cell body and serve in a negative self-regulatory role in serotonergic signalling, via the reduction of cell firing as a result of membrane hyperpolarisation. Initial treatment with drugs targeting the serotonergic system, particularly SSRIs, results in an acute increase in the level of serotonin in the synapse. As a result, the 5-HT_{1A} auto-receptors become activated, which in turn reduces the levels of 5-HT released from the nerve terminals. This results in decreased activation of the post-synaptic receptors producing a lower than expected therapeutic response. However, this attenuation of serotonergic signalling is no longer evident after long-term antidepressant treatment indicating the receptors have become desensitised. The role of 5-HT_{1A} receptors in the antidepressant response is supported the demonstration chronic administration 5-HT_{1A} agonist reduced the firing rate of DRN serotonergic neurons, which was followed by a progressive recovery in firing rates, whilst postsynaptic receptors remained normosensitive (Blier and de Montigny, 1987; Dong *et al.,* 1997). In addition, chronic treatment with an SSRI desensitised 5-HT_{1A} autoreceptors (Jolas *et al.,* 1994). The effect on 5-HT_{2A} receptors is similar, in that long term administration of antidepressant results in the downregulation of 5-HT_{2A} receptors (Gray and Roth, 2001).

To this end, given that serotonin plays an important role in the treatment of the disease, the next question is, why? Why, since the discovery of early antidepressants and throughout the development of antidepressants does serotonin continue to play a central role? This will be discussed below, however one factor that makes the serotonergic system so well placed for investigation is the far-reaching effects due to the innervation of large number of key regions believed to be involved in depressive symptoms (Figure 1.5)

1.5 The central serotonergic system

Serotonin, discovered by Rapport, was originally thought to be a vasoconstrictor (Rapport *et al.,* 1948), however since then it has been found to be involved in numerous physiological processes. It is involved in nearly all functionality including cardiovascular, pulmonary, and gastrointestinal as well as the central nervous system. It is found throughout the central nervous system but the majority is found in the periphery, predominantly in the gut (O'Mahony *et al.,* 2015).

Centrally, the serotonergic system originates in the midbrain, particularly in the dorsal (DRN) and the median raphe nuclei (MRN), with the majority of the neurons coming from the DRN. The serotonergic system is involved in the regulation of mood, sleep, fear, sexual behaviour, appetite, aggression, memory as well as thermoregulation, motor control and CNS vascular tone (Berger *et al.,* 2009). As such it is has been implicated in many psychological conditions as well as other physiological conditions, including irritable bowel syndrome (O'Mahony *et al.,* 2015). The wide reaching effects of this neurotransmitter system is more than likely due to the level of innervation of numerous brain regions. Examples of these regions and the associated role(s) it may play include: hippocampus (neurogenesis, memory), the striatum (psychomotor agitation), prefrontal cortex (mood, executive function), nucleus accumbens (anhedonia), amygdala (anxiety, emotionality, suicide) and the hypothalamus (weight, sleep) (Figure 1.5)

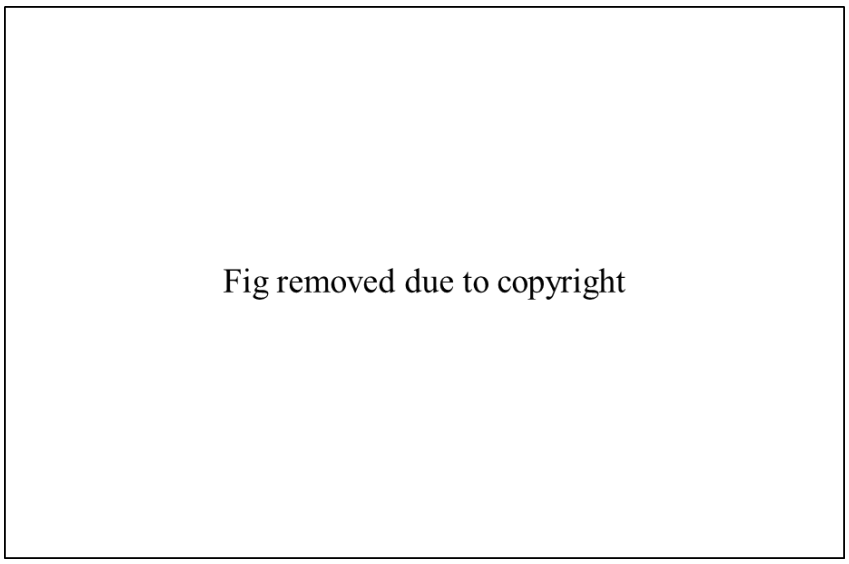


Figure 1.5 Serotonergic pathways and depression related symptoms (Pierz and Thase, 2014)

Serotonin is synthesised from the precursor amino acid, tryptophan, by the rate-limiting enzyme tryptophan hydroxylase. Serotonin is hydrophilic at physiological pH, therefore does not easily cross cellular membranes and the blood brain barrier (Pierz and Thase, 2014). Unable to diffuse back into the neuron, reuptake is mediated by SERT that belongs to family of sodium and calcium dependent transporters. SERT is located on the cellular membrane of the presynaptic neuron and is responsible for the reuptake of the neurotransmitter, which reduces the amount in the synaptic cleft, thus controlling the serotonergic signalling. Once it has been taken into the presynaptic neuron it is then repackaged into vesicles for re-release or is metabolised by monoamine oxidase (MAO) (Pierz and Thase, 2014).

Once the serotonin is released into the synaptic cleft it can bind to numerous receptors, of which 20 subtypes have so far been identified. There are 7 families of receptors (Figure 1.6), which are found presynaptically (autoreceptors) and postsynaptically, as well as heteroceptors (Albert *et al.* 2012), which are found on non-serotonergic neurons such as glutamatergic neurons (Pierz and Thase, 2014). In some cases neurons may express multiple serotonin receptors (Berger *et al.*, 2009).

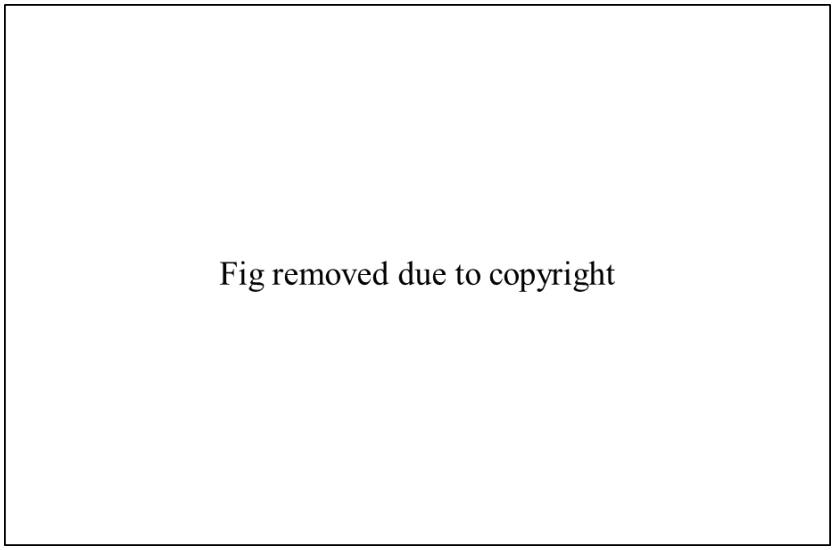


Figure 1.6. Classes of serotonergic receptors and synaptic location. (Adapted from https://www.slideshare.net/amitgajjar85/basic-neurochemistry : Presentation entitled 'Basic Neurochemistry' Presented by Mr. Darmesh Kheni)

As a consequence of the range of areas of the brain innervated by depression and the numerous subtypes of receptors, with both excitatory and inhibitory phenotypes, it is no surprise that this results in the pleiotropic behavioural consequences of serotonin (Berger *et al.,* 2009).

1.5.1 Neuroanatomy of the serotonergic system

The raphe nuclei are a collection of neurons comprising a heterogeneous population of neurons, with distinct morphologies, projections and neurochemical characteristics (Hornung *et al.,* 2003). The serotonergic neurons are located near the midline, with the majority found in the DRN. The human DRN is estimated to contain upwards of 200,000 neurons, of which 70% are serotonergic (Baker *et al.,* 1990; Baker *et al.,* 1991). Most of these serotonergic neurons are located in the five main subdivisions of the DRN - the dorsal DRN (DRD), ventral DRN (DRV), interfasicular DRN (DRI), caudal DRN (DRC) and ventrolateral (DRVL) (Baker *et al.,* 1990). Efferent connections of the DRN are topographically organised; those cells located rostrally project to more rostral areas of the brain; likewise those cells located caudally project to more caudal areas of the brain. Rostral efferent projections innervate numerous forebrain regions, as well as the hypothalamus, amygdala, medial cerebral cortex and the hippocampus (Horung *et al.,* 2003). The caudal projections terminate in such regions as the visceral and somatic motor nuclei (Horung *et al.,* 2003). Afferent projections to the DRN are primarily from limbic structures – the projections from the lateral habenula, hypothalamic nuclei, are glutamatergic and GABAergic in nature (Horung *et al.,* 2003). Thus, given the wide ranging efferent and afferent connection to the serotonergic system, it is of no surprise that it would potentiate many of the symptoms experienced by depressed patients, whilst also playing a role in the therapeutic response associated with antidepressant treatment.

1.5.2 The components of serotonergic neurotransmission

As with all neurotransmitter systems, the serotonergic system comprises of multiple different components (Figure 1.7). Each of these will be discussed in more detail below.

1.5.2.1 Tryptophan

Originally isolated from casein protein, tryptophan was discovered in the early 1900s (Richard *et al.,* 2009) and is found in many common foods such as oats, bananas, chicken and chocolate and was first synthesised in 1949 (Richard *et al.,* 2009). Once consumed it passes around the circulatory system with a large percentage bound to serum albumin, when unbound it is able to pass the blood brain barrier (Richard *et al.,* 2009). As the precursor of serotonin, tryptophan, as an essential amino acid cannot be synthesised endogenously and therefore there is a daily dietary requirement (Keszthelyi *et al.,* 2009). Once absorbed it enters 3 main different pathways 1. Protein synthesis 2. Kynurenine pathway 3. Serotonin synthesis, interestingly this latter pathway only accounts for 1% of the use of tryptophan not used for protein synthesis (Andrews *et al.,* 2015). As reviewed in Davis and Liu (2015) the kynurenine pathway accounts for 99% of the catabolism of tryptophan not used in protein synthesis, the rate limiting step produces kynurenine, which depending on the tissue type can continue down the metabolic pathway in microglial cells or be converted to kynurenic acid in astrocytes. Further modification results in the production of, quinolinic acid, known to excite glutamatergic NMDA receptors, a process that has been implicated in numerous neurological diseases, including depression.

1.5.2.2 Tryptophan hydroxylase

The next stage in the synthetic pathway is the conversion of L-tryptophan to 5-hydroxytryptophan by TPH, which is then further decarboxylated by l-aromatic acid decarboxylase to serotonin (Jenkins *et al.,* 2016). TPH belongs to the family of aromatic amino acid hydroxylases, found peripherally in the enterochromaffin cells, where it is taken up and stored by platelets (Veenstra-VanderWeele *et al.,* 2000). Originally there was thought to be only one form of TPH that was found in the gut (where the majority of 5-HT is made in the enterochromaffin cells) and the CNS. However, Walther *et al.*,(2003) produced the seminal paper which identified two

different isoforms of this enzyme – TPH1 that was predominantly expressed in the periphery, and TPH2 which was expressed centrally (Walther *et al.,* 2003).

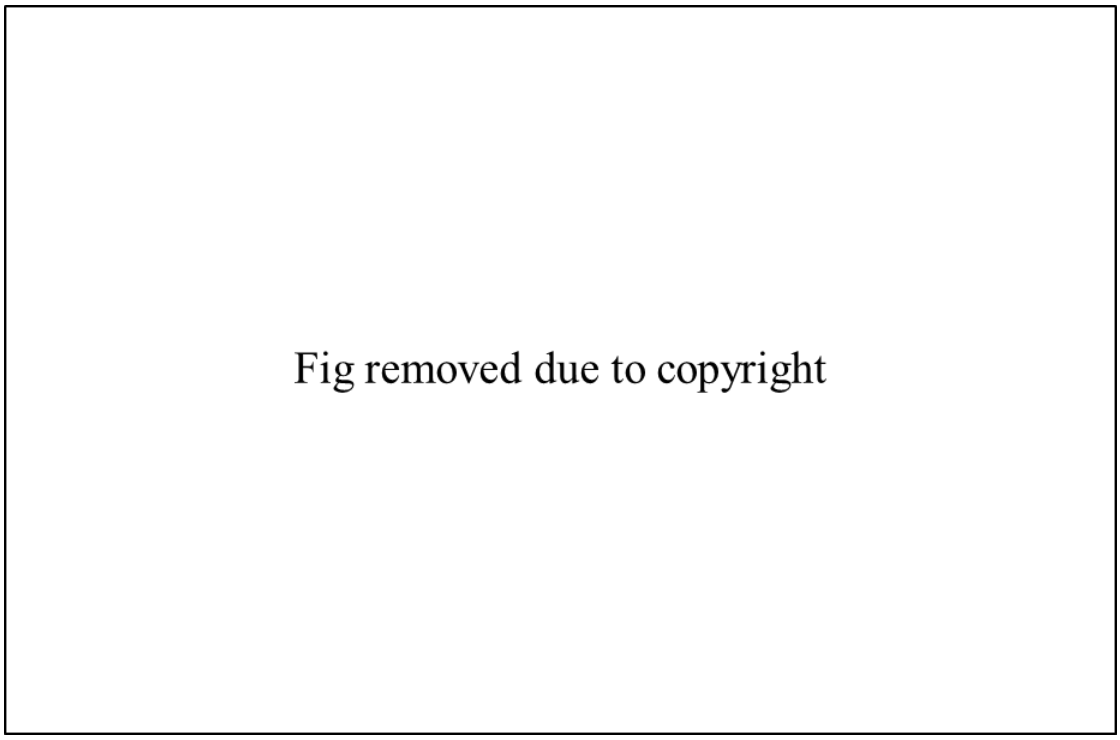


Figure 1.7. Components of 5-HT neurotransmission. (A) Shown are the major components involved in synthesis, vesicular packaging, reuptake, and degradation of serotonin in the brain, and the major receptors that mediate pre- and post-synaptic regulation of 5-HT neurotransmission (Albert *et al.,* 2011). (B) Synthetic (C) Degradation pathway Adapted from pathwayhttps://www.pharmacorama.com/en/Sections/Serotonin_2_1.php)

1.5.2.3 Serotonin

Once serotonin has been made it is then packaged into the vesicles by VMAT 2, which is the primary isoform expressed in the central and peripheral nervous system in rodents, primates and humans (Eiden and Weihe, 2011). It is the primary vesicular transporter for dopamine, noradrenaline, serotonin and histamine (Eiden and Weihe, 2011). In serotonergic cells it is localised to small synaptic vesicles and is controlled by the G-protein, Gα02 (Eiden and Weihe, 2011). It's central role in serotonergic transmission is supported by a deletion study, whereby deletion of the transporter specifically occurs in serotonergic raphe neurons, resulting in a substantial reduction of 5-HT in multiple brain regions (incuding the cortex, striatum, hippocampus and

brain stem) with little alterations in the other monoaminergic systems (Narboux-Neme *et al.,* 2011). Remarkably, DRN TPH and cortical and hippocampal SERT labelling was not altered suggesting that the serotonergic system can compensate in the loss of one aspect of the neurocircuitry (Narboux-Neme *et al.,* 2011). Interestingly, the decrease in 5-HT was not met with a concomitant decrease in 5-HIAA, thus suggesting that serotonin is was being produced in normal amounts (Narboux-Neme *et al.,* 2011). Once serotonin has been packed into vesicles, it is then released into the synaptic cleft where it can interact with and modulate its target receptors.

1.5.2.4 Serotonergic receptors

There are 7 main families of receptors, 5-HT1-7 that are subsequently divided into familial subtypes, due to differential splicing of mRNA (Table 1.4). It wasn't until the 1970s, where the development of radioligand binding further elucidated the various receptor subtypes (Frazer and Hensler, 1999). At the time it was thought there were only 2 types of receptors, termed 5-HT_1 and 5-HT_2, however displacement of [^{3}H] 5-HT by the 5-HT_{1A} antagonist, spiperone, suggested that the 5-HT_1 receptor may actually be a heterogeneous family of receptors (Frazer and Hensler, 1999). The majority are G-protein coupled receptors (GPCRs), with the exception of 5-HT_3 receptors which are ion gated channels. Developments over the years have now found that each of the receptors can be associated with specific physiological processes (Barnes and Sharp, 1999). In depression, the 5-HT_{1A} receptor and the 5-HT_{2A} receptor are the most widely implicated, given that they are each believed to play a role both in the pathophysiology of the disease and also in the therapeutic action of many of the current antidepressants that exert their activity through modulation of the serotonergic system. However, the function and second messenger cascades have remained elusive for some, such as the 5-ht_{5a} receptors and as such have been termed orphan receptors (Glennon *et al.,* 2003). Reviewed by Hoyer *et al.,* (2002) the function of the 5-ht1E receptor is unknown, the 5-ht1F receptor is postulated to have a role as an autoreceptor due to its distribution throughout the brain, but selective agonist have to be developed to further investigate its role. 5-ht6 is another receptor whose role has yet to be established; it is thought to be endogenously expressed on neuronal tissue and play a role in the control of central cholinergic function. 5-ht5A and 5-ht5B are the remaining two orphan receptors, whose roles are still yet to be defined, however it is postulated that they may be expressed in astrocytes

Table 1.4 Serotonin receptors present in the central nervous system

Receptor[a]	Distribution	Effector mechanism
5-HT$_{1A}$	Hippocampus, amygdala, septum, entorhinal cortex, hypothalamus, raphe nuclei	Inhibition of adenylyl cyclase, opening of K^+ channels
5-HT$_{1D\alpha}$	Not distinguishable from 5-HT$_{1D\beta}$	Inhibition of adenylyl cyclase
5-HT$_{1D\beta}$	Substantia nigra, basal ganglia, superior colliculus	Inhibition of adenylyl cyclase
5-ht$_{1E}$	Frontal Cortex	Inhibition of adenylyl cyclase
5-ht$_{1F}$	Cerebral cortex, striatum, hippocampus, olfactory bulb	Inhibition of adenylyl cyclase
5-HT$_{2A}$	Claustrum, cerebral cortex, olfactory tubercle, striatum, nucleus accumbens	Stimulation of phosphoinositide-specific phospholipase C, closing of K^+ channels
5-HT$_{2B}$	Cerebellum, hypothalamus, amygdala	Stimulation of phosphoinositide-specific phospholipase C
5-HT$_{2C}$	Choroid plexus, globus pallidus, cerebral cortex, hypothalamus, septum, substantia nigra, spinal cord	Stimulation of phosphoinositide-specific phospholipase C
5-HT$_3$	Hippocampus, entorhinal cortex, amygdala, nucleus accumbens, solitary tract nerve, trigeminal nerve, motor nucleus of the dorsal vagal nerve, area postrema, spinal cord	Ligand-gated cation channel
5-HT$_4$	Hippocampus, striatum, olfactory tubercle, substantia nigra	Stimulation of adenylyl cyclase
5-ht$_{5A}$	Hippocampus, hypothalamus	Inhibition of adenylyl cyclase
5-HT$_{5B}$	Hippocampus, hypothalamus	Unknown
5-ht$_6$	Striatum, amygdala, nucleus accumbens, hippocampus, cortex	Stimulation of adenylyl cyclase
5-HT$_7$	Cerebral cortex, septum, thalamus, hypothalamus, amygdala, superior colliculus	Stimulation of adenylyl cyclase

Adapted from Frazer and Hensler, 1999;Hoyer et al., 2002 Lower case appellations are used in some cases because the functions mediated by these receptors in intact tissue are presently unknown.

1.5.2.5 5-HT_{1A} and 5-HT_{2A} receptors

As already mentioned the serotonergic system originates in the midbrain – specifically in the DRN and the MRN, however serotonergic neurons are not innate pacemakers and rather depend on external sources to drive their firing (Andrade *et al.,* 2015). The 5-HT_{1A} receptor is commonly referred to as an autoreceptor and is located somatodendritically, with hyperpolarisation of the membrane occurring upon activation, resulting in inhibition of neuronal firing (Andrade *et al.,* 2015). In terms of understanding how these receptors exert their effects, much of the work was carried out in the 1960s-1970s, where neuronal cell firing was regulated *in vivo*, where compounds that increase serotonin inhibited the firing rate of these neurons and vice versa (Andrade *et al.,* 2015). 5-HT_{1A} knockout mice have shown elevated 5-HT in response to fluoxetine, with no effect on basal 5-HT levels when treated with the 5-HT_{1A} agonist 8-OH-DPAT in mice, thus confirming the autoinhibitory nature of these receptors (Bortolozzi *et al.,* 2004).

The 5-HT_{2A} receptor is also a GPCR but unlike the 5-HT_{1A} is only expressed post-synaptically, with activation leading to depolarisation of the membrane due to the closing of potassium channels. Activation of this receptor also leads to activation of phospholipase C through G_q, leading to the accumulation of the IP_3, di-acylglycerol (DAG) and activation of protein kinase C (PKC) (Raote *et al.,* 2007). This receptor also displays function selectivity, whereby the down-stream signalling cascade activated depends on the ligand activating the receptor, a phenomena thought to be due to differential conformations of the receptor (Raote *et al.,* 2007).

The 5-HT_{1A} receptors also expressed postsynaptically in numerous terminal brain regions, many of which have been mentioned earlier for their implication in the pathogenesis of depression. These regions include the hippocampus, amygdala, septum hypothalamus, cortex and entorhinal cortex (Table 1.3). 5-HT_{2A} receptors are highly expressed in other terminal regions, such as the cerebral cortex (particularly layer 5), the olfactory tubercle and striatum (Table 1.3), as well as pyramidal neurons, interneurons and glial cells of the neocortex, amygdala and hippocampus (Fraser and Hensler, 1999; Raote *et al.,* 2007; Zhang and Stackman, 2015). In the normal brain, the 5-HT_{2A} receptor is believed to play a role in learning and memory, with genetic

mutations of this receptor associated with impaired short-term verbal memory (Alfimova *et al.,* 2010), as well as working memory (Blasi *et al.,* 2015).

1.5.2.6 SERT

The final step in the termination of serotonergic signalling is the reuptake of the neurotransmitter via the SERT. It belongs to the solute carrier 6 (SLC6) transport family that also includes the other monoamine transporters, NA transporter (NET) and the dopamine transport (DAT). SERT is found mostly in the brain, as well as on a blood platelets (Kristensen *et al.,* 2011). There are two splice variants resulting in a long and short allele. The primary role of all members of this family, including SERT, is to rapidly transport substrate across cellular membranes against very large gradients (Pramod *et al.,* 2013).

1.6 Evidence for the involvement of serotonin in clinical depression

The decreased levels of serotonergic metabolites 5-HIAA in the CSF of depressed patients, coupled with the mood lowering effects of tryptophan depletion in susceptible patients and the serotonergic modulating therapies, all support the theory that a dysfunctional serotonergic system is a vulnerability factor for depression and affective disorders (Jans *et al.,* 2007). In the past, depression was considered to be due to reduced serotonergic signalling. However, it is now thought low serotonin increases the vulnerability to mood changes (Jans *et al.,* 2007) and that impaired serotonergic function, alone, is neither necessary nor sufficient to cause clinical depression (Sharp and Cowen, 2011). In other words, either innate and/or environmental factors, alone, are not sufficient to cause the disease but when combined together result in alterations in mood (Jans *et al.,* 2007). As such abnormalities in the serotonergic system could be present at one or several levels, incorporating tryptophan availability, synthesis (TPH), release, pre and post-synaptic receptors, as well as reuptake and metabolism.

Some of the sources of criticism of the serotonin theory of depression derive from the fact that antidepressants cause changes in the system within a few hours but the therapeutic effects can take several weeks to appear, tryptophan depletion is only effective in those who have the disease or those who are at risk (Ruhe *et al.,* 2007), the placebo effect is widely reported and finally that a substantial proportion of patients do not respond to treatment.

The control of the serotonergic system is homeostatically regulated (Andrews *et al.,* 2015), with the equilibrium of serotonin transmission rising in situations where limited energetic resources are required and transferred to metabolically expensive processes (incl. growth, physical activity and cognitive functions). In positive and negative mood stages, serotonergic transmission is increased in the hypothalamus as energy must be reallocated for prolonged periods of time. One alternative theory is that serotonin's symptomatic effect is due to the organism's state, in so much as whether it is tired, hungry, physically exhausted (Andrews *et al.,* 2015). Given that measurement of serotonin in the living brain is difficult to achieve, as it does not cross the blood brain barrier, peripheral measurements do not accurately reflect the levels in the brain.

The next sections will describe the evidence that the various stages of serotonergic transmission are altered in clinical depression

1.6.1 Tryptophan depletion

As already mentioned, tryptophan is the crucial precursor for the synthesis of serotonin. It cannot be endogenously synthesised but must be acquired from the diet (Keszthelyi *et al.,* 2009). A meta-analysis has revealed reduced tryptophan plasma concentration in depressed patients compared to healthy controls (Ogawa *et al.,* 2014). One way to modulate serotonergic function *in vivo* is through acute tryptophan depletion. In healthy volunteers, without a personal or familial history of the disease, acute tryptophan depletion had either a mild or no significant effect on mood (Barr *et al.,* 1997; Allen *et al.,* 2006; Young *et al.,* 2013; Hughes *et al.,* 2003), which is unaffected by fluoxetine treatment (Barr *et al.,* 1997). In depressed patients receiving SSRI or SNRI treatment there was a transient return of symptoms upon tryptophan depletion (Booij *et al.,* 2005), indicating that an intact serotonergic system is needed to maintain the antidepressant response to some drugs more strongly than others (Delgado *et al.,* 1999). Patients who have no history of the disease themselves but come from a high familial risk background have impaired emotional processing (Feder *et al.,* 2011). Although the behavioural outcomes of tryptophan depletion is different between healthy controls and those who have some association with the disease, this is not reflected in the neurochemistry, with CSF levels of 5-HIAA not differing between remitted (drug naïve and under treatment with paroxetine) and healthy

controls (Moreno *et al.,* 2010) but this was not accompanied by a decrease in mood (Moreno *et al.,* 2010).

1.6.2 Tryptophan hydroxylase

As mentioned before there are two TPH isoforms, of which TPH2 is the centrally acting form and is the rate limiting step in the serotonergic synthetic pathway. Increased TPH mRNA in the DRN of drug free depressed suicide subjects has been proposed to be a compensatory mechanism to reduced serotonergic signalling (Bach-Mizrachi *et al.,* 2006). This is believed to be due to an increase in transcriptional regulation at the level of individual neurons, with greater expression at the more caudal level of the DRN (Bach-Mizrachi *et al.,* 2008), with increased protein also found in suicide victims compared to healthy age matched controls, again possibly considered a compensatory upregulation due to reduced serotonergic functioning (Boldrini *et al.,* 2005).

A meta-analysis has identified genetic polymorphisms (Gao *et al.,* 2012), with genetic variants associated with increased risk and protective effects, as well as antidepressant response being found in multiple populations (Tsai *et al.,* 2009; Van den Bogaert *et al.,* 2006; Zill *et al.,* 2004; Haghighi *et al.,* 2008). In a single nucleotide polymorphism in a highly conserved region (1463A allele), results in a 80% loss of function in the enzyme, furthermore this allelic variation was also found in a cohort of patients with unipolar depression but not, interestingly, in patients with manic or bipolar depression (Zhang *et al.,* 2005), whereas others have found associations in manic depressive patients (Bellivier *et al.,* 1998). Although this was only a small percentage of the total cohort of patients assessed, the characteristics among the patients included a familial history of mental illness, suicidal ideation, generalised anxiety disorder (GAD) and as with Tsai *et al.,* (2009) it was also associated with antidepressant response, with some exhibiting a lack of response to SSRI treatment, whilst others only responded to the highest dose (Zhang *et al.,* 2005). What is even more interesting is the fact that a very small percentage of control subjects also displayed this genetic variation and although they did not display depressive symptoms, some were diagnosed with GAD, whereas others had mild depression and a familial history of mental illness, thus suggesting that this mutation may be a predisposing factor for the development of certain neuropsychiatric disorders (Zhang *et al.,* 2005). The carriers of this mutation and the

fact they do not respond to classical SSRI treatment therefore suggests that some fraction of a functioning serotonergic system is needed for the efficacy of SSRI treatment, which is in line with the tryptophan depletion studies.

In other studies, haplotype variations in TPH2 implicated in increased risk of suicide was associated with reduced CSF 5-HIAA in healthy controls, though suicide attempters showed no relationship between the two, thus this mutation is not associated with altered 5-HT turnover in suicide attempters (Zhou *et al.,* 2005). It has also been shown that genetic variations in the TPH2 gene are predictive of antidepressant efficacy, including SSRIs such as paroxetine, as well venlafaxine (Tzvetkov *et al.,* 2008; Serretti *et al.,* 2001). Highlighting the heterogeneous nature of this disorder, others have shown that there is no relationship between TPH2 alterations and stress induced depression (Gizatullin *et al.,* 2008) or MDD, with no accompanying association with altered CSF 5-HIAA (Mann *et al.,* 2008).

1.6.3 5-HT and 5-HIAA levels

1.6.3.1 Post-mortem analysis

Given the length of time for which the serotonergic theory of depression has been established, investigations into these alterations date back numerous years. Altieri *et al.,* (2011) have reviewed the findings of these parameters published in 'Neurobiology of depression'. The authors acknowledge that the majority of post-mortem analysis has taken place in suicide victims or from the brains of elderly patients and thus may not represent the general population of depressed patients.. Given that this book was published in 2011, many of the studies relating to post-mortem analysis of serotonin and its metabolite levels are considerably older, with reports of significant reductions of serotonin in the brain stem and amygdala of depressed patients (Lloyd *et al.,* 1974; Birkmayer and Riederer, 1975). This deficit was not however found to be present in the case of remitted patients (Birkmayer and Riderer, 1975). However, Altierei *et al.,* (2011) report that others have found no detectable changes in serotonin (Beskow *et al.,* 1976) or 5-HIAA (Ferrier *et al.,* 1986; McKeith *et al.,* 1987). The authors also note that the collection of post-mortem tissue is lengthy and therefore serotonin and its metabolites may undergo variable degradataion and this may contribute to discrepancies in results found. This was acknowledged by Beskow *et al.,* (1976) who

noted that their patient cohort was from depressed suicide victims and as such there was a greater lag in time between death and tissue analysis compared to healthy controls. Without this factor of time, there was a significant reduction in the levels of 5-HIAA, however when the condition of time was included, there was no significant alteration (Beskow *et al.,* 1976). It was surprising the lack of investigations into levels of serotoin or 5-HIAA in post-mortem tissue in recent times, however it may reflect recent advances in our knowledge of depression and the acknowledgment that depression is a not unifactorial disease.

1.6.3.2 CSF analysis

Lester (1995) carried out a meta-analysis regarding CSF levels, with the aims being to 1. assess changes in serotonin and 5-HIAA in the CSF of suicidal vs non-suicidal patients 2. comparing serotonin and 5-HIAA levels in those who attempted suicide using violent vs non-violent methods. Interestingly, however it was decided to only include studies whereby the controls also exhibited psychiatric conditions as it was concluded that studies which used healthy subjects failed to control for the presence of other psychiatric conditions. The resulting analysis found that in those who attempted suicide and those who attempted suicide via violent methods, had reduced levels of CSF 5-HIAA compared to controls. A significant limitation, also acknowledged by the authors themselves, was the absence of information regarding current pharmacological treatment.

No change in 5-HT, 5-HIAA or 5-HT turnover was found in the CSF between healthy and depressed subjects (Hou *et al.,* 2006) or turnover in drug free patients, with 5-HT levels lower in those patients with suicidal intents compared to those with none (Hou *et al.,* 2006). In drug free depressed women with co-morbid panic disorder a significant increase in CSF 5-HIAA was found in those with co-morbid panic disorder compared to those with MDD and healthy subject, while those with MDD and no comorbid conditions did not differ from healthy controls (Sullivan *et al.,* 2006). The same pattern for no change between 5-HIAA levels of CSF between healthy patients and those with comorbid diseases are supported by other studies which demonstrate that drug naïve suicide attempters with co-morbid alcoholism 5-HIAA was lower in high-lethality attempters compared to those low lethality attempters, but no correlation between 5-HIAA and depression

scores (Sher *et al.,* 2007). Prior to antidepressant treatment, 5-HT CSF was higher in depressed patients prior to treatment with venlafaxine or bupropion but this was no effected by either treatment (Little *et al.,* 1999). The opposite is true for 5-HIAA, whereby there was no difference at baseline prior to treatment but venlafaxine and not bupropion significantly decreased the level of 5-HIAA, whilst venlafaxine significantly increased and bupropion significantly decreased 5-HIAA/5-HT (Little *et al.,* 1999).

1.6.3.3 Blood analysis

Although serotonin does circulate in the periphery, the choice of sample selection is important as plasma contains a smaller proportion of 5-HIAA (Barton *et al.,* 2008) and therefore blood samples taken from the jugular vein give more accurate readings (Barton *et al.,* 2008). Although measurement of 5-HT and 5-HIAA in the CSF is commonly reported as an assessment of serotonergic functioning in depressive states, CSF is an unreliable measure as it is contaminated with peripheral sources. However plasma from the jugular vein drain directly from the brain and therefore it is less contaminated. Serotonergic turnover relates to the rate of synthesis of the neurotransmitter but is difficult to access in humans (Barton *et al.,* 2008), given that the brain is the source of less than 10% of the 5-HIAA found in arterial plasma (Lambert *et al.,* 1995). In a comparison between healthy and MDD patients, who had been newly diagnosed or drug free during a relapse period, there was no significant difference in arterial plasma 5-HIAA levels compared to healthy controls, even though some of those with depression also had a familial history of the disease (Barton *et al.,* 2008) but there was a significant increase in jugular 5-HIAA levels in unmedicated patients, with a trend for a correlation between increased levels and depression severity but not the risk of suicide. A significant increase in serum 5-HT and 5-HIAA compared to controls, this was in patients who were on antidepressants (Mitani *et al.,* 2006). In peripheral venous blood it has been shown that in medication free suicide attempters decreased 5-HIAA and platelet 5-HT (Spreux-Varoquax *et al.,* 2001). However, significantly greater levels of 5-HT and 5-HIAA has been reported in the plasma of depressed patients and in contrast to Barton *et al., (*2008) this was positively correlated to depression severity (Mitani *et al.,* 2006). 5-HIAA of arterial plasma of depressed patients compared to healthy controls (Barton *et al.,* 2008), however jugular

venoarterial 5-HIAA levels were higher, thus indicating an increased rate of turnover (Barton *et al.,* 2008).

1.6.4 5-HT receptors

As already mentioned, the main receptors implicated in depression are the 5-HT_{1A} and the 5-HT_{2A}. Activation of the 5-HT_{1A} receptor results in hyperpolarisation of the cellular membrane, thus reducing signalling and thereby reducing the firing of serotonergic neurons (Celada *et al.,* 2004). The role of these receptors both in the pathophysiology and the treatment of the disease have been reviewed numerous times, however one of the most common ways in which these receptors are assessed is through imaging studies, such as PET imaging and post-mortem studies. Post-mortem analysis however is difficult to interpret, as the degree of the disease, as well as the treatment state cannot be controlled (Lewis, 2002).

1.6.4.1 5-HT_{1A} receptors

The role of the 5-HT_{1A} receptor in depression has been reviewed numerous times (Kaufman *et al.,* 2016) with functionality (Savitz *et al.,* 2009), polymorphisms and genetic alterations (Neumeister *et al.,* 2004) as well as their role in the action of antidepressant treatments (Celada *et al.,* 2004). There are numerous ways in which the 5-HT_{1A} receptor alteration in depression is assessed, however the main ones include functionality studies, post-mortem analysis and *in vivo* PET imaging.

Activation of this receptor results in various physiological responses including hypothermia as well as prolactin, cortisol and growth hormone release. Human functional assessment of the 5-HT_{1A} receptors demonstrate a blunted response to buspirone treatment after citalopram treatment (Navines *et al.,* 2007) and imipramine (Gomez-Gil *et al.,* 2010) suggesting receptor desensitisation. In contrast, *in vivo* PET imaging demonstrates decreased binding potentials for 5-HT_{1A} receptors located in raphe, limbic and cortical regions (Drevets *et al.,* 1999; Drevets *et al.,* 2007; Hirvonen *et al.,* 2008), a decrease that is not attenuated by fluoxetine treatment (Sargent *et al.,* 2000). However, others have shown reductions in the raphe and hippocampus but not the amygdala (Wang *et al.,* 2016). A decrease in binding potential 5-HT_{1A} receptors has been shown to positively correlate with disease severity in late life depression (Meltzer *et al.,* 2004). Together these results could suggest that although the number

of available receptors are reduced, and that those that remain have altered functionality. Post-mortem analysis in depressed suicide victims also demonstrate a reduction in the binding capacity (Boldrini *et al.*, 2008; Arango *et al.*, 2001), without a change in the concentration of receptors in the DRN (Arango *et al.*, 2001). However, no changes in binding sites were detected in cortical regions, hippocampus and amygdala of depressed suicide patients (Lowther *et al.*, 1997).

1.6.4.2 5-HT_{2A} receptors

The 5-HT_{2A} receptor is expressed post-synaptically in the terminal regions of the serotonergic system. Unlike the 5-HT_{1A} receptors, activation of the 5-HT_{2A} receptors results in depolarisation of the cellular membrane. As with the 5-HT_{1A} receptors, 5-HT_{2A} receptor alteration in depression is assessed, using the same techniques. In the assessment of the functionality of this receptor in antidepressant free suicide victims, a reduction in hippocampal receptor binding sites accompanied an increase in binding affinity and subsequent IP_3 concentration in suicide victims (Rosel *et al.*, 2004). This may suggest that, as with 5-HT_{1A} receptors the number available is reduced but the functionality of those receptors is heightened. This reduction in receptor binding was also found in *in vivo* imaging in drug naïve depressives, with hippocampal receptor binding significantly decreased (Mintun *et al.*, 2004). Moreover, in patients who were drug free at the time of testing but had previously been on treatment, there appears to be a long lasting protective effect of antidepressant treatment, evident as an upregulation of 5-HT_{2A} receptors (Sheline *et al.*, 2004; Massou *et al.*, 1997). This is further supported by the increased binding of 5-HT_{2A} receptors in unipolar patients who are fully recovered (Bhagwagar *et al.*, 2006). With regards to post-mortem analysis, increased cortical 5-HT_{2A} mRNA has been found in depressed suicide victims (Escriba *et al.*, 2004).

1.6.5 SERT

A meta-analysis of studies investigating the association between different SERT alleliac variations and their interaction with stressful life events found that there was no significant difference between genotype and depression and also no interaction between genotype and stressful life events on depression (Risch *et al.,* 2009). This was found to be equivalent among the sexes, when assessed together and also separately (Risch *et al.*, 2009). Whereas another meta-analysis study found reduced SERT availability in the major limbic regions (Kambeitz and Howes, 2015). However there appears to be some discrepancy between *in vivo* imaging and post mortem brains, where a significant decrease in SERT was found in the brainstem in depression *in vivo* imaging, but no significant changes found in post-mortem studies (Kambeitz and Howes, 2015). Additionally, reductions in depressed patients have been found in the striatum, amygdala and PFC (Nikolaus *et al.,* 2016) Reasons for the discrepancies could also be due to the heterogeneous nature of the disease whereby the patients in the *in vivo* imaging studies are alive and therefore may have been in a current depressive episode or may have been on treatment. The majority of patients in post-mortem cohorts may have come from suicide victims, as well as those who have recurrent depression or who may be on therapy. Thus this particular cohort may reflect the most severe cases of depression. However, there may be a familial risk as children of parents who suffer from the disease are more likely to have two copies of the short SERT allele (Talati *et al.,* 2015). SERT availability in the midbrain was found to be lower in drug free MDD patients compared to healthy controls, however the availability was significantly lower in patients who responded to antidepressant treatment compared to non-responders (Tsai *et al.,* 2015). This relationship did not predict treatment outcome in a linear manner however (Tsai *et al.,* 2015). Other studies have no significant difference in SERT binding potential between MDD patients and healthy controls (Hahn *et al.,* 2014). Reduced neuronal density and SERT binding has been found both in the ventral and dorsal PFC suicide patients compared to healthy controls (Underwood *et al.,* 2012).

1.7 Summary of the role of serotonergic system in depression

From the literature, there is no denying that serotonin still plays a central role in the therapeutic action of many of antidepressants. The role of serotonin was originally derived based on the mechanism of action of these drugs, however summarising the data with regard to serotonergic changes in depression it can be seen that there are alterations in the system (Table 1.5). The methodologies used to assess each of parameters differed slightly depending on the parameter being measured, however three main themes emerged – the use of peripheral measurements, *in vivo* imaging and post-mortem analysis. Whilst each method has its merits, the very nature of the clinical disease means that they are not without limitations. With peripheral measurements, the source has proved to be vital, as the danger of contamination with peripheral serotonin could increase the risk of false positive or negative results. With post-mortem analysis although useful, the cohort of patients tends to be victims of suicide, thus making the characteristics of the subjects difficult to standardise. For example, the severity of the disease, the duration and the presence of antidepressant or other psychoactive drugs are hard to control and in some cases may be unknown. Thus, animal models of depression are a significant advantage in furthering knowledge as numerous parameters can be controlled and standardised, such as the subtype of depression (i.e. model based on stress, endogenous depression or lesion based) and selection of antidepressant treatment.

Table 1.5 Brief summary of main alterations in components of central serotonergic signalling in depression

Parameter	Finding	Reference
Tryptophan depletion	Affects patients who are recovering from a major depressive episode, or those who have a family history of the disease are susceptible to effects	Barr *et al.,* 1997; Allen *et al.,* 2006; Young *et al.,* 2003; Feder *et al.,* 2011
TPH2	mRNA increased in depressed suicide patients	Bach-Mizrachi *et al.,*2006
	Genetic variations commonly assessed	Tsai *et al.,* 2009; Van den Bogaert *et al.,* 2006; Zill *et al.,* 2004; Haghighi *et al.,* 2008
5-HT & and 5-HIAA	no change in CSF or plasma of depressed patients	Hou *et al.,* 2006; Barton *et al.,* 2008
5-HT$_{1A}$ receptors	Increased functionality attenuated by antidepressant treatment	Navines *et al.,* 2007
	Decreased binding potential not attenuated by antidepressant treatment	Drevets *et al.,* 1999; Drevets *et al.,* 2007; Hirvonen *et al.,* 2008; Sargent *et al.,* 2005
5-HT$_{2A}$ receptors	Decreased hippocampal receptor binding with increased functionality	Mintun *et al.,*2004; Rosel *et al.,* 2004
SERT	Genetic alterations not associated with stress but associated with risk	Risch *et al.,* 2012 ; Talati *et al.,* 2015
	Altered levels and availability	Kambeitz *et al.,* 2015 ; Tsai *et al.,* 2014

1.8 Animal models

Animal models are extremely useful in the further advancement of our knowledge of many neurological and neuropsychiatric diseases, including depression. However, unlike other diseases that have clear pathophysiological, epidemiological and symptomatic characteristics, the aetiology of depression is not fully known, therefore it is very difficult to develop a model that will completely encapsulate all of the features disease. Compounding the difficulty in establishing a model that encompasses all the facets of the disease is the fact there are no biological markers that can objectively assess whether the animal is "depressed" and if so, to what degree. It is also difficult to predetermine what kind of symptom profile the animal will have, given the heterogeneous nature of the clinical disease. In other words that depression may be in fact be made up of different disorders that exhibit different pathophysiology but a similar symptomatic profile, leading to a diagnosis of depression (Czeh *et al.*, 2016).

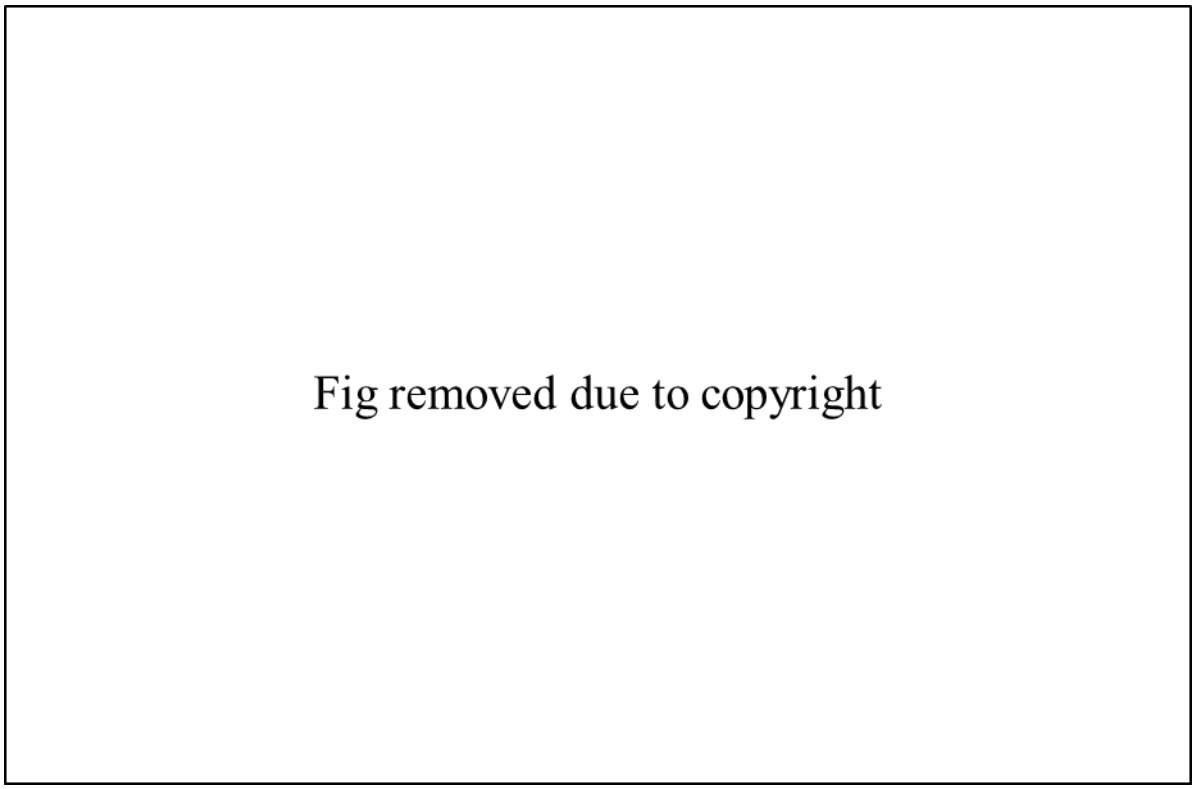
Animal models provide a valuable avenue in assessing the neural circuitry encompassing molecular and cellular pathways that may be crucial in the pathogenesis of depression, as well as overcoming limitations such as ethical concerns and sample sizes that are common obstacles in clinical investigations (Wang *et al.*, 2017). In order for an animal model to be considered useful and valuable for the study of the disease it must meet three criteria – constructive, face and predictive validity. This means that the aetiology of the disease must be the same as the clinical scenario, that the symptomology must be the same and that it must react in a similar fashion to drug treatment as in the clinic but also be non-responsive to ineffective treatments tested in the clinic. McKinney and Bunney (1969), in their seminal paper proposed that the minimal requirement for an animal model of depression is that it:

1. Has symptoms analogous to the clinical disease
2. Has behavioural changes that can be observed and objectively measured
3. Should be robust enough to allow interobserver agreement
4. Demonstrates activity with treatments that are effective in the clinic
5. Is reproducible

An animal model of depression is considered valid when the constructive, face, etiological and predictive validity reflect those seen in the clinic (Willner, 1984). However, given the heterogeneous nature of the disease and its symptomology it must also be acknowledged that animal models may only represent or manifest a subset of these or they may be caused by one aetiological factor (e.g. genetic models)

There have been numerous reviews published on the types and validity of animal models of depression (Cryan and Slattery, 2007; Krishnan and Nestler, 2011; O'Leary and Cryan, 2013). Animal models can be categorised into stress based models (acute or chronic), genetically predisposed models, dysfunction of limbic circuitry and as well as others (Table 1.6). Each model has advantages and disadvantages, which have been detailed by Wang *et al.,* (2017). For example, learned helplessness has the ability to create depression susceptible and depression resilient groups for comparison but it requires specialist equipment. The chronic mild stress model is considered to have strong validity, is good for drug screening but its establishment is labour intensive and is often difficult to reproduce. One of the main limitations in animal models is the inability to model some of the core depressive symptoms such as feelings of guilt and suicidal thoughts (Wang *et al.,* 2017). In this vein, it has been suggested that rather than future studies should be less focused on drug response and instead focus on the underlying mechanisms (Wang *et al.,* 2017). It could be suggested however, that they not be considered of as exclusive entities, as assessment novel compounds and their mechanism of action could shed light on the underlying pathophysiology.

Table 1.6. Animal models commonly used in depression (O'Leary and Cryan, 2013)



1.8.1 Investigation of clinically-relevant parameters in rodent models of depression

1.8.1.2 Evidence for the involvement of BDNF in rodent models of depression

The CMS model resulted in decreased hippocampal BDNF in young and aged animals (Shi *et al.,* 2010), with similar decreases found in the Wistar-Kyoto model (Kyeremanteng *et al.,* 2012). Interestingly, over expression of BDNF in the dentate gyrus caused resistance to depressive-like behaviour in CMS rats (Taliaz *et al.,* 2011) therefore suggesting a protective mechanism. In the OB model of depression the opposite occurs however, BDNF is increased in the hippocampus and frontal cortex (Freitas *et al.,* 2013; Hellweg *et al.,* 2007) and is attenuated by chronic fluoxetine treatment (Freitas *et al.,* 2013). Genetic models of BDNF null mice prove lethal in the postnatal period, therefore heterozygous mice which still have some BDNF, in their system but at a substantially reduced rate, have shown reduced hippocampal dependent learning and long term potentiation (Monteggia *et al.,* 2004). It also seems to play a role in the antidepressant treatment, as knockout mice showed no reduction in forced swim (FST) behaviour but they did not respond to desipramine (Monteggia *et al.,* 2004).

1.8.1.3 Evidence for the involvement of HPA-axis dysregulation in rodent models of depression

Chronic restraint stress results in a depressive like phenotype, with accompanying increase in serum corticosterone and down regulation of GR in the PFC, as well increased immobility time in the forced swim test (FST) (Chiba *et al.,* 2012). Genetically modified mice which express GR receptors at a level 50% lower than normal exhibit disturbed negative feedback of the HPA system in response to restraint stress as evident by a significant increase in corticosterone compared to wildtype control (Ridder *et al.,* 2005). Mice lacking GR in the forebrain show increased depressive like phenotype and anhedonia (Boyle *et al.,* 2005), which was attenuated by chorionic imipramine treatment, with an associated reduction in corticosterone levels (Boyle *et al.,* 2005). Chronic corticosterone treatment in rats results in increased immobility in the FST (Fenton *et al.,* 2015; Lussier *et al.,* 2013; Zhao *et al.,* 2008), which is attenuated by chronic imipramine treatment (Fenton *et al.,* 2015). The CMS

model significantly increases serum corticosterone levels, with desipramine reducing the stress induced increase in corticosterone levels (Liu *et al.,* 2014).

1.8.1.4 Evidence for the role of inflammation in rodent models of depression

The CMS model significantly increased plasma and cerebral cortical TNF-α, as well as serum TNF-α, IL-6 and corticosterone (Guan *et al.,* 2014). Interestingly, pre-treatment with minocycline and the TNF-α antagonist infliximab, reduces immobility time in the force swim test (FST) (Liu *et al.,* 2015). Infliximab also reversed emotional and learning memory deficits, as well as the associated decrease in BDNF in the hippocampus of CMS rats (Sahin *et al.,* 2015). Aspirin is a nonsteroidal anti-inflammatory drug (NSAID) has been shown to decrease immobility time in the FST, whilst concomitantly reducing the increase in the pro-inflammatory cytokines (Guan *et al.,* 2014). This therefore suggests that conventional antidepressants (Guan *et al.,* 2014) may exert part of their effect through anti-inflammatory mechanisms, whilst anti-inflammatory drugs may have antidepressant qualities

1.8.1.5 Evidence for the role of glutamate in rodent models of depression

Ketamine administration had antidepressant like effects in the learned helplessness paradigm as well as the FST (Maeng *et al.,* 2008), an effect that lasted for 2 weeks but the same phenomena was not found with imipramine (Maeng *et al.,* 2008). Interestingly, treatment with an AMPA receptor antagonist abolishes the antidepressant effects of ketamine in the FST, whilst having no effect on its own or in conjunction with imipramine treatment (Maeng *et al.,* 2008; Koike and Chaki *et al.,* 2014). Although the effects appear to be long lasting, the activity of ketamine could be regionally dependent as injection of the R-enantiomer of ketamine into the PFC and hippocampus resulted in antidepressant like behaviour in the learned helplessness paradigm, whereas infusion in the basolateral amygdala and nucleus accumbens did not (Shirayma and Hashimoto, 2017). Ketamine also appears to have an effect on the other systems already mentioned, such as increasing hippocampal BDNF (Yang *et al.,* 2013) and preventing CMS increases in IL-1β, IL-6 and TNF-α in the hippocampus (Wang *et al.,* 2015).

1.8.2 Investigation of the central serotonergic system in rodent models of depression

The neuroanatomy of the serotonergic system in rats is identical to that in humans – the cell bodies are located primarily in the DRN and project to numerous regions, including the PFC, hippocampus, amygdala, striatum, and hypothalamus amongst other regions. Numerous alterations in the serotonergic system can be measured in the rat including 5-HT levels in the brain (Musumeci *et al.,* 2015), effect of TPH knock out/in (Angoa-Perez *et al.,* 2014; Sachs *et al.,* 2014; Jacobsen 2012), receptor functionality and interaction with other neurotransmitter systems (Haleem and Khan, 2003), together with behavioural responses such as agonist-induced hypothermia, serotonin syndrome (Heslop and Curzon, 1999; Granoff and Ashby, 2001; Gaggi *et al.,* 1997) and alterations in SERT (Beaulieu *et al.,* 2008). Therefore rats are an ideal candidate to determine how the serotonergic system works in normal rats and how they can be manipulated with experimental compounds and antidepressants.

1.8.2.1 Neonatal clomipramine

Depression, as already discussed is a complicated and multifaceted disease. It has been shown that depressed patients show cognitive impairment, with particular deleterious effects seen in the hippocampus leading to impaired learning and memory function. Endogenous depression is often thought of as depression that exists from birth i.e. nothing has happened in life to cause it e.g. undue stress or grief. Neonatal clomipramine (CLI) treated rats show changes reflective of the clinical condition including behavioural alterations as well as changes in the monoaminergic system, cellular signal transduction (Bhagya *et al.,* 2008), depressive like behaviour (Feng *et al.,* 2008; Hansen *et al.,* 1997; Vazquez-Palacios *et al.,* 2005; Limon-Moarles *et al.,* 2014), anxiety (Andersen *et al.,* 2002) and sexual performance (Bonilla-Jaime *et al.,* 1998). In this model the pups are treated with clomipramine from PND8-21 (Bhagya *et al.,* 2008), resulting in altered REM sleep in adult rats, depressive like behaviour and enhanced sensitivity to stress (Andersen *et al.,* 2002). Interestingly pups do not show deficits in physical or reflex characteristics (de Souza *et al.,* 2004). Increased depressive like behaviour and anhedonic behaviour was attenuated by adult administration of chronic escitalopram (Bhagya *et al.,* 2011). The increased

immobility time in the FST was reversed by subchronic and chronic fluoxetine treatment (Vazquez-Palacios *et al.,* 2005).

In the prefrontal cortex there was no change in 5-HT levels but there was increase in the 5-HT turnover (Andersen *et al.,* 2002). In the hippocampus there was no change in 5-HT or 5-HIAA levels, however in the amygdala there was an increase in 5-HT turn over in the left amygdala (Andersen *et al.,* 2002). Significant increases in SERT in the DRN of postnatal clomipramine treated animals, without any effect on 5-HT_{1A} autoreceptors (Hansen and Mikkelesen, 1998) has been shown. However in adulthood, SERT mRNA in the DRN was actually significantly decreased but there was no significant change in 5-HT_{1A} receptors in the DRN or hippocampus (Hansen and Mikkelesen, 1998). This change in SERT expression from neonates to adulthood suggests that an alteration in serotonin levels in the neonatal development period persists into later life. The firing rate of the serotonergic neurons in the DRN is also decreased in CLI treated animals, with an altered discharge pattern in adulthood, leading the authors to conclude that there is a role for serotonin in behavioural disturbances in depression (Kinney *et al.,* 1997).With regards to changes in the rate limiting enzyme of serotonergic synthesis, TPH is significantly decreased in the DRN and the MRN of CLI treated neonates, persisting into adulthood (Maciag *et al.,* 2006). Serotonin levels were significantly decreased in a number of key brain areas including the hippocampus, frontal cortex, hypothalamus and brainstem, which were attenuated with chronic escitalopram treatment (Bhagya *et al.,* 2011).

5-HT_{1A} mRNA expression was significantly decreased in the DRN, whilst a significant increase was found in the hypothalamus and hippocampus (Limon-Morales *et al.,* 2014), however changes in functional alterations in the receptors may not be responsible for behavioural changes as 8-OH-DPAT administration reduced time spent immobile in the FST to relatively equal magnitudes in both the CMI and the control rats (Limon-Morales *et al.,* 2014). 5-HT_{1A} mRNA in the DRN was significantly reduced, whilst the 5-HT_{2A} was not altered in any of the other regions (Kim *et al.,* 2013). Electrophysiological studies found that there may be a desensitisation of 5-HT_{1A} receptors in this model (Maudhuit *et al.,* 1995).

Neonate and adult expression of SERT were increased in the somatosensory and medial prefrontal cortex (Maciag *et al.,* 2006). Although not significant, there was a

trend for CLI to increase SERT expression in the hypothalamus and the hippocampus (Kim *et al.,* 2013).

1.8.2.2 Chronic mild stress rat model of depression

The chronic mild stress model (CMS) is one where the animals are exposed to mild stressors in a sequential manner, such as 24 hr light exposure, wet bedding, cage tilt, change of cage mate etc. They are generally exposed to each stressor for matter of hours and alternate between the stressors over a number of weeks. One of the main behavioural parameters used in this model, is the sucrose preference test, which is a model of anhedonia (Willner *et al.,* 1997; Christensen *et al.,* 2012), a behaviour that can be reversed by escitalopram treatment in about 50% of animals (responders) (Christensen *et al.,* 2012). This model has been criticised for its reproducibility and variation among lab groups, with a review by Hill *et al.,* (2012) highlighting both the housing conditions of the animals, ranging from singly housed to 4 per cage but more importantly, highlighting the duration of time the animals are exposed to the procedure – varying from 1 week to 11 weeks, further highlighting the inconsistency in methodologies used, and therefore may account for the conflicting results found.

5-HT levels in the frontal cortex are reduced (Li *et al.,* 2003; Ahmad *et al.,* 2010; Mao *et al.,* 2014) an effect that is reversed by chronic imipramine treatment, but does not reach significance (Vitale *et al.,* 2009). Decrease in total brain 5-HT is reversed by chronic venlafaxine treatment (Darwish *et al.,* 2013). However others find no difference in levels in the frontal cortex (Bekris *et al.,* 2005 and Johnson and Yamamoto *et al.,* 2009). The same pattern of inconsistency is also found in the hippocampus, where some find decreases in 5-HT (Li *et al.,* 2003; Mao *et al.,* 2014), whereas others find no change (Gronli *et al.,* 2007; Dang *et al.,* 2009).

5-HT_{1A} mRNA levels are found to be increased (Li *et al.,* 2009) or decreased (Pan *et al.,* 2010) in the frontal cortex. In the hippocampus mRNA levels are decreased (Lopez *et al.,* 1998; Wang *et al.,* 2009), whilst others find increased levels (Li *et al.,* 2009), with no change in the receptor binding (Haidkind *et al.,* 2003) and decreased sensitivity in the raphe (Bambico *et al.,* 2009). 5-HT_{2A} receptors are also altered in the model, with both increased and decreased binding found in the frontal cortex (Ossowska *et al.,* 2001, Pan *et al.,* 2010).

The discrepancy in the results could be due to the variation in methodologies employed, thus highlighting the need for robust and reproducible model for which to investigate serotonergic changes in depression.

1.8.2.3 Wistar-Kyoto rat model of depression

The Wistar-Kyoto (WKY) rat model of depression is a genetic model that is endogenously depressed, i.e. it does not rely on stressful situations to induce stress nor does it rely on surgical or chemical intervention. It exhibits a depressive like phenotype, with hormonal, behavioural and physiological changes reflective of the clinical scenario (Will *et al.,* 2003). In the FST, the rats exhibit increased time spent immobile, which is attenuated by subchronic desipramine but not fluoxetine treatment (Tejani-Butt *et al.,* 2003; Will *et al.,* 2003).

In terms of the serotonergic system, serotonin levels were lower in the DRN, PFC, basolateral amygdala, lateral and dorsal hippocampus, substantia nigra when compared to Wistar (Yamada *et al.,* 2013) or Sprague-Dawley rats (Scholl *et al.,* 2010). An effect that was reversed by chronic escitalopram treatment with the magnitude of the increase being less in the WKY-rat (Yamada *et al.,* 2013). WKY rats also displayed an increase in 5-HT_{1A} binding sites in the hippocampus and hypothalamus (Pare and Tejani-Butt, 1996). WKY rats showed no difference in hypothalamic 5-HT content compared to a non-anxious rat but did have a reduced level of 5-HIAA, both parameters were reduced by chronic fluoxetine treatment (Durand *et al.,* 1999). Chronic fluoxetine also significantly decreased 5-HT_{2A} in the cortex, with no effect on 5-HT_{1A} (Durand *et al.,* 1999). WKY rats also displayed a reduced SERT binding capacity in the hippocampus, which was further reduced by chronic fluoxetine treatment (Durand *et al.,* 1999).

1.9 The olfactory bulbectomized rat model of depression

The olfactory bulbectomized rat model of depression is a well validated model of depression that shows behavioural, neurochemical and endocrine changes that are reflective of the clinical scenario. One of the primary advantages of this model is the response to chronic but not acute antidepressant treatment. This is very reflective of the clinical condition whereby the drugs take about 4-6 weeks before patients experience therapeutic effects. This is in contrast to other models or behavioural parameters such as the forced swim test, where acute antidepressant treatment is effective.

1.9.1 Anatomy of the olfactory bulbs

The olfactory bulbs are bilateral projections of the rostral telencephalon and make up 4% of the total brain weight in an adult rat (Cain, 1974). They can each be split into two separate, albeit interacting regions, known as the main olfactory bulb (MOB) and the accessory olfactory bulb (AOB). The MOB is organised into 6 well defined layers (Switzer *et al.,* 1985 in Kelly *et al.,* 1997) with the outermost layer containing predominately dopaminergic and GABAergic glomeruli and tufted cells (Kelly *et al.,* 1997), with the glomeruli forming glutamatergic synapses with juxtaglomerular neurons expressing both NMDA and non-NMDA receptors (Keller *et al.,* 1998). Whilst the innermost layers contain tufted and mitral cells, which are believed to be the main projection neurons projecting to regions such as the piriform cortex, amygdala and entorhinal cortex (Kelly *et al.,* 1997).

1.9.2 Physiological changes in the OB rat

OB rats gain less weight compared to sham lesion rats (Skelin *et al.,* 2008). There is an overall reduction in glucose utilisation in OB rats compared to sham (Skelin *et al.,* 2008), which may be due to increased 5-HT content in the brain, such as increased 5-HT synthesis and down regulation of 5-HT_{1A} receptors (Skelin *et al.,* 2008). This global reduction in glucose utilisation, indicative of neuronal function, may be due to regional differences in regions directly connected to the bulbs (e.g. amygdala) as well as those indirectly connected (Skelin *et al.,* 2008). Interestingly, it was the median raphe, rather than the dorsal that was considered to be important for the distinction between sham and OB rats (Skelin *et al.,* 2008). Chronic treatment with citalopram,

rather than restoring abnormal glucose utilisation to the same levels as sham levels, results in a new pattern of regional cerebral glucose utilisation (Skelin *et al.*, 2009).

OB rats also respond differently to stress compared to shams exhibiting a decrease in body temperature in response to the open field (Gigliucci *et al.*, 2014; Roche *et al.*, 2007) with an accompanying increase in heart rate (Roche *et al.*, 2007), both of which may be due to an impairment in their ability to regulate basic functions.

1.9.3 Neuroendocrine changes in the OB rat

OB rats display a significant decrease in GR levels in the hippocampus (Yang *et al.*, 2014), with a significant increase in serum corticosterone (Yang *et al.*, 2014; Rinwa and Kumar, 2014; Rinwa and Kumar, 2013) and CRH mRNA levels in the hypothalamus (Yang *et al.*, 2014). All of these parameters were reversed by chronic amitriptyline treatment (Yang *et al.*, 2014). However Jastrzebska *et al.*, (2015) found no change in corticosterone plasma levels. Although it did not reach significance, OB mice had a doubling in serum corticosterone levels compared to sham, however what is interesting is that chronic fluoxetine treatment increased corticosterone levels in both sham and OB mice (Machado *et al.*, 2012). Interestingly, acute and subchronic treatment with fluoxetine was found to increase brain and plasma corticosterone levels (Weber *et al.*, 2006). The authors fail to address why this might be the case or the molecular mechanisms behind it, however given that this phenomena occurred in mice it may highlight a species difference. Serum corticosterone levels were also increased in OB animals (Breivik *et al.*, 2006) therefore demonstrating the basal HPA axis activity in OB rats is increased. OB rats also have a heighted HPA axis response to LPS (Breivik *et al.*, 2006). They also showed a concomitant significant reduction in hippocampal GR mRNA levels (Breivik *et al.*, 2006). At basal conditions OB rats had significantly higher corticosterone levels (Marcilhac *et al.*, 1999)

1.9.4 Inflammatory changes in the OB rat

TNF-a, IL-1B, IL-6 was increased in the hippocampus of OB animals (Yang *et al.*, 2014; Rinwa and Kumar, 2014; Rinwa and Kumar, 2013) and was also increased in the cerebral cortex (Rinwa and Kumar, 2014) which was reversed by chronic amitriptyline treatment (Yang *et al.*, 2014). IL-1 serum concentration was also increased in OB rats (Song *et al.*, 2009). OB rats exhibit a significant reduction in

TNF-α in response to the LPS challenge (Breivik *et al.,* 2006). Minocycline resulted in an attenuation of the OB of hyperactivity, without affecting locomotion in sham animals, an effect which was evident a week after cessation of treatment (Borre *et al.,* 2012). However in the aforementioned study, minocycline treatment began before surgery and therefore it could be suggested that it has some neuroprotective effects. OB lesion may cause the activation of microglia, resulting in harmful by products (Borre *et al.,* 2012) and therefore minocycline may prevent this from occurring.

1.9.5 Behavioural changes in the OB rat

It is thought that the OB syndrome is due to a retrograde neuronal degeneration, in addition to the non-physiological circuitry that results from removal of the bulbs (Skelin *et al.,* 2008). Hyperactivity in the open field is one of the hallmarks of this model of depression (El Mansari *et al.,* 2014, Roche *et al.,* 2007), a characteristic that is reversed by chronic fluoxetine treatment (Roche *et al.,* 2007) however it has been found that with repeated exposure the rats can become habituated and the hyperactivity is no longer evident (Gigliucci *et al.,* 2014). It also appears that attenuation of the hyperactivity in photocell cages by chronic antidepressant treatment may depend on the length of treatment, as 14-day sertraline treatment does not reverse the hyperactivity but 21-days of dosing does (Bissette *et al.,* 2001). In many cases, drug treatment has no effect on the behaviour of the sham rats (Roche *et al.,* 2007; Mar *et al.,* 2000) however, during the literature review for this introduction it was noticed that many papers do not include a sham treatment group. Given that any drug effects in OB animals would be considered therapeutic, it is therefore prudent to consider any effects in sham animals as side effects. Thus if sham animals are not treated the same as OB animals in drug studies then it cannot be said with confidence that resulting alteration in OB animals is due to a therapeutic effect, rather than a lack of side effects in the sham animals.

One of the common issues with the OB model is related to the loss of the sense of smell – how can it be definitively known that any behavioural changes are not simply due to the animals inability to smell. ZnO_4 is used to render animals anosmic but the hallmark behaviour is not evident in these animals (Mar *et al.,* 2000) and therefore it can be said the changes exhibited in the OB are due to the retrograde degeneration in the brain stem and limbic areas which are heavily innervated by the bulbs.

1.9.6 Central serotonergic investigations in the OB model

1.9.6.1 Serotonin and 5-HIAA (Table 1.7)

Serotonin is one of the most widely studied neurochemical systems (Steinfeld *et al.,* 2015). The DRN and the MRN both project to the olfactory bulbs (McLean and Shipley, 1987). Studies in mice have shown that the DRN mostly projects to the granule cell layer, whereas the MRN projects to the glomerular layer (Steinfeld *et al.,* 2015).

The serotonergic system has long been implicated in the pathophysiology of depression and the OB has also shown to have alterations in the system in numerous brain regions including the frontal cortex, hippocampus, amygdala, DRN, substantia nigra (Nakagawasi *et al.,* 2003, Watanabe *et al.,* 2003, Zhou *et al.,* 1998), however some studies have found there are no differences in this neurotransmitter (Burke *et al.,* 2010). However, given the variability between studies, including housing conditions and the length of time following lesion at which the animals are sacrificed for analysis, these could all have an effect on the levels of neurotransmitter detected.

Studies have looked at numerous regions implicated in depression in an effort to determine the effects of lesion on the levels of the neurotransmitter, its metabolite and its turnover. The majority of studies looking at these parameters in the OB use rats and tend to look at regions of the brain that have been implicated in depression such as the hippocampus, amygdala, prefrontal cortex, frontal cortex (Chang *et al.*, 2016, Jastrzebska *et al.,* 2015, Burke *et al.,* 2010) as well as areas that may be considered less commonly associated such as the striatum (Maturana *et al.,* 2015) and hypothalamus (Saitoh *et al.,* 2008). It has been proposed that measuring these parameters of the serotonergic system are good indicators of the presynaptic functioning of the system and together with SERT and TPH expression is the best way to provide a comprehensive analysis of any presynaptic changes that occur in this model (Zhou *et al.,* 1998; Huether *et al.,* 1997).

When discussing changes in the levels of serotonin it is also important that it is not considered to be an independent factor but the relationship between it and its metabolite must also be considered. This in turn allows the turnover of the neurotransmitter to be assessed, therefore even if the levels of 5-HT are increased but

so is the level of the turnover, then this would also cancel out the effect of the increased 5-HT. It is interesting the number of studies that do not look at each stage in transmission i.e. they may only investigate serotonin and 5-HIAA alterations or serotonin, TPH and SERT without investigating alterations in the receptors (Watanabe *et al.,* 2006; Prins *et al.,* 2011, Haswegawa *et al.,* 2005, Watanabe *et al.,* 2003, Harkin *et al.,* 1999, Van der Stelt *et al.,* 2005, Garris *et al.,* 2003, Jastrzebska *et al.,* 2015, Jimenez-Sanchez *et al.,* 2016, Prins *et al.,* 2010, Redmond *et al.,* 1997). What is also striking about some of these studies, that in those that use drug treatment, not all treat the sham groups with the antidepressant and only treat the OB animals (Marcilhac *et al.,* 1999), thus discounting the possibility that a treatment could alter serotonin levels in normal animals. This therefore throws suspicion on any changes found in the OB animals as it could be a drug effect as opposed to a lesion effect or drug x lesion interaction. Given that serotonin levels can also be influenced by many other factors, taken alone it is considered to be the least reliable measure of changes in serotonin terminal density (Huether *et al.,* 1997)

When reviewing the literature concerning the levels of the 5-HT, 5-HIAA and the turnover it was striking the discrepancies that can exist in the results, using the same analytical techniques and regions. In the frontal cortex no significant changes between sham and OB animals have been found (Zhou *et al.,* 1998, Hellweg *et al.,* 2007, Roche *et al.,* 2012, Jastrzebska *et al.,* 2015, Wang *et al.,* 2012 and Xu *et al.,* 2005), whereas others have found that OB levels have significantly decreased (Saitoh *et al.,* 2008, Redmond *et al.,* 1997, Chang *et al.,* 2016). The same can also be said for the amygdala and hippocampus where some have found no change in the levels (Burke *et al.,* 2010; Wang *et al.,* 2012), whilst others report a decrease (Marcilhac *et al.,* 1999, Saitoh *et al.,* 2008, Chang *et al.,* 2016; Jastrzebska *et al.,* 2015).

The changes observed could be due to the time of day the animals are sacrificed, as serotonin is known to be involved in the regulation of the circadian rhythm, behavioural tests the animals have gone through before sacrifice and even possibly housing conditions. This reinforces the need to also measure 5-HIAA levels as well as 5-HT so that the turnover can also be measured. Just looking at serotonin and if the levels are decreased it could lead to the incorrect conclusion that there may be

hypoinnervation in that particular region, however if the levels of 5-HIAA and turnover are increased it may actually suggest a hyperinnervation (Zhou *et al.,* 1998).

The circadian rhythm is controlled by a pacemaker located in the suprachiasmatic nucleus (SCN), controlling the secretion of the primary circadian hormone, melatonin (McKenna *et al.,* 2017). In addition, specific genes, such as the Circadian Locomotor Output Cycles Kaput (CLOCK) have also been reviewed (Schuch *et al.,* 2017). In a review by Ciarleglio *et al.,* (2011), the interaction between the serotonergic and circadian systems are interconnected at a neuroanatomical and genetic level, with the SCN receiving direct input from the MRN. With regards to the OB model, in rats it causes an increase in mean plasma corticosterone levels and dampens the circadian rhythm of body temperature and motor activity (Marcilhac *et al.,* 2008). In mice, fluoxetine normalises chronically elevated mean activity levels and shorten the free running circadian period (Possidente *et al.,* 1996). The authors suggest that this may be a hyposerotonergic mediated effect of OB lesion on activity levels and circadian period (Possidente *et al.,* 1996). Studies in rodents have revealed that serotonin release in the SCN of rats maintained in a standard 12 hr light:dark cycle are characterised by a sharp increase in the light dark transition from low daytime levels to peak values, with peak values of extracellular 5-HIAA occurring 2-3 hr after the serotonin peak in the dark period (Barassin *et al.,* 2002). Central hippocampal 5-HT levels are significantly higher in the dark phase compared to the light, peaking 1 hr after lights off, oscillating throughout the dark period (Sanchez *et al.,* 2008). CSF levels of 5-HIAA are also reduced, though not significantly, in the dark phase compared to the light phase (Egashira *et al.,* 2000). This appears to be in agreement with Wada et al., (1998) who demonstrate that although 5-HT levels could not be detected in the CSF, 5-HIAA levels were minimal during the dark phase, peaking at the beginning of the light phase. Plasma concentration of 5-HT was assessed in rats under the same light conditions, however it was not possible to say if the highest levels were found in the light or dark period as peaks were identified 2 hr after lights off and again 1 hr after lights on (Sanchez *et al.,* 2008).

With regards to sex differences, the rate of 5-HT synthesis averaged over different brain areas, is found to be higher in men compared to women (Nishizawa et al., 1997). It was suggested that this could be related to a high incidence of major unipolar depression in women (Nishizawa et al., 1997). OB female rats exhibit significant

hyperactivity in the open field, whilst also spending less time in the centre of the arena in the initial test period (Stepanichev et al., 2016). In a recent study "rats of either sex" were used, whereby a significant reduction in hippocampal and cerebral cortical levels of 5-HT in OB rats (Thakare *et al.,* 2017). Given that the ratio of males and females used was not given, it cannot be said whether there could have been an effect of gender. No alteration in open field locomotor activity has been reported in both male and female rats (Stock *et al.,* 2000). Sex differences with regards to changes in the serotonergic system, to our knowledge, has not been measured in the one study. The majority of studies assessing serotonergic changes in OB rats do so in males. Of those using female rats (Shin *et al.,* 2017) the same pattern of TPH alterations were found compared to those using male rats (Saitoh *et al.,* 2007; Saitoh *et al.,* 2008).

Raphe 5-HT neurons are present at embryonic day 10-12 in rodents, with maturation of 5-HT innervation protracted to PND 21 in rodents (reviewed in Teissier *et al.,* 2017). OB lesions carried out in neonatal rats (PND 7) and tested 4 or 7 weeks post lesion, hyperactivity in a novel environment was evident in those rats tested at 4 weeks post lesion (pre-pubertal) but not those tested 7 weeks post lesion (post-pubertal) (Flores *et al.,* 2014). In addition to assessing behavioural changes, the size of the bulbs in pre- and post-pubertal rats revealed reduced OB size compared to sham and control rats (Flores *et al.,* 2014). This would suggest that subventricular zone postnatal neurogenesis may play a key role in the increased size of the bulbs (Flores et al., 2014). With regards to the serotonergic system, OB lesion carried out on male rats at 10 weeks or 9 months of age, had no effect on 5-HT_{1A} receptor binding in the cerebral cortex or striatum, regardless of age or lesion (Slotkin *et al.,* 2005). 5-HT_{2A} receptor binding however was affected by age, with cerebral binding decreased in young but not old OB rats, whilst in the striatum OB rats demonstrate a decrease in old but not young rats (Stotkin *et al.,* 2005). Assessment of SERT binding revealed that in the frontal/parietal cortex, in young animals, levels were increased, whilst in older rats a decrease was found (Slotkin *et al.,* 1999). A similar pattern was found in the hippocampus, with no loss of hippocampal tissue weight in OB rats, the authors concluded that the changes seen were not due to atrophy of the hippocampus (Slotkin *et al.,* 1999). The receptor studies, together with investigations into adenylate cyclase stimulation, led the authors to conclude that 5-HT system adaptations in response to

OB lesion differs between the young and the old brain, that emerge with neuronal damage but are not evident under basal conditions (Slotkin *et al.,* 2005).

Thus it is important that all parameters are analysed to take into account the how easily influenced serotonin levels can be. To further enhance our knowledge as to the changes that occur in serotonergic innervation in this model, it is important that other parameters are also measured including TPH the rate limiting step in the serotonergic synthetic pathway.

Although it is important to assess aspects of the serotonergic circuitry by looking at how the various stages in transmission is affected by the lesion, determining how active the neurons are is also important. Up to 18-days following lesion the average rate of firing of serotonergic neurons in the DRN was significantly decreased in OB compared to sham (El Mansari *et al.,* 2014) and subsequently returned to normal levels 4 weeks following lesion (El Mansari *et al.,* 2014). However paroxetine did not have any effect on the normalisation of the firing rate and the firing rate remained significantly reduced but was normalised with the addition of bupropion (El Mansari *et al.,* 2014).

The role serotonin has in the OB model can be supported by toxicity studies, whereby local injection of the 5-HT toxin 5, 7-DHT, resulted in a syndrome identical to the OB, whereas injection with the dopaminergic toxin 6-OHDA did not (Cairncross *et al.,* 1977). Thus, highlighting the central role that serotonin plays in the syndrome – however that is not to say that the serotonergic system is solely responsible for the changes shown in the model, as it is known that the serotonergic system is integrative and interacts with numerous other neurobiological systems.

In conclusion, neurotoxic studies provide evidence that aberrant serotonergic signalling plays a role a underlying cause of the OB rat model. However the literature is conflicting as to whether it is a model of hyposerotonergic or hyperserotonergic signalling. This could be due to the methodologies employed, the regions investigated or the length of time the lesion has been established.

Table 1.7 Alterations in 5-HT, 5-HIAA and 5-HIAA/5-HT in the OB model

Species	Sex	Methodology	Regions	5-HT	5-HIAA	5-HIAA/5-HT	Reference
SD	Male	Autoradiography (5-HT Synthesis)	FC, Amygdala, Hippocampus (Dorsal and Ventral), DRN	N/S	N/S	↑OB	Watanabe *et al.*, 2006
Wistar	Male	HPLC	FC	↔	↑OB	↑OB	Zhou *et al.*, 1998
			Hippocampus, Midbrain	↔	↔	↔	
SD	Male	HPLC	Hypothalamus	↔	↔	↔	Marcilhac *et al.*, 1999
			Amygdala	↓OB	↔	↔	
SD	Male	Micro dialysis & HPLC	PFC, Hippocampus (Dorsal)	↔	N/S	N/S	Prins *et al.*, 2011
Wistar	Male	HPLC	FC	↓OB	↔	↑OB	Saitoh *et al.*, 2008
			Hippocampus	↓OB	↓OB	↔	
			Hypothalamus	↑OB	↔	↓OB	
			Amygdala	↓OB	↓OB	↑OB	
C57Bl/6N Mice	Male	HPLC	Hippocampus	↔	↓OB	↔	Hellweg *et al.*, 2007
			FC	↔	↓OB	↓OB	
			STR	↔	↔	↔	

Table 1.7 Alterations in 5-HT, 5-HIAA and 5-HIAA/5-HT in the OB model (cont'd)

Species	Sex	Methodology	Regions	5-HT	5-HIAA	5-HIAA/5-HT	Reference
SD	Male	Autoradiography	FC, Amygdala, Hippocampus	N/S	N/S	↑OB	Hasegawa *et al.*, 2005
		(5-HT Synthesis)	DRN	N/S	N/S	↓OB	
SD	Male	Autoradiography	FC, Amygdala, Hippocampus	N/S	N/S	↑OB	Watanabe *et al.*, 2003
		(5-HT Synthesis)	DRN	N/S	N/S	↓OB	
SD	Male	HPLC	Amygdala	↔	↔	N/S	Harkin *et al.*, 1999
SD	Male	Microdialysis & HPLC	Amygdala, Hippocampus (2 weeks and 5 months)	↓OB	↔	N/S	Van der Stelt *et al.*, 2005
Wistar	Male	HPLC	FC	↔	N/S	N/S	Huether *et al.*, 1997
Wistar	Male	HPLC	Hippocampus	↓OB	↓OB	↓OB	Pudell *et al.*, 2014
Mice	Male	HPLC	FC	↔	↓OB	↔	Roche *et al.*, 2012
			Amygdala	↑OB	↔		
			Hippocampus, Hypothalamus	↔	↔	↔	
Listerhooded	Male	HPLC	PFC, Hypothalamus, Hippocampus, Amygdaloid cortex, Thalamus, Cerebellum	↔	↔	↔	Burke *et al.*, 2010
Mice	Male	HPLC	Olfactory tubercules	↑OB	N/S	N/S	Garris *et al.*, 2003
			Hypothalamus	↔	N/S	N/S	

Table 1.7 Alterations in 5-HT, 5-HIAA and 5-HIAA/5-HT in the OB model (cont'd)

Species	Sex	Methodology	Regions	5-HT	5-HIAA	5-HIAA/5-HT	Reference
Wistar	Male	LC/MS	PFC	↓OB	↔	N/S	Jastrzebska *et al.*, 2015
			FC	↔	↔	N/S	
			Hippocampus	↓OB	↓OB	N/S	
			Nucleus accumbens, Cerebellum	↑OB	↔	N/S	
			Dorsal STR	↔	↑OB	N/S	
Wistar	Male	Microdialysis	Medial PFC	↓OB	N/S	N/S	Jimenez-Sanchez *et al.*, 2016
SD	Male	Microdialysis & HPLC	PFC	↔	↔	N/S	Prins *et al.*, 2010
SD	Male	HPLC	FC	↓OB	N/S	N/S	Redmond *et al.*, 1997
SD	Male	HPLC	Cortex	↔	↑	↔	Wang *et al.*, 2012
			STR, Hippocampus, Hypothalamus, Thalamus, Midbrain	↔	↔	↔	
			Medulla (medulla, pons and oblongata)	↓OB	↔	↑OB	
Wistar	Male	HPLC	Hippocampus, FC	↓OB	↑OB	↔	Chang *et al.*, 2016
Wistar	Male	HPLC	STR	↔	↔	↔	Maturana *et al.*, 2015
			Hippocampus	↓OB	↔	↑OB	

Table 1.7 Alterations in 5-HT, 5-HIAA and 5-HIAA/5-HT in the OB model (cont'd)

Species	Sex	Methodology	Regions	5-HT	5-HIAA	5-HIAA/5-HT	Reference
Albino	Male	Microdialysis	Nucleus accumbens shell	↓OB	↓OB	↑OB	Ruda-Kucerova *et al.*, 2015
SD	Male	Microdialysis & HPLC	Olfactory tubercle	N/S	↑OB	N/S	Masini *et al.*, 2004

N/S = Not specified. SD = Sprague-Dawley ↑ OB = Increased in OB lesion group vs sham counterparts; ↓OB = Decreased in OB lesion group vs sham counterparts; ↔ = no change between sham lesion and OB lesion; DRN = dorsal raphe nucleus; FC = frontal cortex; STR = striatum; PFC = prefrontal cortex

1.9.6.2 TPH (Table 1.8)

As already discussed TPH is the rate limiting step in the synthesis of serotonin with the majority of this enzyme being found in the DRN and MRN. There is a paucity of studies investigating changes in TPH in this model of depression with the DRN, MRN and the frontal cortex being the most commonly investigated. In general, there have been no changes found in the MRN between sham and OB animals (Saitoh *et al.,* 2007, Saitoh *et al.,* 2008). However, in the DRN it has been shown that there are reduced numbers of TPH positive cells in OB animals compared to sham (Saitoh *et al.,* 2007, Saitoh *et al.,* 2008), whereas increases have been found in the frontal cortex (Grecksch *et al.,* 1997, Huether *et al.,* 1997). Zhou *et al.,* (1998) investigated TPH levels in the occipital cortex, hippocampus, hypothalamus, midbrain, brain stem and cerebellum and found that there were no significant changes between sham and OB animals. Decreased levels of TPH in the DRN was found to be reversed by subchronic fluvoxamine and desipramine (Saitoh *et al.,* 2007; Saitoh *et al.,* 2008) but not restored to sham equivalent levels (Saitoh *et al.,* 2007). The same can be said for the increase found in the FC, where they were significantly decreased with imipramine treatment (Grecksch *et al.,* 1997, Huether *et al.,* 1997).

In conclusion, the literature is conflicting with regards to altered TPH expression in the OB rat, which is responsive to certain antidepressants. A reduction is found at the level of the cell bodies in the DRN but the results of the terminal regions vary. This could suggest selective regional differences, however differences in methodologies could also be a factor. TPH positive cells in the DRN were measured immunohistochemically, whilst those in the terminal regions were measured via ELISA.

Table 1.8 Alterations in TPH in the OB model

Species	Sex	Methodology	Regions	TPH	Reference
SD	Female	IHC - 40µm Western blot	DRN	↓OB	Shin *et al.*, 2017
Wistar	Male	IHC - 20 µm	DRN MRN	↓OB ↔	Saitoh *et al.*, 2007
Wistar	Male	ELISA	FC	↑OB	Greksch *et al.*, 1997
Wistar	Male	ELISA	FC	↑OB	Huether *et al.*, 1997
Wistar	Male	IHC - 20µm	DRN MRN	↓OB ↔	Saitoh *et al.*, 2008
Wistar	Male	ELISA	FC Occipital cortex, Hippocampus, Hypothalamus, Midbrain, Brain stem, Cerebellum	↑OB ↔	Zhou *et al.*, 1998

OB = Increased in OB lesion group vs sham counterparts; SD = Sprague-Dawley; ↓OB = Decreased in OB lesion group vs sham counterparts; ↔ = no change between sham lesion and OB lesion; DRN = dorsal raphe nucleus; MRN = Median raphe nucleus; FC = frontal cortex; PFC = prefrontal cortex

1.9.6.3 Investigation of 5-HT receptors in the OB model

The two main receptors which have been implicated in depression are the 5-HT_{1A} and the 5-HT_{2A} receptors, as antidepressants are believed to exert some of their therapeutic effects through these two receptors. In order to assess how these receptors are altered in this model of depression, agonists or antagonists for the receptors can be used to determine their functionality or autoradiography can be used to assess the level of receptors found in numerous brain regions.

1.9.6.3.1 5-HT_{1A} receptor in the OB model (Table 1.9)

This receptor is expressed both pre and post-synaptically, where it is thought that chronic antidepressant treatment, results in the desensitisation of this autoreceptor. The 5-HT_{1A} receptor is abundantly expressed in the brain in many areas that received input from the DRN, including the prefrontal cortex, limbic system and hypothalamus (Jiang *et al.,* 2014). Many of the papers investigating the aberrations of the receptor in the model have done so with the interest of looking at increasing the efficacy of current antidepressants, the theory being that if antidepressants work by desensitising these receptors, then combing them with an antagonist may accelerate the therapeutic response.

In terms of the number of receptors, autoradiographic studies show that there is a significant decrease in OB animals in numerous areas, including the prelimbic cortex, cingulate cortex, hippocampus, dorsal DRN (Sato *et al.,* 2008). It is hypothesised that the decrease in 5-HT_{1A} receptor in the OB model could be due to an increase of serotonin found in the OB, as a result on increase 5-HT synthesis (Sato *et al.,* 2008). However the increase in the serotonin synthesis would correlate with an already down regulated 5-HT_{1A}, therefore the antidepressant induced alterations observed here is proposed to be fine regional balancing between the different regions (Sato *et al.,* 2008). Based on the results in this study it was suggested that it is inadequate to look at the 5-HT_{1A} receptors alone (Sato *et al.,* 2008).

However by combining these studies with the addition of a 5-HT_{1A} agonist, it also provides insights into the functionality of the receptor in the model. One commonly used agonist is 8-OH-DPAT, where the only reported result of treatment with this drug is the induction of hypothermia. Some find that there is no effect of lesion (Cryan *et*

al., 1999, Harkin *et al.*, 1999; McGrath and Norman, 1998) and with 14-days of treatment with Paroxetine there was a significant reversal of the hypothermic response in both sham and OB rat (Cryan *et al.,* 1999) but none with venlafaxine treatment (McGrath and Norman, 1998). It is thought that this hypothermic effect may be mediated by the postsynaptic receptors (O'Connell *et al.*, 1992). With 7 and 14 days treatment with sertraline + reboxetine, the hypothermia in the OB rat was reversed when compared to the OB treated controls (Harkin *et al.*, 1999). This suggests that antidepressants targeting the serotonergic system could alter the 5-HT_{1A} receptor sensitivity (Harkin *et al.*, 1999)

Acute treatment with the 5-HT_{1A} agonist 8-OH-DPAT resulted in a significant increase in OF locomotion in the sham rat but had no effect in the OB (Jiang *et al.*, 2014), whereas chronic treatment significantly reduced the OB hyperactivity, when compared to the OB vehicle-treated group but had no effect on sham animals (Jiang *et al.,* 2014), therefore suggesting that chronic 8-OH-DPAT treatment had normalised the hyperactivity in the OB rats.

In conclusion, reductions in 5-HT_{1A} receptor number could be due to altered serotonin levels but it does not appear that the alteration in levels affects the functionality of the receptors , but antidepressant treatment may increase the sensitivity regardless of lesion type.

Table 1.9 Alterations in 5-HT_{1A} receptor in the OB model

Species	Sex	Methodology	Regions	Drug	1A	Reference
SD	Male	Hypothermia	N/S	8-OH-DPAT	↔	Cryan *et al.*, 1999
SD	Male	Hypothermia	N/S	8-OH-DPAT	↔	Harkin *et al.*, 1999
SD	Male	Autoradiography	Anteriol olfactory nucleus, Entorhinal cortex, DRN (ventral)	[3H]-8-OH-DPAT	↔	Sato *et al.*, 2008
			Cortices (Prelimbic, Cingulate, Entorhinal, Primary motor, Primary somatosensory), Medial amygdaloidal nucleus, Hippocampus, Ventromedial hypothalamic nucleus, DRN (dorsal), Raphe Magnus, Pontine Raphe nuclues	[3H]-8-OH-DPAT	↓OB	

SD = Sprague-Dawley ↑ OB = Increased in OB lesion group vs sham counterparts; ↓OB = Decreased in OB lesion group vs sham counterparts; ↔ = no change between sham lesion and OB lesion; DRN = dorsal raphe nucleus; MRN = median raphe nucleus

1.9.6.3.2 5-HT_{2A} receptor in the OB model (Table 1.10)

In terms of the densities of receptors, it has been found that in nearly all regions of the brain there is an overall increase in 5-HT_{2A} densities in OB animals compared to sham, including the amygdaloid cortex, hippocampus, dorsal raphe and prefrontal cortex (Sato *et al.,* 2010). In addition there was no significant difference in the frontal or occipital cortex in sham and OB animals the number of 5-HT_{2A} receptors in sham and OB rats (Mudunkowtuwa and Horton, 1996). However treatment with desipramine for 7, 14 and 21-days significantly decreased the numbers of receptors, regardless of lesion in the frontal cortex (Mudunkowtuwa and Horton, 1996), with the magnitude of the decrease becoming more pronounced the longer the treatment time (Mudunkowtuwa and Horton, 1996).

OB rats exhibit learning deficits in the step-through passive avoidance task when compared to sham counterparts (Takeuchi *et al.,* 1997), however an acute treatment with a 5-HT_{2A} antagonist improved this learning deficit (Takeuchi *et al.,* 1997). LSD, the non-selective 5-$HT_{1A/2A}$ agonist, has also been shown to reverse learning deficits in OB rats (Buchborn *et al.*, 2014), whilst reducing the increase in 2A binding in the hippocampus (Buchborn *et al.,* 2014) and not affecting the levels in the sham rats (Buchborn *et al.,* 2014). This is accompanied by a reduction in signalling of the 2A receptor in OB rats, which was reversed by LSD treatment but the opposite happened in sham rats (Buchborn *et al.,* 2014). Whilst in the frontal cortex the increase in OB rats was not normalised by LSD treatment (Buchborn *et al.,* 2014) It is thought that LSD induced normalisation of the avoidance learning may be a re-balancing of the hippocampal 2A vs 1A signalling (Buchborn *et al.*, 2014).

5-HT_{2A} antagonists have also been shown to reduce hyperactivity in the open field after a 14-day treatment (Pandey *et al.,* 2010) an effect that manifests earlier when paired with amitriptyline for 7-days (Pandey *et al.,* 2010). Given the OB model is known for only responding to chronic antidepressant treatment, the fact that the hyperactivity in the open field was attenuated after 7-days of treatment with a 5-HT_{2A} + SNRI combination treatment would suggest that perhaps targeting this receptor would lead to an early onset of antidepressant action (Pandey *et al.,* 2010). Chronic treatment with the 5-HT_{2A} agonist, TCB-2, did not result in a reduction in immobility time in the FST in OB mice (Islam *et al.,* 2014).

It is known depression can occur at any point in life, however the symptoms and the recurrence of the disease is different between young, adolescent and geriatric patients (Fiske *et al.,* 2009) Therefore it was interesting to find that 5-HT_{1A} receptor binding was not altered in young and aged OB rats in the cerebral cortex or the striatum but the 5-HT_{2A} receptor was regionally affected, being significantly decreased in the striatum of OB rats but not in the cerebral cortex of aged rats, whilst significantly decreased in the cerebral cortex of adolescent OB rats (Slotkin *et al.,* 2005). Therefore this would suggest that the ability of the serotonergic system to adapt to OB lesions differs markedly with age and may give insight into the varying responses to antidepressant treatment in elderly patients compared to those who experience depression earlier in life (Slotkin *et al.,* 2005).

The functionality of the receptor has also been investigated in terms of behaviour in OB mice. DOI dose dependently increased head twitches 14 days following lesion but not 3 and 7 days following lesion (Oba *et al.,* 2013) and this was inhibited by that the 2A antagonist ketanserin and was reduced by chronic fluvoxamine treatment (Oba *et al.,* 2013). Stimulation with 5-HT induces a significant increase in head twitches in OB animals (Nakagawasi *et al.,* 2003) and it was proposed that this could be due increased sensitivity of the receptor as a result of degeneration in regions of the brain that have direct and indirect connections with the olfactory bulbs (Nakagawasi *et al.,* 2003).

In conclusion, alterations in the number of receptors appears to be dependent on the region assessed. This alteration in cell number is accompanied by regionally dependant sensitivities of the receptors, which are altered by LSD treatment. With regards to behaviour, a role in antidepressant treatment is postulated, which also suggest receptor sensitivity.

Table 1.10 Alterations in 5-HT_{2A} receptor in the OB model

Species	Sex	Methodology	Regions	Drug	2A	Reference
SD	Male	Radioligand binding	FC, Occipital cortex	[3H]-ketanserin	↔	Mudunkowtuwa and Horton, 1996
SD	Male	Radioligand binding	FC	[3H]-ketanserin	↔	McGrath and Norman, 1998
SD	Male	Autoradiography	Amygdala, Hippocampus, FC, Dorsal raphe	[3H]-ketanserin	↑OB	Sato *et al.*, 2010
SD	Male	Radioligand binding	STR, Cerebral cortex	[3H]-ketanserin	↓OB	Slotkin *et al.*, 2005
ddY Mice	Male	Functionality	N/S	5-HT	↑OB	Nakagawasi *et al.*, 2003

SD = Sprague-Dawley N/S = not specified ↑ OB = Increased in OB lesion group vs sham counterparts; ↓OB = Decreased in OB lesion group vs sham counterparts; ↔ = no change between sham lesion and OB lesion; DRN = dorsal raphe nucleus; MRN = median raphe nucleus; FC = frontal cortex; STR = striatum

1.9.6.4 SERT in the OB model (Table 1.11)

There was a significant increase in the SERT density in the FC of OB rats compared to sham (Grecksch *et al.,* 1997) and this was not altered by subchronic imipramine treatment (Grecksch *et al.,* 1997). OB mice exhibit significantly reduced SERT binding in the lateral globus pallidus and the lateral septum (Licht *et al.,* 2010) which is could be due to decreased expression or increased degradation. Sato *et al.,* (2010) found increases in the hippocampus and frontal cortex, among others, effects that were attenuated by the 5-HT_{1A} receptor agonist, buspirone (Sato *et al.,* 2010) .The increases in these regions may be due to neuronal sprouting or perhaps an adaptive response to altered 5-HT levels.

In conclusion, SERT density in the OB model is altered on a regional basis, which may be due to adaptive changes in response to abherant serotonergic signalling.

Table 1.11 Alterations in SERT in the OB model

Species	Sex	Methodology	Regions	SERT	Reference
Mice	Male	Autoradiography	Caudate putamen (Frontal), Hippocampus (Dorsal and Ventral), Basolateral amygdala, FC, NAC Shell, Olfactory tubercle	↔	Licht *et al.*, 2010
			Lateral globus pallidus, Lateral Septum	↓OB	
SD	Male	Autoradiography	Anterior olfactory nucleus, Hippocampus, FC, Lateral geniculate	↑OB	Sato *et al.*, 2010
Albino Mice	Female	PCR	Hypothalamus	↓OB	Poretti *et al.*, 2015

↑ OB = Increased in OB lesion group vs sham counterparts; ↓OB = Decreased in OB lesion group vs sham counterparts; ↔ = no change between sham lesion and OB lesion; FC = frontal cortex

1.10 Limitations of current literature

As has been mentioned on numerous occasions the role of serotonin in therapeutic action of antidepressant drugs has been known since their early development. Numerous clinical cases have shown a variety of changes at each stage of serotonergic neurotransmission. These observations have been based on genetic investigations, but more frequently through peripheral measurements (biomarkers), *in vivo* imaging as well as post-mortem analysis. Although these methods give valuable insights into alterations, especially in regard to clinically relevant brain regions, they are not without limitations. The heterogeneous nature of the disease means that in is often difficult to stratify patient cohorts based on the subtype of depression, thus confounding results in the literature could be due differing types of depression in the patients. Post-mortem analysis, although once again useful, is still subject to difficulty of standardising the sample population due to the unfortunate circumstances samples can be collected. It could also be argued that this population represents those suffering from a severe depressive episode.

The advantage of animal models is the ability to control many of factors that clinical investigations cannot – such as depressive subtype, life exposures as well as treatment type and duration. Given that the OB model is one of the most well validated model of depression, it was striking the lack of investigations into the serotonergic system that have taken place. It cannot be denied that each aspect of the serotonergic signalling cascade has been investigated but often these aspects are measured in isolation. This eliminates the opportunity to assess if changes in one parameter has consequences on the functioning or expression of another parameter, in other words are there compensatory mechanism. Another major gap was the lack of investigations into the functionality of the two main receptors implicated in depression.

In addressing this gap in the literature, not only is it important to select the right elements of serotonergic transmission but it also crucial that the appropriate neuroanatomical regions and methodological approaches are selected. Neuroanatomical selection was based on regions that are relevant to depression, are known to have high levels of serotonergic innervation but will also be affected by the retrograde degeneration that occurs after bulbectomy, To this end, four main regions of interest were select – DRN, prefrontal cortex, hippocampus and amygdala.

With regards to methodological techniques, many were chosen based on previous investigations looking the parameters, whilst also considering the breadth of information that could be acquired. For example analysis of TPH expression via immunohistochemical staining was not only proven to be a common method using in clinical and non-clinical research but it would also further in-depth analysis with regards to the topography of the DRN to be assessed.

1.11 Hypothesis

The hypothesis under investigation was that the OB rat model of depression would have alterations in the central serotonergic structure and functionality and to assess whether such changes could be attenuated by chronic antidepressant treatment.

1.12 Research objectives

To that end, the primary objective of the work herein was to assess multiple aspects of central serotonergic transmission, in clinically relevant brain regions using the most appropriate methodological approaches. The goal was to first identify the components that would be assessed and through literature review it was decided that each stage of neurotransmission would be assessed represents the main components to be assessed, encompassing the serotonergic cell bodies as well as terminal projection regions (Figure 1.8).

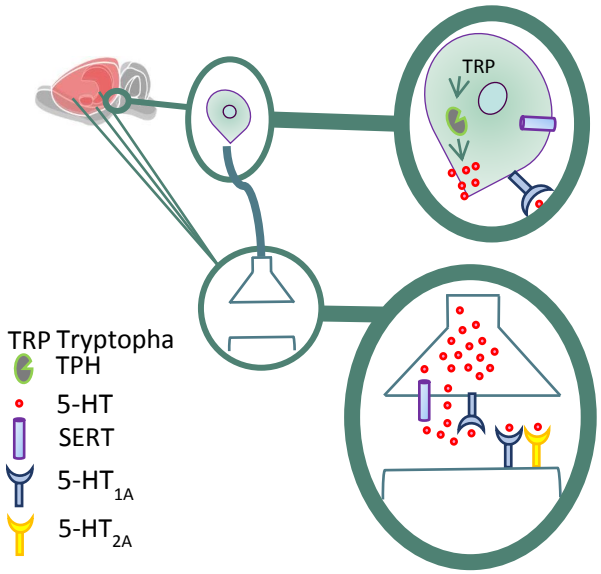


Figure 1.8 Schematic representation of the central serotonergic transmission

Having selected the main components to be assessed in the previously mention regions, the second primary goal was to determine how these parameters would be measured. As mentioned TPH is to be assessed via immunohistochemical analysis, with SERT also measured this way. This was chosen as it had been shown previously that immunohistochemical staining of SERT gave a better characterisation of the serotonergic fibres than 5-HT staining (Neilsen *et al.,* 2006) combined the fact that the commonly used method of autoradiography was not available this was the next best option. Receptor functionality is assessed via the acute challenge of receptor specific agonists, which have been shown to elicit a characteristic behavioural syndrome.5-HT and 5-HIAA was determined using HPLC with brain tissue, as this abolished the risk of peripheral serotonin contamination.

The secondary goal was to assess how antidepressant treatment augmented each of these parameters. To achieve this, 3 antidepressants were chosen, representing 3 different classes and thus distinct methods of modulating the serotonergic system.

The specific aims in undertaking these studies are as follows:

1. Temporal analysis of behavioural and central serotonergic changes in the OB model, with 2, 4 and 6 weeks following lesion chosen as the time points
2. To determine the optimal dose of the two serotonergic receptor agonists needed to elicit the stereotyped behaviours of the serotonin syndrome
3. Once optimal time course and agonist dose had been determine, to use three established antidepressants (fluoxetine, venlafaxine and imipramine) and assess their ability to attenuate lesion induced behavioural and serotonergic signalling alterations.

Chapter 2:

Materials and Methods

This chapter describes the general Materials and Methods used in the studies that comprise this thesis. Where necessary, more detailed information pertaining to individual studies is provided in the Materials and Methods sections of the Results chapters.

2.1 Materials

2.1.1 Rat husbandry

Rats: male Sprague-Dawley strain, bred in-house (CNS Pharmacology Laboratory, NUI Galway) and housed in cages with plastic cage bottoms (42cm x 25.5cm x 13cm) with a metal cage top and plastic water bottles: North Kent Plastics (Coalville, Leicestershire, United Kingdom)

Bedding: 3 Rs Paper Bedding, FibreCycle Ltd. (Scunthorpe, Lincolnshire, United Kingdom)

Diet: Harlan Teklad global diets chow, Envigo RMS (UK) Ltd., (Bicester, Oxfordshire, United Kingdom)

Temperature/Humidity Monitor: Radionics Ltd. (Galway, Ireland)

Weighing scales: Mason Technology (Dublin, Ireland) or Ohaus, CS Series: Radionics Ltd. (Dublin, Ireland)

2.1.1.1 Specific materials for breeding purposes

Rats: male and female Sprague-Dawley strain: Charles River (Margate, Kent, United Kingdom)

Bedding: Goldflakes Bedding, LBS Ltd., (Surrey, United Kingdom)

Nesting Material: Safe Bed fluff bedding (Petworld, Galway, Ireland)

2.1.1.2 Nutritional Enrichment

Cocopops©: Tesco (Galway, Ireland); *Muesli (Sugar free):* Centra (Galway, Ireland); *Hazelnuts (in shell)* : Dunnes Stores (Galway, Ireland)

2.1.2 Behavioural Equipment

2.1.2.1 Open Field

Circular arena (75cm diameter with 41cm high walls): Constructed by Mr Ambrose O'Halloran (Discipline of Pharmacology and Therapeutics, NUI Galway, Galway, Ireland)

2.1.2.2 Homecage locomotor tracking

The homecage locomotor apparatus was designed and constructed by Mr. Ambrose O'Halloran (Discipline of Pharmacology and Therapeutics, NUI Galway, Galway, Ireland) below is a list of the components of this system:

- Arena: Rat's home cage (25cm x 28cm x 13cm): North Kent Plastics (Coalville, United Kingdom)
- White cage insert (for under food hopper space), domestic water drain pipe (68mm outer diameter x 64mm internal diameter), Egeplast Ireland Ltd., (Waterford, Ireland) cut to 7cm in diameter.
- Black corriboard plastic (3mm in depth) for base of cage bottom : McMahons Builders providers (Galway, Ireland)
- Array of free standing infrared lights: Abus TV6700, IR illuminator, Radionics (Dublin, Ireland)
- CCTV Cameras: CCTV Miniature Camera 700TVL 3.6mm lens, Radionics (Dublin, Ireland)

2.1.2.3 Recording equipment

Video Cameras: Sanyo Digital Colour LCD Camera, Radionics (Dublin, Ireland)

DVD Recorder: Panasonic DMR-EX75, Currys (Galway, Ireland)

DVD+R Recorder: Panasonic DMR-EX83, Currys (Galway, Ireland)

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

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

2.1.3 Surgery

Anaesthetic machine: Matrx, Grays Medical (Biggar, Lanarkshire, United Kingdom) Isoflurane: Chanelle Veterinary (Loughrea, Galway, Ireland) and oxygen: BOC (Galway, Ireland)

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

Handheld vacuum cleaner: Simple H: Argos (Galway, Ireland)

Stereotaxic frame: Harvard Apparatus (MA, USA)

Eye drops: Blink®Tears (Polyethylene 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: Supplied by 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, UK)

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

Swabs: Gauze Mediswabs: Supplied by Lohan's Pharmacy (Galway, Ireland)

Cotton Buds: Johnson & Johnson: Boots (Galway, Ireland)

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

Surgical Clips: 7.5mm x 1.75mm: Aesculap AG & Co. KG (Tullingen, Germany)

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

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

Needles: 25G x 5/8" BD Microlance (Oxford, United Kingdom)

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

Hot bead steriliser: Steri 250

Milupa® Pure Baby Rice: Tesco (Galway, Ireland)

2.1.4 Drugs and drug administration

Sodium chloride (NaCl): Cat# 10274392, Sigma Aldrich (Dublin, Ireland)

Fluoxetine hydrochloride: Cat # 11PM00777PL, Pinewood Laboratories (Tipperary, Ireland)

Venlafaxine hydrochloride: Cat # V0110, Tokyo Chemical Industry (TCI) UK Ltd., (Oxford, United Kingdom)

Imipramine hydrochloride: Cat # I10971: TCI UK Ltd., (Oxford, United Kingdom)

DOI hydrochloride: Cat # D101, Sigma Aldrich (Dublin, Ireland)

8-OH-DPAT: RBI Research Biochemicals International (RBI), (Natick, MA, USA)

Sodium Pentobarbital: Chanelle Veterinary (Loughrea, Galway, Ireland)

Syringes and needles: 1ml, 2ml, 5ml syringes and 25G x 5/8" needles, BD Microlance (Oxford, United Kingdom)

2.1.5 Perfusion apparatus

Sodium chloride (NaCl): Cat# 10274392, Sigma Aldrich (Dublin, Ireland)

Heparin: 5000U/L: Wockhardt UK Ltd., (Wrexham, United Kingdom)

Paraformaldehyde: Cat# 10131580, Fisher Scientific (Dublin, Ireland)

Sucrose: Cat# 10346150, Fisher Scientific (Dublin, Ireland)

Sodium Azide: Cat # S2002, Sigma Aldrich (Dublin, Ireland)

Perfusion pump: Watson Harlow 325: Lennox Laboratory Supplies (Dublin, Ireland)

2.1.6 Immunohistochemistry

Freezing stage-sled microtome: Bright Instruments (Cambridgeshire, United Kingdom)

24-well plates: Cat # 83.3922.300, Sarstedt (Dublin, Ireland)

Twin Frosted microscopic slides: Cat # 7107, Fisher Scientific (Dublin, Ireland)

Stoppered Grenier pots: Cruinn Diagnostics (Dublin, Ireland)

Stoppered pots: ThermoFisher (Dublin, Ireland)

Phosphate buffered saline tablets: Cat# 101826844, Sigma Aldrich (Dublin, Ireland)

Triton-X: Cat # T9284, Sigma Aldrich (Dublin, Ireland)

Polyclonal sheep anti-tryptophan hydroxylase antibody: Cat# AB1541, Merck Millipore (Cork, Ireland)

Biotin-SP-AffiniPure donkey anti-sheep IgG (H+L): Cat# 713-065-003, Jackson ImmunoResearch (Suffolk, United Kingdom)

Polyclonal rabbit anti- SERT antibody: Cat# ab44520, Abcam (Cambridge, United Kingdom)

Biotin-SP-AffiniPure goat anti-rabbit IgG: Cat# 111-065-144, Jackson ImmunoResearch, Suffolk (United Kingdom)

Monocloncal mouse anti-serotonin transporter antibody, clone 17-7A4: Cat# MAB1564:, Merck Millipore (Cork, Ireland)

Biotinylated horse anti-mouse IgG: Cat#BA-2001, Vector Laboratories Ltd., (Peterborough, United Kingdom)

Normal donkey serum: Cat# D9663, Sigma Aldrich (Dublin, Ireland)

Normal goat serum: Cat# G9023, Sigma Aldrich (Dublin, Ireland)

Normal horse serum: Cat# H0146, Sigma Aldrich (Dublin, Ireland)

Hydrogen peroxide: Cat# 216763, Sigma Aldrich (Dublin, Ireland)

Vectastain ABC Kit: Cat# PK6100, Vector Laboratories Ltd., (Peterborough, United Kingdom)

3,3-Diaminobenzidine tetrahydrochloride (DAB): Cat# D8001, Sigma Aldrich (Dublin, Ireland)

Industrial Methylated Spirits (IMS): Cat# BX2428258, Lennox Laboratory Supplies (Dublin, Ireland)

Xylene: Cat# SX-002-1612, Lennox Laboratory Supplies (Dublin, Ireland)

DPX mountant: Cat# 100503-834, VWR (Dublin Ireland); Cat# 44581, Sigma Aldrich (Dublin, Ireland); Cat# 10050080, Fisher Scientific (Dublin, Ireland)

Coverslips: Fisher Scientific (Dublin, Ireland)

Microscope & digital Cameras: BX40 and C5060, Olympus UK (London, United Kingdom)

2.1.7 RT-PCR (5-HT_{1A} and 5-HT_{2A} receptor mRNA)

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

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

Molecuar 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 spectrophometer: Medical Supply Company (Dublin, Ireland)

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

5-HT_{1A} probe: Rn00561409_s1 Htr1a_rat (Fab-MGB): Cat# 4331182, Bio Sciences Ltd., (Dublin, Ireland)

5-HT$_{2A}$ probe: Rn00568473_m1 Htr2a_rat (Fab-MGB): Cat# 4331182, Bio Sciences Ltd., (Dublin, Ireland)

β-actin: Cat# 4352340E, Applied Biosystems, (Warrington, United Kingdom)

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)

2.1.8 High Performance Liquid Chromatography (HPLC)

Citric acid monohydrate: Cat# 331144, Sigma Aldrich, (Dublin, Ireland)

Sodium dihydrogen phosphate monohydrate: Cat# A13737, Sigma Aldrich (Dublin, Ireland)

1-Octane Sulfonic acid sodium salt: Cat# O0133, Sigma Aldrich (Dublin, Ireland)

Ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA): Cat# 34549, Sigma Aldrich (Dublin, Ireland)

N-Methyl-5-HT: Sigma Aldrich (Dublin, Ireland)

1,3,4 Dihydroxyphenylamine (L-DOPA): Sigma Aldrich (Dublin, Ireland)

Noradrenaline (NA): Sigma Aldrich (Dublin, Ireland)

5-hydroxyindole-3-acetic acid (5-HIAA): Sigma Aldrich (Dublin, Ireland)

Homovanillic acid (HVA): Sigma Aldrich (Dublin, Ireland)

Serotonin (5-HT): Sigma Aldrich (Dublin, Ireland)

Reverse phase C18 analytical column: Licrosorb RP-18 column, Phenomenex (Cheshire, United Kingdom)

Electrochemical detector: L-ECD-6A Shimadzu, Mason Technology (Dublin, Ireland)

Merck-Hitachi D-2000 Integrator: Agilent Technologies (Cork, Ireland)

Integration software: Shimadzu Class VP 4.2

2.1.9 Computer Software

Microsoft Office: Microsoft Ireland (Dublin, Ireland)

IBM SPSS Statistics 22: SPSS Inc., (Chicago, IL, USA)

Image J® software: National Institute of Health (Bethesda, MD, USA)

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

EthoVision ® XT 8.5 or XT 11: Noldus (Wageningen, The Netherlands)

Step One software v2.3: Applied Biosystems (Warrington, United Kingdom)

2.2 Methods

2.2.1 Rats

All rat experimental work was carried out with the approval of the Animal Care and Research Ethics Committee (ACREC) of NUI Galway (Application ID: 12/NOV/07), and under a project authorisation form the Health Products Regulatory Authority (HPRA, authorisation ID: AE19125/P006), and in compliance with European Communities Council directive 86/609 guidelines.

Male (300-325g) and female (250-275g) Sprague-Dawley rats were obtained from Charles River, UK and used to create a breeding colony. Such rats were obtained periodically during the project so that only F1 generation rats were used in the experimental studies.

All rats were housed in a light (12 hr light/dark cycle; lights on at 08:00 hr), temperature (20-24°C) and humidity (45-65%) controlled environment. Changing regimes for the rats varied depending on the point in the study i.e. mating, gestational, pre-natal and during study. These will be specified in the relevant sections below. Food and water for all studies and for breeding was available *ad libitum.* Once enrolled in the experiments, the body weight, food and water consumption was recorded daily.

2.2.2 Breeding

Approximately 3-4 days prior to mating males were singly housed and allowed to acclimatise to their new environment. Females remained in cages of 3 until they were introduced into the male's cage for mating. Male and female rats were housed together for 2 weeks, at a ratio of 1:2 or 1:3 during which time the bedding was changed once per week. After 2 weeks, females were removed from the male's cage and singly housed, with nesting material and environmental enrichment (cardboard tubes) to enable nest building.

During the gestational period, females were left undisturbed, except for during cage changes. When the cages were changed a handful of females' bedding material was placed back into the cage in order to maintain scent and familiarity. Environmental enrichment was replenished if required. On gestational day (GD) 21 cages were monitored for the presence of pups – postnatal day (PND) 0 was determined if the

pups were born before 17:00 hr. Once littered, the mother and pups were left undisturbed until PND 2-3 and at this point the pups were counted and sexed. Any deaths or unexpected pup sacrifices were recorded, on average accounting for 14-20% of the number of pups recorded at littering. During the postnatal period cages were changed once weekly, beginning from when the pups were at least 5 days old. During the postnatal period until weaning, the pups were counted daily and their health monitored.

2.2.2.1 Weaning

On PND 21 pups were weaned from their litter. As only male pups were required, the female pups were either allocated to another study/research group or culled if surplus to requirements. The male pups were housed in groups of six (with their litter mates wherever possible), and at this stage the number of male pups was finalised and the required number selected. If there was a surplus, litters that were born as close to each other were kept. Once the selected litters were 4 weeks old, pups were weighed and housed in groups of four according to body weight, and remained in these groups of 4 until they were 7 weeks old, at which time they were enrolled into the study.

2.2.3 Housing

One week prior to surgery all rats were singly housed (8-OH-DPAT/DOI dose response study and antidepressant studies) or group housed (time course study). Certain aspects of environmental enrichment (such as nesting material or plastic tubing) were not provided as it was felt that these might interfere with home cage tracking. However, nutritional enrichment was provided from day 14 following lesion, after the open field (detailed in 2.2.5), providing foraging opportunities. The nutritional enrichment was provided on a weekly basis, coinciding with when cages were changed, using an enrichment protocol developed in the laboratory. This was given in the following order 1.Cocopops 2.Museli (Sugar free) 3. Hazelnuts in shells. Rats remained in their housing conditions for the duration of the study.

2.2.4 Surgical procedures

2.2.4.1 Aseptic technique

The surgical procedure required the adherence to aseptic techniques which will be briefly described. Surgical packs were prepared, each consisting of surgical instruments wrapped in two sheets of tinfoil and then autoclaved. Other materials that were required to be autoclaved included cotton buds, gauze, tissue, surgical instruments, bulldog clips, and suture clips. Haemostatic sponge did not need to undergo autoclaving as it came pre-packed in sterile blister packs.

Prior to the surgical procedure the surgical space was made sterile. Once the rat was rendered anaesthetised, an assistant shaved the area where the incision was to be made. The rat was then placed in the stereotactic frame and the ear bars were positioned. Gloves were then replaced with sterile surgical gloves, which were changed when practical to preserve sterility. For the duration of the surgery, only sterile materials were used. In between the surgery for each rat, the utensils were washed in Rapidex® and placed in a hot bead steriliser. Once removed from the steriliser, they were placed on the sterile tinfoil of the autoclaved surgical packs. This helped to ensure that the surgical area and the procedure remained sterile throughout the surgical session.

2.2.4.2 Olfactory bulbectomy

Rats were handled daily for the week prior to surgery during which time, body weight and food and water consumption were monitored. Based on their body weight rats were randomly assigned to their surgery groups. At approximately 8 weeks old (approx. 200-300g), male Sprague-Dawley rats underwent sham or bilateral olfactory bulbectomy (OB) surgery. On the night before surgery, rats were given a small ramekin of diet that had been softened with some water, in order to acclimatise them to this novel presentation of their food, which they would then receive for 48 hr following surgery to promote a more rapid recovery.

Bilateral olfactory bulbectomy surgery was carried out as previously reported (Roche *et al.*, 2007; Burke *et al.*, 2010). Rats were weighed and anaesthetised with isoflurane (Cat# CP0009, Chanelle, Ireland, 5% induction, 2.5% maintenance in 0.5 L/min O_2), the incision area was shaved and placed in ear bars in a stereotaxic frame (Harvard Apparatus, MA, USA). Eye drops (Blink®, Boots, Galway) were applied to each eye

to prevent drying out during surgery and the head was sterilised with Betadine (7.5% w/v, iodinated povidone, Videne®, Ecolab Ltd., Leeds UK) along the incision area. A midline sagittal incision was made in the skin overlying the skull using a sterile scalpel blade and the incision was held open with 2 bulldog clips so that bregma and the frontal sutures were visible. After the application of the local anaesthetic Norocaine® (Lidocaine Hydrocholoride, Cat# NB0041, Chanelle Veterinary, Ireland), the periosteum was cleared from the skull and the skull rinsed with sterile saline. A sterile scalpel blade was used to make two marks on the skull, approximately 5 mm rostral to bregma and 2 mm lateral to the midline (Figure 2.1). Using these markers, two burr holes of 2 mm diameter were drilled into the skull using a sterilised drill bit (no. 8) until the skull was penetrated; the drill was lowered into the vicinity of the olfactory bulbs, which were slightly macerated as a result to aid in their removal. The olfactory bulbs were then removed with a blunt hypodermic needle (16G) attached to a vacuum pump, with care taken not to damage the frontal cortex. Once the bulbs were removed the holes were plugged with haemostatic sponge (Septodont, supplied by Lohans Pharmacy, Galway, Ireland) until bleeding from the drilled holes ceased. Sham-operated rats were treated in the same manner, with the dura being pierced but the bulbs left intact. The wound was closed with sterile wound clips (7.5 x 1.75 mm), isoflurane flow was stopped and the rat continued to receive oxygen until they became responsive. At this point, the rats received 1 ml of sterile saline administered intraperitoneally (i.p.) to promote rehydration and rats were placed in a recovery cage, positioned on a heating pad. Once the rats had become fully awake and were moving, pain relief was provided with Rimadyl® (Carprofen) (Chanelle Veterinary, Loughrea, Galway, Ireland) (5 mg/kg at a dose volume of 1 ml/kg, s.c.) and rats were returned to their home cage. Rats received another dose of Rimadyl® (Carprofen, Cat# BE0067, Chanelle Veterinary, Loughrea, Galway, Ireland) (5 mg/kg at a dose volume of 1 ml/kg, s.c.) 24 hr following surgery. Rats were monitored closely for 48 hr following surgery, with softened chow being provided for the duration and Milupa® (Tesco, Galway) given if a rat was not recovering at the expected rate. Milupa® was made with lukewarm water until it reached a paste like consistency, it was placed in a shallow petri dish and placed in the base of the rat's cage. This was generally removed after approx. 24 hr but if needed, a fresh batch would be provided for a further 24hr.

Body weight, food and water consumption were monitored for the duration of all studies, ensuring that all rats were handled daily. If a rat progressively deteriorated or lost weight (amounting to 20% of pre-operative body weight) in the post-operative period the rat was killed (accounting for 1.2% of all studies). Lesions were verified upon sacrifice at completion of a study and any rats that had incomplete bulb removal or damage to the frontal cortex were removed from further analysis (accounting for 6.3% of all studies). Sham-operated rats were removed if any were found to have damage to the bulbs, however this did not occur as a result of any studies.

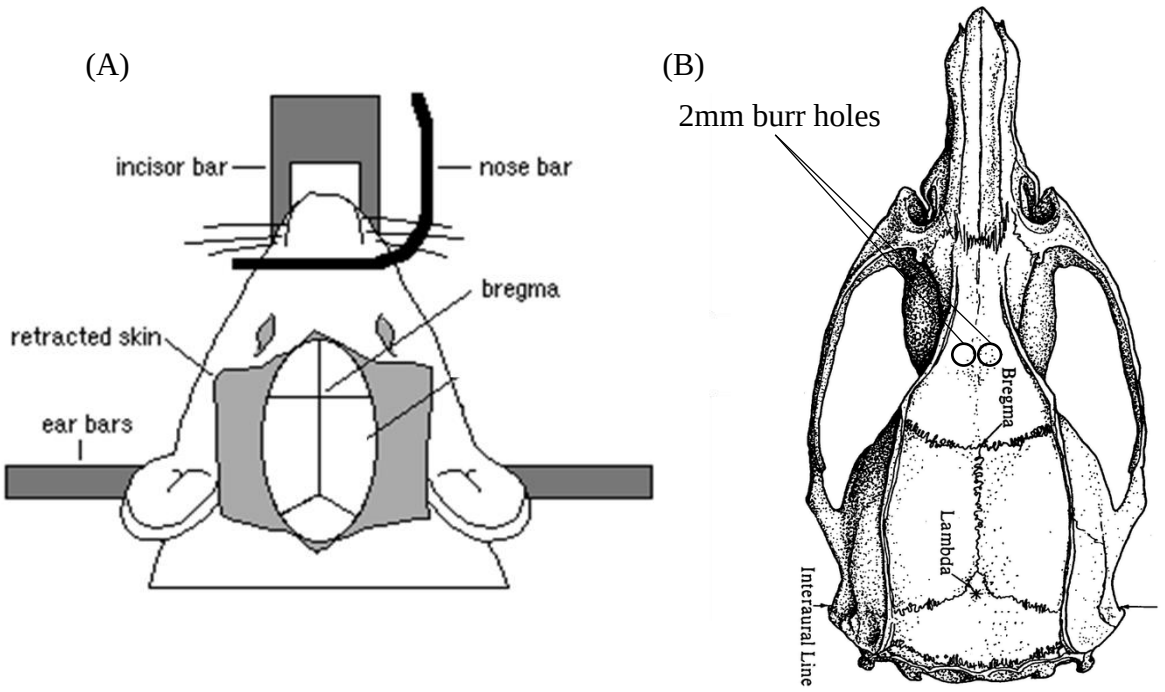


Figure 2.1 Representation of surgical field (A) Schematic representation of surgical field (adapted from www.currentprotocols.com) (B) Illustration of location of burr holes

2.2.5 Behavioural measurements

2.2.5.1 Homecage locomotor activity (HCA)

Homecage locomotor activity was carried out to assess the nocturnal activity of the rats in their natural home environment for the duration of the study. The rats were housed in 3 racks of 24 cages that enabled 72 cages to be monitored simultaneously (Figure 2.2 and 2.3). A black plastic sheet (3mm) was placed at the base of each cage, which was covered with bedding. This ensured that the animals could be tracked at all times as it allowed a continuous degree of contrast from the cage base at all times. A white plastic insert was placed under the food hopper to prevent the rats from disappearing from view and thus not being tracked (Figure 2.2).

Each of the 3 place racks was illuminated by a free standing infrared light array (2 lights per array) with 1 array per place rack, placed on the room floor. This enabled nocturnal measurements to be evaluated making use of the infrared nature of the recording camera.

A camera positioned above the cage captured the rat's activity for the entire cage floor. The video feed from the camera was recorded onto a DVR, which in turn recorded onto a central DVR consisting of 9 channels with 8 cameras per channel. The rat's 12 hr nocturnal homecage activity, as measured by the distance moved (cm), was then scored using EthoVision® XT 8.5 or EthoVision® XT 11 at a later date. A known measurement of the arena was calibrated and the 'detection determines speed' setting was used to assess the distance moved (Figure 2.3)

Figure 2.2 Home cage apparatus (A) Homecage racks (B) Homecage with the black sheet on base of cage and white cage insert (under food hopper) highlighted

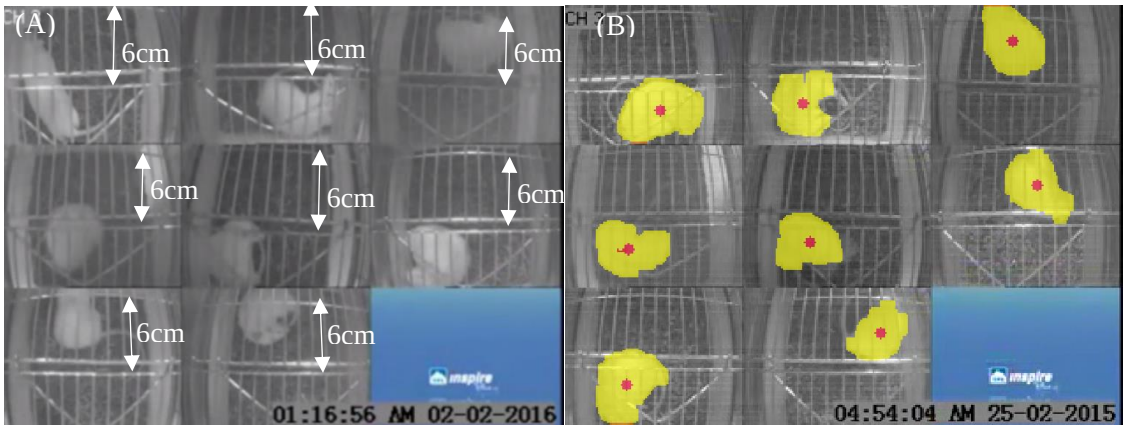


Figure 2.3 Home cage tracking parameters (A) Nocturnal Homecage with arena size calibrated (B) Tracking of 8 arenas using-EthoVision® tracking technology, the body of the rat is highlighted in yellow. The red dot represents a marker of 'center point detection', denoting the point which is tracked.

2.2.5.2 Open Field

This test involves removing the rat from its home cage (familiar environment) and being placed in an open field, an aversive novel environment from which it cannot escape. The open field used in this project was a circular arena (75cm in diameter) with a white wooden floor and reflective aluminium walls (41cm high). Four 60 watt bulbs are placed above the arena. Each bulb was attached to a dimmer switch allowing the intensity of the light to be set to between 200-250 lux at the base and which was evenly distributed around the arena, avoiding any 'bright spots' (Figure 2.4)

Rats were placed in the centre of the open field and were allowed to freely explore for 5 min. After removal of the rat, the arena was cleaned with warm soapy water, to remove any residual odours between each rat and then thoroughly dried with paper tissue. A camera located 92cm above the base of the arena recorded the rats activity. All trials were recorded onto a DVD or DVR recorder and scored at a later date using EthoVision® XT 8.5 or Ethovision® XT 11. The parameters of interest were distance moved (cm) and inner zone duration (sec) (Figure 2.5). The diameter of the arena was calibrated and the setting 'detection determines speed' was used to assess the above parameters. On some occasions further parameters were measured such as speed of bout, grooming and rearing, these were scored manually using EthoVision® XT 11.

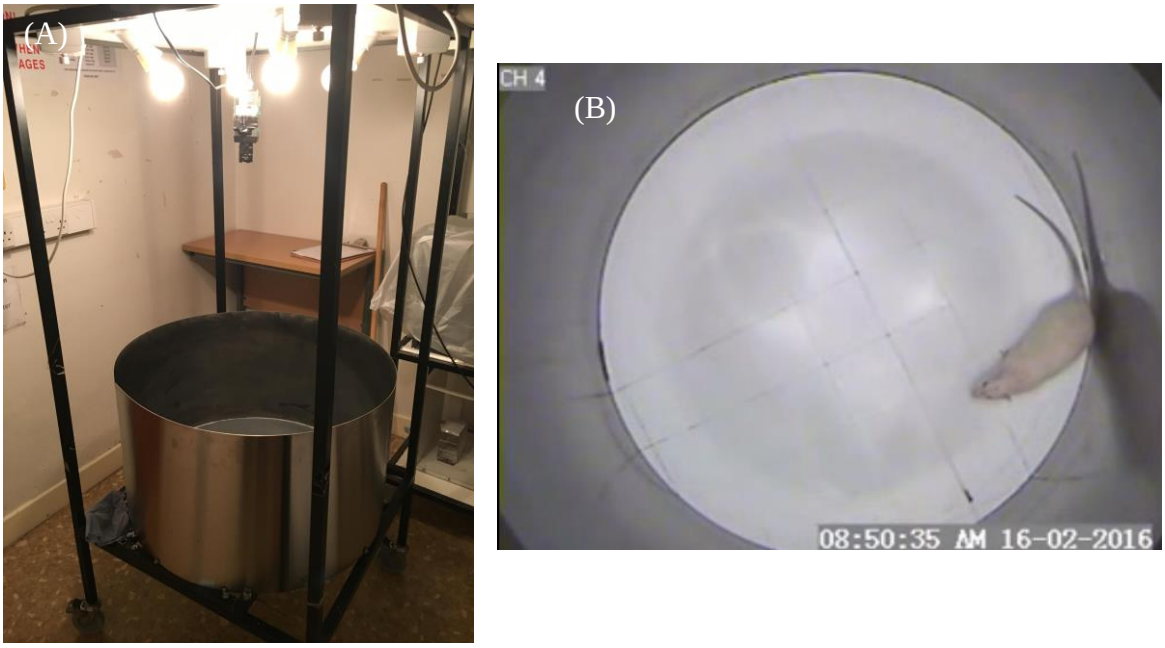


Figure 2.4 Open field apparatus (A) Open field (B) Rat in the open field

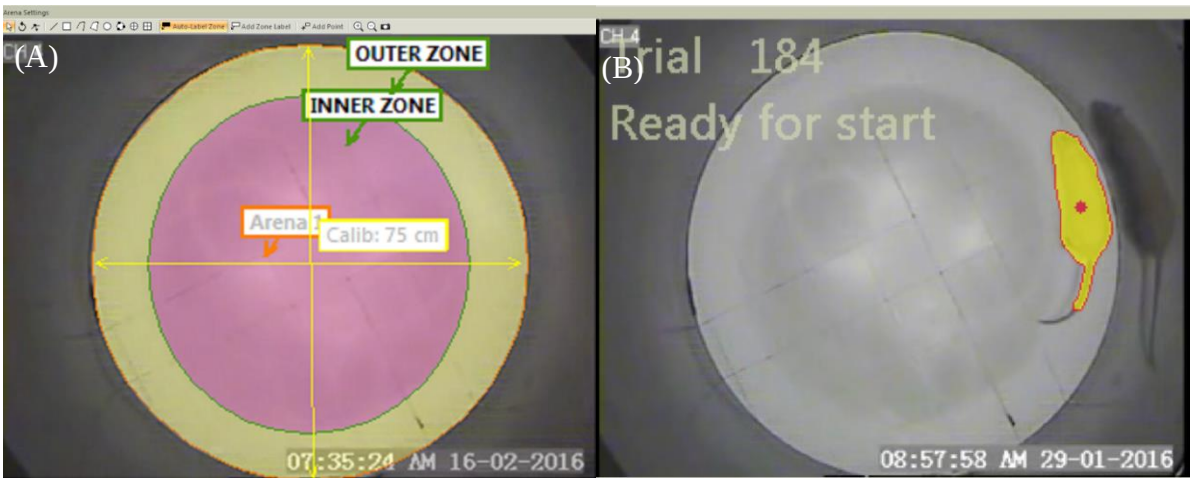


Figure 2.5 Open field tracking parameters (A) Open field with arena size calibrated (B) Tracking of a rat in the open field using EthoVision® tracking technology. The red dot represents a marker of 'center point detection', denoting the point which is tracked.

2.2.5.3 8-OH-DPAT and DOI induced behaviours

8-hydroxy-2-dipropylaminotetralin hydrobromide (8-OH-DPAT) and 2-[2,5-dimethoxy-4-iodophenyl]-2-aminopropane (DOI) and are 5-HT_{1A} and 5-HT_{2A} receptor agonists, respectively. Upon administration of these drugs, they elicit stereotyped behaviours (Table 2.1) that can be scored and counted. The individual experimental conditions for each study will discussed in chapters 4 and 5, thus only the common elements will be discussed here.

All dosing took place approximately 4 weeks following lesion. Rats were weighed from 09:00 to 10.30-11am and left undisturbed for 2 hr – this was the baseline period. Once the 2 hr baseline period had elapsed, rats received a dose of either DOI or 8-OH-DPAT (s.c.) and were once again left undisturbed for 2 hr. The rat's behaviour was recorded onto a central DVR recorder and was scored at a later date using EthoVision® XT 8.5 or EthoVision® XT 11.

The rat's total locomotor activity (distance moved cm) was measured for the 2 hr baseline and the 2 hr post dose period using the detection determines speed setting. The stereotyped behaviours elicited by both agonists, visible via individual cameras (Figure 2.6) above the cages, were manually scored for the first hour post dosing, beginning immediately when the rat's home cage was placed on the rack. There was no vehicle group included with this dosing regime, as the behaviours elicited are specific to the drugs and therefore would not be present post dosing.

Figure 2.6: Overview of individual cage for scoring of behaviours

Table 2.1 Description of stereotyped behaviours elicited by DOI and 8-OH-DPAT

Behaviour	Description
Rearing	Rat is on its hind legs
Flat body posture	Rat's ventral surface is on the cage floor (visible via bedding moving), resulting in a flattened and elongated posture
Hind limb abduction	One (predominantly) or both hind legs are transiently stretched out backwards and to the side, away from the rats body; can occur when rat is rearing
Forepaw treading	Rat makes consecutive reciprocal movements with the front paws
Wet dog shakes	Rat's entire body shakes (head and back). It was not included if the rat began scratching at injection site following injection, as this could be due to the drug being found as aversive
Head twitches	Rat's head moves forwards and back (towards dorsal side)
Head shakes	Rat's head shakes, similar to whisker twitch
Head weaving	Rat moves head from side to side, absence of whole body movement or investigative behaviour
Backward walking	Rat makes a series of consecutive backward moves
Hunched back	Rat's back hunches over, often accompanied by a stretching motion
Piloerection	Rat's hair stands on end - can be difficult to distinguish from poor grooming, which can be a side effect of the OB syndrome
Skin Jerks	Skin on back twitches

Behaviours adapted from: Haberzettl *et al.,* 2014; Kleven *et al.,* 1997; Darmani and Ahmad, 1999

2.2.6 Drug dosing (Table 2.2)

Table 2.2 Route of administration, dose volumes and vehicle used

Drug	Dose	Route	Dose Volume	Vehicle	Frequency
8-OH-DPAT DR study	0.1, 0.25 and 0.5 mg/kg	s.c.	1 ml/kg	Sterile Saline (0.9%)	1
AD Studies	0.5 mg/kg				
DOI DR study	0.3,1and 3 mg/kg	s.c.	1 ml/kg	Sterile Saline (0.9%)	1
AD studies	3 mg/kg				
Fluoxetine hydrochloride	10 mg/kg	s.c.	2 ml/kg	dH_2O	21
Venlafaxine hydrochloride	20 mg/kg	s.c.	4 ml/kg	dH_2O	21
Imipramine hydrochloride	10 mg/kg	s.c.	4 ml/kg	dH_2O	21

DR = dose response; AD = antidepressant; s.c. = subcutaneous

All doses selected were based on the literature for both DOI (Kawakami *et al.,* 2005; Kitamura *et al.,* 2007; Amano *et al.,* 2007; Kozuru *et al.,* 2000; Biezonski *et al.,* 2009; Xu and Miller, 1998) and 8-OH-DPAT (Cryan *et al.,* 1997; Cryan *et al.,* 1999; Nalivaiko *et al.,* 2009; Dabrowska *et al.,* 2007; Haleem *et al.,* 2007; Rodriguez-Manzo *et al.,* 2011; Inam *et al.,* 2006; Batool and Haleem, 2008). 8-OH-DPAT and DOI was made up fresh on the morning of dosing, for the dose response study, whilst 8-OH-DPAT was made up fresh on the morning for the venlafaxine and imipramine study. For the dose response study, a stock solution of the highest dose of each of the drug was made up and serial dilutions of this stock were made to achieve the lower doses.

For the fluoxetine study, the 8-OH-DPAT and DOI were made prior to dosing and frozen at -20°C until needed.

All doses selected were based experiments carried out previously in our laboratory or in the literature. Fluoxetine (Roche *et al.,* 2007; Rodriguez-Gaztelumendi *et al.,* 2014; Mar *et al.,* 2000; Mar *et al.,* 2002). Venlfaxine (Uzunova *et al.,* 2004; Czubak *et al.,* 2009; Martisova *et al.,* 2015; Szkutnik-Fielder *et al.,* 2012); Imipramine (Breuer *et al.,* 2009 ; Roche *et al.,* 2008; van Hoomissen *et al.,* 2003). For chronic antidepressant study, fluoxetine hydrochloride, venlafaxine hydrochloride and imipramine hydrochloride were dissolved in the specified vehicles to the required concentrations. All were made up to the desired volume and concentration needed for a 2 ml/kg volume dosing. The drugs were then divided in aliquots for each day and frozen at -20°C until needed. The vehicle was also frozen in aliquots to ensure that compounds were treated the same. When needed, an aliquot of drug and vehicle were thawed at room temperature until needed for dosing.

2.2.7 Rat sacrifice and tissue collection

2.2.7.1 Decapitation

For post-mortem investigations (RT-PCR and HPLC) that required fresh-frozen tissue, rats underwent the open field after 21-days of chronic antidepressant dosing and were immediately killed by live decapitation. Immediately following decapitation, the skin was removed from the skull and the optic ridge between the eyes was cracked with rongeurs. An incision was made at the base of the skull and along the midline and the parietal and frontal bones were removed. Care was taken not to damage the cavity where the olfactory bulbs sit so that the validity of the surgery could be confirmed. Once it was established whether the OB or sham surgery was a success, the optic nerve was severed and the brain was removed from the skull cavity using forceps. It was then snap frozen on a flat petri dish submerged in solid CO_2 pellets (to maintain shape and integrity) and was stored at -80°C until analysis.

2.2.7.2 Transcardial perfusion

Rats were terminally anaesthetised with sodium pentobarbitol via i.p. injection and were observed until the rat did not respond to pain reflex (toe pinch) or blink reflex. The rat was then placed in the supine position and an incision was made in the

abdomen, just below the base of the sternum, to remove the overlying skin. The sternum was lifted with a pair of forceps and an incision was made on either side of the ribcage, exposing the tissue and muscle underneath. The diaphragm was then cut as quickly as possible and the rib cage pinned back to expose the chest cavity, exposing the heart. The descending aorta was clamped off using a haemostat so that only the upper body would be perfused. A small incision was made into the base of the left ventricle and a 25G blunted needle, connected to an infusion pump was inserted into the left ventricle and into the ascending aorta, was secured with a small clamp. A small incision was made into the right atrium to reduce pressure. At this point heparinised saline was pumped into the heart at approx. 70 rpm. Once the needle was secured this was increased to approx. 250 rpm and the rat was perfused with heparinised saline until the ears and forepaws had turned white and the water flowing from the hole in the right atrium ran clear. Once the blood had cleared, the reservoir supplying the heparinised saline was switched to 4 % v/v PFA (paraformaldehyde in phosphate buffer pH 7.4) and maintained 30 sec at 250 rpm, with rigorous upper body movements indicating sufficient fixing. Once 30 sec had elapsed, the needle was removed and the reservoir changed to saline and the speed decreased to approx. 70 rpm to avoid bubbles being introduced into the lines. Clamps were removed and the rat was decapitated; brains were removed as indicated above and allowed to post fix in 4% v/v PFA at 4°C overnight. After 24 hr post fixing, the brains were transferred to 25% sucrose (in distilled water with 1% w/v sodium azide) and stored at 4°C until ready for processing.

2.2.7.3 Brain dissection and tissue collection

For RT-PCR and HPLC analysis, brains were gross dissected. Brains were removed from a -80°C freezer and place in a styrofoam box containing solid CO_2 pellets prior to dissection. Each brain was individually taken from the styrofoam box and placed on an ice cold glass plate. When sufficiently (but not fully) thawed, the prefrontal cortex (PFC), hippocampus and amygdala were dissected (Figure 2.7). The right-hand side of all three regions, weighing approximately 20-30 mg per tissue, were taken for PCR analysis. The left-hand side of all three regions were placed into separate eppendorfs, snap frozen on dry ice and frozen at -80°C for HPLC analysis.

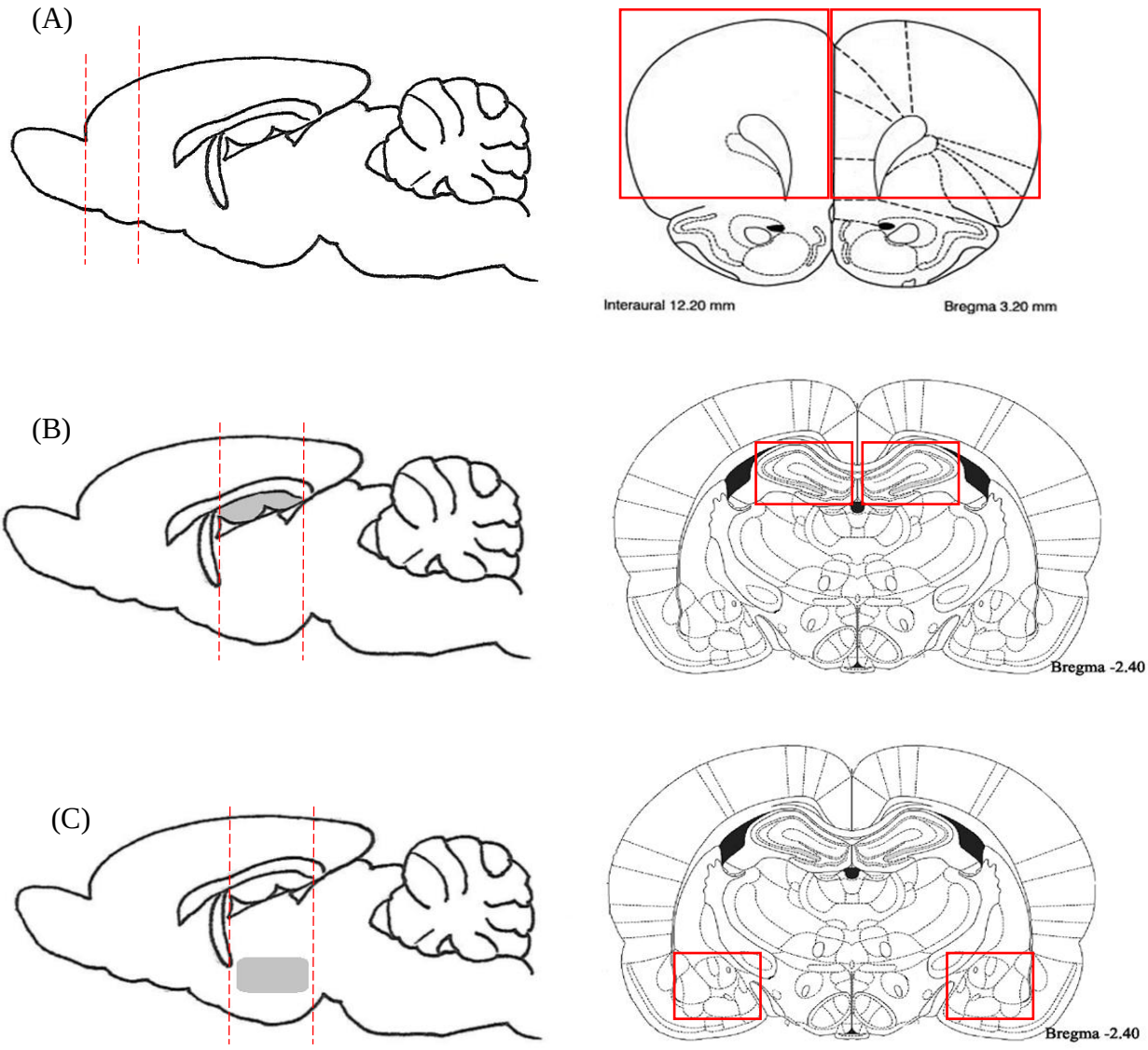


Figure 2.7. Schematic representations of the regions gross dissected for PCR and HPLC analysis. (A) PFC (B) Hippocampus (C) Amygdala. Red lines denote the areas where cuts were made and the grey highlighted regions are the dissected regions for the hippocampus and amygdala; red boxes highlight the approx. regions taken, with both the left and right hand side taken.

2.2.8 Analysis of gene expression using quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

2.2.8.1 RNA isolation

Total RNA was isolated from homogenised discrete PFC, hippocampal and amygdala samples using NucleoSpin RNA II total RNA isolation kit (Macherey-Nagal, Fisher Scientific, Ireland). Once dissected, approximately 20-30 mg of tissue was placed in RNase-free eppendorfs containing 354µl RA1 lysis buffer containing 1% β-mercaptoethanol (Cat# M6250, Sigma-Aldrich, Dublin, Ireland) and homogenised with an Ultra-Turrax Polytron tissue disrupter (Fisher Scientific, Dublin, Ireland). Homogenates were then frozen at -80°C until processed. Homogenates were thawed at room temperature and transferred to Nucleospin filter column (purple) and centrifuged at 11,000 g for 1 min. 350 µl of 70% molecular grade ethanol (Cat# E7023, Sigma Aldrich, Dublin, Ireland) was added to each of the lysates and mixed by pipetting up and down 10 times. The samples were transferred to Nucleospin RNA II columns (blue) and centrifuged at 11,000 g for 30 sec. The columns were then placed in new collection tubes, 350 µl of membrane desalting buffer (MDB) was added and samples were centrifuged at 11,000 g for 1 min. A 10% v/v rDNase solution was prepared using DNase reaction buffer (supplied) and 95 µl was added to the centre of each column and allowed to stand at room temperature for 15 min. 200 µl of RA2 buffer was then added to each column and were centrifuged at 11,000 g for 30 sec. The columns were placed in new collection tubes, 600 µl of RA3 wash buffer was added and centrifuged at 11,000 g for 30 sec. The eluent was discarded and 250 µl of RA3 wash buffer was placed in the centre of the column and centrifuged at 11,000 g for 2 min. The columns were placed in RNase-free collection tubes (supplied) and the RNA was eluted with the addition of 60µl of RNase free water (supplied), followed by centrifugation at 11,000 g for 1 min. The columns were discarded and the eluted RNA was stored on ice until RNA quantification was complete, upon which they were stored at -80°C until RNA equalisation

2.2.8.2 RNA quantification

The quantity, purity and quality of isolated RNA was assessed using a Maestro Nano drop spectrophometer (Medical Supply, Co. Dublin). RNA quantity was determined by measuring optical density (OD) at 260 nm and RNA quality was measured via the OD260/OD280, where a value of 1.6-2.2 was deemed indicative of pure RNA. All RNA was isolated in batches of 12, therefore a blank reading was recorded at the start of each batch of 12 with the same RNase free water used to elute the RNA. All RNA samples with a ratio greater than 1.6 were accepted and were then equalised to a concentration of 2 µg/20 µl or 1 µg/20 µl with the addition of RNase free water (Cat# W45020, Sigma-Aldrich, Dublin, Ireland) to 30 µl of each RNA sample. Samples were then vortexed to ensure homogenisation and subsequently frozen at -80°C until cDNA synthesis.

2.2.8.3 Complementary DNA (cDNA) synthesis

High capacity cDNA kit (Cat# 4368814, Applied Biosystems, Warrington, United Kingdom) was used to reverse transcribe the equalised RNA samples. 10µl of equalised RNA was mixed with equal volume of 2X master mix in RNase-free mini-eppendorf. The 2X master mix was prepared as follows (volumes given are for one sample): 2.0 µl 10X RT buffer, 0.8µl 25X dNTP mix, 2.0 µl 10X RT random primers, 1.0 µl Multiscribe Reverse Transcriptase and 4.2 µl RNase free water (Cat# W45020: Sigma-Aldrich, Dublin, Ireland). Samples were then vortexed and centrifuged in a mini centrifuge to ensure proper mixing and all the sample was at the bottom of the tube. Samples were then placed in an 'MJ research' thermal cycler (Bio-Rad, Fannin, Dublin) and incubated at 25°C for 10 min, 37°C for 120 min and 85°C for 5 min. The final stage of the cycle kept the samples at 4°C and the resultant cDNA samples were then diluted 1:4 with RNase free water (Cat#W45020, Sigma-Aldrich, Dublin, Ireland), vortexed and stored at -80°C until qRT-PCR.

2.2.8.4 Quantitative Real-Time PCR (qRT-PCR)

Gene expression target proteins were determined using the commercially available TaqMan gene expression assays (Applied Biosystems, Warrington, United Kingdom) containing specific forward and reverse target primers and FAM-labelled MGB probes. β-actin was the endogenous control and was used to normalise gene expression

between samples and was quantified using a β-actin endogenous control assay (Cat# 4352340E Applied Biosystems, Warrington, United Kingdom) containing specific primers and VIC-labelled MGB probe. ID's for each of the genes are given in Table 2.3.

The reaction master mix was prepare for each target gene and stored on ice until needed. This consisted of 0.625 µl target primers, 0.625 µl β-actin (Multiplex version) and 6.25 µl TaqMan Universal PCR Master Mix (Cat# 4324018, Applied Biosystems, Warrington, United Kingdom), per sample. 5 µl of each sample was pipetted in duplicate on a MicroAmp® optical 96-well plate (Applied Biosystems, Warrington, United Kingdom), which was kept on ice for the duration. 8 µl of the relevant reaction master mix was then added to each well to give a total reaction volume of 13 µl. Non-template controls containing no cDNA but with the reaction master mix for the relevant target gene was also included in all plates. Plates were then covered with optical adhesive plate covers and spun at 4,000 g to ensure complete mixing and elimination of bubbles in all wells. The plate was then placed back on ice until it was placed in the real time PCR thermocycler (*StepOnePlus*™, Applied Biosystems, Warrington, United Kingdom) pre-set to run the Relative Quantification protocol: step 1: 95°C for 10 min, step 2: 95°C for 15 s, followed by 1 min at 60°C. Step 2 was repeated 40 times and the fluorescence read during the annealing and extension phase (60°C) for the duration of the programme.

Table 2.3: Target genes used in qRT-PCR

Target gene	Assay number
5-HT$_{1A}$	Rn00561409_s1
5-HT$_{2A}$	Rn00568473_m1
Endogenous Control	
β-actin	4352340E

2.2.8.5 Analysis of qRT-PCR Data

Amplification plots and copy threshold (Ct) values were examined using Applied Biosystems StepOne software V2.3. Ct values for each sample were analysed after the threshold was set to the linear exponential phase of the amplification plots and exporting the analysis to Microsoft Excel for 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 that of control samples, thus allowing determination of the fold change in mRNA expression between groups (Figure 2.9). The method comprises of 3 steps:

Step 1: Normalisation to endogenous control (β-actin) where ΔCt is determined:

ΔCt = Ct target gene – Ct endogenous control.

Step 2: Normalisation to control sample where ΔΔCt is determined:

ΔΔCt = ΔCt Sample - average ΔCt of Control group

Step 3: Fold difference is given by $2^{-\Delta\Delta Ct}$. The $2^{-\Delta\Delta Ct}$ values were then expressed as a percentage of the average of the $2^{-\Delta\Delta Ct}$ values of the control group. In this manner the percentage increase or decrease in mRNA expression between experimental groups was determined.

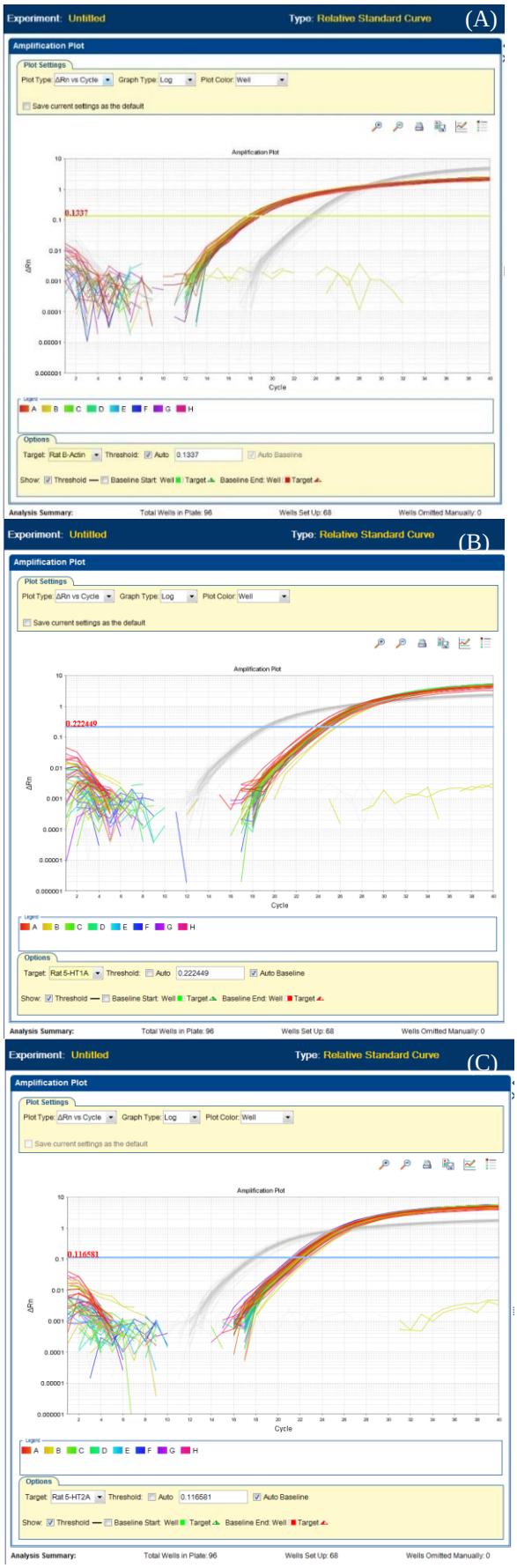


Figure 2.8: Sample Amplification plots (A) Endogenous control rat β-actin (B) Rat 5-HT_{1A} receptor (C) Rat 5-HT_{2A} receptor

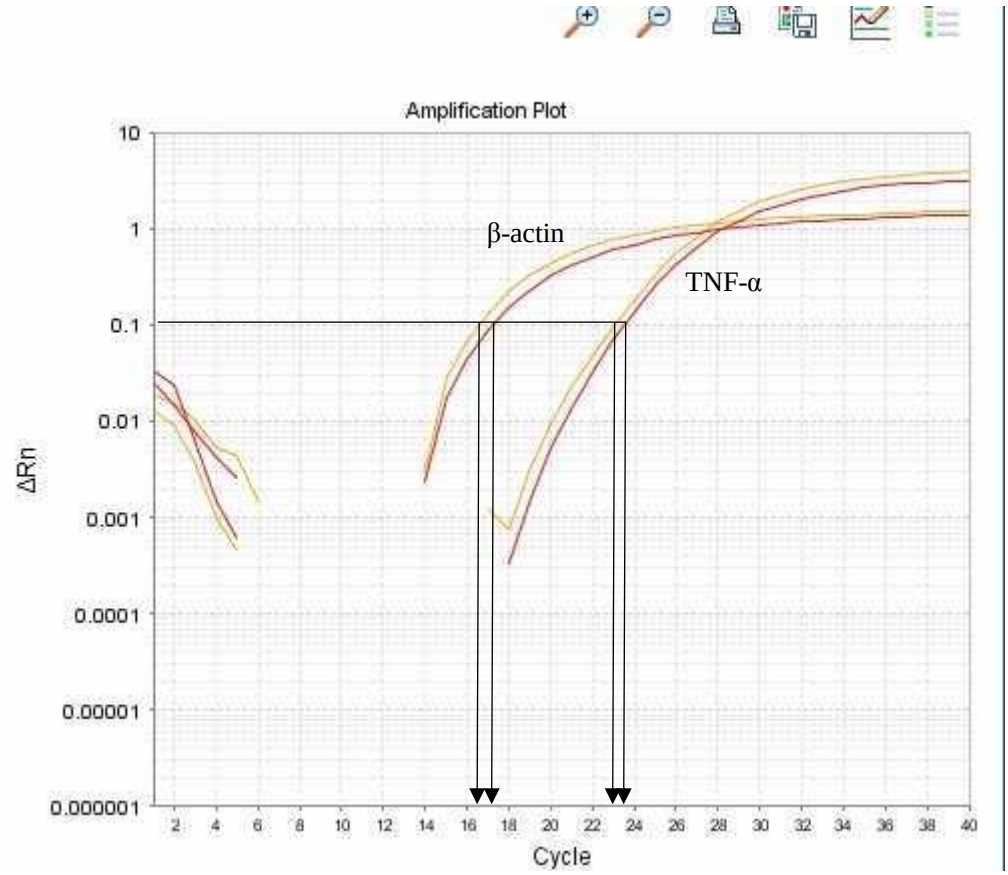


Figure 2.9 Demonstration of determination of Ct values for ΔΔ Ct calculation
Yellow = Vehicle (control) group; Red = Drug group (sample)
ΔΔ Ct = (ΔCt TNFα$_{sample}$ – ΔCt β-actin$_{sample}$) - ΔCt (TNFα$_{control}$l – β-actin$_{control}$)
Rn = fluorescence of the reporter dye divided by the fluorescence of a passive reference dye i.e. Rn is the reporter signal normalised to a fluorescence signal of dye e.g. Applied Biosystems™ ROX ™ Dye
Cycle = cycle number; threshold cycle is the intersection between an amplification curve and a threshold line (black horizontal line). It is the relative measure of the concentration of the target in the PCR reaction.
(Figure constructed with assistance from Dr. Danny Kerr; https://www.thermofisher.com/ie/en/home/life-science/pcr/real-time-pcr/qpcr-education/pcr-understanding-ct-application-note.html)

2.2.9 Immunohistochemistry

2.2.9.1 Tissue processing

Serial coronal sections (30 µm or 40 µm) were cut using a freezing stage sledge microtome (Bright, Cambridgeshire, United Kingdom) and collected in a series of 12. 1:6 series of sections were used for all quantitative immunohistochemistry, in line with laboratory established protocols.

2.2.9.2 Immunohistochemistry

Free floating immunohistochemistry was carried out using the streptavidin-biotin-peroxidase method (Figure 2.10). Sections were placed in Grenier pots, with 1 ml of each solution per pot. All incubations were carried at room temperature under gentle agitation. Sections were initially washed in PBS (3 x 5 min washes), followed by quenching of endogenous peroxidase with a solution of 3% v/v hydrogen peroxide/10% v/v methanol in distilled water. Sections were again washed with PBS (3 x 5 min washes) and then incubated in a solution of 3% normal serum (dependent on the 2° antibody host) in PBS with 0.2% Triton-X 100 (TX-PBS) for 1 hr at room temperature, to block non-specific binding. Excess blocking solution was poured off and sections were incubated overnight in appropriate antibody solution (Table 2.4) diluted in TX-PBS with 1% normal serum (dependant on the 2° antibody host). The following morning sections were washed in PBS (3 x 10 min washes) prior to incubation with corresponding biotinylated 2° antibody diluted in TX-PBS with 1% normal serum (Table 2.5) for 3 hr. Following this, sections were washed with PBS (3 x 10 min washes) during which time a streptavidin-biotin-horseradish peroxidase solution (ABC complex, Vector, UK, (PK 6100)) was prepared and incubated for 30 min. Sections were then incubated in ABC complex for 2 hr, after which they were washed in phosphate buffer ((PB), pH 7.4) and stored at 4°C until incubation with 3, 3- diaminobenzidine tetrahydrochloride ((DAB), Cat# D5637, Sigma, Dublin Ireland). Colour was developed by incubating sections in DAB solution made up in PB with 0.03% v/v hydrogen peroxide, following which they were washed in PB (3 x 5 min) and stored at 4°C while they were mounted.

Sections were mounted onto gelatin-coated slides, allowed to air dry and were then dehydrated by immersing the slides for 5 min in increasing concentrations of alcohol

(50%, 70%, 100%, 100%), clearing with xylene (2 x 5 min immersion) and coverslipped with DPX mountant. Slides were allowed to dry in the fumehood until the DPX was dry and excess could be removed from the slide.

Table 2.4: List of primary antibodies used in analysis

Primary Antibody	Source	Target	Host	Dilution
Trytophan hydroxylase (TPH)	Merck Millipore	TPH	Sheep	1:2000 (DRN) 1:2000/1:1000 (TR)
Serotonin Transporter (SERT)	Abcam	SERT	Rabbit	1:1000
	MerckMillipore	SERT	Mouse	1:1000

Table 2.5: List of secondary antibodies used in analysis

Secondary Antidbody	Source	Host	Reactivity	Dilution
Biotinylated	Jackson ImmunoResearch	Donkey	Sheep	1:200
Biotinylated	Jackson ImmunoResearch	Goat	Rabbit	1:200
Biotinylated	Vector	Horse	Mouse	1:200

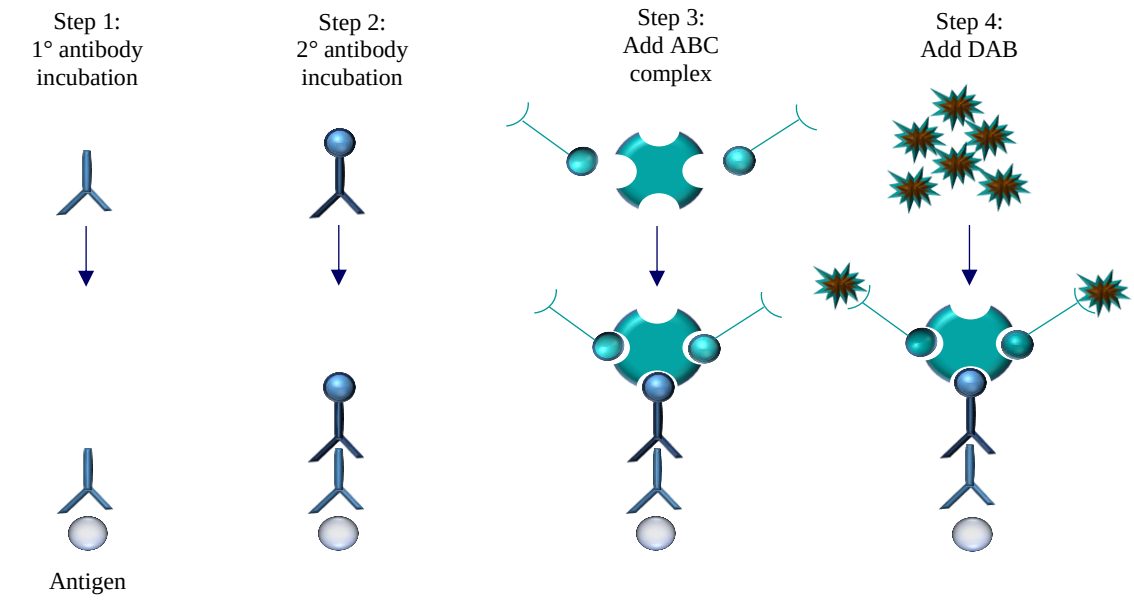


Figure 2.10: Schematic representation of stages in immunohistochemistry

2.2.9.3 Histological Quantification

Images of the DRN were taken on Olympus BX40 digital camera, whist images of the terminal regions was taken on Olympus C5060 digital camera. All image analysis was carried out using ImageJ software® (U.S National Institutes of Health, Bethesda, Maryland, US). Quantification of tryptophan hydroxylase (TPH) positive cells were counted using the cell counter macro, with the counter initialised between each image (Figure 2.11). For analysis of TPH expression in the whole DRN, up to 3 sections from each rat was chosen from analysis comprising of one section from the rostral, middle and caudal portion of the DRN. Given the topographic organisation of the DRN it was felt that this would best represent the entirety of the DRN. For stereological analysis of the dorsal, ventral and lateral wings of the DRN, up to 3 sections for each location was analysed for each rat, with the Paxinos and Watson Brain Atlas (The Rat Brain in Stereotaxic Coordinates, Paxinos and Watson) used as a guide in distinguishing the boundaries.

2.2.9.3.1 Density measurements

TPH positive fibres in terminal regions were quantified using optical density measurement in ImageJ®. For each rat, up to 3 sections per region, were converted to 8-bit black within ImageJ®. The regions of interest were identified using the Paxinos and Watson Atlas (The Rat Brain in Stereotaxic Coordinates, Paxinos and Watson) and delineated using the freehand drawing tool. Mean grey value was measured for all and in the case of SERT analysis, the area was also measured. To assess the specific TPH or SERT staining density, the optical density readings were corrected for non-specific background density. In the case where a region was split over two sides e.g. amygdala and prefrontal cortex, analysis of both sides were taken and averaged. TPH measurements (Figure 2.12) taken were expressed as optical density and SERT measurements (Figure 2.13) were expressed as a percentage of the area measured for terminal regions but not DRN.

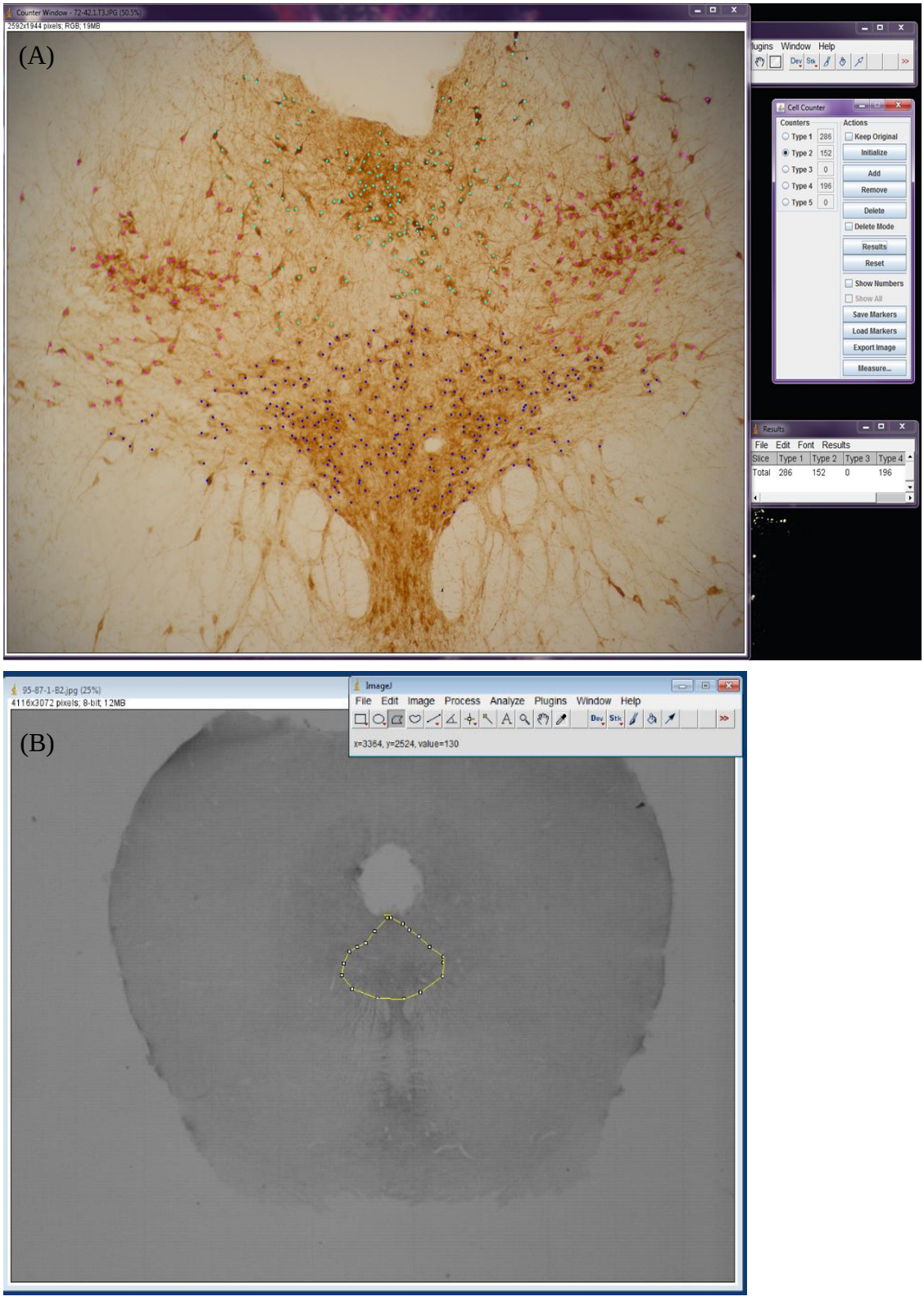


Figure 2.11 Screen grab of ImageJ® software used for quantification (A) TPH-positive cell bodies in the DRN; Each dot represents a cell counted – Blue = Ventral DRN; Turquoise = Dorsal DRN; Pink = Ventrolateral DRN (B) SERT in the DRN

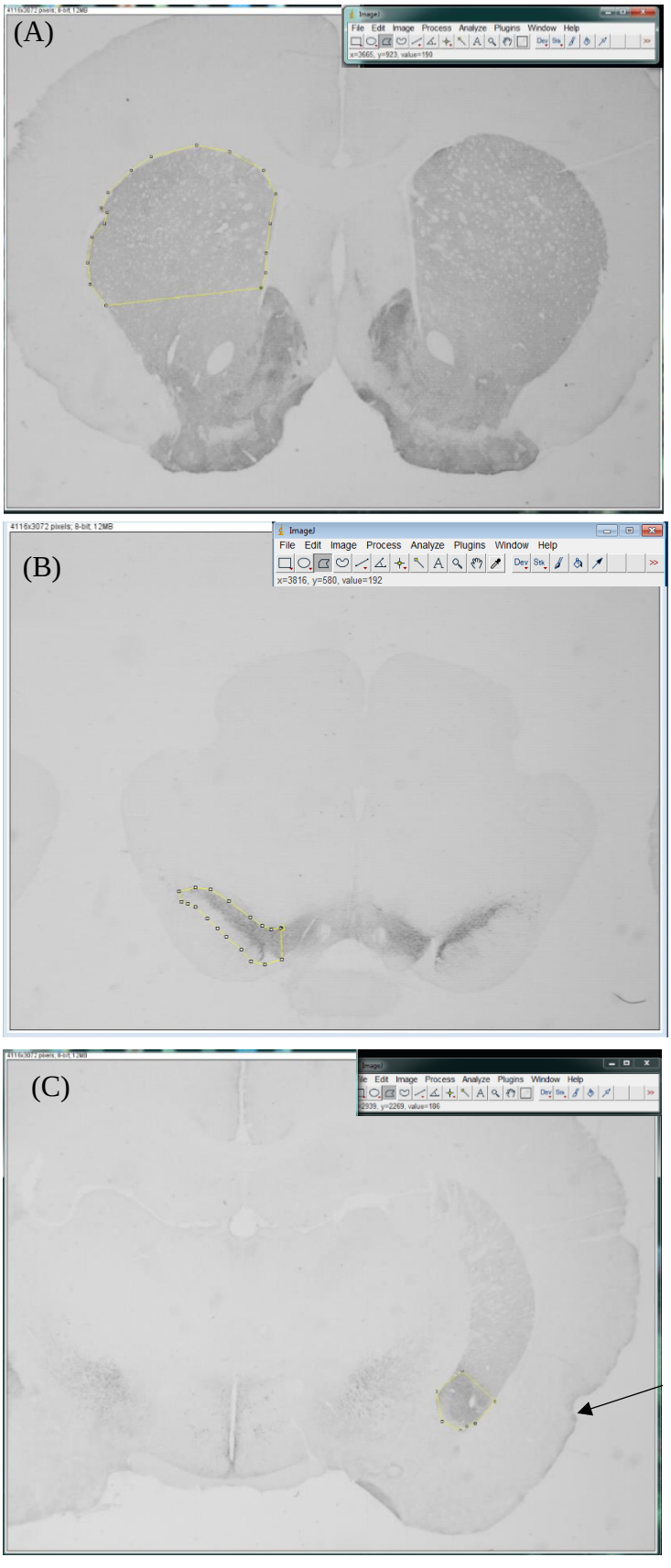


Figure 2.12: Screen grab of ImageJ® software used to quantify optical density of TPH positive staining (A) Striatum (B) Substantia nigra (C) Amygdala – arrow pointing to anatomical marker used to identify amygdala

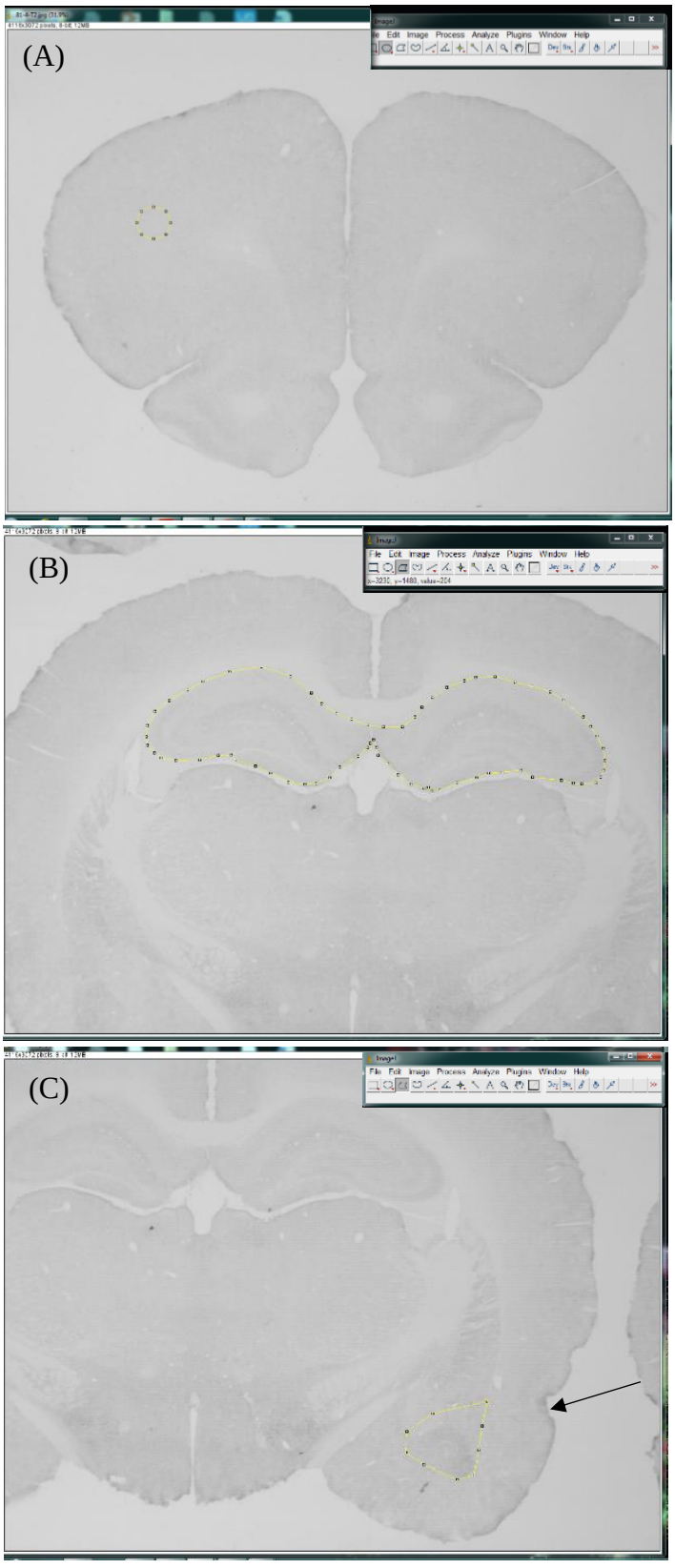


Figure 2.13: Screen grab of ImageJ® software used to quantify optical density of SERT positive staining (A) PFC (B) Hippocampus (C) Amygdala – arrow pointing to anatomical marker used to identify amygdala

2.2.10 High Performance Liquid Chromatography (HPLC) with electrochemical detection for measurement of brain monoamines

2.2.10.1 Preparation of samples

The mobile phase buffer consisted of 0.1 M Citric acid monohydrate, 0.1 M Sodium dihydrogen phosphate, 1.4 mM Octane-1-Sulfonic acid and 0.01 mM Ethylene Diaminetetraacetic acid (EDTA), with 10% HPLC grade methanol, pH adjusted to 2.8. This was spiked with 2 ng/20 µl of internal standard (N-methyl-5-hydroxytryptamine; N-methyl-5-HT). A 1 ml aliquot was added to the amygdala samples, and then sonicated, placed on ice and then centrifuged at 14,000 g at 4°C for 15 min. The supernatant was then removed, placed into fresh eppendorfs and stored at -80°C until sent to Trinity College Dublin for analysis.

2.2.10.2 HPLC analysis of rat brain biogenic amines

100 µl of supernatant from each sample was transferred to glass HPLC assay vials. Mobile phase was circulated via a Shimadzu LC-10AT –pump (Mason Technology Dublin) through the HPLC system at a constant flow rate of 1 ml/min. A 10 µl sample of supernatant was injected onto a reverse-phase C18 column (Licrosorb RP-18 column; Phenomenex, UK) maintained at 30^{o}C and eluting monoamines and their metabolites were detected by an L-ECD-6A Shimadzu electrochemical detector maintained at a potential of +0.8V(Mason Technology Dublin). A 10 µl solution containing a mixture of 1,3,4 Dihydroxyphenylamine (L-DOPA), Noradrenaline (NA), 3,4-dihyroxyphenylacetic acid (DOPAC), dopamine (DA), 5-hydroxyindole-3-acetic acid (5-HIAA) homovanillic acid (HVA), serotonin (5-HT) and N-methyl serotonin (N-M-5HT),(Sigma Aldrich) each at a concentration of 2ng/20µl was also run intermittently between samples. Standard and sample chromatograms peak heights were recorded and analysed using Shimadzu Class VP 4.2 software (Figure 2.14). Concentration of monoamines and their metabolites was determined by ratiometric analysis of standard and sample data and the results expressed an ng neurotransmitter/g of tissue (formulae detailed below).

Step 1. Calculation of RRF_{mix}

$$RRF\ NT_{mix} = \frac{CONC_{IS\ mix}\ X\ PH_{NT\ mix}}{CONC_{NT\ mix}\ X\ PH_{IS\ mix}}$$

RRF: Relative retention factor

$CONC_{IS}$ mix: Weight of the internal standard (1ng) in the volume of the mix (10µl) injected

$CONC_{NT}$ mix: Weight of amine neurotransmitter (1ng) in the volume of the mix (10 µl) injected

PH_{NT} mix: Peak height of the amine neurotransmitter in the mix

PH_{IS} mix: Peak height of the internal standard in the mix

Step 2. Determination of neurotransmitter (NT) concentration per 10µl injected

N-Methyl-5-HT (2ng/20µl) was the internal standard within each sample. To determine the concentration of amines in the brain tissue sample in terms of ng of NT per 10µl, the following equation was used:

$$\text{NT Concentration in sample (ng/10µl):} \quad \frac{PH_{NT\ sample}\ X\ CONC_{IS\ sample}}{PH_{IS\ sample}\ X\ RRF\ of\ the\ NT_{mix}}$$

PH_{NT} sample: Peak height of the neurotransmitter in the sample

$CONC_{IS}$ sample: Concentration of the internal standard in the sample

PH_{IS} sample: Peak height of the internal standard in the sample

RRF of the NTmix: RRF of the NT in the mix (calculated in step 1)

Step 3. To express the results as ng NT/g tissue

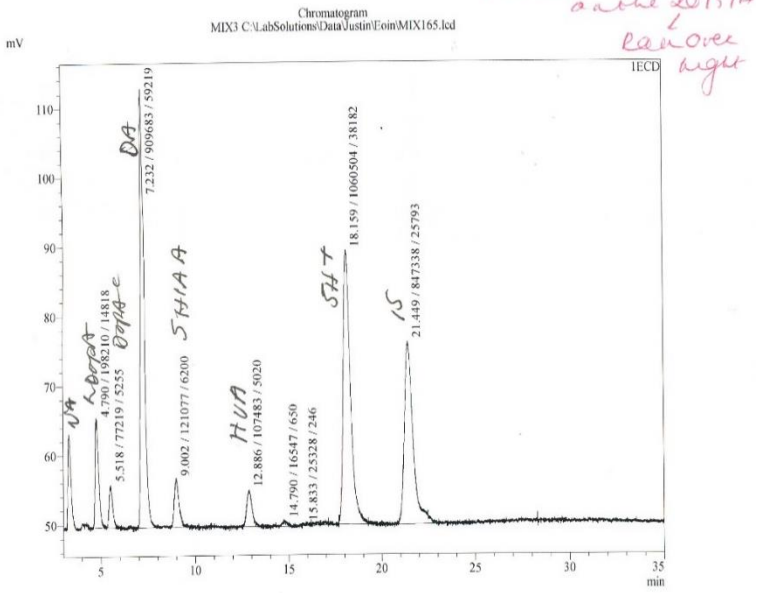
$$\text{NT concentration (ng/g tissue)} = \left(\frac{CONC\ NT_{sample}\ X\ 50}{\text{Weight of sample (mg)}} \right) \text{ X } 1000$$

TCD - School Of Pharmacy (A)

Sample Information MIX3 C:\LabSolutions\Data\Justin\Eoin\MIX165.lcd

Acquired by	: System Administrator
Sample Name	: MIX3
Sample ID	:
Vial#	: 12
Injection Volume	: 10
Data File	: MIX165.lcd
Method File	: ECD Method.lcm
Batch File	: Zara TC NUIG.lcb
Date Acquired	: 19/05/2017 23:31:29

Mix 3 → done on this day last Samples Ran on the 20/5/17 / Ran over night



TCD - School Of Pharmacy (B)

Sample Information IS C:\LabSolutions\Data\Justin\Eoin\IS032.lcd

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Sample ID	:
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Injection Volume	: 10
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Method File	: ECD Method.lcm
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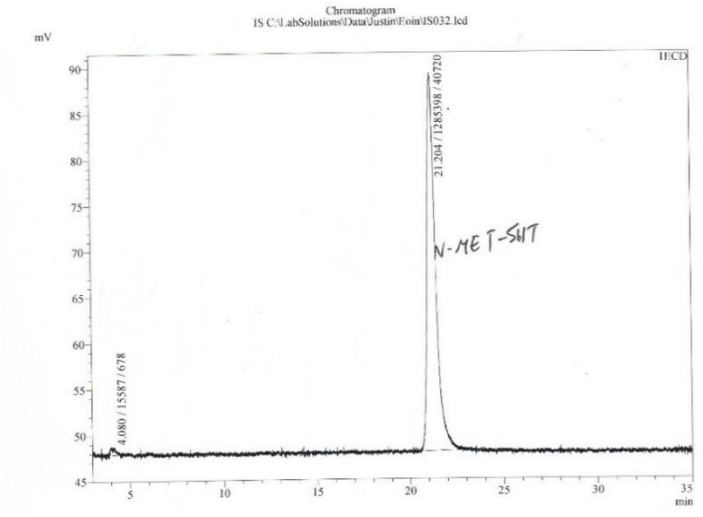


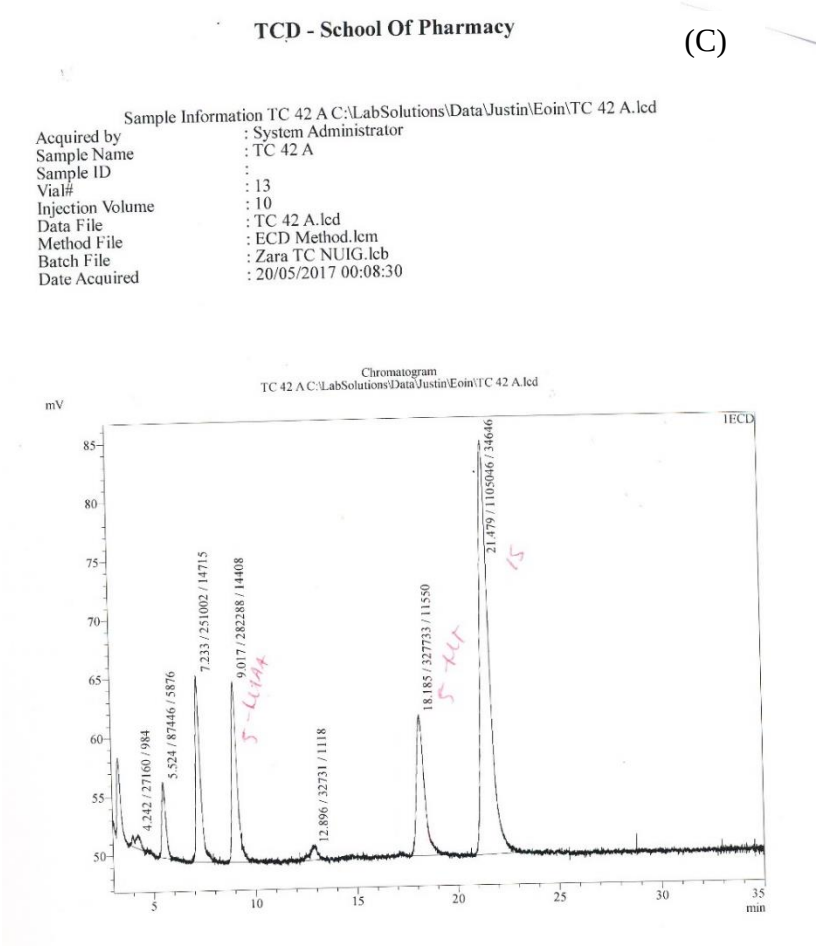


Figure 2.14: Chromatograms from HPLC analysis of 5-HT and 5-HIAA in the amygdala (A) Mix (B) Internal standard – N-Methyl-5-HT (C) Sample. Identification of relevant peaks identified by hand to allow for analysis.

2.2.11 Statistical analysis (Table 2.6)

All statistical analysis was carried out using IBM SPSS computer package. Specific statistical analysis used will be detailed in each results chapter, however in all cases normality and homogeneity of variance (p>0.05) was determine for each data set. If data passed normality and homogeneity, they were analysed parametrically. If normality and homogeneity were not found, data was transformed, in which case data were analsyed parametrically. If data could not be transformed non-parametric analysis was carried out. In all cases p<0.05 was significant.

The following statistical tests were carried out under the following circumstances

Table 2.6: List of statistical analysis carried out

Condition	Statistical test
2 groups • **Sham vs OB**	Parametric: Independent students t-test Non-parametric: Mann-Whitney U
2 Factors • **Lesion & DOI/8-OH-DPAT dose response** • **Lesion & antidpressant**	Parametric : Two-Way ANOVA, followed by One-Way ANOVA and *post-hoc* Student Newman-Keuls Non-parametric: Kruskal-Wallace, followed by all pair-wise comparison
Time as a factor • **Homecage activity with surgery only as only factor** • **Homecage activity as surgery and antidepressant treatment as a factory** • **Open field in time bins and behavioural parmeters**	Parametric: One-Way Repeated measures, followed by *post-hoc* Student Newman-Keuls Parametric: Two-Way Repeated measures, followed by One-Way ANOVA and *post-hoc* Student Newman-Keuls Non-parametric: Kruskall-Wallace followed by all pair-wise comparison

Chapter 3:

Time course investigation of central serotonergic parameters in the OB rat

3.1 Introduction

As was previously reviewed in Chapter 1, serotonergic alterations in depression are associated not only with helping to explain the underlying pathophysiology of the disease but also contribute to the mechanism of action of the majority of antidepressants. In clinical studies, there have been numerous ways in which the various components of the serotonergic system have been evaluated (e.g. peripherally, *in vivo* imaging and post mortem studies). It is not always possible or ethical to recruit or obtain samples from patients who are drug naïve. In cases where it has been possible to recruit such patients, the duration of the illness is oftentimes not specified or if is specified, it is generally when the initial diagnosis is made. Depression is generally self-reported and diagnostic tools such as the Hamilton scale are based on how the patients think they are feeling. Given the current lack of reliable biomarkers, the severity and the length of time the condition has been present, it is often difficult to estimate when the patients have been enrolled in the study. Thus it is difficult to accurately monitor changes in neurocircuitry in the progression of the disease.

Animal models provide controlled conditions where the time that the 'disease' has been established can be accurately measured and monitored. In the case of the OB model, many behavioural and serotonergic parameters have been measured. However, varying experimental designs for assessing behavioural and serotonergic changes have resulted in varying times at which these parameters are assessed. Therein, the period for which the syndrome has been established varies, which in turn may account for some of the disparity that exists in the literature, in respective of central serotonergic changes.

The ability of this model to respond to chronic but not acute antidepressant treatment is one the key characteristics reflective of the clinical disease. Accordingly, the length of time the lesion is established could also have a bearing on the therapeutic effects of antidepressants. Given the therapeutic lag that many antidepressants exhibit assessing their efficacy at the optimal time is crucial. As such, if antidepressant treatment commences 2 weeks following lesion but central serotonergic changes do not manifest until 4 weeks following lesion, treatment may have no effect. Equally, if alterations in the serotonergic system occur at an earlier time point, beginning treatment too late

may also be inconsequential, as the serotonergic system could have already undergone adaptive responses.

Thus, a useful starting point in investigating the central serotonergic system in the OB rat would be to examine in a time course manner whether there are any changes. The aim of this chapter is to determine whether there were changes in many aspects of the serotonergic transmission at 3 different time points, namely 2, 4 and 6 weeks following OB surgery. These time points were chosen as it is known that the behavioural hallmark of hyperactivity in the open field first appears at 1 week following lesion (Burke *et al.,* 2010) and given the therapeutic lag of many of the current antidepressants many studies assesses antidepressant efficacy in the OB rat from 1-3 weeks (Cryan *et al.,* 1999; Jiang *et al.,* 2014; Goyal *et al.,* 2009; Mar *et al.,* 2000; Bissette *et al.,* 2001), resulting in a length of 2-9 weeks following surgery before the rats would normally be killed for post-mortem measurements.

Hyperactivity in the open field is the behavioural hallmark of this model and is indicative of the fact that the syndrome has been successfully established. The most common time point at which hyperactivity in the open field is assessed is 2 weeks following lesion, however others are exposed at earlier time points (Burke *et al.,* 2010; Mar *et al.,* 2002), whilst others are exposed at later time points (Table 3.1).In the cases where the animals have been exposed at 2 weeks following lesion, this can be used to assign rats to treatment groups (Breuer *et al.,* 2008; Breuer *et al.,* 2009), whereas those that are tested later may have undergone antidepressant treatment (Gigliucci *et al.,* 2014; Harkin *et al.,* 1999; Norman *et al.,* 2012) or other behavioural tests (Borre *et al.,* 2012).

Table 3.1 Time following lesion that open field exposure commonly occurs in the OB rat

Time following lesion	Reference
2 weeks	Jiang *et al.*, 2014; Burke *et al.*, 2010 ; Wang *et al.*, 2012 ; El Mansari *et al.*, 2014; Burke *et al.*, 2013 ; Borre *et al.*, 2012 ; Prins *et al.*, 2011 ; Breuer *et al.*, 2008 ; Breuer *et al.*, 2009 ; Rodriguez-Gatzelumendi *et al.*, 2014 ; Gigliucci *et al.*, 2014
3 weeks	Gigliucci *et al.*, 2014; Cryan *et al.*, 1999 ; Prins *et al.*, 2011
4 weeks	Gigliucci *et al.*, 2014; Burke *et al.*, 2013; Morales-Medina *et al.*, 2013; Mar *et al.*, 2000 ; Cryan *et al.*, 1999 ; Harkin *et al.*, 1999 ; Goyal *et al.*, 2009 ; Norman *et al.*, 2012
5 weeks	Borre *et al.*, 2012; Prins *et al.*, 2011
7 or more weeks	Roche *et al.*, 2007; van der Stelt *et al.*, 2005; Borre *et al.*, 2012

As with open field exposure, there are also variations in the time following lesion that rats are sacrificed, resulting in varying increments following lesion in which changes to the serotonergic system are assessed. As mentioned previously, this is often due to experimental design whereby the rats may have undergone additional behavioural testing or antidepressant treatment. Commonly, changes in serotonergic parameters are assessed, approx. 2-3 weeks following lesion but this can extend to time points of over 8 weeks following lesion (Table 3.2) or be as early as one day following lesion (Prins *et al.*, 2010).

Table 3.2 Time following lesion that serotonergic parameters have been measured in the OB rat

Time following lesion	Reference
2 -3 weeks	Burke *et al.*, 2010; McGrath and Norman, 1999; Saitoh *et al.*, 2007; Van der Stelt *et al.*, 2005
4 weeks	Sato *et al.*, 2010 ; Slotkin *et al.*, 2005
5-6 weeks (37 days)	Prins *et al.*, 2011
8 or more weeks	Zhou *et al.*, 1998 ; Grecksch *et al.*, 1997

When considering a time course investigation of behavioural and neurochemical measurements in the OB rat, there are certain facets to be taken into consideration. Reversal of the hyperactivity in the open field by chronic antidepressant treatment is a hallmark sign that the treatment has been successful. However in some studies that look at both the OB hyperactivity and aspects of the serotonergic circuitry the rats are not re-exposed to the open field prior to sacrifice and assessment of serotonergic

parameters (Redmond *et al.*, 1997), therefore it cannot be said if the hyperactivity persists. Thus it is imperative to determine if the hyperactivity in the open field is a characteristic that is present early in lesion establishment or is hyperactivity a robust behavioural parameter in this model. Thus, any evaluation of time course related changes in open field behaviour would need to be investigated in separate cohorts of animals, as opposed to using the same cohort that are repeatedly exposed to the open field. This would enable the true status of the OB rats, as regards hyperactivity, to be determined without the confounding factor of habituation that will occur to some degree when the same animals are exposed to the open field on more than one occasion.

As well as the behavioural consideration, it is also important to determine if the alterations in the serotonergic system known in the model vary depending on how long the syndrome has been established and if so, do all components change in the same direction at each time point or are there variations in how the system changes depending on how long the syndrome has been established.

The ability of the being able to evaluate temporal changes in the OB model of depression will potentially have considerable utility as to provide an opportunity to determine if there is a time window during which animals are exposed to antidepressant treatment, with regard to the establishment of behavioural and central serotonergic measures which may be markers of the syndrome that can be evaluated as to whether they are modified by antidepressant treatment. Such investigations would require a stable collection of markers, or at the least an awareness of whether or how the markers might vary with time following olfactory bulbectomy. To our knowledge, this is the first time a time course analysis has been carried out assessing both the primary behavioural hallmark of this model in conjunction with a cluster of central serotonergic parameters.

The aims of this chapter are to:

1. Determine if the OB-induced hyperactivity in the open field is evident at the three selected time points, namely 2, 4 and 6 weeks following surgery.
2. Examine a range of serotonergic parameters in anatomically relevant regions, namely:
    a. Tryptophan hydroxylase expression in the dorsal raphe nucleus
    b. Tryptophan hydroxylase serotonergic terminal regions
    c. 5-HT_{1A} and 5-HT_{2A} mRNA expression in the hippocampus, amygdala and the prefrontal cortex
    d. 5-HT, 5-HIAA and 5-HIAA/5-HT in the amygdala
    e. Serotonin reuptake transporter expression in the dorsal raphe nucleus

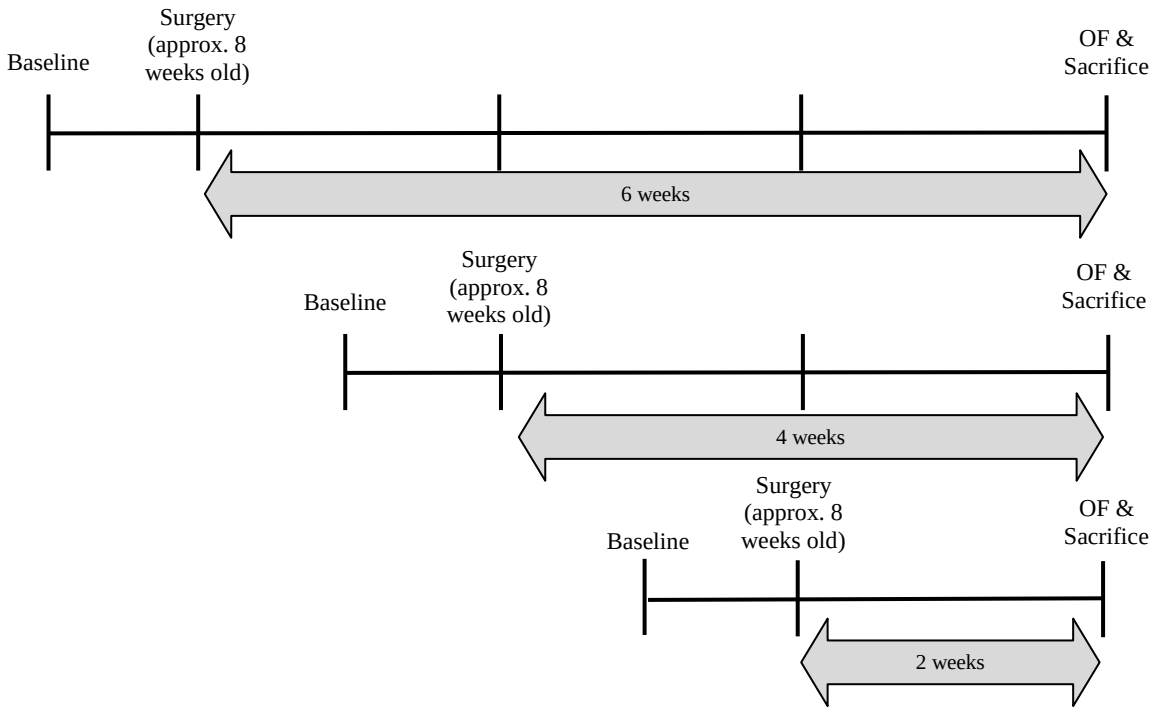
3.2 Methods

All methods have been described in more detail in Chapter 2. Methods specific to this chapter are detailed below.

3.2.1 Study design (detailed below)

All male Sprague-Dawley rats were housed in groups of 4 at 7 weeks old and sham-lesion or OB surgery was carried out at approx.8-weeks old (n=111). In order to maintain the ages of the rats across all 3 time points, rats were bred in house (as detailed in 2.2.2) in staggered fashion to ensure that all were approx. 8 weeks old at the time of surgery. Therefore rats that would remain for 6 weeks following lesion were bred first and underwent surgery first, followed by the 4 and 2 week groups (Study design is detailed below). Body weights at the time of surgery for each cohort are as follows:

- 6 week (190-280g, average pre-lesion body weight for sham-lesion (235g) and OB (239g))
- 4 week (220-330g, average pre-lesion body weight for sham-lesion (276g) and OB (277g))
- 2 week (220-350g, average pre-lesion body weight for sham-lesion (278g) and OB (279g))



Rats underwent the open field at 2, 4 or 6 weeks following lesion and were immediately killed either via decapitation or transcardial perfusion (as detailed in 2.2.7.2 Rat sacrifice and tissue collection). Rats were housed 2 shams and 2 OB per cage and weighed daily to avoid aggression developing (Leonard and Tuite 1981).

3.2.2 Tissue processing

Immunohistochemistry (as detailed in 2.2.9.2), PCR (as detailed in 2.2.8) and HPLC (as detailed in 2.2.10) carried out as detailed in Chapter 2.

TPH staining in the DRN was carried out as detailed in 2.2.9.2, however, in order to maximise the amount of available tissue two 1:6 series were carried out.

3.3 Results

3.3.1 Effect of olfactory bulbectomy over time on total body weight gain

The results are depicted in Figure 3.1. There was a significant effect of lesion [$F_{(1,103)}$ = 20.77, p<0.001)] and time [$F_{(2,103)}$ = 189.49, p<0.001] but no significant lesion x time interaction [($F_{(2,103)}$ = 0.28, p>0.05] was found. A significant increase in body weight gain was observed in the 4 and 6 week sham and OB groups when compared to their 2 week counterparts [p<0.05]. The OB animals exhibited a significant reduction in weight gain at all time points when compared to their respective sham groups [p<0.05].

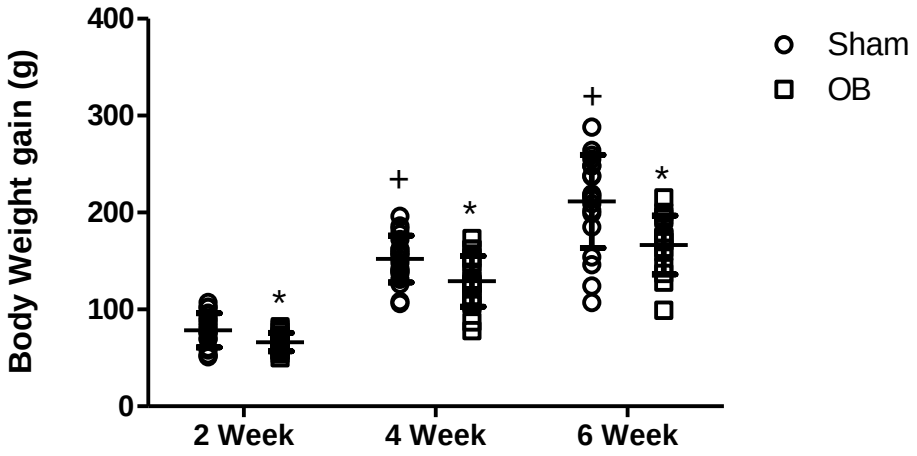


Figure 3.1 Total body weight gain. Data are expressed as mean ± SD (n=15-20). *p<0.05 vs sham counterparts; +p<0.05 vs 2 week sham [Student Newman-Keuls test]

3.3.2 Effect of olfactory bulbectomy over time on locomotor activity in the open field

The results are depicted in Figure 3.2. There was a significant effect of lesion [$F_{(1,103)}$ = 36.46, p<0.001] and time [$F_{(2,103)}$ = 8.36, p<0.001] but no significant lesion x time interaction [$F_{(2,103)}$ = 1.11, p>0.05] was found. At all time points, the OB rats moved significantly more when compared to their sham counterparts at all time points [p<0.05]. Sham rats at 4 weeks moved significantly less compared to their 2 week counterparts [p<0.05]

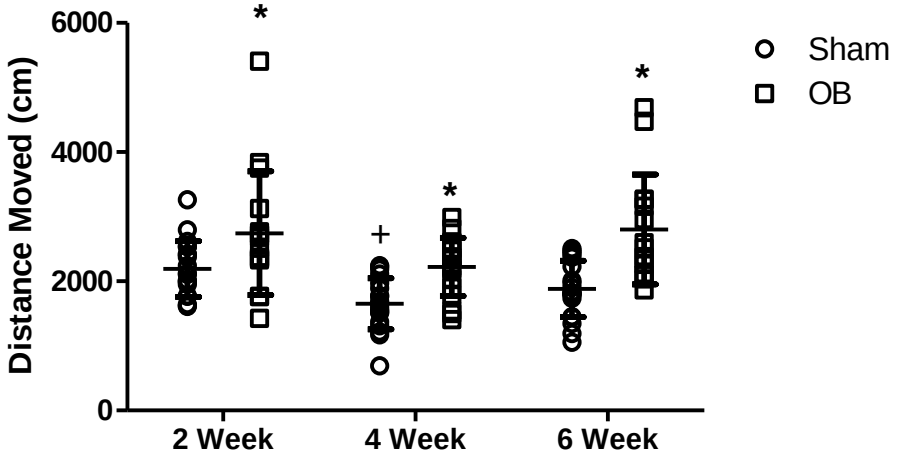


Figure 3.2 Distance moved in the open field. Data are expressed as mean ± SD (n=15-20). *p<0.05 vs sham counterparts +p<0.05 vs 2 week sham counterparts [Student Newman-Keuls test]

3.3.3 Tryptophan hydroxylase immunohistochemistry

3.3.3.1 Effect of olfactory bulbectomy over time on TPH positive cells in the DRN (Table 3.3)

No significant lesion effect was found, a significant time effect was found but no lesion x time interaction effect. A significant effect of time was found but subsequent *post-hoc* analysis revealed this to be between two non-comparable groups (Figure 3.3 for representative images)

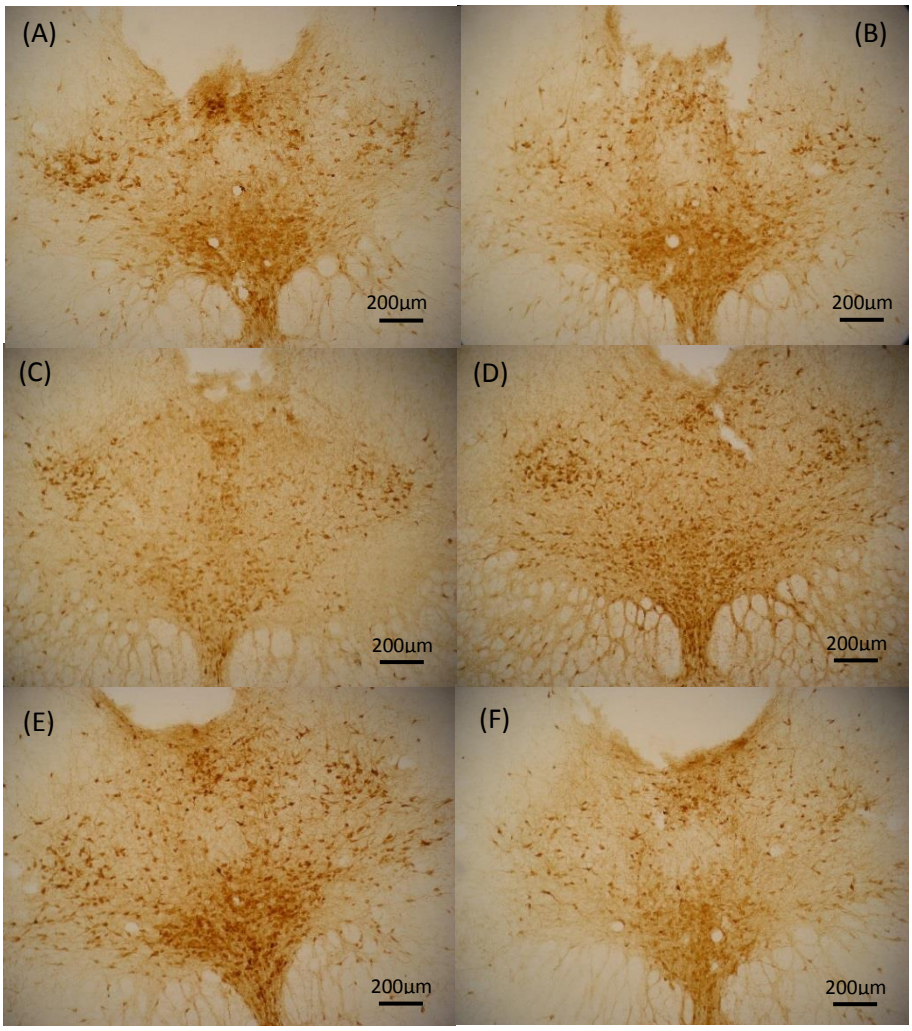


Figure 3.3 Representative images of TPH positive cells in the DRN of sham and OB animals at 2, 4 and 6 weeks following lesion. A - Sham 2 week; B - OB 2 week; C – Sham 4 week; D – OB 4 week; E – Sham 6 week; F – OB 6 week

Table 3.3 Effect of olfactory bulbectomy over time on TPH positive cells in the DRN

Group	Cell count/section
Sham + 2 Week	310 ± 124
OB + 2 Week	309 ± 134
Sham + 4 Week	419 ± 106
OB + 4 Week	383 ± 130
Sham + 6 Week	297 ± 126
OB + 6 Week	263 ± 108
Lesion	$F_{(1,46)} = 0.47$, $p>0.05$
Time	$F_{(2,46)} = 4.41$, ***p*<0.05**
Lesion x Time	$F_{(2,46)} = 0.10$, $p>0.05$

Table 3.3 Data are expressed as mean ± SD (n=8-10)

3.3.3.2 Effect of olfactory bulbectomy over time on TPH expression in terminal regions (Table 3.4)

No significant difference between the groups were found in either the striatum or the amygdala.(Figure 3.4 for representative images)

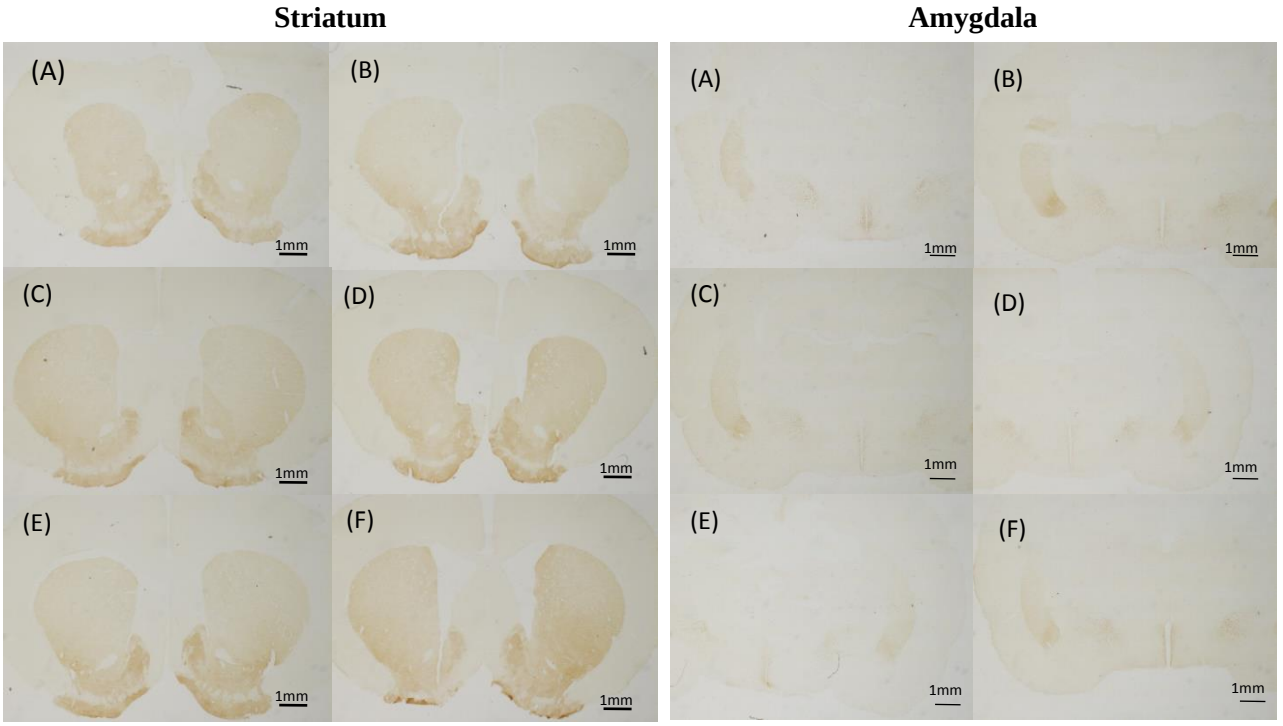


Figure 3.4 Representative images of TPH positive cells in the striatum and amygdala of sham and OB animals at 2, 4 and 6 weeks following lesion. A - Sham 2 week; B - OB 2 week; C – Sham 4 week; D – OB 4 week; E – Sham 6 week; F – OB 6 week

Table 3.4 Effect of olfactory bulbectomy over time on TPH expression in the striatum & amygdala

Group	Striatum	Amygdala
Sham + 2 Week	0.022 (0.013-0.025)	0.014 (0.008-0.020)
OB + 2 Week	0.017	0.020 (0.013-0.027)
Sham + 4 Week	0.023 (0.019 -0.029)	0.018
OB + 4 Week	0.032 (0.022-0.039)	0.019
Sham + 6 Week	0.023 (0.019-0.029)	0.015 (0.008-0.023)
OB + 6 Week	0.022 (0.018-0.030)	0.016 (0.015-0.025)
Group	$K_{(5)} = 6.03$, $p>0.05$	$K_{(5)} = 2.60$, $p>0.05$

Table 3.4 Data are expressed as median (25^{th} – 75^{th} percentile) OD/Section. (Striatum: n=3-7; Amygdala n=3-9) OD = optical density

3.3.4 5-HT receptor mRNA expression in discrete brain regions

3.3.4.1 Effect of olfactory bulbectomy over time on 5-HT$_{1A}$ receptor mRNA expression in discrete brain regions (Table 3.5)

A significant lesion effect was found in the PFC, but not in the hippocampus or amygdala. No significant time or lesion x time effect was found in the PFC, hippocampus or amygdala. Significant lesion effect found in the PFC was found to be non-significant in subsequent *post-hoc* analysis.

Table 3.5 Effect of olfactory bulbectomy over time on 5-HT$_{1A}$ mRNA fold change

Group	PFC	Hippocampus	Amygdala
Sham + 2 Week	1 ± 0.19	1 ± 0.107	1 ± 0.214
OB + 2 Week	0.846 ± 0.123	0.955 ± 0.060	0.978 ± 0.272
Sham + 4 Week	1.048 ± 0.263	1.136 ± 0.228	1.044 ± 0.165
OB + 4 Week	0.954 ± 0.139	1.056 ± 0.190	1.079 ± 0.228
Sham + 6 Week	1.093 ± 0.245	1.040 ± 0.153	0.996 ± 0.152
OB + 6 Week	0.889 ± 0.180	1.018 ± 0.169	1.197 ± 0.299
Lesion	$F_{(1,44)} = 7.00$, ***p*<0.05**	$F_{(1,50)} = 1.29$, $p>0.05$	$F_{(1,38)} = 1.04$, $p>0.05$
Time	$F_{(2,44)} = 0.75$, $p>0.05$	$F_{(2,50)} = 2.58$, $p>0.05$	$F_{(2,38)} = 0.96$, $p>0.05$
Lesion x Time	$F_{(2,44)} = 0.31$, $p>0.05$	$F_{(2,50)} = 0.15$, $p>0.05$	$F_{(2,38)} = 0.96$, $p>0.05$

Table 3.5 Data are expressed as mean ± SD (n=6-10)

3.3.4.2 Effect of olfactory bulbectomy over time on 5-HT_{2A} receptor mRNA expression in discrete brain regions (Table 3.6)

A significant lesion effect was found in the PFC and amygdala but not in the hippocampus. A significant time effect was found in the amygdala but not the PFC or hippocampus. No significant lesion x time effect was found in the PFC, hippocampus, or amygdala. Significant lesion and time effects were found to be non-significant in subsequent *post-hoc* analysis.

Table 3.6 Effect of olfactory bulbectomy over time on 5-HT_{2A} mRNA fold change

Group	PFC	Hippocampus	Amygdala
Sham + 2 Week	1 ± 0.121	1 ± 0.139	1 ± 0.078
OB + 2 Week	0.917 ± 0.184	0.971 ± 0.187	1.124 ± 0.431
Sham + 4 Week	0.984 ± 0.242	0.997 ± 0.257	0.893 ± 0.105
OB + 4 Week	0.866 ± 0.129	0.988 ± 0.221	0.874 ± 0.134
Sham + 6 Week	1.096 ± 0.193	1.090 ± 0.182	0.924 ± 0.067
OB + 6 Week	0.865 ± 0.179	1.082 ± 0.233	1 ± 0.091
Lesion	$F_{(1,44)} = 7.83$, ***p*<0.01**	$F_{(1,49)} = 0.072$, $p>0.05$	$F_{(1,39)} = 4.78$, ***p*<0.05**
Time	$F_{(2,44)} = 0.38$, $p>0.05$	$F_{(2,49)} = 1.325$, $p>0.05$	$F_{(2,39)} = 3.65$, ***p*<0.05**
Lesion x Time	$F_{(2,44)} = 0.71$, $p>0.05$	$F_{(2,49)} = 0.014$, $p>0.05$	$F_{(2,39)} = 0.51$, $p>0.05$

Table 3.6 Data are expressed as mean ± SD (n=6-10)

3.3.5 Effect of olfactory bulbectomy over time on 5-HT, 5-HIAA and 5-HIAA/5-HT content in the amygdala (Table 3.7)

No significant lesion effect was found in any of the parameters. A significant time effect was found 5-HT and 5-HIAA content but subsequent *post-hoc* analysis revealed this to be non-significant or was between non-comparable groups. No significant time or lesion x time interaction effect was found in any of the parameters measured.

Table 3.7 Effect of olfactory bulbectomy over time on 5-HT, 5-HIAA and 5-HIAA/5-HT content in the amygdala

Group	5-HT	5-HIAA	5-HIAA/5-HT
Sham + 2 Week	377.7 ± 107.8	2709.8 ± 757.2	7.2 ± 0.8
OB + 2 Week	428.9 ± 38.2	2701.9 ± 383.9	6.3 ± 0.9
Sham + 4 Week	400.3 ± 118.9	2514.7 ± 630.2	6.5 ± 1.4
OB + 4 Week	370.9 ± 105.3	2458.7 ± 554.7	6.8 ± 1.1
Sham + 6 Week	474.8 ± 61.8	2946.1 ± 561.7	6.2 ± 0.8
OB + 6 Week	505 ± 107.2	3241 ± 804.3	6.4 ± 0.8
Lesion	$F_{(1,50)} = 0.46$, $p>0.05$	$F_{(1,50)} = 0.21$, p>0.05	$F_{(1,50)} = 0.28$, $p>0.05$
Time	$F_{(2,50)} = 6.33$, ***p<0.01***	$F_{(2,50)} = 4.38$, ***p<0.05***	$F_{(2, 50)} = 1.20$, $p>0.05$
Lesion x Time	$F_{(2,50)} = 0.92$, $p>0.05$	$F_{(2,50)} = 0.41$, $p>0.05$	$F_{(2,50)} = 2.13$, $p>0.05$

Table 3.7 Data are expressed as mean ± SD (n=8-10) 5-HT and 5-HIIAA values are ng/g

3.3.6 SERT expression in the whole dorsal raphe nucleus

3.3.6.1 Effect of olfactory bulbectomy over time on SERT positive cells in the DRN (Table 3.8)

No significant lesion, time or lesion x time interaction was found (Representative images Figure 3.5)

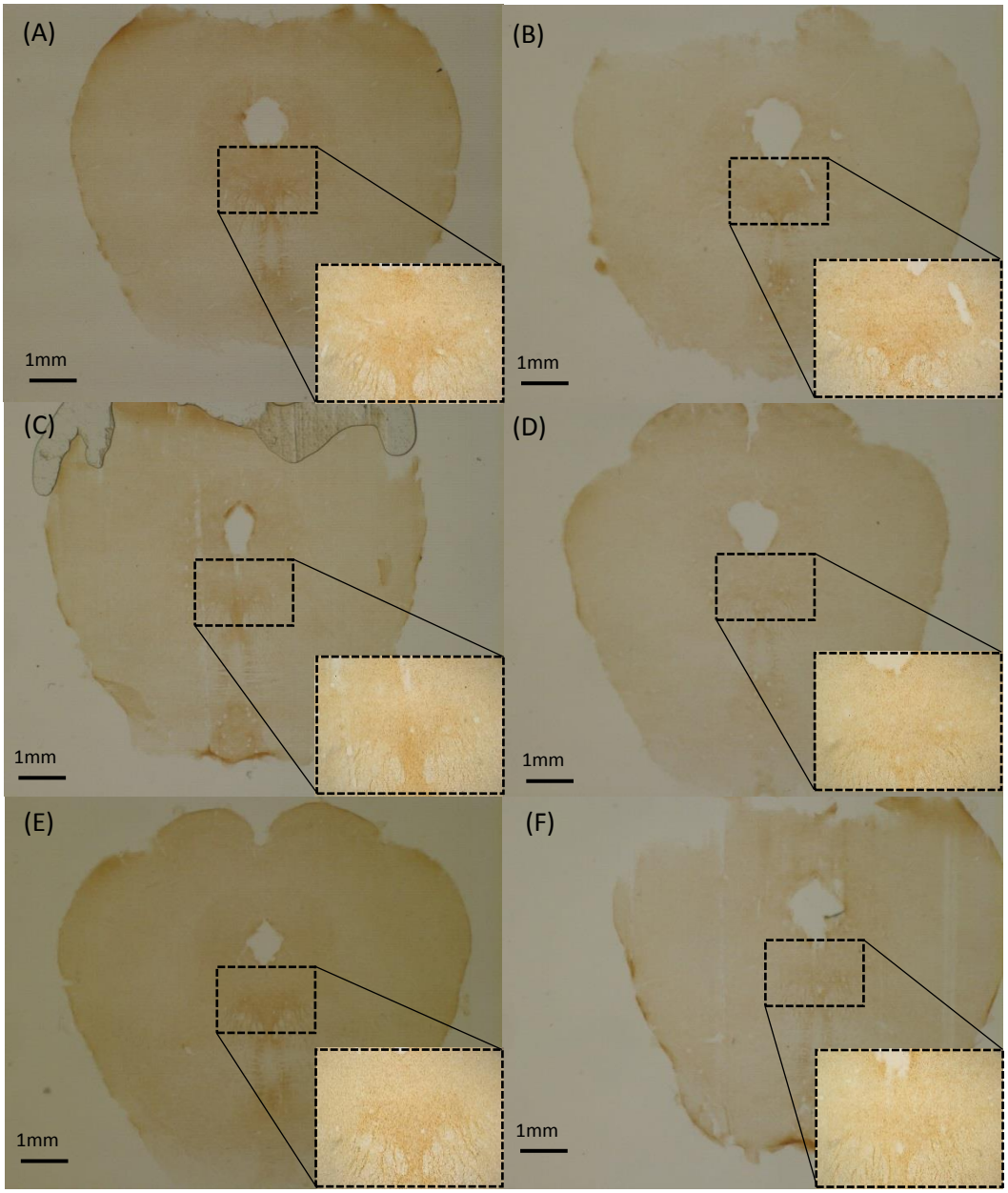


Figure 3.5. Representative images of SERT expression in the DRN of sham and OB animals at 2, 4 and 6 weeks following lesion. A - Sham 2 week; B - OB 2 week; C – Sham 4 week; D – OB 4 week; E – Sham 6 week; F – OB 6 week

Table 3.8 Effect of olfactory bulbectomy over time on SERT positive cells in the DRN

Group	OD/Section
Sham + 2 week	0.022 ± 0.012
OB + 2 Week	0.015 ± 0.013
Sham + 4 Week	0.037 ± 0.061
OB + 4 Week	0.030 ± 0.018
Sham + 6 Week	0.022 ± 0.015
OB + 6 Week	0.023 ± 0.019
Lesion	$F_{(1,37)} = 0.21$, $p>0.05$
Time	$F_{(2,37)} = 0.97$, $p>0.05$
Lesion x Time	$F_{(2,37)} = 0.07$, $p>0.05$

Table 3.8 Data are expressed as mean ± SD (n=5-10)

3.4 Discussion

The main aim of this chapter was to assess temporal changes in the OB rat model of depression, encompassing behavioural and central serotonergic neurocircuitry parameters. To summarise the findings, firstly OB rats gained significantly less weight compared to sham-lesion counterparts, whilst sham-lesion rats continued to gain weight for the duration of the experimental period. OB rats were significantly hyperactive in the open field compared to their sham-operated counterparts at each time point. Interestingly, sham-lesion rats at 4 weeks following lesion were significantly less mobile compared to sham-lesioned rats at 2 weeks following lesion. With regards to the central serotonergic components there was no significant effect of time or lesion in any of the parameters measured in *post-hoc* analysis but there were some main effects of lesion or time in the initial statistical analysis, these will be discussed in more detail below.

OB rats gaining significantly less weight compared to sham-lesion rats at all time points is in line the literature (Redmond *et al.,* 1997). Sham-lesioned rats continually gaining weight over time demonstrates that the surgical procedure itself does not affect the wellbeing of the rats.

OB-induced hyperactivity in the open field was apparent from within 2 weeks of surgery, and its maintenance throughout all of the time points suggests that it is a robust characteristic that is conserved with increasing time following lesion. The appearance of hyperactivity 2 weeks following lesion is supported by numerous other studies (Jiang *et al.,* 2014; Gigliucci *et al.,* 2014; Burke *et al.,* 2013; Borre *et al.,* 2012; Prins *et al.,* 2011) reflecting the standard practice of a 2 week recovery period prior to the initiation of additional behavioural tests or antidepressant treatment. Although, others have found that the hyperactivity is evident 1 week following lesion (Bissette *et al.,* 2001; Burke *et al.,* 2010), persisting for up to 10 weeks following lesion surgery (Borre *et al.,* 2012; Roche *et al.,* 2007; Van der Stelt *et al.,* 2005; Prins *et al.,* 2011; Hendriksen *et al.,* 2012). Demonstrating the robust nature of this behaviour is of great importance in the detection of the antidepressant treatment with this model, with attenuation of hyperactivity in the open field in response to chronic antidepressant being indicative of antidepressant efficacy in this model.

Intriguingly, sham-lesion rats at 4 weeks were found to be significantly less hyperactive compared to their counterparts at 2 weeks. To our knowledge this the first study investigating a temporal change open field hyperactivity, using separate cohorts of rats, whereas others have used the same cohort of rats, introducing the risk of habituation (Gigliucci *et al.,* 2014; Burke *et al.,* 2013; Burke *et al.,* 2010; Borre *et al.,* 2012; van der Stelt 2005; Prins *et al.,* 2011). The distance moved in sham-lesioned rats in the above study is not dissimilar to previous work carried out in our laboratory, using the same apparatus and testing conditions (Burke *et al.,* 2013). In comparison to other data, the results obtained in this study are higher in some instances (Gigliucci *et al.,* 2014; Borre *et al.,* 2012), whilst much lower compared to others (Burke *et al.,* 2010; van der Stelt *et al.,* 2005). However this could be due to differences in exposure times, 5 min vs 15-30 min (Prins *et al.,* 2011; van der Stelt *et al.,* 2005). Although not significant, the activity of sham-lesion rats at 6 weeks looks similar to those at 4 weeks, whilst being lower compared to those at 2 weeks. This could therefore indicate, that the surgical procedure may have some effects on sham rats in the post-operative period that may normalise or settle as time progresses.

With regards to changes in the central serotonergic system, the following applies. TPH expression in the DRN was largely unaffected by surgery, even though at 4 and 6 weeks following lesion OB rats have a slight reduction in TPH positive cell number. Noticeably, although the effect of time was not significant in *post-hoc* analysis, at 4 weeks following lesion, there seems to be an increase in TPH cell number which is unaffected by lesion. The lack of change in TPH positive cell number was unexpected as previous findings suggest that at 3-5 weeks following lesion, there is a significant decrease in the number of TPH positive cells in the DRN (Saitoh *et al.,* 2007; Saitoh *et al.,* 2008; Shin *et al.,* 2017). Similar methodological approaches, utilising immunohistochemical analysis followed by counting of TPH positive cells were used, thus methodological approaches are unlikely to play role in the differing results. This is further supported by similar numbers of cells found in approx. the same region of the DRN of sham rats found in this study and those in the literature, with the range in the literature 250-400 (Shin *et al.,* 2017; Roh *et al.,* 2016; Kim *et al.,* 2015; Saitoh *et al.,* 2008). An additional source of variation could be the sex of rats used, as females (Shin *et al.,* 2017) have higher rates of TPH activity compared to males (Carlsson and

Carlsson, 1988), which could leave them more susceptible to changes or the changes may be evident.

Given that the DRN is split into 3 major levels – rostral, middle and caudal (Abrams *et al.,*2004) the selection of the region of the DRN to be analysed is important. Due to tissue damage, the tissue available for analysis was limited, as such the results in our study come from the analysis of the middle portion DRN encompassing the dorsal, ventral and lateral wing subdivisions (Abrams *et al.*, 2004; Monti, 2010). Given that serotonergic projections from the DRN are wide reaching it was felt that by encompassing as many of the subdivisions as possible, it would allow for a more uniform analysis. Saitoh *et al.,* (2007) however appear to have assessed only the caudal portion of the DRN (from images provided), thus only representing a singular level of the DRN and not taking into the account the whole of the DRN and its projections (Monti, 2010). Potentially, the reduction in the TPH may be evident in particular subdivisions of the DRN.

Given that there was no significant change in TPH expression in the DRN, the next step was to assess changes in the terminal regions. It must be also be noted that from the images (Figure 3.4), the expression of TPH in the amygdala may appear to be unilateral, however TPH expression was found bilaterally. When the images were taken, each side was taken separately as the entirety of the section would not fit in the frame of the microscope. It was felt that taking each side separately would allow anatomical landmarks to identify the correct region to be maintained in the image. In the analysis, TPH expression of both left and right sides were measured to ascertain the level of expression. Interestingly, TPH expression was found in the striatum and the early part of the amygdala but no expression was found in the PFC or the hippocampus, which was surprising given that these are highly innervated by the serotonergic system. However, whilst both regions are highly innervated by the serotonergic system, the DRN projects to the amygdala, striatum, whereas the median raphe nucleus (MRN) projects to the hippocampus (Vertes and Linley, 2008). Therefore it could be suggested that TPH expression in the MRN may be altered, which may be reflected in its terminal regions. Yet, the lack of a lesion or time effect in the striatum or the amygdala may not be unexpected given that no change was found in the DRN.

The other studies that have examined TPH expression in the OB model in the frontal cortex are not directly comparable to our investigation as they utilised an ELISA (Zhou *et al.,* 1998; Grecksch *et al.,* 1997; Huether *et al.,* 1997). Although both techniques allow the expression of a protein of interest to be measured, ELISAs are very sensitive with both the detecting and catching antibodies being specific for the antigen of interest, where as in immunohistochemical (IHC) analysis only one antibody (the primary) is selective for the antigen of interest. In addition, ELISAs estimation of protein expression is discernible even at very high concentrations, whereas in IHC protein expression is determined by the level of staining, meaning that protein levels above maximum staining is not detectable (Ferrier *et al.,* 1999). Yet, the substantial advantage to using IHC analysis is that it allows cell morphology to be assessed and importantly for the study covered in this chapter, it allows subtle differences in expression in DRN sub regions to be assessed.

Analysis of the levels of 5-HT_{1A} and 5-HT_{2A} receptor mRNA in the prefrontal cortex, hippocampus and amygdala were chosen both for the regional relevance of the receptors in terms of the role in the pathophysiology or symptomology in depression but also given the fact that these are regions whereby the receptors are largely expressed. Radioligand binding or autoradiography are the commonly used techniques in assessing 5-HT_{1A} and 5-HT_{2A} receptors in the OB model (Sato *et al.,* 2010; Sato *et al.,* 2008; Slotkin *et al.,* 2005). However, given the number of samples across all studies covered in this thesis and the fact that autoradiography or radioligand binding were not available in our laboratory it was decided to proceed with analysis of mRNA levels, as opposed to the protein expression. It is acknowledged, that assessment of protein level would be preferable as this would have increased correlation to the functionality of the receptors. Insight into alterations into mRNA levels would also be beneficial as it would represent one of the earliest stages that alterations in receptor could occur.

In the above study there was no significant difference between lesion groups or as time following lesion progresses, however there was a trend for a reduction in expression of 5-HT_{1A} mRNA in the PFC in OB rats compared to sham-lesion counterparts at all time points following lesion. To our knowledge, the only other literature pertaining to receptor mRNA expression in this model demonstrated that at 2 weeks following lesion, OB rats exhibit a decrease in 5-HT_{1A} mRNA in the hippocampus with no

change in the PFC (Szewczyk *et al.*, 2014). Autoradiographic studies reveal, 5-HT_{1A} density is decreased in numerous regions, including the amygdala, hippocampus, cortical regions, as well as the dorsal DRN, in OB rats 4 weeks following lesion (Sato *et al.*, 2008).

In terms of 5-HT_{2A} receptor expression again, no significant effect of lesion or time following lesion was found in the regions examined, yet as with the 5-HT_{1A} receptor there was a trend for a reduction in expression of 5-HT_{2A} mRNA in the PFC of OB rats. Once again radioligand binding and autoradiographic studies have demonstrated that at increasing intervals following lesion (3, 4 and 5 weeks) there was no change in the number of 5-HT_{2A} binding sites in the occipital cortex of OB rats (Mudunkotuwa and Horton, 1996), whereas at nearly 4 weeks following lesion a decrease was found in the striatum and cerebral cortex, depending on the age of the rats (Slotkin *et al.*, 2005). Conversely at 9 weeks following lesion there was increased binding in the hippocampus in OB rats, with no change found in the frontal cortex (Buchborn *et al.*, 2014). At 4 weeks following lesion, a significant increase in receptor density in OB rats was found in the amygdala, hippocampus, frontal cortex and all stereological regions of the DRN, with accompanying decrease in the prefrontal cortex (Sato *et al.*, 2010; Earley *et al.*, 1994). In contrast, McGrath and Norman (1998) found no significant lesion effect on receptor binding density in the cortex, though there was a slight trend for an increase. This suggests that regional variations may exist in 5-HT_{2A} receptor alterations in this model and therefore further regions could be assessed in future studies.

With regard to serotonin, its metabolite 5-HIAA and serotonin turnover (5-HIAA/5-HT), no significant lesion or lesion x time interaction effect was found in the amygdala. A significant main effect of time was found but was not evident in subsequent *post-hoc* analysis. HPLC is a common method used to assess changes in these parameters, as it allows direct measurement of central serotonin and 5-HIAA levels. No change in any parameters, regardless of time following lesion, is in agreement with others, where at 3 weeks post-lesion (Burke *et al.*, 2010) and 4 weeks following lesion (Harkin *et al.*, 1999) no change is found. However, Saitoh *et al.*, (2008) found, at 3 weeks following lesion, significant decreases in serotonin and 5-HIAA, with a corresponding decrease in serotonin turnover. Increased 5-HT levels have been found in the amygdala of OB rats compared to sham (O'Connor *et al.*,

1985). In addition to HPLC, levels of serotonin and 5-HIAA have also been assessed via autoradiography, where regional differences in the magnitude of serotonin synthesis has been shown in OB rats (Watanabe *et al.,* 2003). Thus, in future work it is recommended that serotonin, 5-HIAA and turnover be assessed in other terminal regions, such as the PFC and the hippocampus, as although no difference was found in the amygdala it could be that the OB model leads to disproportionate levels of 5-HT available in certain terminal brain regions (Watanabe *et al.,* 2003)

The final stage in serotonergic transmission that was assessed was SERT expression in the DRN, as with the other parameters assessed there was no effect of lesion or time. Due to tissue quality the analysis was carried out in one tissue section per rat. With regards to the expression of SERT in this model, there is a limited amount of data, however radioligand binding and autoradiography are the two most commonly employed assessment methods. As previously stated, these are not available in the laboratory and therefore immunohistochemical analysis was chosen. Autoradiographical analysis of SERT expression in the DRN, reflect no significant changes found in the whole DRN as well as the dorsal, ventral and lateral subdivisions 4 weeks following lesion (Sato *et al.,* 2010). However significant increases were found in numerous terminal regions, suggesting further analysis of terminal regions is needed (Sato *et al.,* 2010).

To summarise, in the above study, hyperactivity in the open field is a robust behaviour that does not wane with increasing time following lesion. This reinforces its importance as a measure of antidepressant activity in this model. Interestingly, the locomotor activity of sham rats declines at 4 weeks following lesion and given that this is not a habituation effect, an argument could be made that the operation process itself has an effect that stabilises over time. Regardless of the time following lesion there were no alterations in any of the aspects of the serotonergic system that were measured, both pre- and post-synaptic measurements. Moreover, there were no changes amongst the sham-lesion rats over time, suggesting that the surgery process itself does not alter the system and that any changes that are found are due to the ablation of the bulbs. Taken together these results imply that, under the experimental conditions employed in the above study, the serotonergic system itself does not play a role in the behavioural hallmark of this model. Nonetheless it does not discount the

possibility that the serotonergic system does play role but perhaps it is through the modulation of other neurotransmitter systems.

For future studies, these results suggest that there is no optimal time window to expose the rats to antidepressant treatment, resulting in the modification of behavioural or serotonergic markers. We believe that this is the first piece of work to assess a multitude of pre and post-synaptic serotonergic parameters over time, however some aspects need to be further evaluated, including the effects of chronic antidepressant treatment on these parameters. Given this, although there is data available regarding 5-HT_{1A} and 5-HT_{2A} receptor expression in the literature, there is very little data regarding their functionality in this model. Agonists targeting these receptors can be used to assess functionality, thus Chapter 4 will assess dose responses to these agonists to determine an optimal dose that will be capable of detecting potential antidepressant effects.

Chapter 4:

Behavioural consequences of activation of 5-HT_{1A} and 5-HT_{2A} receptors in the OB rat

4.1 Introduction

One of the shortcomings of the literature with regards to the serotonergic system in the OB rat model is the lack of a comprehensive evaluation of multiple aspects of the neurochemistry and neurocircuitry. Whilst many published papers look at individual aspects of the system, an investigation of more than one element of the system has been lacking. One area that has not been adequately evaluated in the OB model is the functionality of the two main serotonergic receptors that have been implicated in depression – namely the 5-HT_{1A} and 5-HT_{2A} receptors.

The 5-HT_{1A} receptor is a GPCR, whose functionality (Barnes and Sharp, 1999), role in mood disorders (Garcia-Garcia *et al.,* 2014) and in antidepressant response (Celada, 2004) have been reviewed extensively. 5-HT_{1A} receptors are expressed on the on the soma and dendrites of serotonergic neurons in the DRN, where coupling $G_{\alpha i}$ proteins leads to numerous downstream signalling effects, such as partial inhibition of adenylate cyclase, opening of potassium channels and closing of calcium channels (Garcia-Garcia *et al.,* 2014; Barnes and Sharp, 1999; Raymond *et al.,* 2001). These receptors are also considered to be autoinhibitory and are thought to play a role in the delayed therapeutic action of antidepressants by limiting the initial increase in serotonin induced by SSRIs (Celada *et al.,* 2004). They are also expressed post-synatpically in terminal regions, with high concentrations found in the amygdala, hippocampus and frontal cortex, as well as being moderately expressed in the piriform cortex and hypothalamic nuclei (Polter and Li, 2010). The 5-HT_{2A} receptor is also a GPCR, which like 5-HT_{1A} receptor has been reviewed many times (Celada *et al.,* 2004; Zhang and Stackman, 2015). It is coupled to the G_q and excitatory in nature (Puig and Gulledge, 2011). It is highly expressed in the cortex, with lower levels in the hippocampus, amygdala, striatum and hypothalamus (Celada *et al.,* 2004).

8-OH-DPAT is a preferential 5-HT_{1A} agonist (Millan *et al.,* 1997), which acts as a full and potent agonist at this receptor (Dabrowska and Brylinksi, 2006). When administered systemically to rodents, it produces a range of stereotyped behaviours, known collectively as serotonin syndrome (Haberzettl *et al.,* 2013). This encompasses such behaviours flat body posture, hind limb abduction and forepaw treading (Haberzettl *et al.,* 2013), which are believe to be due to activation of the post-synaptic receptors (Bill *et al.,* 1991). DOI is a 5-HT_{2A} receptor agonist and systemic

administration also elicits stereotyped behaviours in rodents, including wet dog shakes, head shaking and head twitches (Haberzettl *et al.,* 2013). Use of these two agonists gives vital insight into the functionality of these two receptors.

With regards to investigations into these receptors in the OB model, the data is rather limited. In relation to the 5-HT_{1A} receptor, the density of the receptor in numerous brain regions has been assessed, with OB rats showing reduced densities in limbic areas, cortical regions, as well as in the raphe nucleus, including the dorsal but not the ventral portion of the DRN (Sato *et al.,* 2008). With regards to the assessment of functionality, research has been largely confined to examining the consequences of acute administration of 8-OH-DPAT on the hypothermic responses. No lesion induced changes were observed when examined at 2, 3 and 4 weeks following lesion (Cryan *et al.,* 1998; Harkin *et al.,* 1999; McGrath and Norman, 1998). With respect to the 5-HT_{2A} receptor, densitometric analysis is the primary form of analysis in this model, with OB rats exhibiting increases in the amygdala, hippocampus and DRN (Sato *et al.,* 2010). Investigations into the functionality of this receptor is scarce, one being, where the 5-HT_{2A} receptor agonist DOI elicited enhanced head-twitch responses in OB mice at doses of 0.5 and 1 mg/kg at 2 and 4 weeks following lesion (Oba *et al.,* 2013).

Given the lack of studies looking at the stereotyped behaviours elicited by both 8-OH-DPAT and DOI in OB rats, doses required to achieve the adequate effects needed to be established. However, in carrying out a literature search it was found that in studies assessing the effects of these two compounds on the stereotyped behaviours, there was a wide range of doses that could be used. With regards to the doses it would appear that much lower doses were required to elicit a drop in body temperature than was needed to reliably elicit the group of behaviours known as serotonin syndrome. Therefore it was decided that a dose response study was required to determine the optimal doses.

As there are limited published data available regarding to doses best used to assess serotonin syndrome in the OB model induced by 8-OH-DPAT, with the only studies to our knowledge reporting a hypothermic response, it was decided to start with a low dose of 0.1 mg/kg as doses of 0.15 mg/kg and upwards have been shown to induce hypothermia in sham and OB rats (Cryan *et al.,* 1997; McGrath and Norman, 1998;

Cryan *et al.*, 1999), with a dose as low as 0.05mg/kg also demonstrating hypothermia in the OB rat (Harkin *et al.*, 1999). The doses chosen are detailed in Table 4.1, as each one has been shown to elicit aspects of the serotonin syndrome in 'normal' rats.

Table 4.1 8-OH-DPAT doses selected for dose response analysis

Dose	Parameter	Reference
0.1 mg/kg	Hypothermia	Wright *et al.*, 2012; Nalivaiko *et al.*, 2009
	Increased locomotor activity	Nalivaiko *et al.*, 2009
	Head weaving Forepaw treading Flat body posture	Dabrowska *et al.*, 2007;Piper *et al.*, 2006
0.25 mg/kg	Hypothermia	Forster *et al.*, 1995; Chueh *et al.*, 2004
	Flat body posture	Rodriguez- Manzo *et al.*, 2011
	Hindlimb abduction Locomotor effects Forepaw treading Flat body posture	Granoff and Ashby, 2001; Samad *et al.*, 2007;
0.5 mg/kg	Forepaw treading Head weaving	Haleem *et al.*, 2007; Piper *et al.*, 2006; Granoff and Ashby, 2001;
	Flat body posture	Inam *et al.*, 2006; Batool and Haleem, 2008; O'Connell and Curzon, 1996

In selection of the doses of DOI for a dose-response study, it was important to look at studies which investigated the effects of DOI in other behavioural phenotypes or under different experimental conditions. The doses chosen are detailed in Table 4.2 and as with 8-OH-DPAT were chosen based on their ability to elicit aspects of the serotonin syndrome in 'normal' rats.

Table 4.2 DOI doses selected for dose response analysis

Dose	Parameter	Reference
0.3 mg/kg	Wet dog shakes	Kawakami *et al.*, 2005; Kitamura *et al.*, 2007
1 mg/kg	Locomotor effects	Bull *et al.*, 2004
	Wet dog shakes	Hill *et al.*, 2006; Bull *et al.*, 2004; Gorzalka *et al.*, 2005;Amano *et al.*, 2007; Kozuru *et al.*, 2000
	Rearing	Bull *et al.*, 2004
3 mg/kg	Head twitches	Biezonski *et al.*, 2009
	Wet dog shakes	Kawakami *et al.*, 2005; Kitamura *et al.*, 2007; Biezonski *et al.*, 2009; Xu and Miller, 1998

It was also important to select a time following olfactory bulbectomy at which the functional responses would be examined. As the data is limited on assessing the functionality of these receptors in this model of depression it was decided that animals would be treated 4 weeks following lesion, as this would be in line with the previous literature investigating the functionality of this receptor in this model (Cryan *et al.*,1998; Harkin *et al.,* 1999; Oba *et al.,* 2013). However, it would also tie in with a commonly using dosing regime of antidepressants, where by many studies assess the efficacy of antidepressant treatment after a period of 14-days (Breuer *et al.,* 2008; Breuer *et al.,* 2009) , and thus could be used in future investigations to assess the impact of chronic antidepressant treatment on these responses.

Therefore the aim of this study was:

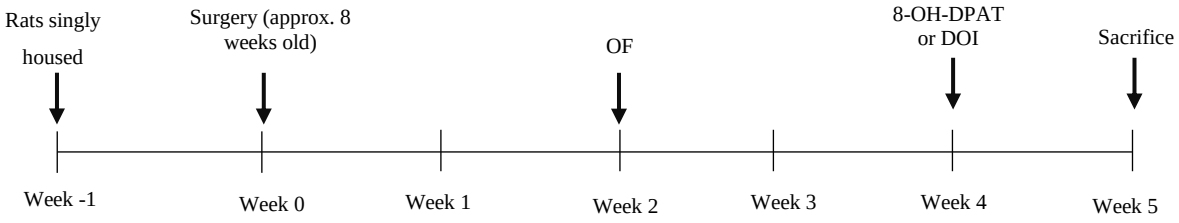
1. To determine the behavioural consequences of acute administration of 8-OH-DPAT and DOI by employing a dose response paradigm to investigate whether there are functional alterations in the 5-HT_{1A} and 5-HT_{2A} receptors in the OB model
2. To select a single dose of each serotonergic agonist which can be used for further studies where the effect of chronic antidepressant treatment on the functionality of the 5-HT_{1A} and 5-HT_{2A} receptors can be evaluated in the OB model

4.2 Methods

All methods have been described in more detail in Chapter 2. Methods specific to this chapter are detailed below.

4.2.1 Study design (detailed below)

All male Sprague-Dawley rats were singly housed for a week prior to surgery and sham or OB surgery was carried out at approx.8-weeks old (n=60). Following surgery, rats were handled and weighed daily, together with food and water weights, to avoid aggression developing and underwent the open field 2 weeks following lesion to confirm the syndrome had developed. At 4 weeks following surgery, rats were given a subcutaneous acute injection of either 8-OH-DPAT (0.1, 0.25 and 0.5 mg/kg) or DOI (0.3, 1 and 3 mg/kg) and their locomotor and stereotyped behaviour was assessed (as detailed in 2.2.5.3).



4.2.2 Animal sacrifice

One week post testing, approx. 5 weeks following lesion animals were sacrificed via decapitation or transcardial perfusion (as detailed in 2.2.7) to confirm lesion status.

4.2.3 Scoring of behaviours

Locomotor activity and post-dosing behaviour were analysed as detailed previously (2.2.5.3). This study design did not include a vehicle treated control group, as the agonist-elicited behaviours are specific to the agonist are largely absent from the normal repertoire of rat behaviour. To verify this, rat baseline behaviour was scored for the first hour of the baseline period for all of the behaviours (description of behaviours detailed in 2.2.5.3).

4.3 Results

4.3.1 Effect of olfactory bulbectomy on locomotor activity in the open field

The results are depicted in Figure 4.1. There was a significant effect of lesion, with OB rats being significantly hyperactive compared to their sham counterparts [$t_{(58)}$ = 7.59, p<0.001].

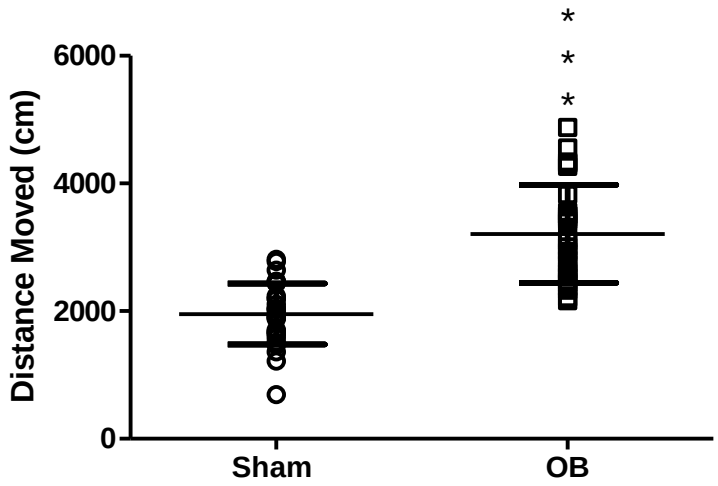


Figure 4.1. Locomotor activity in the open field in sham and OB animals 2 weeks following surgery. Data are expressed as mean ± SD, ***p<0.001 vs sham (n=30)

4.3.2 Functionality of 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors

4.3.2.1 Effect of 8-OH-DPAT on locomotor activity in OB rats (Table 4.3)

A significant lesion and drug effect was found during the first hour post dosing and for the duration of the 2 hour dosing period, with OB rats in the 0.5 mg/kg showing significantly greater locomotor activity when compared to their sham-operated counterparts receiving the same dose of 8-OH-DPAT [$p<0.05$]. Significant lesion x drug interaction was found not to be significant in subsequent *post-hoc* analysis.

Table 4.3 Dose response effect of 8-OH-DPAT on locomotor activity in the 1st hr and 2 hr trial duration in OB rats

Group	1st hr	Total (2 hr)
Sham + 0.1 mg/kg	-841 ± 96	-164 ± 292
Sham + 0.25mg/kg	474 ± 430	391 ± 1014
Sham + 0.5 mg/kg	399 ± 501	420 ± 352
OB + 0.1 mg/kg	930 ± 716	888 ± 678
OB + 0.25mg/kg	1401 ± 612	1316 ± 251
OB + 0.5 mg/kg	2409 ± 837*	2949 ±1234*
Lesion	$F_{(1,24)} = 27.40$, ***p*<0.001**	$F_{(1,24)} = 30.97$, ***p*<0.001**
Drug	$F_{(2,24)} = 5.07$, ***p*<0.05**	$F_{(2,24)} = 8.16$, ***p*<0.01**
Lesion x Drug	$F_{(2,24)} = 1.9$, $p>0.05$	$F_{(2,24)} = 3.63$, ***p*<0.05**

Table 4.3 Data are expressed as mean ± SD (n=5) *$p<0.05$ vs sham [Student Newman-Keuls test]

4.3.2.2 8-OH-DPAT induced behaviours in OB rats

4.3.2.2.1 8-OH-DPAT induced behaviours in OB rats at baseline (Table 4.4)

Certain behaviours, such as flat body posture, hindlimb abduction, head shakes and head twitch were not detected and therefore are not included. No significant lesion, dose or lesion x dose interaction effect was found in any of the behavioural parameters assessed.

Table 4.4 Frequency of behaviours in OB rats at baseline

Group	Rearing	Forepaw treading	Head weaving	Wet dog shakes
Sham	33 ± 12	1 ± 1	0 ± 1	0 ± 0
OB	30 ± 27	0 ± 1	1 ± 2	0 ± 1
Lesion	$t_{(28)} = 0.49$, $p>0.05$	$t_{(28)} = 0$, $p>0.05$	$t_{(28)} = 1.31$, $p>0.05$	$t_{(28)} = 1.18$, $p>0.05$

Table 4.4 Data are expressed as mean ± SD (n=5)

4.3.2.2.2 8-OH-DPAT induced behaviours in OB rats (Table 4.5)

Significant lesion and drug effects were found to be non-significant or between non-comparable groups in subsequent *post-hoc* analysis. No lesion x drug interaction effect was found in any of the behavioural parameters assessed. However a general reduction in flat body posture, hindlimb abduction, and increase in head weaving was observed in the OB groups when combined together, with greater rearing observed in the OB groups when compared to shams.

Table 4.5 Frequency of 8-OH-DPAT induced behaviours in OB rats

Group	Rearing	Flat body posture	Hindlimb abduction	Forepaw treading	Head weaving	Wet dog shakes	Head shakes	Head twitch
Sham + 0.1 mg/kg	32 (19-81.5)	9 (1-12)	5 ± 5	10 (0-12)	4 ± 4	4 ± 6	3 ± 2	0 ± 0
Sham + 0.25 mg/kg	17 (17-26)	4 (1.5-7)	27 ± 17	1 (0-1.5)	7 ± 5	2 ± 1	2 ± 2	0 ± 0
Sham + 0.5 mg/kg	7 (14.5-23)	8 (4.5-22.5)	53 ± 59	0 (0-0.5)	5 ± 5	1 ± 2	1 ± 2	1 ± 0
OB + 0.1 mg/kg	51 (40-62.5)	1 (1-1.5)	0 ± 0	4 (2.5-8.5)	13 ± 14	2 ± 2	2 ± 2	0 ± 0
OB + 0.25 mg/kg	29 (19.5-34.5)	2 (1.5-11)	6 ± 5	0 (0-0)	18 ± 10	2 ± 1	2 ± 3	0 ± 0
OB + 0.5 mg/kg	39 (15.5-50.5)	2 (1-21.5)	15 ± 18	1 (0-17.5)	23 ± 36	5 ± 8	5 ± 8	0 ± 0
Lesion			$F_{(1,24)} = 4.73$, ***p*<0.05**		$F_{(1,24)} = 5.61$, ***p*<0.05**	$F_{(1,24)} = 0.02$, p>0.05	$F_{(1,24)} = 0.29$, *p*>0.05	$F_{(1,24)} = 0.66$, *p*>0.05
Drug	$K_{(5)} = 12.98$, ***p*<0.05**	$K_{(5)} = 6.34$, *p*>0.05	$F_{(2,24)} = 3.60$, ***p*<0.05**	$K_{(5)} = 10.20$, *p*>0.05	$F_{(2,24)} = 0.56$, *p*>0.05	$F_{(2,24)} = 0.04$, *p*>0.05	$F_{(2,24)} = 0.65$, *p*>0.05	$F_{(2,24)} = 1.16$, *p*>0.05
Lesion x Drug			$F_{(2,24)} = 0.99$, *p*>0.05		$F_{(2,24)} = 0.07$, *p*>0.05	$F_{(2,24)} = 1.41$, *p*>0.05	$F_{(2,24)} = 1.44$, *p*>0.05	$F_{(2,24)} = 0.16$, *p*>0.05

Table 4.5 Data are expressed as mean ± SD for parametric analysis and median (25^{th} – 75^{th} percentile) for non-parametric analysis (n=5)

4.3.2.3 Effect of DOI on locomotor activity in OB rats (Table 4.6)

No significant lesion, drug or lesion x drug interaction was found in the first hour. A significant lesion effect was found in the total 2 hr period, but no significant drug or lesion x drug interaction was found. *Post-hoc* tests revealed that the OB group that received the 3 mg/kg dose of DOI moved significantly more compared to their sham counterparts [$p<0.05$]

Table 4.6 Dose response effect of DOI on locomotor activity in the 1st hr and 2 hr trial duration in OB rats

Group	1st hr	Total (2 hr)
Sham + 0.3 mg/kg	775 ± 512	1449 ± 567
Sham + 1 mg/kg	778 ± 358	1720 ± 486
Sham + 3 mg/kg	526 ± 311	788 ± 427
OB + 0.3 mg/kg	1198 ± 656	1939 ± 629
OB + 1 mg/kg	820 ± 656	2081 ± 592
OB + 3 mg/kg	1553 ± 1158	3288 ± 622*
Lesion	$F_{(1,24)} = 3.96$, $p>0.05$	$F_{(1,24)} = 6.31$, **$p<0.05$**
Drug	$F_{(2,24)} = 0.71$, $p>0.05$	$F_{(2,24)} = 0.20$, $p>0.05$
Lesion x Drug	$F_{(2,24)} = 1.31$, $p>0.05$	$F_{(2,24)} = 2.42$, $p>0.05$

Table 4.6 Data are expressed as mean ± SD (Post-dose – Baseline) (n=5). *$p<0.05$ vs Sham + 3 mg/kg [Student Newman-Keuls test]

4.3.2.4 DOI induced behaviours in OB rats

4.3.2.4.1 DOI induced behaviours in OB rats at baseline (Table 4.7)

There was a significant effect of lesion in the frequency of wet dog shakes [$p<0.05$]. No significant lesion effect was found in the frequency of rearing, forepaw treading and head weaving. Flat body posture, hindlimb abduction, head shakes and head twitches were scored, however in all cases, either no behaviour was detected, or was minimally detected and therefore it was not analysed.

Table 4.7 Frequency of behaviours in OB rats at baseline

Group	Rearing	Forepaw treading	Head weaving	Wet dog Shakes
Sham	31 ± 15	0 ± 1	0 ± 1	1 ± 1
OB	37 ± 23	1 ± 1	0 ± 1	0 ± 1
Lesion	$t_{(28)} = 0.81$, $p>0.05$	$t_{(28)} = 1.26$, $p>0.05$	$t_{(28)} = 0.42$, $p>0.05$	**$t_{(28)} = 2.25$, $p<0.05$**

Table 4.7 Data are expressed as mean ± SD (n=5) *$p<0.05$ vs Sham

4.3.2.4.2 DOI-induced behaviours in OB rats (Table 4.8)

A significant lesion effect was found in the frequency of rearing and wet dog shakes, with a significant drug effect also found in the frequency of rearing. A significant lesion and drug effect was found in the frequency of rearing, with 1 and 3 mg/kg significantly reducing the frequency of rearing in sham and OB rats when compared to their 0.3 mg/kg treated counterparts [$p<0.05$]. In addition, there was a significant lesion effect on wet dog shakes, which was not confined to a specific group on *post-hoc* analysis, but was more an overall reduction in the OB model, irrespective of dose of DOI employed, Lesion effect on rearing was found to be non-significant in subsequent *post-hoc* analysis. No significant lesion x drug interaction effect was found in any of the behavioural parameters measured.

Table 4.8 Frequency of DOI induced behaviours in OB rats

Group	Rearing	Flat body posture	Hindlimb abduction	Forepaw treading	Head weaving	Wet dog shakes	Head shakes	Head twitch
Sham + 0.3 mg/kg	58 ± 18	0 (0-10.5)	1 (0-4)	5 ± 8	1 ± 1	13 ± 7	1 ± 1	0 (0-1)
Sham + 1 mg/kg	27 ± 10*	0 (0-8.5)	0 (0-0.5)	12 ±15	1 ± 1	21 ± 15	3 ± 4	0 (0-3)
Sham + 3 mg/kg	23 ± 6*	0 (0-2)	0 (0-0)	11 ± 8	4 ± 2	14 ± 6	6 ± 6	0 (0-0.5)
OB + 0.3 mg/kg	68 ± 12	0 (0-7)	0 (0-0)	7 ±7	3 ± 4	8 ± 6	2 ± 3	0 (0-0.5)
OB + 1 mg/kg	36 ± 14*	0 (0-6)	0 (0-3)	8 ±5	1 ± 2	6 ± 4	3 ± 3	0 (0-0)
OB + 3 mg/kg	34 ± 16*	0 (0-0.5)	0 (0-0)	9 ± 12	3 ± 4	7 ± 7	6 ± 8	0 (0-1)
Lesion	$F_{(1,24)} = 4.25$, **p=0.05**			$F_{(1,24)} = 0.07$, $p>0.05$	$F_{(1,24)} = 0.008$, $p>0.05$	$F_{(1, 24)} = 8.64$, ***p<0.01***	$F_{(1,24)} = 0.01$, $p>0.05$	
Drug	$F_{(2,24)} = 20.89$, ***p<0.001***	$K_{(5)} = 0.91$, $p>0.05$	$K_{(5)} = 9.27$, $p>0.05$	$F_{(2,24)} = 0.6$, $p>0.05$	$F_{(2,24)} = 1.64$, $p>0.05$	$F_{(2,24)} = 0.33$, $p>0.05$	$F_{(2,24)} = 2.08$, $p>0.05$	$K_{(5)} = 3.69$, $p>0.05$
Lesion x Drug	$F_{(2,24)} = 0.036$, $p>0.05$			$F_{(2,24)} = 0.283$, $p>0.05$	$F_{(2,24)} = 4.92$, $p>0.05$	$F_{(2,24)} = 1.05$, $p>0.05$	$F_{(2,24)} = 0.08$, $p>0.05$	

Table 4.8 Data are expressed as mean ± SD for parametric analysis and median (25^{th} – 75^{th} percentile) for non-parametric analysis (n=5).*p<0.05 vs 0.3 mg/kg treated counterparts [Student Newman-Keuls test]

4.4 Discussion

The main aim of this study was to determine the optimal dose of 8-OH-DPAT and DOI in order to assess the functionality of the 5-HT_{1A} and 5-HT_{2A} receptors respectively in the OB rat model. As expected, hyperactivity of OB rats was established 2 weeks following lesion, confirming that the syndrome has been successfully established.

With regards to 8-OH-DPAT changes, the highest dose of 0.5 mg/kg produced a significant increase in locomotor activity in OB rats compared to sham-lesion counterparts. This therefore suggests that the 5-HT_{1A} receptor functionality is greater in OB rats. Increased sensitivity of this receptor has been shown (via GTP-gamma-S binding) in the frontal cortex of OB rats (Buchborn *et al.,* 2014).This increase in locomotor activity was seen in the first hour as well as the total 2 hr observation period, indicating that 5-HT_{1A} mediated effects are present at an early stage. Increases in locomotor activity in response to a range of doses of 8-OH-DPAT, similar to the ones in the above study, have been reported previously (Hillegaart *et al.,* 1989; Blanchard *et al.,* 1993; Evenden and Angeby-Moller, 1990).

As the rats acted as their own control, it was important to assess the presence of any of the stereotyped behaviours prior to injection. Therefore in the first hour of the baseline period, rats were removed from the cage and weighed as normal to replicate the process that would take place with dosing. It was found that very few stereotyped behaviours were evident, as would be expected. Of those that were detected, rearing is normal aspect of a rats behaviour and therefore it would be expected that this would be present. In terms of the frequency of forepaw treading detected, head weaving and wet dog shakes, these were minimal in occurrence and therefore any increase after drug administration would more than likely be due to the effects of the drug. However, in an attempt to limit any false positives, forepaw treading was only recorded if the rat was not in the vicinity of the food hopper; head weaving was only recorded if the animal was on all fours and was not looking at anything in particular (e.g. the tester in the room or a rat in an adjacent cage); wet dog shakes were only recorded if they were not accompanied by grooming, biting or licking at the injection site to remove any sign of irritation being misconstrued as a particular behaviour. Given this, we were confident in proceeding with the rats acting as their own controls.

In relation to stereotyped behaviour, there was no significant response exhibited. However, there was a trend for a dose response in incidences of hindlimb abduction in both sham and OB rats, with the incidence being lower in OB rats when combined as a whole. At a dose of 0.25 mg/kg a reduction in rearing was found in the first 10 min when the animals were tested in their home cage, as was the condition here, but for the remainder of the 2 hr testing period there was no change in rearing frequency (Blanchard *et al.,* 1993). Therefore the fact that the frequency of rearing represents a 1 hr testing period could be why there were no significant effects found in response to 8-OH-DPAT. Future work could investigate the behaviours in time bins to see if there is an optimal time to capture a heightened response. The robust appearance of some but not all the expected behaviours at the doses given are in line with the current literature (Rogriguez-Manzo *et al.,* 2011)

With regards to DOI-induced changes in locomotor activity, no significant effect was found within the first hour, however in the total 2 hr observation period OB rats treated with 3 mg/kg were significantly hyperactive compared to their sham-lesion counterparts. Given the lack of studies investigating the effect of DOI in the OB model it is hard to determine if the effects found are to be expected, however DOI has been shown to decrease locomotor activity in normal rats (Krebs-Thomson *et al.,* 1998), albeit at a lower dose, this would concur with the decrease in locomotor activity observed in sham-lesioned rats. In 5-HT_{2A} knockout mice, DOI at a dose of 5 mg/kg was found to cause a delayed increase in the locomotor activity (Halberstadt *et al.,* 2009). Our results would be similar, whereby the increase of hyperactivity was only evident in total 2 hr observation period. As with 8-OH-DPAT, the hyperactivity exhibited in OB rats, would be suggestive that the receptor functionality is greater in this model. Given that these receptors are known to become internalised in response to agonist stimulation, it could be suggested that could there could be a decrease in the number of receptors expressed but those that are remaining may exhibit increased functionality. An argument could also be made, that in the OB model, 5-HT_{2A} receptors have reduced functionality when compared to 5-HT_{1A} receptors, as the hyperactive response is only evident in a 2 hr observation period.

The presence of baseline behaviours were evaluated in the same manner described for 8-OH-DPAT above. Once again no significant differences were found in the frequency of rearing, forepaw treading or head weaving in sham and OB rats. OB rats showed a significant decrease in the frequency of wet dog shakes detected, this was marginal however and could simply have been a reflex in the rat.

In relation to stereotyped behaviours, a significant effect was found in the frequency of rearing, with 1 and 3 mg/kg resulting in a decrease in sham and OB rats when compared to their 0.3 mg/kg treated counterparts. However there was an absence of significant changes in stereotyped behaviours in either sham or OB rats. The dose response effect of DOI on the frequency of rearing is independent of the surgery group, therefore suggesting that this is a characteristic of the drug rather than a reflective change in the functionality of the receptors. Wet dog shakes are considered to be primary behavioural consequence of DOI administration and given that in all doses it was evident in both lesion groups, albeit at to a higher degree in sham animals. Previously, doses of 0.5 and 4 mg/kg have elicited this response (Darmani and Ahmad, 1999), with others showing the same response (Hill *et al.,* 2006; Kawakami *et al.,* 2005; Kitamura *et al.,* 2007) at doses used in this dose response study. Given that locomotor induced changes and the stereotyped behaviours are post-synaptically mediated, it may be possible that different receptors are involved in the different responses i.e. 5-HT_{2A} receptors expressed in the motor cortices may exhibit greater functionality compared to those in other regions, thus accounting for the alterations in locomotor activity but not in the expected stereotyped behaviour.

In conclusion, locomotor activity suggests that both 5-HT_{1A} and 5-HT_{2A} receptors may have increased functionality in the OB rat compared to sham-lesion counterparts and suggests that the 5-HT_{1A} receptors demonstrate greater functionality compared to the 5-HT_{2A} receptors. Despite the lack of significance in the frequency of stereotyped behaviour elicited with both agonists but trends for the highest dose tested having the most profound effect on the behavioural hallmarks of the syndrome it was decided to proceed with 3 mg/kg of DOI and 0.5 mg/kg of 8-OH-DPAT for the remaining studies. It is still important to ascertain how chronic antidepressant treatment may affect these receptors as they are thought to play a crucial role in the mechanism of action of many antidepressants (Celada *et al.,* 2004).

Chapter 5:

Effects of chronic antidepressant treatment on central serotonergic and agonist-induced behavioural parameters in the OB rat

5.1 Introduction

The aim of the previous two chapters was to assess temporal changes in the central serotonergic system and to determine the optimal dose of two serotonergic agonists, 8-OH-DPAT and DOI in order to assess the functionality of the 5-HT_{1A} and the 5-HT_{2A} receptors. With regards to temporal changes in the serotonergic system, no significant lesion or temporal changes were found in any of the parameters measured, even though OB hyperactivity in the open field was found at each time point, thus suggesting that the syndrome had been established. In relation to the functionality of the two receptors, locomotor activity revealed a significant lesion effect in response to acute doses of 8-OH-DPAT and DOI. Results demonstrated the highest doses measured reveal a potential heightened functionality of these two receptors in this model. 8-OH-DPAT induced stereotyped responses, when combined together, OB rats exhibited a general reduction in a number of responses compared to shams with a concomitant increase in rearing. DOI induced a dose response decrease in rearing, regardless of lesion. In addition there was an overall reduction in wet dog shakes in OB rats, irrespective of dose employed.

Given that the most common treatment for depression is pharmacological intervention, the next step was to determine if chronic antidepressant treatment would affect the parameters measured in the first two chapters. Given that the majority of therapies still rely on modification of the serotonergic system as a component of their mechanism of action, fluoxetine (SSRI), venlafaxine (SNRI) and imipramine (TCA) were chosen, as although they all modulate the serotonergic system, they each have a different affinities for SERT. Owens *et al.*, (1997) assessed the affinity for each of these drugs for both human and rat SERT, in transfected HEK-293 cells. Fluoxetine demonstrated highest affinity with for both human and rat SERT (Ki = 0.9 and 2.0 respectively). Imipramine demonstrated the next highest affinity for human and rat SERT (Ki = 8.7 and 1.3 respectively), with venlafaxine demonstrating the lowest affinity for the human and rat SERT (Ki = 19 and 7.5 respectively) when compared to the other drugs. However, although affinities for SERT differ between the three drugs, each demonstrate greater affinity for SERT compared to NET. In this case imipramine has the highest affinity for NET out of the three compounds (Ki = 11 (human NET) and 20 (rat NET)) but still displays greater affinity for SERT compared to NET.

One of the main characteristics of the OB model is its responsiveness to chronic but not acute antidepressant treatment (Wang *et al.,* 2012; Pandey *et al.,* 2014, Kelly *et al.,* 1997; Song and Leonard, 2005). OB induced hyperactivity in the open field is attenuated by chronic fluoxetine (Kalshetti *et al.,* 2015; Roche *et al.,* 2007; Mnie-Filai *et al.,* 2011; Aswar *et al.,* 2012), venlafaxine (McGrath and Norman, 1998) and imipramine treatment (Aswar *et al.,* 2012; Goyal *et al.,* 2009; Roche *et al.,* 2008; Breuer *et al.,* 2007). Imipramine treatment has also been shown to reverse depressive like behaviours in rats (Rinwa *et al.,* 2013), with chronic fluoxetine and venlafaxine attenuating depressive like behaviours in mice (Portetti *et al.,* 2016). With regards to molecular parameters, in contrast to behavioural measurements acute fluoxetine (Wang *et al.,* 2012), desipramine (Saitoh *et al.,* 2008) and chronic fluoxetine treatment (Marcilhac *et al.,* 1999) reverse OB induced changes in 5-HT, 5-HIAA and serotonin turnover (Wang *et al.,* 2012). Imipramine treatment has also been shown to reverse OB induced alterations in TPH expression (Grecksch *et al.,* 1997; Huether *et al.,* 1997).

One of the other parameters to consider when undertaking an antidepressant regime is the length of treatment time for the rats. Commonly with regards to the three drugs discussed above, the dosing schedule ranges from 14 days (Kalshetti *et al.,* 2015; Roche *et al.,* 2008); 16-17 days (McGrath and Norman, 1998; Grecksch *et al.,* 1997), 21 days (Mnie-Filali *et al.,* 2011; Uzonva *et al.,* 2004) and 35 days (Roche *et al.,* 2007). Given that 17 days of treatment has been described as subchronic by some (Grecksch *et al.,* 1997) it was decided that a dosing regimen of 21 days would be employed, as this has been used in the literature. In addition, with regards to other models of the disease, a longer dosing regime appears favourable. A 21 day dosing regime has also been employed for venlafaxine in the CMS model (Xing *et al.,* 2013), with longer treatment times of 5 weeks also reported for imipramine and fluoxetine in the CMS model (Papp *et al.,* 2003).

However, several gaps in the literature have been identified, primarily the lack of studies investigating multiple components of the serotonergic system in this model and how they are modulated by chronic antidepressant treatment. Particularly striking was the lack of studies investigating the effects of antidepressant therapy on the functionality of the 5-HT_{1A} and 5-HT_{2A} receptors, which are believed to play a major role in the therapeutic action of antidepressants (Celada *et al.,* 2004). Another aspect

that was found in antidepressant studies so far conducted, was the absences in some studies to include sham-lesion groups that had also been treated with the antidepressant being investigated (Chang *et al.,* 2016; Saitoh *et al.,* 2007). This is a vital group to include in order to determine if the compound of interest has an effect sham rats as well as OB rats.

Therefore the aims covered in this chapter are to:

1. Assess the effect of chronic antidepressant treatment on TPH expression in the dorsal raphe nucleus, both the whole dorsal raphe nucleus and also its stereological regions
2. Assess the effect of chronic antidepressant treatment on TPH expression in terminal regions
3. Assess the effect of chronic antidepressant treatment on 5-HT_{1A} and 5-HT_{2A} receptor functionality and mRNA expression in discrete brain regions
4. Assess the effect of chronic antidepressant treatment on 5-HT, 5-HIAA and 5-HT turnover in the amygdala
5. Assess the effect of chronic antidepressant treatment on SERT expression in terminal regions.

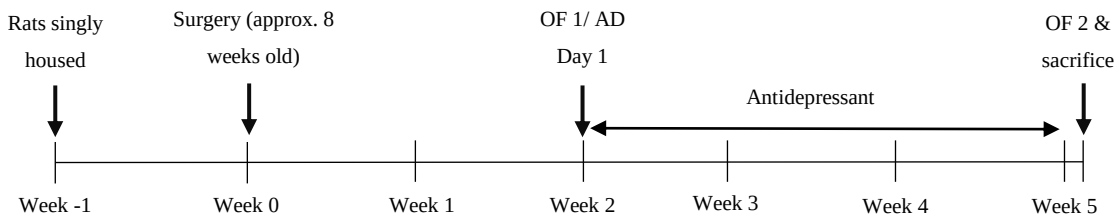
5.2 Methods

All methods have been described in more detail in Chapter 2. Methods specific to this chapter are detailed below.

5.2.1 Study design (detailed below)

All male Sprague-Dawley rats were singly housed for a week prior to surgery and sham or OB surgery was carried out at approx.8 weeks old (n= 87 and 135). Following surgery, rats were handled and weighed daily, together with food and water weights, to avoid aggression developing and underwent the open field 2 weeks post-lesion to confirm the syndrome had been successfully established. This first open field exposure was carried out from approx. 08:00 to 12:00 hr after which the locomotor activity was scored (as detailed in 2.2.5.2). Rats were then assigned to drug treatment groups based on their locomotor activity. Rats received once daily subcutaneous injections of assigned treatment (as detailed in 2.2.6).

The antidepressant investigations were divided into two studies. The first one involved fluoxetine (10 mg/kg s.c) (n=87), whilst the second one involved venlafaxine (20 mg/kg s.c) and imipramine (10 mg/kg s.c) (n=135). After 2 weeks of antidepressant treatment, rats received an acute injection of either DOI (3 mg/kg s.c) or 8-OH-DPAT (0.5 mg/kg s.c) and their behaviour was assessed as previously detailed (as detailed in 2.2.5.3). DOI was not possible to include in the study involving venlafaxine or imipramine as it could not be sourced at the time of the conduction of this experiment. Rats continued on treatment with the antidepressants for a further 7 days (i.e. a total of 21 days of treatment), and after an washout period of 16-22 hr, they again underwent the open field and were immediately sacrificed via decapitation or transcardial perfusion (as detailed in 2.2.7)



5.2.2 Tissue processing

All post-mortem analysis – Immunohistochemistry (as detailed in 2.2.9.2) , PCR (as detailed in 2.2.8) and HPLC (as detailed in 2.2.10) were carried out as detailed in Chapter 2.

5.3 Results

5.3.1 Functionality of 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors

5.3.1.1 Effect of 8-OH-DPAT on locomotor activity in fluoxetine treated OB rats

Results are depicted in Figure 5.1. A significant effect of lesion [$F_{(1,27)}$ = 28.23, p<0.001] but no significant effect of drug [$F_{(1,27)}$ = 2.23, p>0.05] or lesion x drug interaction [$F_{(1,27)}$ = 0.39 p>0.05] was found. Both OB groups that received 8-OH-DPAT moved significantly more compared to their sham counterparts [p<0.05].

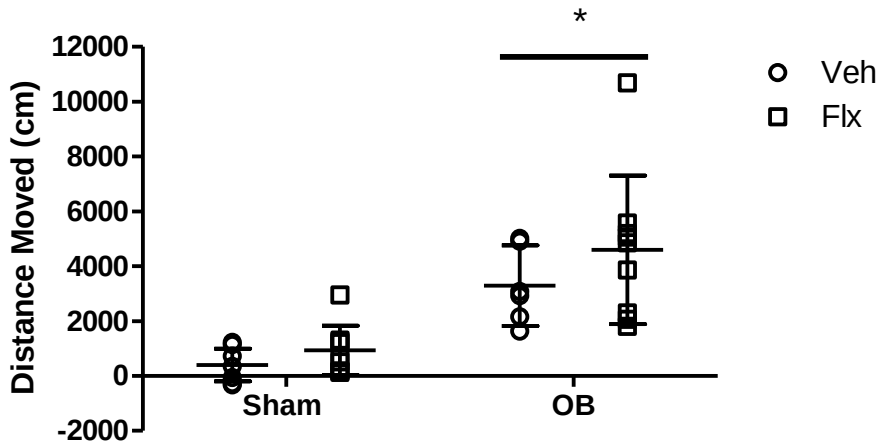


Figure 5.1. 8-OH-DPAT induced changes in homecage locomotor activity (post-dose-baseline) Data are expressed as mean ± SD (n=6-9). *p<0.05 vs sham counterpart [Student Newman-Keuls test]

5.3.1.2 8-OH-DPAT induced behaviours in fluoxetine treated OB rats (Table 5.1)

No significant lesion effect was found in any of the parameters measured. A significant drug effect was found in the frequency of flat body posture, hindlimb abduction and head weaving. No significant lesion x drug interaction effect was found in any of the parameters measured. Fluoxetine significantly reduced the frequency of flat body posture in both sham-lesion and OB rats, while also increasing the frequency of head weaving in OB rats compared to vehicle treated counterparts [$p<0.05$]. A significant effect of drug was found in the frequency of hindlimb abduction but this was not significant in subsequent *post-hoc* analysis

Table 5.1 Frequency of 8-OH-DPAT induced behaviours in the OB rat following chronic fluoxetine treatment

Group	Rearing	Flat body posture	Hindlimb abduction	Forepaw treading	Head weaving	Wet dog shakes	Head shakes	Head twitch
Sham + Veh	39 ± 24	16 ± 10	44 ± 18	0 (0-0)	0 ± 0	1 ± 1	1 ± 0	3 ± 2
Sham + Flx	53 ± 43	1 ± 3*	30 ± 42	0 (0-0)	1 ± 1	0 ± 1	5 ± 5	5 ± 7
OB + Veh	28 ± 15	27 ± 16	94 ± 114	0 (0-0)	0 ± 0	0 ± 0	2 ± 2	4 ± 2
OB + Flx	89 ± 75	1 ± 2*	19 ± 28	0 (0-0)	2 ± 2*	0 ± 1	7 ± 6	2 ± 4
Lesion	$F_{(1,26)}$ = 0.08, p>0.05	$F_{(1,26)}$ = 2.07, p>0.05	$F_{(1,26)}$ = 0.15, p>0.05		$F_{(1,26)}$ = 0.21, p>0.05	$F_{(1,26)}$ = 1.27, p>0.05	$F_{(1,26)}$ = 1.19, p>0.05	$F_{(1,26)}$ = 0.161, p>0.05
Drug	$F_{(1,26)}$ = 3.06, p>0.05	$F_{(1,26)}$ = 76.69, ***p*<0.001**	$F_{(1,26)}$ = 7.59, ***p*<0.01**	$K_{(3)}$ = 4.00, p>0.05	$F_{(1,26)}$ = 15.61, ***p*<0.01**	$F_{(1,26)}$ = 0.03, p>0.05	$F_{(1,26)}$ = 3.92, p>0.05	$F_{(1,26)}$ = 0.03, p>0.05
Lesion x Drug	$F_{(1,26)}$ = 0.76, p>0.05	$F_{(1,26)}$ = 1.33, p>0.05	$F_{(1,26)}$ = 0.88, p>0.05		$F_{(1,26)}$ = 0.75, p>0.05	$F_{(1,26)}$ = 0.72, p>0.05	$F_{(1,26)}$ = 0.01, p>0.05	$F_{(1,26)}$ = 0.72, p>0.05

Table 5.1 Data are expressed as mean ± SD in parametric analysis and median (25^{th} – 75^{th} percentile) in non-parametric analysis. *p<0.05 vs vehicle treated surgery counterparts [*post-hoc* Student Newman-Keuls] (n=6-9)

5.3.1.3 Effect of DOI on locomotor activity in fluoxetine treated OB rats

Results are depicted in Figure 5.2. A significant effect of lesion was found [$F_{(1,31)}$ = 23.34, p<0.001] but no significant effect of drug [$F_{(1,31)}$ = 2.90, p>0.05] or lesion x drug interaction was found [$F_{(1,31)}$ = 0.38, p>0.05]. OB rats moved significantly more compared to sham equivalents [p<0.05].

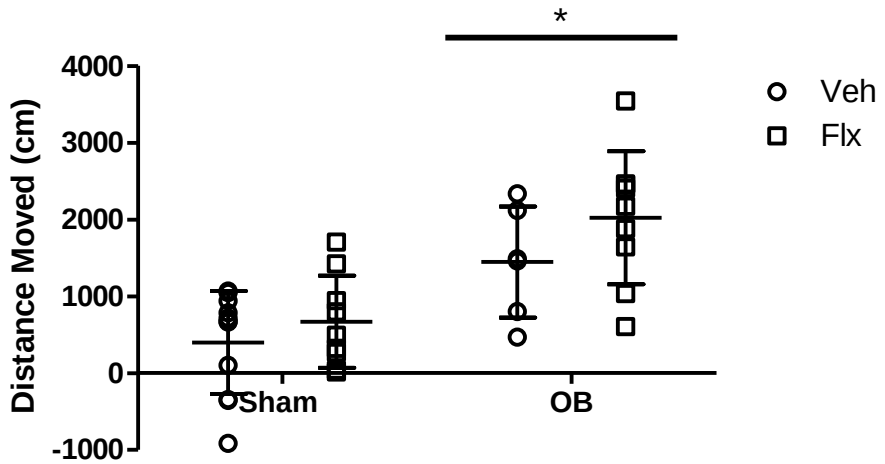


Figure 5.2. DOI induced changes in homecage locomotor activity (post-dose – baseline) Data are expressed as mean ± SD (n=6-11). *p<0.05 vs sham counterparts [Student Newman-Keuls test]

5.3.1.4 DOI induced behaviours in fluoxetine treated OB rats (Table 5.2)

No significant lesion, drug or lesion x drug interaction effect was found in any of the behaviours analysed.

Table 5.2 Frequency of DOI induced behaviours in the OB rat following chronic fluoxetine treatment

Group	Rearing	Wet dog shakes	Hindlimb abduction	Forepaw treading	Head weaving	Flat body posture	Head Shakes
Sham + Veh	27 (20-33)	7 ± 5	0 (0-0.3)	1 ± 1	0 ± 1	0 ± 0	10 ± 7
Sham + Flx	21 (16-36)	8 ± 6	0 (0-0)	2 ± 4	0 ± 0	0 ± 1	13 ± 11
OB + Veh	40 (10-70)	9 ± 7	0 (0-16)	4 ± 5	0 ± 1	1 ± 2	7 ± 8
OB + Flx	37 (37-72)	13 ± 13	0 (0-1)	4 ± 6	0 ± 1	0 ± 0	12 ± 7
Lesion		$F_{(1,30)} = 1.85$, $p>0.05$		$F_{(1,30)} = 1.72$, $p>0.05$	$F_{(1,30)} = 0.35$, $p>0.05$	$F_{(1,30)} = 0.10$, $p>0.05$	$F_{(1,30)} = 0.38$, $p>0.05$
Drug	$K_{(3)} = 3.81$, $p>0.05$	$F_{(1,30)} = 0.94$, $p>0.05$	$K_{(3)} = 0.36$, $p>0.05$	$F_{(1,30)} = 0.07$, $p>0.05$	$F_{(1,30)} = 0.00$, $p>0.05$	$F_{(1,30)} = 0.07$, $p>0.05$	$F_{(1,30)} = 1.50$, $p>0.05$
Lesion x Drug		$F_{(1,30)} = 0.45$, $p>0.05$		$F_{(1,30)} = 0.00$, $p>0.05$	$F_{(1,30)} = 0.00$, $p>0.05$	$F_{(1,30)} = 83$, $p>0.05$	$F_{(1,30)} = 0.80$, $p>0.05$

Table 5.2 Data are expressed as mean ± in parametric analysis and median (25^{th} – 75^{th} percentile) in non-parametric analysis. (n=6-11)

5.3.1.5 Effect of 8-OH-DPAT on locomotor activity in venlafaxine or imipramine treated OB rats

Results are depicted in Figure 5.3. A significant effect of lesion [$F_{(1,62)}$ = 17.11, p<0.001] but no significant effect of drug [$F_{(2,62)}$ = 0.63, p>0.05] or lesion x drug interaction [$F_{(2,62)}$ = 0.24, p>0.05] was found. OB rats treated with venlafaxine or imipramine moved significantly more compared to their sham counterparts when treated with acute injection of 8-OH-DPAT [p<0.05].

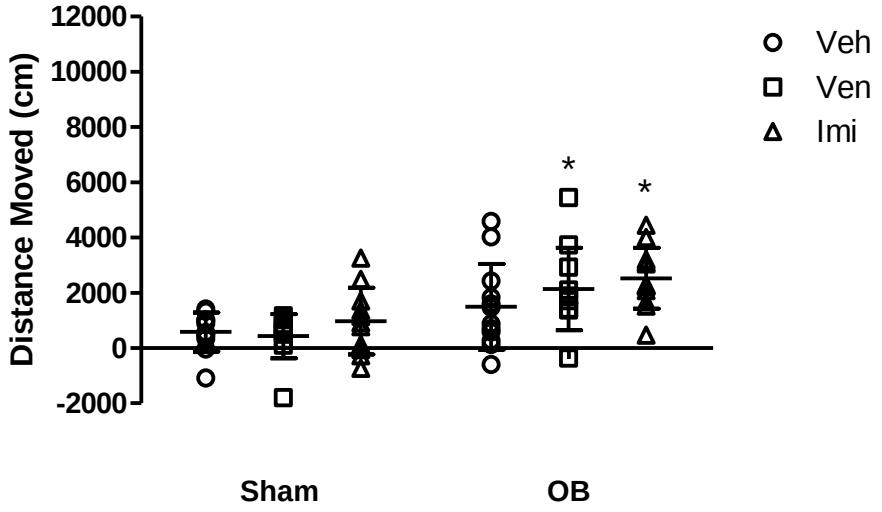


Figure 5.3. 8-OH-DPAT induced changes in home cage locomotor activity (post-dose – baseline). Data are expressed as mean ± SD (n=11-12). *p<0.05 vs sham counterparts [Student Newman-Keuls test]

5.3.1.6 8-OH-DPAT induced behaviours in venlafaxine or imipramine treated OB rats (Table 5.3)

A significant lesion effect was found in the frequency of forepaw treading, with a significant drug effect found in the frequency of rearing and flat body posture. No significant lesion x drug interaction effect was found in any of the parameters measured. Imipramine significantly increased the frequency of rearing in the sham-lesion rats compared to vehicle treated counterparts [$p<0.05$]. Significance found in flat body posture and forepaw treading, respectively, but was found not to be significant in subsequent *post-hoc* analysis.

Table 5.3 Frequency of 8-OH-DPAT induced behaviours in the OB rat following chronic venlafaxine or imipramine treatment

Group	Rearing	Flat body posture	Hindlimb abduction	Forepaw treading	Head weaving	Wet dog shakes	Head shakes
Sham + Veh	31 ± 23	20 ± 17	22 ± 22	1 ± 1	3 (2-8)	2 ± 2	4 ± 4
Sham + Ven	23 ± 14	7 ± 3	28 ± 28	1 ± 1	1 (0-4)	2 ± 2	4 ± 3
Sham + Imi	63 ± 45*	5 ± 5	43 ± 36	1 ± 0	1 (1-2)	1 ± 0	5 ± 2
OB + Veh	27 ± 15	18 ± 16	20 ± 23	1 ± 1	5 (.25-16)	1 ± 2	3 ± 3
OB + Ven	24 ± 18	11 ± 11	19 ± 20	2 ± 3	3 (1-6)	2 ±1	2 ± 2
OB + Imi	40 ± 37	4 ± 5	32 ± 25	1 ± 1	8 (2.5-10.5)	1 ± 1	4 ± 3
Lesion	$F_{(1,62)} = 2.01$, $p>0.05$	$F_{(1,62)} = 0.28$, $p>0.05$	$F_{(1,62)} = 1.29$, $p>0.05$	$F_{(1,62)} = 8.72$, **$p<0.01$**		$F_{(1,62)} = 1.00$, $p>0.05$	$F_{(1,62)} = 1.61$, $p>0.05$
Drug	$F_{(2,62)} = 6.48$, **$p<0.01$**	$F_{(2,62)} = 7.8$, **$p<0.01$**	$F_{(2,62)} = 2.68$, $p>0.05$	$F_{(2,62)} = 0.26$, $p>0.05$	$K_{(5)} = 9.53$, $p>0.05$	$F_{(2,62)} = 2.08$, $p>0.05$	$F_{(2,26)} = 1.15$, $p>0.05$
Lesion x Drug	$F_{(2,62)} = 1.33$, $p>0.05$	$F_{(2,62)} = 0.41$, $p>0.05$	$F_{(2,62)} = 0.15$, $p>0.05$	$F_{(2,62)} = 1.92$, $p>0.05$		$F_{(2,62)} = 0.84$, $p>0.05$	$F_{(2,26)} = 0.08$, $p>0.05$

Table 5.3 Data are expressed as mean ± SD in parametric analysis and median (25^{th} – 75^{th} percentile) in non-parametric analysis. *p<0.05 vs vehicle treated surgery counterparts [*post-hoc* Student Newman-Keuls] (n=11-12)

5.3.2 Trytptophan hydroxylase (TPH) immunohistochemistry

5.3.2.1 TPH expression in the dorsal raphe nucleus (DRN) (representative images Figure 5.4)

5.3.2.1.1 Effect of chronic fluoxetine treatment on TPH expression in the whole DRN in OB rats (Table 5.4)

No significant lesion, drug or lesion x drug interaction effect was found.

Table 5.4 Number of TPH positive cells in the DRN in OB rats after chronic fluoxetine treatment

Group	Cell count/section
Sham + Veh	348 ± 81
Sham + Flx	325 ± 78
OB + Veh	324 ± 85
OB + Flx	348 ± 66
Lesion	$F_{(1,39)} = 0.00$, $p>0.05$
Drug	$F_{(1,39)} = 0.00$, $p>0.05$
Lesion x Drug	$F_{(1,39)=} 0.99$, $p>0.05$

Table 5.4 Data are expressed as mean ± SD. (n=9-12)

5.3.2.1.2 Effect of chronic fluoxetine treatment on TPH expression in distinct stereological regions of the DRN in OB rats (Table 5.5; Figure 5.5)

No significant lesion, drug or lesion x drug interaction effect was found

Table 5.5 Number of TPH positive cells in the dorsal, ventral and lateral subdivisions of the DRN in OB rats after chronic fluoxetine treatment

Group	Cell count/section		
	Dorsal	Ventral	Lateral
Sham + Veh	92 ± 25	149 ± 41	145 ± 41
Sham + Flx	77 ± 19	151 ± 25	147 ± 32
OB + Veh	94 ± 32	151 ± 41	133 ± 41
OB + Flx	83 ± 22	144 ± 36	150 ± 31
Lesion	$F_{(1,39)} = 0.24$, $p>0.05$	$F_{(1,39)} = 0.05$, $p>0.05$	$F_{(1,39)} = 0.00$, $p>0.05$
Drug	$F_{(1,39)} = 2.9$, $p>0.05$	$F_{(1,39)} = 0.07$, $p>0.05$	$F_{(1,39)} = 0.18$, $p>0.05$
Lesion x Drug	$F_{(1,39)} = 0.07$, $p>0.05$	$F_{(1,39)} = 0.15$, $p>0.05$	$F_{(1,39)} = 0.00$, $p>0.05$

Table 5.5 Data are expressed as mean ± SD (n=9-12)

5.3.2.1.3 Effect of chronic venlafaxine or imipramine treatment on TPH expression in the whole DRN in OB rats (Table 5.6)

No significant lesion, drug or lesion x drug interaction effect was found.

Table 5.6 Number of TPH positive cells in the DRN in OB rats after chronic venlafaxine or imipramine treatment

Group	Cell count/section
Sham + Veh	323 ± 101
Sham + Ven	313 ± 73
Sham + Imi	331 ± 91
OB + Veh	337 ± 94
OB + Ven	331 ± 81
OB + Imi	339 ± 68
Lesion	$F_{(1,58)} = 0.41$, $p>0.05$
Drug	$F_{(2,58)} = 0.12$, $p>0.05$
Lesion x Drug	$F_{(2,58)} = 0.16$, $p>0.05$

Table 5.6 Data are expressed as mean ± SD. (n=10-11)

5.3.2.1.4 Effect of chronic venlafaxine or imipramine treatment on TPH expression in distinct stereological regions of the DRN in OB rats (Table 5.7; Figure 5.5)

No significant lesion, drug or lesion x drug interaction effect was found.

Table 5.7 Number of TPH positive cells in the dorsal, ventral and lateral subdivisions of the DRN in OB rats after chronic venlafaxine or imipramine treatment

Group	Cell count/section		
	Dorsal	Ventral	Lateral
Sham + Veh	110 ± 35	147 ± 48	132 ± 47
Sham + Ven	105 ± 29	155 ± 32	150 ± 35
Sham + Imi	101 ± 34	143 ± 35	141 ± 51
OB + Veh	116 ± 38	143 ± 35	160 ± 56
OB + Ven	100 ± 47	152 ± 36	157 ± 43
OB + Imi	117 ± 40	153 ± 38	152 ± 28
Lesion	$F_{(1,58)} = 0.31$, $p>0.05$	$F_{(1,58)} = 0.02$, $p>0.05$	$F_{(1,58)} = 1.98$, $p>0.05$
Drug	$F_{(2,58)} = 0.47$, $p>0.05$	$F_{(2,58)} = 0.27$, $p>0.05$	$F_{(2,58)} = 0.17$, $p>0.05$
Lesion x Drug	$F_{(2,58)} = 0.40$, $p>0.05$	$F_{(2,58)} = 0.22$, $p>0.05$	$F_{(2,58)} = 0.35$, $p>0.05$

Table 5.7 Data are expressed as mean ± SD (n=10-11)

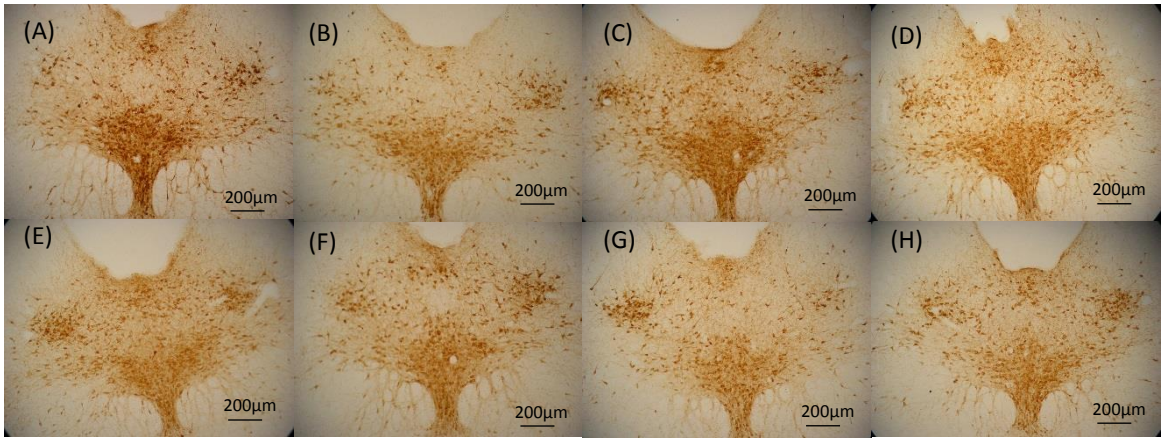


Figure 5.4 Representative images of TPH positive cells in the DRN. A = Sham + Veh; B = Sham + Flx; C = Sham + Ven; D = Sham + Imi; E = OB + Veh; F = OB + Flx; G = OB + Ven; H = OB + Imi

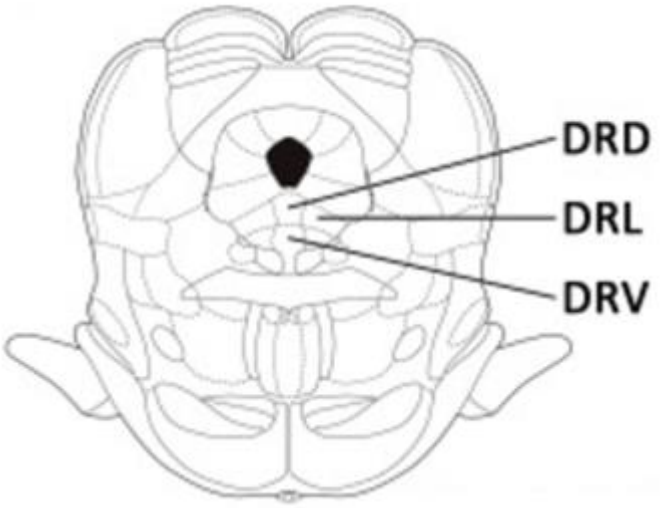


Figure 5.5 Outline of subdivisions for stereological analysis. DRD = Dorsal DRN; DRL = Lateral DRN; DRV = Ventral DRN

5.3.2.2 TPH expression in serotonergic terminal regions (Representative images Figure 5.6)

5.3.2.2.1 Effect of chronic fluoxetine on TPH expression in the OB rat in discrete brain regions (Table 5.8)

A significant effect was found between the groups in the striatum but this was between non-comparable groups. No significant lesion, drug or lesion x drug interaction effect was found in the substantia nigra. No significant lesion or drug effect but a significant lesion x drug interaction effect was found in the amygdala. Fluoxetine significantly decreased the level of TPH expression the OB in the amygdala compared to vehicle treated counter parts [$p<0.05$].

Table 5.8 TPH expression in the striatum, substantia nigra and amygdala of OB rats after chronic fluoxetine treatment

Group	Striatum	Substantia nigra	Amygdala
Sham + Veh	0.025 (0.02-0.027)	0.126 ± 0.056	0.051 ± 0.011
Sham + Flx	0.028 (0.025-0.03)	0.137 ± 0.036	0.056 ± 0.016
OB + Veh	0.024 (0.02-0.031)	0.134 ± 0.018	0.056 ± 0.018
OB + Flx	0.019 (0.016-0.023)	0.091 ± 0.052	$0.039 \pm 0.009^{+}$
Lesion		$F_{(1,31)} = 1.17$, $p>0.05$	$F_{(1,36)} = 2.09$, $p>0.05$
Drug	$K_{(3)} = 11.34$, ***p*<0.05**	$F_{(1,31)} = 0.85$, $p>0.05$	$F_{(1,36)} = 1.76$, $p>0.05$
Lesion X Drug		$F_{(1,39)} = 2.26$, $p>0.05$	$F_{(1,36)} = 5.78$, ***p*<0.05**

Table 5.8 Data are expressed as median (25^{th} - 75^{th} interquartile range) for non-parametric analysis ; mean ± SD parametric analysis. All data are expressed as OD/Section. (Striatum n=9-12; Substantia nigra n=9-12; Amygdala n = 9-10) OD = optical density; +p<0.05 vs OB + Veh [Student-Newman Keuls test]

5.3.2.2.2 Effect of chronic venlafaxine or imipramine on TPH expression in the OB rat in discrete brain regions (Table 5.9)

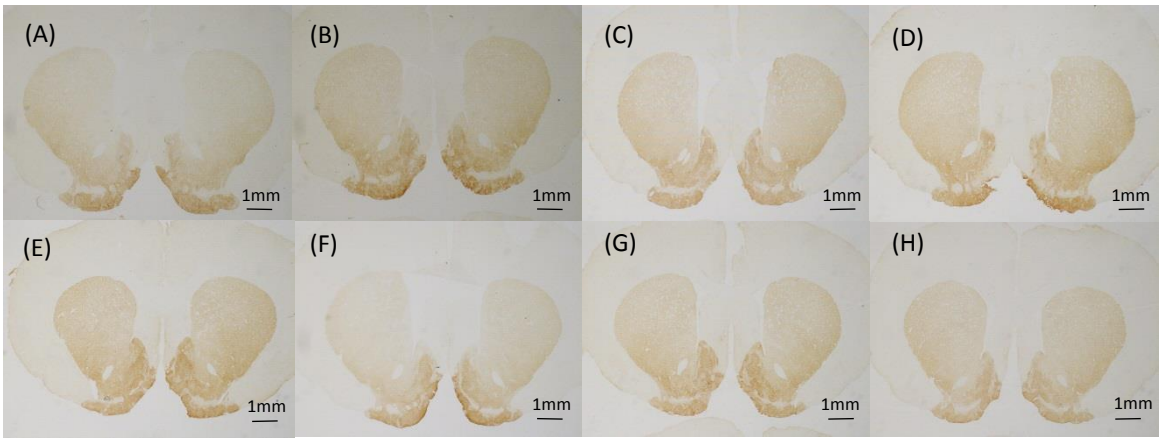
No significant lesion, drug or lesion x drug interaction effect was found in any of the regions assessed

Table 5.9 TPH expression in the striatum, substantia nigra and amygdala of OB rats after chronic venlafaxine or imipramine treatment

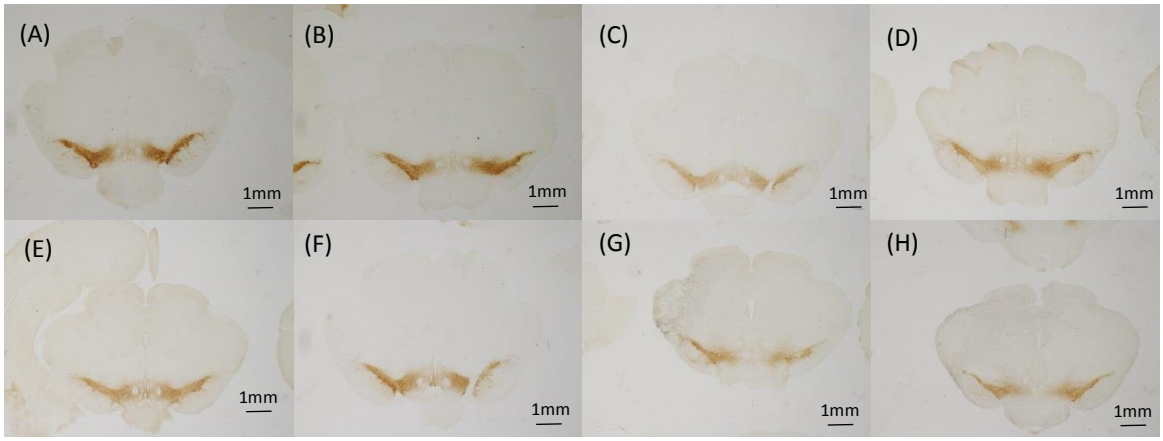
Group	Striatum	Substantia nigra	Amygdala
Sham + Veh	0.029 (0.024-0.036)	0.100 (0.084-0.118)	0.046 ± 0.014
Sham + Ven	0.038 (0.035-0.044)	0.098 (0.082-0.112)	0.054 ± 0.014
Sham + Imi	0.033 (0.031-0.037)	0.110 (0.102-0.126)	0.061 ± 0.016
OB + Veh	0.030 (0.029-0.036)	0.094 (0.082-0.113)	0.056 ± 0.020
OB + Ven	0.033 (0.03-0.05)	0.110 (0.067-0.304)	0.053 ± 0.018
OB + Imi	0.031 (0.028-0.036)	0.100 (0.087-0.103)	0.046 ± 0.010
Lesion	$K_{(5)} = 0.83$, $p>0.05$	$K_{(5)} = 6.55$, $p>0.05$	$F_{(1,52)} = 0.03$, $p>0.05$
Drug			$F_{(2,52)} = 0.67$, $p>0.05$
Lesion X Drug			$F_{(2,52)} = 1.29$, $p>0.05$

Table 5.9 Data are expressed as median (25^{th} – 75^{th} percentile) for non-parametric analysis ; mean ± SD parametric analysis . All data are expressed as OD/Section (Striatum n=9-11; Substantia nigra n=7-11; Amygdala n = 8-12) OD = optical density; OD = optical density

Striatum



Substantia nigra



Amygdala

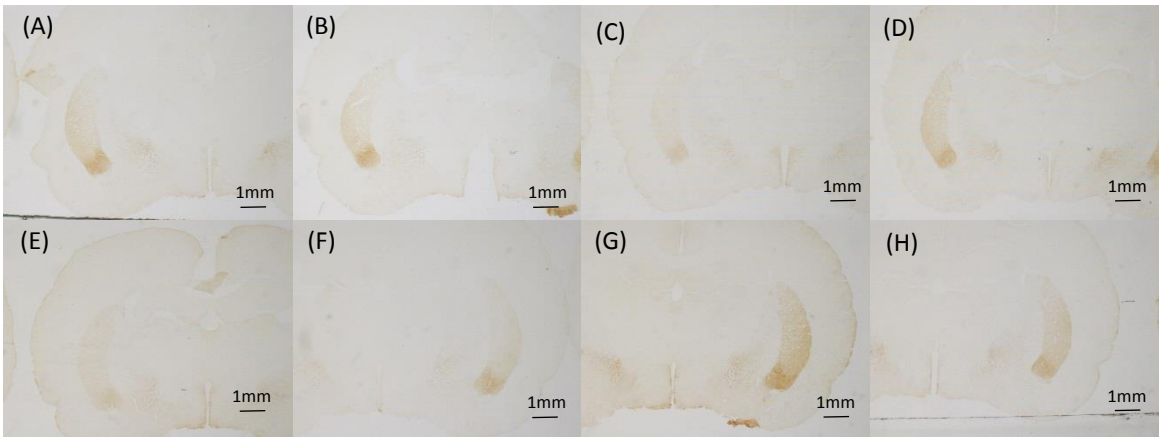


Figure 5.6 Representative images of TPH expression in the striatum, substantia nigra and amygdala. A = Sham + Veh; B = Sham + Flx; C = Sham + Ven; D = Sham + Imi; E = OB + Veh; F = OB + Flx; G = OB + Ven; H = OB + Imi

5.3.3 5-HT receptor mRNA expression in discrete brain regions

5.3.3.1 Effect of chronic fluoxetine treatment on 5-HT$_{1A}$ receptor mRNA in OB rats (Table 5.10)

No significant lesion or drug effect was found in the PFC, hippocampus or amygdala. No significant lesion x drug interaction was found in the hippocampus or the amygdala but a significant interaction was found in the PFC. Fluoxetine significantly reduced mRNA expression in sham lesion rats compared to vehicle treated rats [$p<0.05$].

Table 5.10 5-HT$_{1A}$ receptor mRNA fold change in the OB rat following chronic fluoxetine treatment

Group	PFC	Hippocampus	Amygdala
Sham + Veh	1 ± 0.159	1 ± 0.238	1 ± 0.324
Sham + Flx	0.721 ± 0.261*	1.040 ± 0.147	0.971 ± 0.179
OB + Veh	0.804 ± 0.213	1 ± 0.328	1.070 ± 0.271
OB + Flx	0.919 ±0.213	1.032 ± 0.236	1.095 ± 0.228
Lesion	$F_{(1,39)} = 0.00$, $p>0.05$	$F_{(1,40)} = 0.00$, $p>0.05$	$F_{(1,38)} = 1.50$, $p>0.05$
Drug	$F_{(1,39)} = 1.50$, $p>0.05$	$F_{(1,40)} = 0.22$, $p>0.05$	$F_{(1,38)} = 0.00$, $p>00.5$
Lesion x Drug	$F_{(1,39)} = 8.72$, $\mathbf{p<0.01}$	$F_{(1,40)} = 0.00$, $p>0.05$	$F_{(1,38)} = 0.11$, $p>0.05$

Table 5.10 Data are expressed as mean ± SD (n=8-12). *$p<0.05$ vs Sham + Veh [Student Newman-Keuls]

5.3.3.2 Effect of chronic fluoxetine treatment on 5-HT_{2A} receptor mRNA in OB rats (Table 5.11)

No significant drug or lesion x drug interaction effect was found and the significant lesion effect in the PFC was found to be non-significant in subsequent *post-hoc* analysis.

Table 5.11 5-HT_{2A} receptor mRNA fold change in the OB rat following chronic fluoxetine treatment

Group	PFC	Hippocampus	Amygdala
Sham + Veh	1 ± 0.136	1 ± 0.185	1 (0.979-1.021)
Sham + Flx	0.873 ± 0.268	0.833 ± 0.223	0.990 (0.977-1.043)
OB + Veh	0.789 ± 0.212	0.973 ± 0.270	0.978 (0.962-0.999)
OB + Flx	0.803 ± 0.212	0.902 ± 0.178	0.995 (0.982-1.008)
Lesion	$F_{(1,39)} = 4.527$, ***p*<0.05**	$F_{(1,40)} = 0.10$, $p>0.05$	$K_{(3)} = 2.82$, p>0.05
Drug	$F_{(1,39)} = 0.728$, $p>0.05$	$F_{(1,40)} = 3.43$, $p>0.05$	
Lesion x Drug	$F_{(1,39)} = 1.140$, $p>0.05$	$F_{(1,40)} = 0.54$, $p>0.05$	

Table 5.5 Data are expressed as mean ± SD in parametric analysis and median (25^{th} – 75^{th} percentile) in non-parametric analysis. (n=8-12)

5.3.3.3 Effect of chronic venlafaxine or imipramine treatment on 5-HT$_{1A}$ receptor mRNA in OB rats (Table 5.12)

No significant lesion, drug or lesion x drug interaction effect was found.

Table 5.12 5-HT$_{1A}$ receptor mRNA fold change in the OB rat following chronic venlafaxine or imipramine treatment

Group	PFC	Hippocampus	Amygdala
Sham + Veh	1 ± 0.393	1 ± 0.269	1 ± 0.343
Sham + Ven	1.038 ± 0.295	0.956 ± 0.219	1.161 ± 0.211
Sham + Imi	1.048 ± 0.429	0.901 ± 0.168	1.230 ± 0.347
OB + Veh	1.060 ± 0.300	0.897 ± 0.172	1.300 ± 0.528
OB + Ven	1.003 ± 0.385	0.938 ± 0.196	1.203 ± 0.337
OB + Imi	0.863 ± 0.295	0.912 ± 0.141	1.044 ± 0.271
Lesion	$F_{(1,60)} = 0.37$, $p>0.05$	$F_{(1,63)} = 0.61$, $p>0.05$	$F_{(1,58)} = 0.33$, $p>0.05$
Drug	$F_{(2,60)} = 0.29$, $p>0.05$	$F_{(2,63)} = 0.35$, $p>0.05$	$F_{(2,58)} = 0.08$, $p>0.05$
Lesion x Drug	$F_{(2,60)} = 0.68$, $p>0.05$	$F_{(2,63)} = 0.52$, $p>0.05$	$F_{(2,58)} = 2.42$, $p>0.05$

Table 5.12 Data are expressed as mean ± SD. (n=9-13)

5.3.3.4 Effect of chronic venlafaxine or imipramine treatment on 5-HT$_{2A}$ receptor mRNA in OB rats (Table 5.13)

A significant lesion effect was found in the PFC. No significant drug or lesion x drug interaction effect was found the significant lesion effect in the PFC was found to be non-significant in subsequent *post-hoc* analysis.

Table 5.13 5-HT$_{2A}$ receptor mRNA fold change in the OB rat following chronic venlafaxine or imipramine treatment

Group	PFC	Hippocampus	Amygdala
Sham + Veh	1 ± 0.2	1 ± 0.352	1 ± 0.113
Sham + Ven	0.994 ± 0.179	0.814 ± 0.24	0.968 ± 0.102
Sham + Imi	0.962 ± 0.155	0.82 ± 0.239	0.942 ± 0.069
OB + Veh	0.957 ± 0.169	1.088 ± 0.393	1.011 ± 0.139
OB + Ven	0.893 ± 0.113	0.944 ± 0.272	1.029 ± 0.14
OB + Imi	0.835 ± 0.208	0.909 ± 0.29	0.956 ± 0.148
Lesion	$F_{(1,60)} = 4.37$, ***p*<0.05**	$F_{(1,63)} = 1.94$, $p>0.05$	$F_{(1,58)} = 0.85$, $p>0.05$
Drug	$F_{(2,60)} = 1.15$, $p>0.05$	$F_{(2,63)} = 2.37$, $p>0.05$	$F_{(2,58)} = 1.34$, $p>0.05$
Lesion x Drug	$F_{(2,60)} = 0.33$, $p>0.05$	$F_{(2,63)} = 0.03$, $p>0.05$	$F_{(2,58)} = 0.26$, $p>0.05$

Table 5.13 Data are expressed as mean ± SD. (n=9-13)

5.3.4 Serotonin neurotransmitter content in the amygdala in OB rats

5.3.4.1 Effect of chronic fluoxetine treatment on 5-HT, 5-HIAA and 5-HIAA/5-HT content in OB rats (Table 5.14)

No significant lesion, drug or lesion x drug interaction effect was found with respect to 5-HT levels. A significant lesion and drug effect were found, but no significant lesion x drug interaction effect was found in the levels of 5-HIAA. OB + Veh treated animals had significantly reduced levels of 5-HIAA compared to their sham counterparts, whilst fluoxetine significantly decreased 5-HIAA compared to their vehicle treated counterparts [$p<0.05$]. A significant lesion effect was found in 5-HIAA/5-HT but no drug or lesion x drug interaction effect was found. The lesion effect was not significant in subsequent *post-hoc* analysis.

Table 5.14 5-HT, 5-HIAA and 5-HIAA/5HT content in the amygdala of OB rats after chronic fluoxetine treatment

Group	5-HT	5-HIAA	5-HIAA/5-HT
Sham + Veh	287.4 ± 65.9	2530.6 ± 249.4	9.3 ± 2.3
Sham + Flx	281.4 ± 42.1	1537.6 ± 294.4^{+}	5.3 ± 1.8
OB + Veh	266.3 ± 63.9	2191.5 ± 311.2*	8.8 ± 3.2
OB + Flx	328.9 ± 50.5	1369.8 ± 211.2^{+}	4.3 ± 0.9
Lesion	$F_{(1,37)} = 0.56$, $p>0.05$	$F_{(1,36)} = 8.73$, ***p*<0.01**	$F_{(1,36)} = 0.17$, ***p*<0.001**
Drug	$F_{(1,37)} = 2.6$, $p>0.05$	$F_{(1,36)} = 111.88$, ***p*<0.001**	$F_{(1,36)} = 1.16$, $p>0.05$
Lesion x Drug	$F_{(1,37)} = 3.82$, $p>0.05$	$F_{(1,36)} = 0.99$, $p>0.05$	$F_{(1,36)} = 0.17$, $p>0.05$

Table 5.14 Data are expressed as mean ± SD (n= 7-12). All values are expressed as ng/g. *p<0.05 vs Sham + Veh; +p<0.05 vs veh treated counterparts [Student Newman-Keuls test]

5.3.4.2 Effect of chronic venlafaxine or imipramine treatment on 5-HT, 5-HIAA and 5-HIAA/5-HT content (Table 5.15)

There was no significant lesion, drug or lesion x drug interaction effect found in the levels of 5-HT or 5-HIAA. Although a significant drug effect was found in the levels of 5-HIAA this was found to be between non-comparable groups in subsequent *post-hoc* analysis. A significant lesion and drug but no lesion x drug interaction effect was found in 5-HIAA/5-HT turnover. Venlafaxine significantly increased turnover in sham animals when compared to vehicle treated sham groups [$p<0.05$]. The drug effect found in 5-HIAA/5-HT was found to be between non-comparable groups.

Table 5.15 5-HT, 5-HIAA and 5-HIAA/5HT content in the amygdala of OB rats after chronic venlafaxine or imipramine treatment

Group	5-HT	5-HIAA	5-HIAA/5-HT
Sham + Veh	361.3 ± 52.7	2321.7 ± 231.9	6.5 ± 1.0
Sham + Ven	337.5 ± 44.3	2634.2 ± 376	8 ± 0.9*
Sham + Imi	391 ± 74.6	2306.4 ± 299.5	6.2 ± 0.9
OB + Veh	379.9 ± 107.8	2300.7 ± 332.8	5.99 ± 1.5
OB + Ven	362.7 ± 41.4	2397.7 ± 352.3	6.6 ± 1.0
OB + Imi	373.6 ± 29.3	2108.6 ± 281.9	5.7 ± 0.9
Lesion	$F_{(1,53)} = 0.3$, $p>0.05$	$F_{(1,53)} = 3.5$, $p>0.05$	$F_{(1,53)} = 7.5$, $\mathbf{p<0.01}$
Drug	$F_{(2,53)} = 1.38$, $p>0.05$	$F_{(2,53)} = 5$, $\mathbf{p<0.05}$	$F_{(2,53)} = 9$, $\mathbf{p<0.001}$
Lesion x Drug	$F_{(2,53)} = 0.66$, $p>0.05$	$F_{(2,53)} = 0.69$, $p>0.05$	$F_{(2,53)} = 1.05$, $p>0.05$

Table 5.15 Data are expressed as mean ± SD (n=8-12). All values are expressed as ng/g *p<0.05 vs Sham + Veh [Student Newman-Keuls test]

5.3.5 SERT expression in serotonergic terminal regions (representative images Figure 5.7)

5.3.5.1 SERT expression in OB rats after chronic fluoxetine treatment in discrete brain regions (Table 5.16)

No significant lesion, drug or lesion x drug interaction effect was found in any of the regions measured.

Table 5.16 SERT expression in the PFC, Hippocampus and Amygdala of OB rats after chronic fluoxetine treatment

Group	PFC	Hippocampus	Amygdala
Sham + Veh	5.496 ± 2.841	0.09 ± 0.016	1.116 ± 0.377
Sham + Flx	5.806 ± 2.828	0.092 ± 0.033	1.259 ± 0.308
OB + Veh	6.461 ± 2.874	0.092 ± 0.034	1.299 ± 0.255
OB + Flx	6.304 ± 1.294	0.069 ± 0.058	1.034 ± 0.499
Lesion	$F_{(1,35)} = 0.79$, $p>0.05$	$F_{(1,36)} = 0.81$, $p>0.05$	$F_{(1,35)} = 0.03$, $p>0.05$
Drug	$F_{(1,35)} = 0.00$, $p>0.05$	$F_{(1,36)} = 0.74$, $p>0.05$	$F_{(1,35)} = 0.25$, $p>0.05$
Lesion X Drug	$F_{(1,35)} = 0.08$, $p>0.05$	$F_{(1,36)} = 1.12$, $p>0.05$	$F_{(1,35)} = 2.79$, $p>0.05$

Table 5.16 Data are expressed as mean ± SD (n=8-12). All values are expressed as OD%Area OD = optical density

5.3.5.2 SERT expression in OB rats after chronic venlafaxine or imipramine treatment in discrete brain regions (Table 5.17)

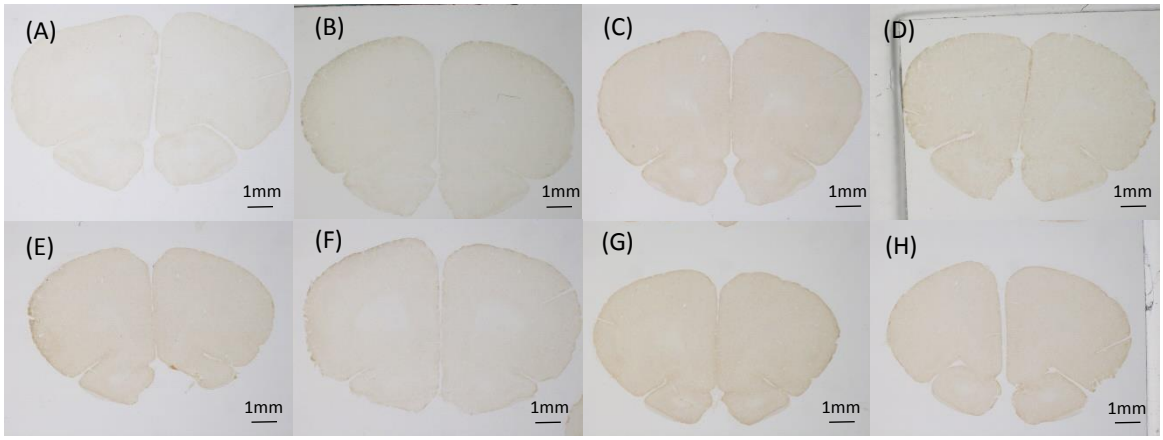
No significant lesion, significant drug but no significant lesion x drug interaction was found in the PFC. No significant lesion, drug or lesion x drug interaction effect was found in the other regions. The significant drug effect in the PFC was found to be non-significant in subsequent *post-hoc* analysis.

Table 5.17 SERT expression in the PFC, Hippocampus and Amygdala of OB rats after chronic venlafaxine or imipramine treatment

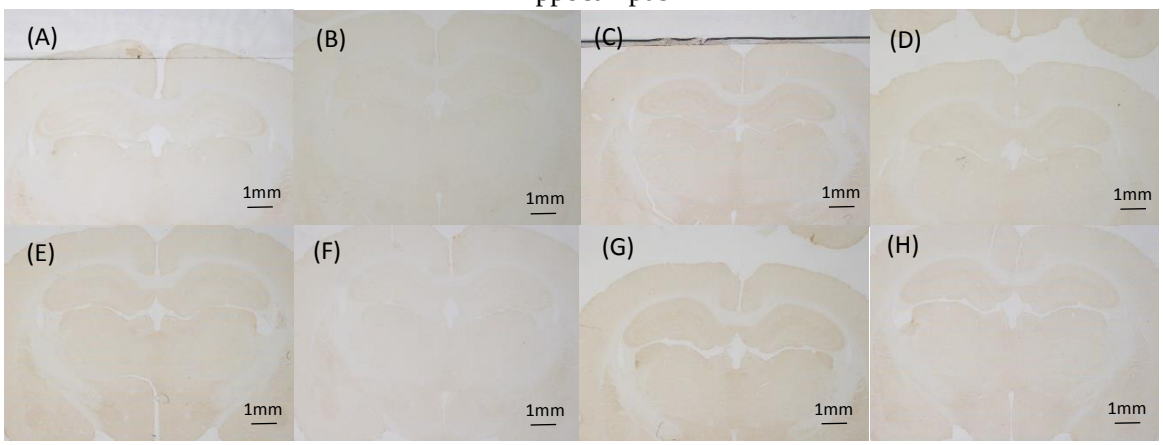
Group	PFC	Hippocampus	Amygdala
Sham + Veh	10.764 ± 2.813	0.163 ± 0.046	1.594 ± 0.853
Sham + Ven	12.270 ± 2.724	0.178 ± 0.05	1.737 ± 0.786
Sham + Imi	10.220 ± 2.766	0.146 ± 0.07	1.012 ± 0.660
OB + Veh	9.490 ± 2.604	0.168 ± 0.052	1.798 ± 0.990
OB + Ven	11.457 ± 1.980	0.170 ± 0.072	1.894 ± 0.579
OB + Imi	9.885 ± 2.001	0.159 ± 0.064	1.463 ± 0.620
Lesion	$F_{(1,58)} = 1.50$, $p>0.05$	$F_{(1,52)} = 0.04$, $p>0.05$	$F_{(1,52)} = 0.38$, $p>0.05$
Drug	**$F_{(2,58)} = 3.27$, $p<0.05$**	$F_{(2,52)} = 0.66$, $p>0.05$	$F_{(2,52)} = 1.10$, $p>0.05$
Lesion X Drug	$F_{(2,58)} = 0.17$, $p>0.05$	$F_{(2,52)} = 0.15$, $p>0.05$	$F_{(2,52)} = 0.92$, $p>0.05$

Table 5.17 Data are expressed as mean ± SD (PFC n=9-12; Hippocampus n=9-11; Amygdala n=8-12); All values are expressed as OD%Area; OD = optical density

PFC



Hippocampus



Amygdala

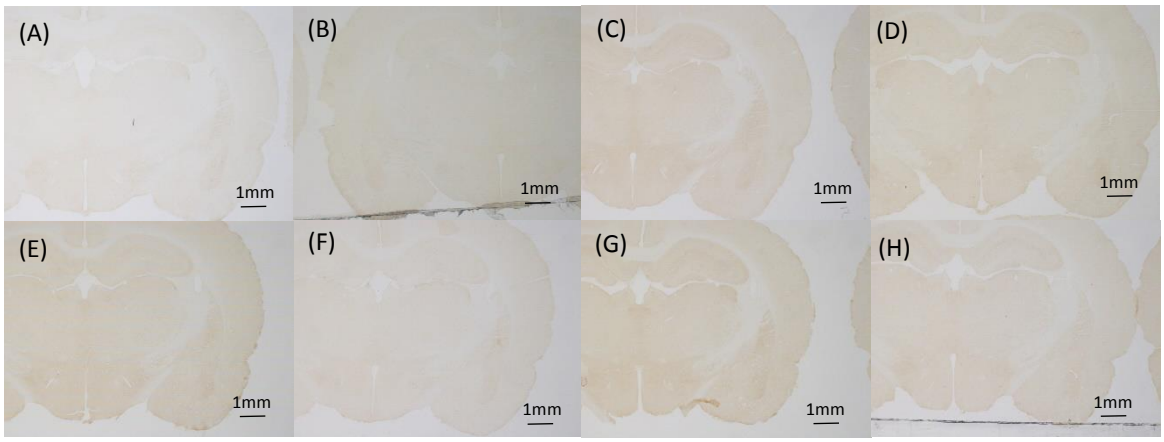


Figure 5.7 Representative images of SERT expression in the striatum, substantia nigra and amygdala. A = Sham + Veh; B = Sham + Flx; C = Sham + Ven; D = Sham + IMI; E = OB + Veh; F = OB + Flx; G = OB + Ven; H = OB + IMI

5.4 Discussion

The aim of this chapter was to assess the effects of chronic treatment with three different antidepressants on numerous stages of central serotonergic transmission. One drug from each of the main classes of antidepressant was chosen, namely the SSRI fluoxetine, the SNRI venlafaxine and the TCA imipramine. By choosing a drug from each of the different classes, and therefore with different affinities and selectivities for the noradrenaline and/or serotonin reuptake sites, it gave further insight into how the serotonergic system in this model may be differentially modulated depending on the drug. The main findings of this chapter are summarised, briefly, below (Figure 5.8)

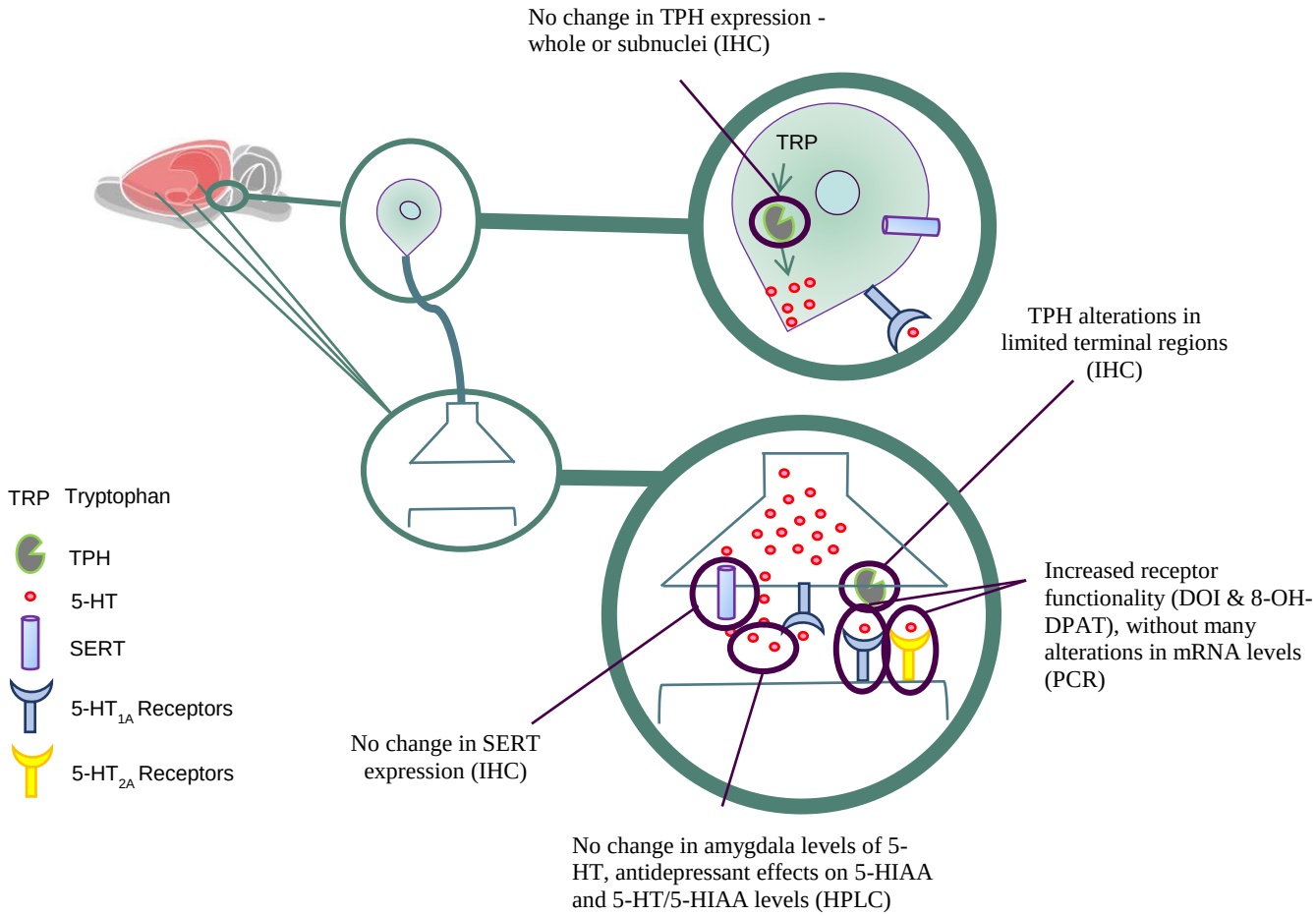


Figure 5.8 Summary of findings with respect to chronic antidepressant treatment on numerous components of the central serotonergic system

Although there have been limited studies in the OB model, those that have been conducted have found a reduced numbers of TPH positive cells in the DRN (Saitoh *et al.,* 2007; Saitoh *et al.,* 2008; Shin *et al.,* 2017), with a reduction also found in adult rats exposed to neonatal clomipramine (Maciag *et al.,* 2006). Although our findings contradict those found in the literature, they are consistent with previous findings within this thesis, with the temporal changes also revealing no change in OB DRN expression at any time point when compared to sham-lesion controls (Chapter 3).

Moreover, chronic fluoxetine, venlafaxine or imipramine treatment had no effect on TPH expression in the whole DRN. This is in contrast to the literature where fluvoxamine, desipramine and milacipran have been shown to attenuate OB induced decreases in the DRN compared to vehicle treated OB rats (Saitoh *et al.,* 2007; Saitoh *et al.,* 2008). Discrepancies between our analysis and the literature are unlikely to be due to methodological approaches as counting the number of cells is a commonly reported method (Saitoh *et al.,* 2008; Roh *et al.,* 2016). However, differences could also be due the regions of the DRN selected. In our analysis of the DRN, a section from the rostral, middle and caudal portion of the DRN was selected in order to assess TPH activity in the whole DRN, whereas, from the images provided, the middle (Saitoh *et al.,* 2008) and caudal (Saitoh *et al.,* 2007) regions have been assessed in these OB studies. The duration of antidepressant treatment was also much shorter, 8 days (Saitoh *et al.,* 2007; Saitoh *et al.,* 2008) compared to 21 days used in our study, which may also have had an impact.

Given that no alteration in TPH expression was found in the DRN as a whole, alterations in specific subregions were evaluated. The DRN can be divided into 5 main subregions - the interfasicular, ventral, lateral, dorsal and caudal (Michelsen *et al.,* 2008). Each of these subregions receive input and project to distinct regions of the brain. Monti (2010) has provided a detailed description of the afferent and efferent connections associated with the rodent DRN. However, as with analysis of TPH expression in the whole DRN, no lesion or drug effects were found with analysis of the dorsal, ventral and lateral wings of the DRN. The lack of effect of fluoxetine is in contrast to Klomp *et al.,* (2014) who report fluoxetine alterations in TPH positive cells in the ventral but not dorsal or lateral wings of the DRN.

The lack of an antidepressant effect, regardless of lesion type in the DRN, is in agreement with others where 5 or 14 days of fluoxetine treatment have no effect on TPH optical density (Choi *et al.,* 2012) or on mRNA expression after 20 days of fluoxetine treatment (Zhou *et al.,* 2006). Contradictory findings suggest a fluoxetine induced decrease in TPH cell number in rats housed under standard conditions (MacGillivray *et al.,* 2012). This effect remains after 28 days of fluoxetine and citalopram treatment, with the citalopram induced reduction persisting from 24 hr and 7 day treatment (MacGillivray *et al.,* 2010). mRNA levels are also decreased after 4 weeks of citalopram (Abumaria *et al.,* 2007) and 2 weeks of fluoxetine treatment (Dygalo *et al.,* 2006). This suggests that SSRI treatment leads to rapid and long lasting reductions in TPH expression in the DRN, and in turn SERT, may be important in the regulation of TPH (MacGillvray *et al.,* 2010). However, in our data presented above, as well as in the literature reported, the level of TPH positive cells does not give an insight into the activity of the enzyme. Chronic clomipramine treatment has been shown to increase activity (Sinei and Redfern, 1993) and thus assessing the activity of the enzyme could be the next recommended parameter to assess. Interestingly, in researching antidepressant effects on TPH expression in the DRN, the majority of the literature deals with SSRI effects, with limited mention of TCA or SNRI effects.

In order to further elucidate changes in TPH expression, given no lesion or drug effect was found at the level of the cell body and given the wide reaching innervation of the serotonergic system, TPH expression in terminal regions was subsequently assessed. As with Chapter 3, individual images of both sides were taken in order to maintain anatomical markers to correct region location; in analysis both sides were assessed. Robust staining was found in the striatum, substantia nigra and early part of the amygdala but none was observed in the hippocampus or PFC. This was unexpected as TPH protein has been detected immunohistochemically in the hippocampus (Choi *et al.,* 2012) and via western blot analysis in the hippocampus and PFC (Duan *et al.,* 2016). However as optimisation of the staining in primary and secondary negative antibodies revealed no staining, it was concluded that the staining was specific and it was decided to proceed with the analysis of the regions that demonstrated staining.

No effect of lesion was found in either cohort in any of the regions assessed, with chronic venlafaxine or imipramine treatment having no significant effect in any of the regions measured. In the amygdala, fluoxetine significantly reduced expression in OB

rats compared to their vehicle treated counterparts. In the OB model, the levels of TPH apoenzyme in terminal regions have been assessed in the frontal cortex, with imipramine attenuating the increased level of TPH in the frontal cortex (Grecksch *et al.,* 1997; Huether *et al.,* 1997). Therefore it is difficult to compare our findings in the OB model with those in the literature. Fluoxetine has been shown to have no effect on increased TPH mRNA expression in the nucleus accumbens in mice exposed to chronic restraint stress (Zhao *et al.,* 2013). In the absence of a TCA or SNRI effect, via imipramine and venlafaxine respectively, on TPH expression at the terminal regions, tentatively suggests that this parameter may be more sensitive to manipulation by fluoxetine.

Both 5-HT_{1A} and 5-HT_{2A} receptors appear to exhibit increased functionality or sensitivity in OB rats, reflected by increased locomotor activity, after an acute injection of their respective agonists 8-OH-DPAT and DOI. In both receptor types this was unaffected by antidepressant treatment. Given that this parameter has not been measured in OB rats before, it is difficult to say whether the increased locomotor activity, followed by a lack of effect of SSRI, SNRI or TCA is a characteristic of this model.

Previous studies involving the administration of 8-OH-DPAT in this model have primarily focused primarily on another post-synaptic response, 8-OH-DPAT induced hypothermia. In these cases there was no lesion effect but interestingly, there was a reversal of the hypothermic response with chronic SSRI treatment alone and in combination with the receptor antagonism, suggesting a densitisation of the receptors (Cryan *et al.,* 1999). Acute 8-OH-DPAT, increases open field locomotor activity in sham-leison rats and not OB rats, whilst chronic 8-OH-DPAT reduced activity in OB rats only (Jiang *et al.,* 2014). The dose used in this study was 6 times higher compared to the 0.5 mg/kg used in this chapter and as the open field was the test arena the results cannot really be comparable.

With regards to the stereotyped behaviours scored, fluoxetine decreased the incidence of flat body posture, irrespective of lesion group, whilst also increasing the frequency of head weaving in OB rats treated with 8-OH-DPAT. Reduction of flat body posture by 7 day treatment with fluoxetine has also been shown (Rogoz and Skuza, 2009). Imipramine increased rearing in sham-lesion rats treated with 8-OH-DPAT without

having an effect on any of the other parameters measured. Venlafaxine had no impact on the parameters assessed, which would be in line with previous work whereby venlafaxine had no effect on 8-OH-DPAT induced hypothermia in OB or sham rats (McGrath and Norman, 1998). 8-OH-DPAT, albeit at a higher dose, has been shown to elicit serotonin syndrome in rats but in contrast to the results found in our study this was unaffected by 14 (Maj and Moryl, 1993) or 21 days of pre-treatment with fluoxetine (Freo *et al.,* 2010) in normal rats. The authors suggest thus that the post-synaptic 5-HT_{1A} receptors mediating the motor effects of 8-OH-DPAT were unaffected by chronic antidepressant treatment (Freo *et al.,* 2010).

Interestingly, although DOI had a stimulatory effect on locomotor activity regardless of drug group, there was no effect found on any of the stereotyped behaviour. This was unexpected as previous studies have shown that at the same dose used in the above study (Kawakami *et al.,* 2005; Kitamura *et al.,* 2007; Biezonski *et al.,* 2009) or at a similar dose of 2.5 mg/kg (Kleven *et al.,* 1997) the expected stereotyped behaviours were elicited. No effect of fluoxetine treatment is also in contrast with the literature, where 21 days of fluoxetine treatment attenuated a DOI induced increase in head shakes (Freo *et al.,* 2010), whilst imipramine reduces wet dog shakes (Kitamura *et al.,* 2008; Kawkami *et al.,* 2005). It is known that these behavioural characteristics of receptor stimulation are due to post-synaptic activation, however given the discrepancy between behaviours it is possible that they are modulated through different subtypes of receptors expressed in different regions of the brain. The lack of a robust effect across multiple behaviours in response to both agonists could be due to the dose chosen, thus a higher dose may have revealed greater differences in antidepressant response. In addition, the results were measured over a 1 hr period, thus it would be interesting to assess these parameters in time bins.

Alterations in receptor functionality appears to be in the absence of changes in receptor mRNA levels, as no lesion effects were found with respect to either receptor. However, fluoxetine significantly reduces 5-HT_{1A} mRNA expression in the PFC in sham-lesion rats. Fluoxetine had no effect on 5-HT_{1A} receptor mRNA expression in the hippocampus or amygdala, whilst neither venlafaxine or imipramine had an effect on expression in any of the 3 regions assessed. Autoradiographic studies have found widespread decreases in 5-HT_{1A} density in numerous brain regions of the OB rat, including the hippocampus and amygdala (Sato *et al.,* 2008). Therefore, it suggests

that future analysis should focus on exploring changes in protein levels of these receptors. Although not directly highlighted in the paper, Shishkina *et al.,* (2012) demonstrated that 8 weeks of fluoxetine treatment decreased 5-HT_{1A} mRNA in the frontal cortex of normal rats, which would be in line with our findings. This is in contrast to fluoxetine treatment of 2 weeks having no impact on cortical expression (Le Poul *et al.,* 2000). The discrepancies in the literature could be due to the length of antidepressant treatment. The absence of an effect of imipramine on 5-HT_2 mRNA levels has been shown previously in the cortex and hippocampus of non-depressed rats (Burnet *et al.,* 1994), whilst others have a significant decrease in 5-HT_{2A} mRNA the frontal cortex in response to 14 day treatment with imipramine (Kitamura *et al.,* 2008). Although no effect of antidepressant treatment on 5-HT_{2A} mRNA levels was found, chronic venlafaxine treatment has been shown to significantly reduce 5-HT_{2A} binding in the frontal cortex of both sham and OB rats compared to vehicle treated counterparts (McGrath and Norman, 1998).

There was no change in 5-HT levels with respect to lesion type or antidepressant treatment. 5-HIAA levels were reduced OB rats treated with vehicle in the fluoxetine cohort of rats but not in the venlafaxine or imipramine cohort. Fluoxetine decreased 5-HIAA content regardless of lesion type, whilst no significant effect was found with venlafaxine or imipramine treatment. 5-HIAA/5-HT levels were unaltered by fluoxetine treatment but venlafaxine increased levels in sham-lesion compared to sham + veh. Previous studies looking at the impact of chronic antidepressant treatment on the levels of 5-HT, 5-HIAA and turnover reveal that fluoxetine (14 day treatment) decreases 5-HIAA in the amygdala of OB rats (Marcilhac *et al.,* 1999). In contrast, fluoxetine has been shown to increase 5-HT and reduce 5-HT/5-HIAA levels in the amygdala of OB rats compared to vehicle treated controls (Marcilhac *et al.,* 1999), which is reflective for a trend in raised levels of 5-HT in the amygdala in OB rats treated with fluoxetine. Desipramine (14 days treatment) (the active metabolite of imipramine) significantly increased 5-HT and 5-HIAA, with no effect on turn over (Saitoh *et al.,* 2008) which is contrast to the results found in this chapter. With sertraline (14 days treatment), 5-HIAA concentrations were reduced in sham animals (Harkin *et al.,* 1999). Together this is suggestive, that the data present in the literature is, as of yet, inconclusive regarding changes in 5-HT, 5-HIAA and 5-HIAA/5-HT

turnover. Noticeably, antidepressant efficacy was reached with 14 days of treatment, suggesting that a shorter period of dosing would be more suitable.

The final stage in serotonergic neurocircuitry is the cessation of the signalling cascade via the reuptake of 5-HT through SERT, the reuptake transporter. Given that there was no significant effect of lesion found in the DRN of the temporal study (Chapter 1) it was decided that perhaps there may be changes in clinically relevant terminal regions. As the antibody targeting SERT used in Chapter 3 was discontinued, the antibody used this chapter was different. Therefore optimisation was carried out prior to analysis to determine optimal concentration and confirm specificity. Thus sections were incubated with varying concentrations of antibody, as well as the inclusion of primary and secondary negative antibody sections. The concentration and primary antibody incubation time were in line with the literature also using this antibody (Abe *et al.*, 2016; Shin *et al.,* 2012; Carlsson *et al.,* 2007). The resulting staining was diffuse in nature, corresponding to the pattern of staining obtained by Abe *et al.,* (2016) and in Brown and Gerfe, (2006) using a different antibody. In addition, the antibody negative sections remained unstained, thus it was decided to proceed.

In the above results, no significant effect of lesion or drug was found, thus suggesting that SERT expression in this model is not altered, in the PFC, hippocampus or amygdala. This is in agreement with previous autoradiographic findings, with no change in the amygdala or hippocampus (Licht *et al.,* 2010), whereas others have found an increase in OB rats in the hippocampus and frontal cortex, with no change found in the amygdala (Sato *et al.,* 2010). Autoradiography is a technique that was not available in our laboratory, thus IHC was the next best available option as it also provided the opportunity to gain more insight into regional variation and selectivity as opposed to gross anatomical locations. SERT reactivity in the frontal cortex has been shown to be increased with fluoxetine but not desipramine treatment (Zhou *et al.*, 2006), with paroxetine but not venlafaxine significantly reducing density in the hippocampus (Gould *et al.,* 2006). Although no change in antidepressant treatment was found in the above studies, it has been shown that chronic fluoxetine reduces SERT density in the hippocampus (Nadgir and Malviya, 2008), whilst also reducing the OB induced increases in membrane plasma levels, suggestive of protein shifting between cellular compartments (Riad *et al.,* 2017). Although a lack of effective fluoxetine treatment was found, given that the primary target is SERT. Imipramine

and venlafaxine also target noradrenergic signalling, with increasing doses venlafaxine having a greater affinity for NET (Millan *et al.*,2001) but neither having an effect on hippocampal SERT density (Gould *et al.,* 2006).

Taking the results together as a comprehensive evaluation of multiple components of the serotonergic system in this model and the effects of chronic antidepressant treatment on each parameter, it suggests that there little change in the serotonergic system in the regions assessed. Those studies that examine more than one aspect of the system (Zhou *et al.,* 1998; Sato *et al.,* 2010; Riad *et al.,* 2017) allow a more robust conclusion as to the changes in 5-HT innervation, such as hyperinnervation of the frontal cortex. Similarly, our study allows us to draw the same inferences – however in this case, there appears to be very little lesion induced changes and or compensatory mechanisms. Having said that, there are some limitations to the current study design; firstly, TPH expression was only measured in the DRN, given the topographic organisation of the raphe nuclei and relative specificity of the origin of the projection regions, analysis of TPH in the median raphe nucleus (MRN) should also be investigated. Secondly, the effect of bulbectomy on serotonin, 5-HIAA and 5-HIAA/5-HT was only analysed in one region of serotonergic innervation, it is suggested that more regions be assessed – such as the hippocampus and PFC, as OB rats have reduced basal levels of 5-HT in the medial PFC (Jimenez-Sanchez, *et al.,* 2016). It could be postulated however, that the reduction in 5-HIAA found in OB vehicle treated animals in the fluoxetine cohort, may possibly be reflective of altered SERT functioning. Thirdly, with regards to the two receptors, only mRNA analysis was carried out, therefore it is recommended that protein levels also be assessed. This is highlighted by a recent study by Riad *et al.,* (2017) who demonstrated that OB rats exhibit increased plasma membrane labelling of 5-HT_{1A} receptors in the DRN, with no change in total labelling, thus suggesting protein levels may be altered. Interestingly, this was reversed by chronic fluoxetine treatment, with a concomitant increase in cytoplasmic concentration, suggesting that decrease in membrane expression may be due to proteins shifting between cellular compartments (Riad *et al.*, 2017).

Chapter 6:

The search for robust behavioural measurements following chronic antidepressant treatment in the OB rat

6.1 Introduction

In previous chapters it has been shown that hyperactivity is present from 2 week following lesion and persists until 6 weeks following lesion. Hyperactivity in the open field is the behavioural hallmark of this model and the hyperactivity is believed to be due the rat's inability to adapt to a novel environment (Mar *et al.,* 2002). It demonstrates that the syndrome has been established, sufficient time has lapsed for molecular changes to be detected and for the assessment of antidepressant activity. It is the attenuation of the hyperactivity by chronic and not acute antidepressant treatment that makes this model one of the most relevant and valid models of disease to the clinical scenario.

Hyperactivity in the open field is often expressed as 'distance moved' but can also be depicted as changes in number of line crossings but in general it is considered a measure of ambulation. The open field was designed by Hall in 1934, with defaecation as a measure of emotionality or timidity (Standford, 2007; Walsh and Cummins 1976). Walsh and Cummins (1976) proposed that the behaviours elicited in the open field are dependent upon a number of parameters including stimulation arising from removal from the home/familiar environment, stimulation in transferring an animal to the arena, exposure to not only the open field but also the surroundings and finally prior experience to the arena or habituation to the arena. In order for hyperactivity in OB animals in the open field to manifest, the level of aversion in the open field is important as the animals needs to find the arena sufficiently aversive, without being excessively so. The shape of the arena, lux and wall material has been found to be essential in producing the hyperactivity (Kelly *et al.*, 1997). In our laboratory, a circular arena 75cm in diameter, with light reflecting walls and a lux of 200-250 have been found to be the optimal conditions. In low luminance, OB rats were not significantly hyperactive compared to sham rats, but were in a high luminance open field with reflective walls (Mar *et al.*, 2002). What is noteworthy about this is that in the low luminance, sham rat's activity increases, whereas OB rats exhibit approximately the same degree of locomotor activity, thus it is thought that they are unable to adapt to a change in environment (Mar *et al.*, 2002).

Although ambulation/distance moved/hyperactivity are the primary counts that are measured in this paradigm, inner and outer zone patterns can also be assessed, rearing and grooming, as well as faecal boli number can also be assessed. OB rats in general spend less time in the inner zone of the open field (Burke *et al.,* 2013), exhibiting increased number of rearings and faecal boli, with decreased frequency of grooming (O'Connor *et al.,* 1985).

Given that one of the assessment parameters in the open field is habitation to the open field upon re-exposure, as already mentioned this may cause issues in the detection of antidepressant activity if the attenuation of the hyperactivity can no longer be detected. Habituation to the open field in OB rat has not been consistently shown with some exhibiting habituation with repeated exposure (Gigliucci *et al.,* 2014) whilst others show that there is no impact on repeated exposure on the extinction of hyperactivity in the open field (Cryan *et al.,* 1999; Burke *et al.,* 2010).

Another behavioural paradigm that can be assessed in the OB rat is hyperactivity in the home cage, whereby there is no chance that habituation may occur as the animal is in its familiar environment. Previous work in our laboratory has shown that monitoring HCA is superior in detecting dose response effects for both stimulant and sedative drugs over a short period of time, whilst also detecting differences in potency and efficacy (Dunne *et al.,* 2007). To our knowledge, HCA in this model is not commonly reported with Vinkers *et al.,* (2009) reporting increased nocturnal activity in OB rat from 3 days following lesion continuing to 5 days following lesion. One of the limitations to this study is the short duration in which the behaviour is monitored, given that the majority of studies assessing locomotor activity in the OB model do so at 2 weeks following lesion, it is not known whether this increase in HCA activity persists, becomes stronger or even wanes. Another drawback is the use of telemetry devices inserted into the abdominal cavities of the rats, which exposes them to more stressors and unnecessary procedures.

Given the value of the attenuation of OB hyperactivity in the open field in the detection of antidepressant action and the evidence that rats do become habituated to the arena, it is necessary to determine if there are other behaviours that can be measured in the open field that may not be susceptible to habituation. More so, given that our laboratory has previously shown the superiority of home cage monitoring (Dunne *et*

al., 2007), if this paradigm could be applied in the OB model, hyperactivity and antidepressant activity may be more accurately measured.

Therefore the aims of the studies in this chapter are to:

1. Assess locomotor activity in the open field following lesion and post chronic antidepressant treatment
2. Assess various behavioural parameters in the open field not subject to locomotor activity
3. Assess nocturnal homecage locomotor activity using a system that was designed in our laboratory at baseline, following lesion and duration of chronic antidepressant treatment.

6.2 Methods

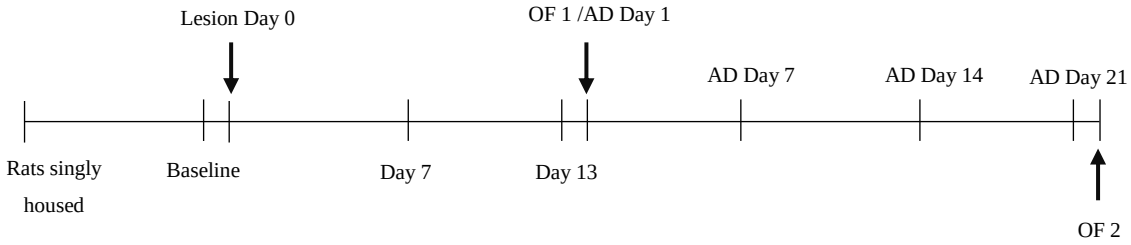
All methods have been detailed in Chapter 2. Methods specific to this chapter are detailed below

6.2.1 Study design

The open field and homecage locomotor profiles of rats in Chapter 5 are addressed in this chapter. Two separate experiments were carried out: Experiment 1 – rats received either vehicle (dH_2O @ 2ml/kg s.c.) or fluoxetine (10mg/kg @ 2ml/kg s.c.) for 21-days; Experiment 2 – rats received either vehicle (dH_2O @ 4ml/kg s.c.), venlafaxine (20mg/kg @ 4ml/kg s.c.) or imipramine (10mg/kg @ 4ml/kg s.c.). Throughout this chapter the experiments will be referred to as experiment 1 and experiment 2. Although separate cohorts of rats were used, the study design was identical throughout. At 2 weeks following lesion rats went through the open field (approx. 8am-12pm) and were assigned treatment groups based on this data. On the same day, rats began antidepressant treatment with dosing beginning at approx. 2pm each day and lasting for 21 days. After a 24 hr wash out period from the last dose, rats underwent the open field again and were immediately sacrificed via decapitation or transcardial perfusion (as detailed in 2.2.7)

6.2.2 Behavioural measurements

The open field and HCA were carried out as detailed in Chapter 2 (as detailed in 2.2.5.2 and 2.2.5.1). Video files of 24 hr HCA were recorded for the week prior to surgery to establish baseline activity (when rats were singly housed) and every day for the duration of the study. However due to time constraints, 12 hr nocturnal HCA was assessed for designated experimental time points, timeline given below:



6.3 Results

6.3.1 Locomotor activity in the open field at 2 weeks following lesion

The results are depicted in Figure 6.1. OB rats in experiment 1 [$t_{(86)} = 4.19$, $p<0.001$] and experiment 2 [$t_{(133)} = 5.72$, $p<0.001$] were significantly hyperactive compared to sham-operated rats in the open field.

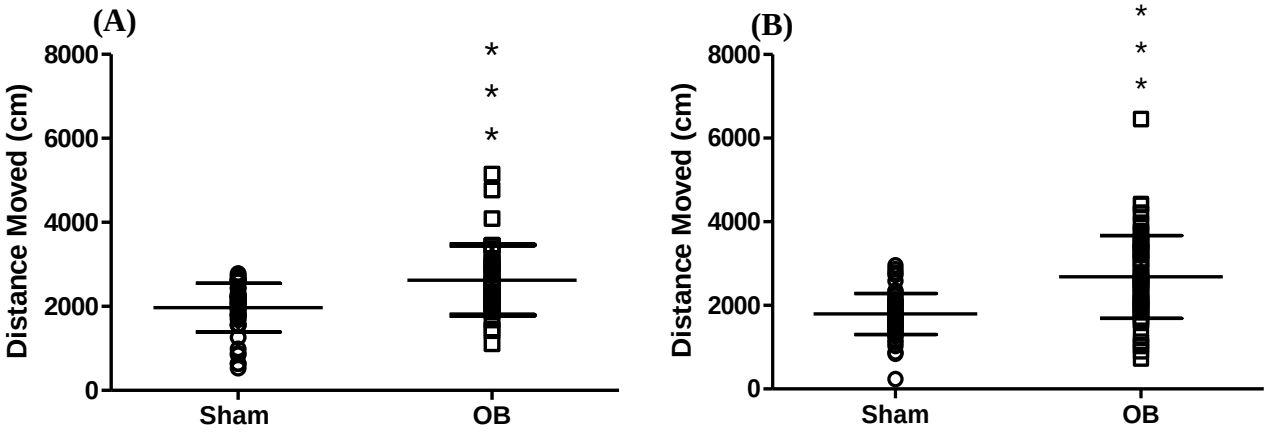


Figure 6.1 Distance moved in the open field 2 weeks following lesion (Experiment 1 n=40-48; Experiment 2 (n=64-71)**.** Data are expressed as mean ± SD ***$p<0.001$ vs sham animals (A) Rats allocated to experiment 1 (B) Rats allocated to experiment 2

6.3.2 Time spent in the inner zone in the open field 2 weeks following lesion (Table 6.1)

OB rats in experiment 1 and experiment 2 spend significantly less time in the inner zone compared to sham operated rats in the open field (Arenas are depicted in Figure 6.2)

Table 6.1 Open field inner zone duration in OB rats 2 weeks following lesion

	Experiment 1	Experiment 2
Sham	42 ± 22	81 ± 40
OB	24 ± 12***	65 ± 34*
	$t_{(85)} = 4.47$, **$p<0.001$**	$t_{(133)} = 2.5$, **$p<0.05$**

Table 6.1 Data are expressed as mean ± SD (Experiment 1: n=40-48; Experiment 2: n=64-71) ***$p<0.001$ vs sham *$p<0.05$ vs sham

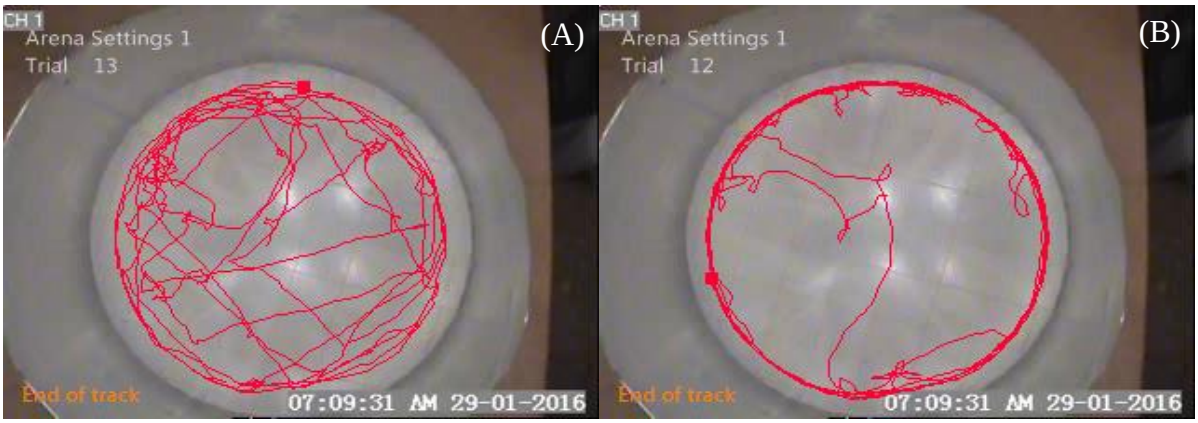


Figure 6.2 Representative images of pattern of movement in the open field 2-weeks following lesion in (A) sham and (B) OB. OB spend less time in the inner zone compared to sham rats.

6.3.3 Allocation of rats into treatment groups

Results are depicted in Figure 6.3. Rats were assigned into their respective treatment for experiment 1 and experiment 2 based on their hyperactivity in the open field. OB rats allocated to vehicle or fluoxetine (experiment 1), a significant effect of lesion [$F_{(1,84)}$ = 18.05, p<0.001] but no significant drug [$F_{(1,84)}$ = 0.40, p>0.05] or lesion x drug interaction [$F_{(1,84)}$ = 1.69, p>0.05] was found (Figure 6.2). OB rats groups were significantly different compared to their sham lesion counterparts [p<0.05]. OB rats allocated to vehicle, venlafaxine or imipramine (experiment 2) a significant lesion [$F_{(1,129)}$ = 32.41, p<0.001] but no significant effect of drug [$F_{(2,129)}$ = 0.65, p>0.05] or lesion x drug interaction [$F_{(2,129)}$ = 0.52, p>0.05] was found (Figure 6.2 B.). OB rats assigned to venlafaxine or imipramine treatment were significantly hyperactive compared to their sham lesion counterparts, however OB rats assigned to vehicle treated group were not significantly hyperactive compared to their sham surgery counterparts [p<0.05].

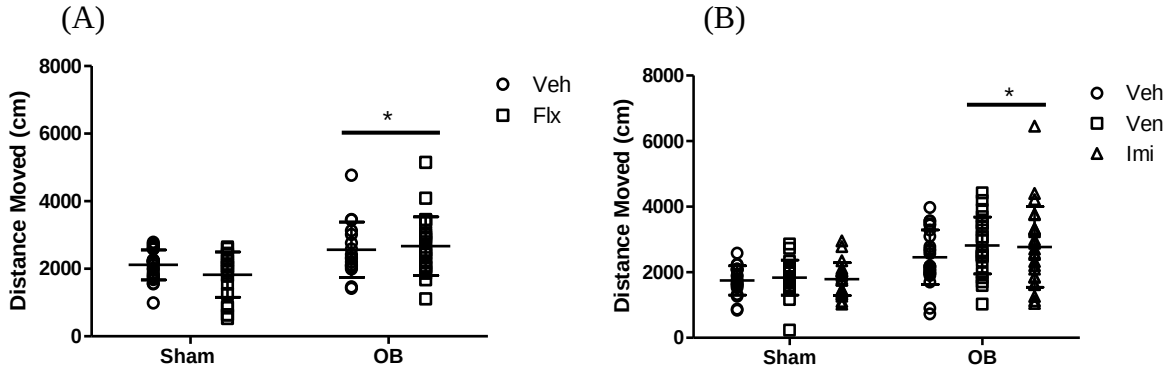


Figure 6.3 Distance moved in the open field in allocated treatment groups. (A) Rats allocated to vehicle or fluoxetine treatment**.** Data are expressed as mean ± SD (n= 17-24)*p<0.05 vs sham lesion counterparts [Student Newman Keuls test] (B) Rats allocated to vehicle, venlafaxine or imipramine treatment. Data are expressed as mean ± SD (n=22-24) *p<0.05 vs sham lesion counterparts [Student Newman Keuls test]

6.3.4 Locomotor activity in the open field after chronic antidepressant dosing

Results are depicted in Figure 6.4. In experiment 1, where rats underwent chronic fluoxetine treatment, no significant lesion [$F_{(1,83)} = 1.79$, $p>0.05$], drug [$F_{(1,83)} = 0.09$, $p>0.05$] or lesion x drug interaction [$F_{(1,83)} = 0.90$, $p>0.05$] effect was found (Figure 6.3 A). The same pattern was also found in experiment 2, where rats underwent chronic venlafaxine or imipramine treatment no significant lesion [$F_{(1,129)} = 0.97$, $p>0.05$], drug [$F_{(2,129)} = 1.94$, $p>0.05$] or lesion x drug interaction effect [$F_{(2,129)} = 0.01$, $p>0.05$] was found.

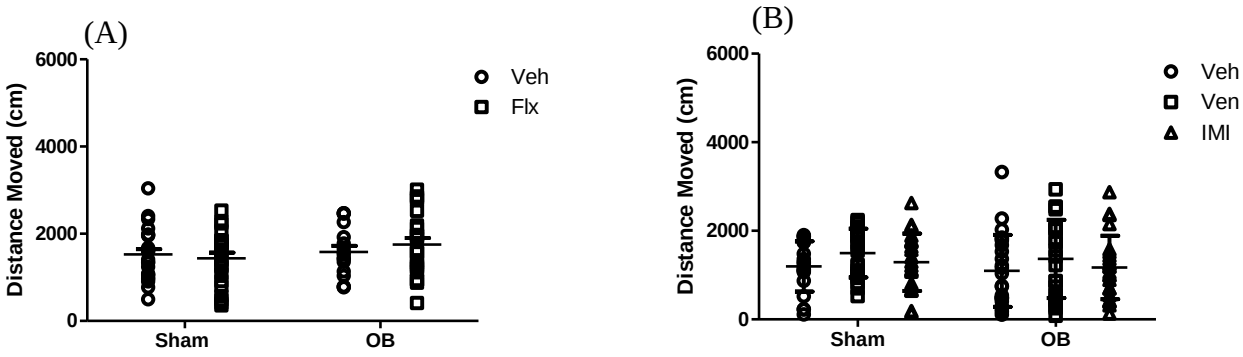


Figure 6.4 Effect of chronic antidepressant treatment on hyperactivity in the open field. Data are expressed as mean ± SD (A) Animals allocated to vehicle or fluoxetine treatment (n=17-24) **(**B) Animals allocated to vehicle, venlafaxine or imipramine treatment (n=22-24)

6.3.5 Time course of locomotor activity in the open field after chronic antidepressant dosing

6.3.5.1 Time bin analysis of locomotor activity in rats chronically treated with fluoxetine

Results are depicted in Figure 6.5. A significant time [$F_{(30.12, 259.72)} = 35.87$, $p<0.001$] and lesion x time interaction [$F_{(3.12, 259.72)} = 9.93$, $p<0.001$] but no significant time x drug interaction [$F_{(3.12, 259.72)} = 1.53$, $p>0.05$] or lesion x time x drug interaction effect [$F_{(3.12, 259.72)} = 1.31$, $p>0.05$] was found. However, there was no significant lesion [$F_{(1,83)} = 1.80$, $p>0.0$], drug [$F_{(1,83)} = 0.09$, $p>0.05$] or lesion x drug interaction [$F_{(1,83)} = 0.90$, $p>0.05$] main effect found between the groups (Figure 6.4). Significant main effects were not found in subsequent *post-hoc* analysis.

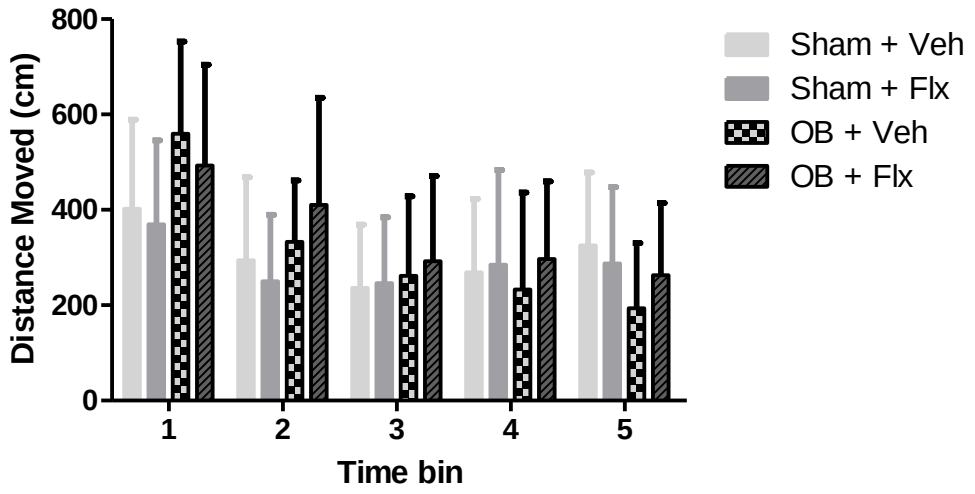


Figure 6.5 Effect of chronic fluoxetine treatment on distance moved in the open field in 5 x 1min time bins. Data are expressed as mean ± SD (n= 17-24).Time bins (min) 1 = 0-1.00; 2 = 1.00-2.00; 3 = 2.00-3.00; 4 = 3.00-4.00; 5 = 4.00-5.00

6.3.5.2 Time bin analysis of locomotor activity in rats chronically treated with venlafaxine or imipramine

The results are depicted in Figure 6.6. A significant time [$F_{(3.13,\ 404.14)}$ = 11.83, p<0.001] and time x lesion interaction [$F_{(3.133,\ 404.14)}$ = 22.54, p<0.001] but no significant time x drug interaction [$F_{(6.26,\ 404.14)}$ = 22.54, p>0.05] or time x lesion x drug interaction [$F_{(6.26,\ 404.14)}$ = 1.31, p>0.05] effect was found. No significant lesion [$F_{(1,\ 129)}$ = 0.96, p>0.05], a significant drug [$F_{(2,129)}$ = 3.39, p>0.05] effect but no lesion x drug interaction [$F_{(2,\ 129)}$ = 0.04, p>0.05] main effect was found between the groups. In all time bins no significance was found or significance was found between non-comparable groups [p>0.05].

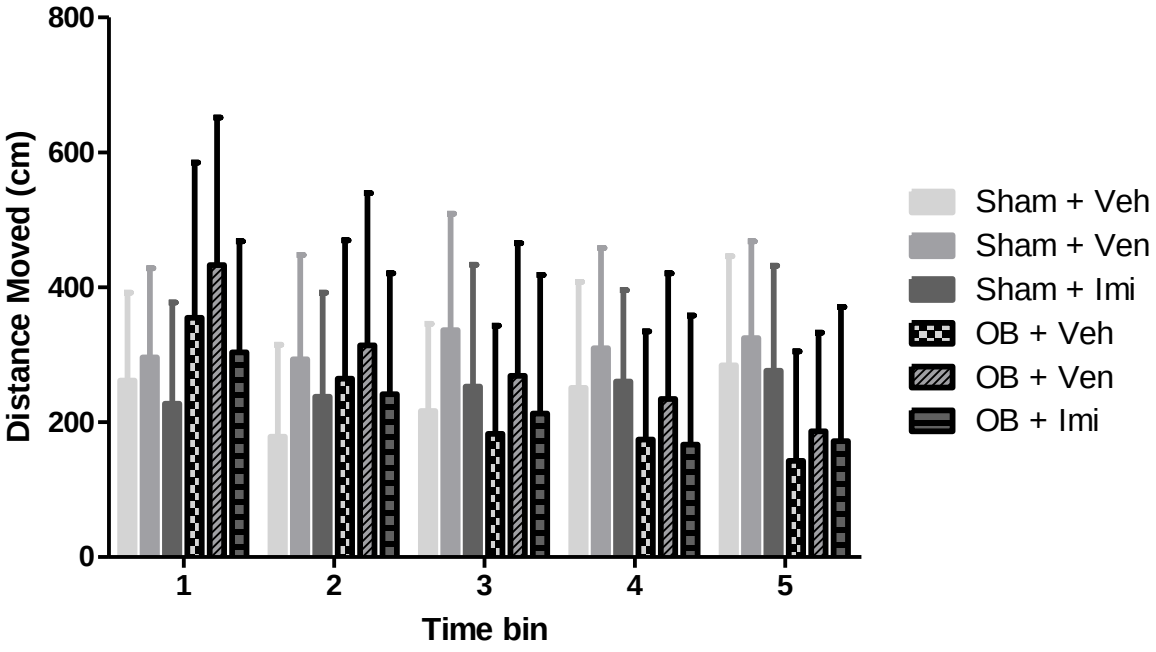


Figure 6.6 Effect of chronic venlafaxine or imipramine treatment on distance moved in the open field in 5 x 1min time bins. Data are mean ± SD (n= 22-24). Time bins (min) 1 = 0-1.00; 2 = 1.00-2.00; 3 = 2.00-3.00; 4 = 3.00-4.00; 5 = 4.00-5.00

6.3.6 Further characterisation of behavioural parameters in the open field

6.3.6.1 Speed of bout

The results are depicted in Figure 6.7. Speed of bout is classified as rapid bursts of locomotor activity (Gigluicci *et al.,* 2014). A significant lesion [$F_{(1,82)} = 5.30$, $p<0.05$] but no significant drug [$F_{(1,82)} = 2.41$, $p>0.05$] or lesion x drug interaction [$F_{(1,82)} = 0.51$, $p>0.05$] effect was found. Significance was found between non-comparable groups in subsequent *post-hoc* analysis

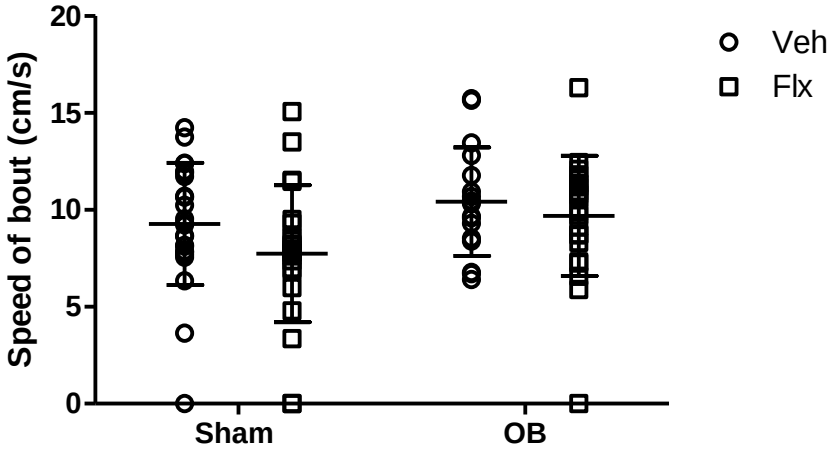


Figure 6.7 Effect of chronic fluoxetine treatment on speed of bout in the open field. Data are expressed as mean ± SD (n=17-24)

6.3.6.2 Classification of degrees of movement (Table 6.2)

No significant lesion effect was found in any of the parameters assessed. A significant drug effect was found in the time spent mobile. No significant lesion x drug interaction effect was found in any of the parameters assessed. Significant drug effect in the duration spent mobile was found to be non-significant in subsequent *post-hoc* analysis.

Table 6.2 Effect of chronic venlafaxine or imipramine treatment on time spent moving, not moving and time spent highly mobile, mobile and immobile in the open field

Group	Moving	Not moving	Highly mobile	Mobile	Immobile
Sham + Veh	30 ± 13	253 ± 42	0.09 ± 0.09	2.36 ± 1.34	295 (293-299)
Sham + Ven	39 ± 12	260 ± 13	0.18 ± 0.16	1.46 ± 2.29	295 (292-299)
Sham + Imi	32 ± 16	266 ± 16	0.12 ± 0.25	1.53 ± 1.69	299 (295-300)
OB + Veh	28 ± 16	271 ± 17	0.11 ± 0.19	2.48 ± 4.19	298 (295-299)
OB + Ven	33 ± 19	266 ± 19	0.13 ± 0.26	3.58 ± 3.28	296 (293-299)
OB + Imi	27 ± 18	260 ± 57	0.12 ± 0.26	1.67 ± 1.81	297 (294-299)
Lesion	$F_{(1,129)} = 2.37$, $p>0.05$	$F_{(1,129)} = 1.12$, $p>0.05$	$F_{(1,129)} = 0.09$, $p>0.05$	$F_{(1,129)} = 1.57$, $p>0.05$	
Drug	$F_{(2,129)} = 2.76$, $p>0.05$	$F_{(2,129)} = 0.02$, $p>0.05$	$F_{(2,129)} = 0.76$, $p>0.05$	$F_{(2,129)} = 6.2$, $\mathbf{p<0.05}$	$K_{(5)} = 9.61$, p>0.05
Lesion x Drug	$F_{(2,129)} = 0.12$, $p>0.05$	$F_{(2,129)} = 1.59$, $p>0.05$	$F_{(2,129)} = 0.34$, $p>0.05$	$F_{(2,129)} = 0.67$, $p>0.05$	

Table 6.2 Data are expressed as mean ± SD in parametric analysis and median (25-th – 75th percentile for non-parametric analysis (n=17-24). Data are expressed as sec.

6.3.6.3 Frequency of rearing and grooming (Table 6.3)

In a cohort of rats the frequency of rearing and grooming was assessed after chronic fluoxetine treatment. No significant lesion, drug or lesion x drug interaction effect was found.

Table 6.3 Effect of chronic fluoxetine treatment on the frequency of rearing and grooming in the open field

Group	Rearing	Grooming
Sham + Veh	9 ± 4	1 ± 2
Sham + Flx	7 ± 6	3 ± 2
OB + Veh	16 ± 15	1 ± 1
OB + Flx	17 ± 11	3 ± 3
Lesion	$F_{(1,14)} = 3.09$, $p>0.05$	$F_{(1,14)} = 0.01$, $p>0.05$
Drug	$F_{(1,14)} = 0.02$, $p>0.05$	$F_{(1,14)} = 2.81$, $p>0.05$
Lesion x Drg	$F_{(1,14)} = 0.11$, $p>0.05$	$F_{(1,14)} = 0.01$, $p>0.05$

Table 6.3 Data are expressed as mean ± SD (n=8-10)

6.3.7 Nocturnal homecage locomotor activity

6.3.7.1 Effect of lesion on nocturnal HCA

The results are depicted in Figure 6.8. In rats assigned to treatment with fluoxetine (experiment 1), a significant time [$F_{(3,180)}$ = 39.06, p<0.001] and time x lesion interaction [$F_{(3,180)}$ = 41.78, p<0.001] was found. OB rats were significantly hyperactive compared to sham-lesion counterparts at day 7 and day 13 following lesion [p<0.05]. The same pattern was observed in rats assigned to venlafaxine or imipramine treatment (experiment 2). A significant time [$F_{(1.56,\ 103.29)}$ = 14.56, p<0.001] and time x lesion interaction [$F_{(1.56, 103.29)}$ = 20.25, p<0.001] effect was found. OB rats were not significantly hyperactive at baseline or day 0 compared to sham-lesion rats, however they were significantly different day at 7 and day 13 post surgery [p<0.05]. Interestingly, sham animals at day 0, day 7 and day 13 were significantly less hyperactive compared to shams at baseline [p<0.05].

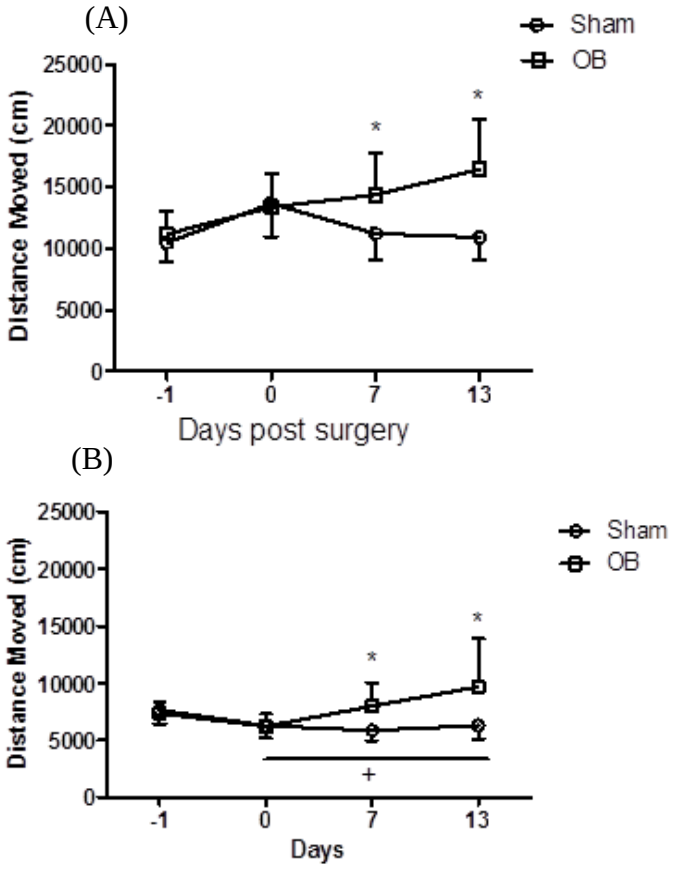


Figure 6.8 Effect of lesion on 12 hr nocturnal HCA**.** (A) OB hyperactivity in rats to be assigned to treatment with fluoxetine. Data are expressed as mean ± SD (n=27-35) * p<0.05 vs sham-lesion counterparts [Student Newman-Keuls test] (B) OB hyperactivity in rats to be assigned to venlafaxine or imipramine treatment. Data are expressed as mean ± SD (33-35) *p<0.05 vs sham-lesion counterparts; +p<0.05 sham lesion baseline vs sham day 0, 7, 13 [Student Newman-Keuls test].

6.3.7.2 Effect of chronic antidepressant treatment on nocturnal HCA

6.3.7.2.1 Effect of chronic fluoxetine treatment

The results are depicted in Figure 6.9. Significant time [$F_{(4.69, 267.77)} = 28.79$, $p<0.001$] and time x lesion [$F_{(4.69, 267.77)} = 19.88$. $p<0.001$] but no significant time x drug [$F_{(4.69, 267.77)} = 2.03$, $p>0.05$] or time x lesion x drug [$F_{(4.69, 167.77)} = 1.95$, $p>0.05$] effect was found. Between subject effects revealed a significant lesion [$F_{(1,57)} = 21.15$, $p<0.001$] effect but no significant drug [$F_{(1,57)} = 0.006$, $p>0.05$] or lesion x drug interaction [$F_{(1,57)} = 0.23$, $p>0.05$] effect was found. OB rats in both treatment groups were significantly hyperactive compared to their sham-lesion counterparts on day 1 of dosing but this waned over time with only OB + Veh treated rats being significantly different compared to sham-lesion counterparts at day 7 of dosing, no significant changes in hyperactivity on day 14 of dosing was found and OB + Flx animals were significantly hyperactive from sham-lesion counterparts on day 21 of dosing [$p<0.05$]

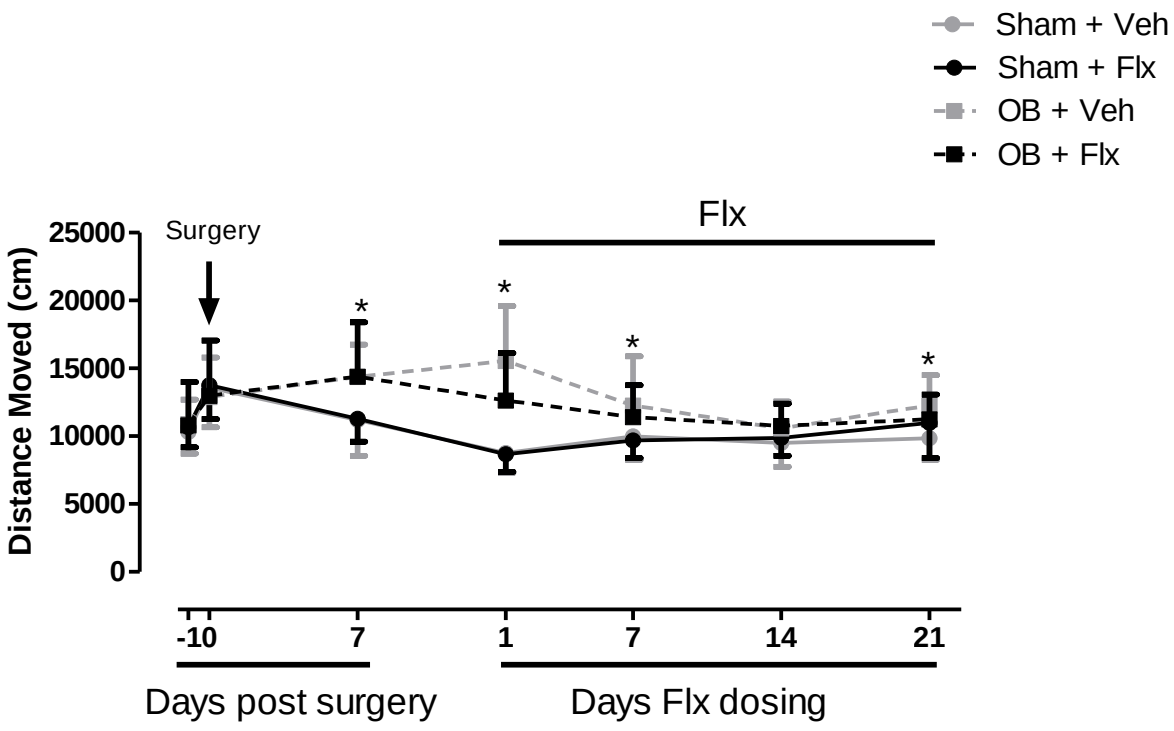


Figure 6.9 Effect of chronic fluoxetine treatment on nocturnal HCA**.** Data are expressed as mean ± SD (n = 12-18). *p<0.05 OB vs Sham-lesion counterparts.

6.3.7.2.2 Effect of chronic venlafaxine or imipramine treatment

The results are depicted in Figure 6.10. Significant time [$F_{(1.82,113.15)} = 11.89$, $p<0.001$], time x lesion [$F_{(1.82,113.15)} = 15.02$. $p<0.001$], significant time x drug [$F_{(3.65,113.15)} = 2.6$, $p<0.05$] but no signficant time x lesion x drug effect [$F_{(3.65,113.15)} = 1.69$, $p>0.05$] effect was found. Between subjects revealed a significant effect of lesion [$F_{(1,62)} = 9.22$, $p<0.01$] and drug [$F_{(2,62)} = 12.2$, $p<0.001$] but no significant lesion x drug interaction [$F_{(2,62)} = 0.16$, $p>0.05$] effect was found. On day 1 there was no significant effect between groups. On day 7 OB vehicle treated rats were significantly hyperactive compared to sham-lesion vehicle treated rats, whilst imipramine significantly reduced locomotor activity in sham-lesion and OB rats compared to their vehicle treated counterparts [$p<0.05$]. On day 14 imipramine treated sham and OB rats remained significantly less mobile compared to vehicle treated counterparts [$p<0.05$]. On day 21 no difference was found between the groups.

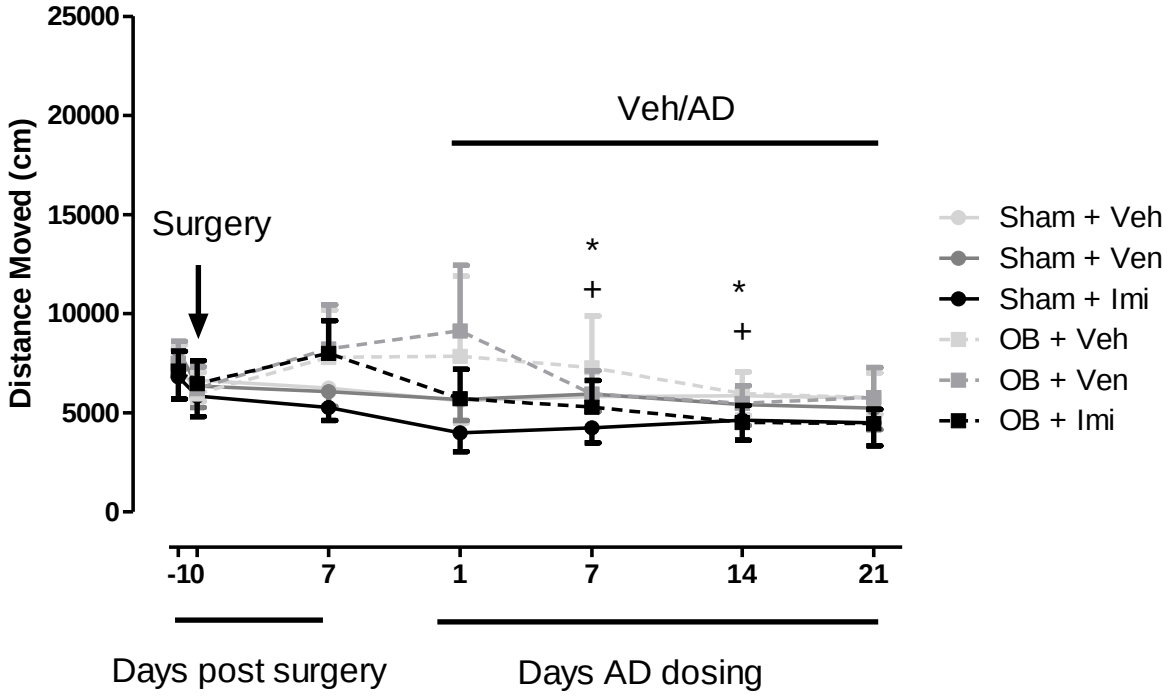


Figure 6.10 Effect of chronic venlafaxine or imipramine treatment on nocturnal HCA**.** Data are expressed as mean ± SD (n=11-12) *p<0.05 vs Sham + Veh; +p<0.05 vs OB + Veh

6.4 Discussion

The aims of this chapter were to assess OB hyperactivity in the open field following lesion and again after chronic antidepressant treatment. Given that OB rats have been shown to become habituated to the open field with repeated exposure, classification of other open field parameters were assessed to determine if they would be subject to habituation. Finally in order to assess locomotor activity in rats without exposure to a novel or stressful environment, thus eliminating the need for repeated exposure, nocturnal HCA activity was assessed.

As expected 2 weeks following lesion, upon initial exposure to the open field OB, rats exhibited the behavioural hallmark of hyperactivity (Roche *et al.,* 2007: Burke *et al.*, 2010) and as with previous studies OB rats also spend less time in the inner zone (Burke *et al.,* 2010), a phenomenon which is clearly visible when the rats activity path in the open field is recorded (Figure 6.2). This is an example of increased anxiety in OB rats. This hyperactivity is indicative that the syndrome has been established and as a result the rats can be assigned to their treatment groups. It is important that the hyperactivity is evident in all groups to ensure that potential antidepressant activity (attenuation of the hyperactivity) is detected. In nearly all groups, hyperactivity in the open field was obtained prior to antidepressant dosing, except for rats that were assigned to vehicle treatment in experiment 2. It is evident that the rats do exhibit hyperactivity and therefore it was decided to proceed with treatment. It could also be indicative of the degree of lesion that occurs, as although rats with incomplete bulb ablation or damage to the cortex are discounted from analysis upon post-mortem lesion confirmation, it could be that in certain individual cases the degree of ablation is not sufficient for the syndrome to fully develop in a particular rat.

Upon re-exposure to the open field the OB induced hyperactivity is no longer evident and this suggests the rats have become habituated to the arena (Gigliucci *et al.,* 2014; other work in the laboratory); this is in contrast to other studies (Cryan *et al.,* 1999; Burke *et al.,* 2010; Breuer *et al.,* 2009). This means it is not possible to say whether the ability of the antidepressants to attenuate OB hyperactivity is lost because the antidepressants have been ineffective or if it is due to the habituation. Thus, further analysis was carried out to assess other parameters that could be used to detect antidepressant activity. The duration the rats are exposed to the open field varies, the

most common being 5 min but some studies report exposure for 3 min, whilst others it is for 15 min. Therefore, the activity was split into 1 min time bins to assess if the changes occur at an earlier time point, before habituation. No difference was found between any groups at any time point measured. However, there does appear to be a trend that OB rats are hyperactive in the first minute, decreasing as time continues, whilst sham-lesion rats remain largely stable.

Gigliucci *et al.,* (2014) is one group who have devised a new parameter in the open field which is not subject to habituation and termed this 'speed of bout', which they defined as 'rapid bursts of locomotion'. This was trialled in the rats of experiment 1 in their second exposure to the open field. No significant lesion or drug effect was evident, however this may not be due to habituation or lack of antidepressant response. Instead it may have been due to the difficulty in standardising the behaviour and tracking it accurately using EthoVision. It was often the case that the rat would move in short bursts of activity, which lasted only a few seconds thus was not enough time for the tracking software to begin tracking. Another parameter that can be measured automatically in EthoVision, is the degree of movement and the type of movement exhibited. There is a trend that vehicle treated OB rats spent more time highly mobile compared to vehicle treated sham rats, but this did not reach significance.

Grooming, rearing and fecal boli are other parameters that have been assessed in the open field. This was scored in a cohort of rats from experiment 1 but again there was no significant change in the frequency of rearing or grooming, which is an agreement with previous literature (McGrath and Norman, 1998).

Given that habituation to the novel and stressful environment of the open field is evident in the OB rats tested in our laboratory, previous work has shown that HCA is superior in detecting the effects of stimulant and depressive drugs (Dunne *et al.,* 2007) and as such nocturnal HCA was assessed in OB rats. To our knowledge only one other study has assessed HCA in OB rats, which showed that hyperactivity was evident at 3 days following lesion and persisted until day 5 (Vinkers *et al.,* 2009). However, in rats assessed in activity monitors OB rats were also significantly hyperactive during the nocturnal cycle, an effect that was attenuated by imipramine (Giardina and Radek, 1991). Results from both cohorts show that OB rats are significantly hyperactive compared to sham-lesion rats at 7 and 13 days following lesion, thus giving credence

to the fact that hyperactivity may not be a maladaptive response to stress but may be an inherent characteristic of the model. This provided a paradigm that detected a clear behavioural difference between sham and OB rats, which was not subject to habituation. Given the design of the apparatus it was possible to continually monitor the rat's activity meaning in future work it would be possible to detect changes at any interval during the duration of the study.

One of the characteristics of this model that gives it a high degree of validity is lack of response to acute antidepressant treatment, with attenuation of behaviour only seen after chronic treatment (Harkin *et al.*, 1999; Roche *et al.*, 2007). The results of experiment 1 failed to detect an antidepressant effect through attenuation of hyperactivity, with the hyperactivity appearing to wane as no hyperactivity was evident between the groups after 14 days of dosing but reappears between certain groups at 21-days of dosing. In experiment 2, imipramine treatment appears to reduce the locomotor activity in sham and OB rats on day 7and day 14, which could be indicative of a modulatory effect of imipramine that is independent of a depressive phenotype. The effect of imipramine on locomotor activity in a familiar environment has also demonstrated attenuation of OB hyperactivity, although this was after 7 days of treatment (Gardina and Radek, 1991).

Like experiment 1, the hyperactivity appears to wane as at 21 days post-dosing there is no hyperactivity in any of the groups. Given that this is the first study to assess the effect of chronic antidepressant treatment on nocturnal HCA, it cannot be said how this compares to the literature however it does provide a promising alternative to the open field in the detection of antidepressant activity. Given that all three antidepressants have different modes of action, it is interesting that imipramine, a TCA, yielded detectable changes consistently for numerous time points, whilst the others did not. To this end it could be proposed that the detectability of antidepressant activity depends on the arena in which they are tested.

In conclusion, hyperactivity, measured by distance moved in the open field is a parameter that is subject to habituation. This increases the risk of false negative results with regards to antidepressant treatment. It is perhaps suggestive of a cognitive deficit in the animals, whereby perhaps, upon re-exposure to animals no longer recognise the arena as being aversive. Nocturnal HCA detects OB hyperactivity both at the same

time point as the first exposure but also at 7 days following lesion but as time progresses this hyperactivity wanes. Thus this potentially indicates that there is optimal time to detect antidepressant effects in this parameter; beginning treatment at an earlier time point, for example 7 days following lesion and treating for 14-21 days may help in the detection of an antidepressant response.

Chapter 7:

General Discussion

The work presented in this thesis aimed to investigate the central serotonergic system in the olfactory bulbectomized rat model of depression. The OB model is considered to be a well validated model, primarily due its response to chronic but not acute antidepressant treatment. Behavioural, physiological and neurochemical changes that occur in this model have been extensively reviewed (Song and Leonard, 2005; Kelly *et al.,* 1997). However, in assessment of the literature a requirement was found for an investigation into multiple aspects of serotonergic transmission to be carried out within the one study. In addition there is a paucity of data relating to the functionality of the two main receptors implicated in depression, the 5-HT_{1A} and the 5-HT_{2A} receptors. The aim of the work presented was to address these limitations.

Acute injections of agonists targeting 5-HT_{1A} and 5-HT_{2A} receptor elicited increased locomotor activity in the OB rats, which were largely unaffected by chronic antidepressant treatment. Alterations in locomotor activity was the predominant behavourial change, with general observations of reduced rearing, in line with (Hillegaart *et al.,* 1989 and Evenden and Angeby-Moller, 1990) with regards to 8-OH-DPAT. In addition there was a generalised reduction in flat body posture, hindlimb abduction and increase in head weaving in response to 8-OH-DAPT. In addtion there was also a general decrease in rearing and in wet dog shakes in response to DOI. This therefore suggests that receptors responsible for mediating the locomotor effects may have an increased level of functionality in this model, whilst those responsible for mediating the stereotyped behaviours may not be altered. One of main differences with regards to methodological approaches taken in this thesis and those in the literature relates to the arena in which the animals were tested. In many of the studies presented in the literature assessing the stereotyped behaviours the rats are placed in a novel arena (Dabrowska *et al.,* 2007; Samad *et al.,* 2007; Biezonski *et al.,* 2009; Bull *et al.,* 2004), however the rats in our studies were assessed in their homecage. In our opinion this represents a substantial methodological advantage, as the rats could be tracked for extended periods of time without the need to expose them to novel arenas. Additionally, the design of the apparatus involves recording the rat's behaviour to a central DVR, which allows for additional analysis, such as time course evaluation of the behaviours. As such the same rats could be used for the assessment of multiple parameters, such as locomotor as well as stereotyped responses. Also the tracking methodology enables a continuous locomotor response to be objectively measured,

which differs from others studies whereby activity is recorded at specific intervals (Carey *et al.*, 2004; Granoff and Ashby 2001; Samad *et al.*, 2007) or a more subjective approach via observer rating is employed (Sorensen *et al.*, 2001; Gaggi *et al.*, 1997).

Furthermore, assessing these parameters in the homecage reduces the number of times rats are exposed to novel experimental arenas. It also allows the rats to be used for additional behavioural tests, whereas in other scenarios it may have been necessary to include an additional cohort of rats, thus increasing the numbers used. This supports the "Three Rs" – replacement, reduction and refinement, which is a set of principles that had been developed to guide the ethical evaluation of animal use (Fenwick *et al.*, 2009). As such, the methodologies employed for the assessment of receptor functionality will contribute to this philosophy by reducing the number of rats that are needed, not only to assess one parameter, but in the experimental design as a whole.

With regards to a comprehensive evaluation of multiple components of the serotonergic system, this was addressed via an investigation of a time course of the changes, as well as modulation of chronic antidepressant treatment. No significant alteration in any of the parameters assessed was detected in OB rats (when compared to sham-lesion counterparts) over time. In addition, antidepressant alterations were primarily mediated by fluoxetine, with limited effects by venlafaxine. The absence of a robust lesion effect suggests that under the conditions in our laboratory there is no change in the serotonergic circuitry, whilst the limited antidepressant response found may be due to the duration of treatment, as 17 and 14 days of imipramine treatment has been shown to attenuate the increased TPH levels in the frontal cortex (Grecksch *et al.*, 1997; Huether *et al.*, 1997)

An explanation for the lack of alteration of serotonergic components could arise from the pattern of innervation from the DRN to the olfactory bulbs. The DRN is the primary source of serotonergic innervation in the olfactory bulbs (Michelsen *et al.*, 2008), with viral mediated investigation in mice demonstrating that the DRN innervates the granule cell layer whilst the MRN the source of fibres in the glomerular layer (Steinfeld *et al.*, 2015). Analysis of the number of neurons or the magnitude of raphe input into the main olfactory bulb via retrograde analysis suggested that as many as 1300 raphe neurons project to the main olfactory bulb (McLean and Shipley, 1987). Of those, it is estimated that 600-1000 neurons originate in the DRN, with the majority

of these having detectable 5-HT content (McLean and Shipley, 1987). The DRN contains approx. 34,500 neurons, with specific serotonergic cell bodies accounting for 11,500 of the neurons (Descarries *et al.,* 1982). Therefore, given that removal of olfactory bulbs in the rat is known to cause retrograde degeneration (Kelly *et al.,* 1997) it could be cautiously postulated that given the limited number of serotonergic neurons which innervate the olfactory bulbs that OB induced changes are specific. In other words, although there was no alteration in the levels of SERT expression in the DRN or in terminal regions for example, it could be that the functionality is altered or the cellular location has changed.

As well as assessing changes in the central serotonergic system, behavioural parameters in the OB model were also assessed. The main finding from Chapter 6 highlighted the occurrence of habituation upon re-exposure to the open field resulting in the loss of OB exhibited hyperactivity. This resulted in the inability to detect antidepressant attenuation of the hyperactivity. Given that the hyperactivity was no longer present it cannot be said if this was as a consequence of inadequate antidepressant efficacy or was due to the disappearance of the hyperactivity. However, the doses used in this study were based on previous literature demonstrating OB induced hyperactivity in the open field being attenuated by fluoxetine (Jindal *et al.,* 2012; Roche *et al.,* 2007) and imipramine (Roche *et al.,* 2008; Breuer *et al.,* 2009). The dose of venlafaxine used in this study was chosen based on that used in the OB model (Uzunova *et al.,* 2004) as well as in other rodent studies as there is limited data on the use of venlafaxine in the OB model. Pandey *et al.,* (2008) and McGrath and Norman (1998) assessed the efficacy of venlafaxine at 10 mg/kg, as such a 20 mg/kg dose may have been too high. In addition, none of the other open field parameters assessed showed a significant effect. Given the open field results in Chapter 3 the disappearance of the hyperactivity is not due to the length of time the syndrome was established as OB rats were significantly hyperactive at all timepoints assessed. When compared to open field exposure on a single occasion.

Although the main focus of this thesis was the serotonergic system, other neurotransmitters may also play in the hyperactivity exhibited by this model. OB mice hyperactive in the open field, demonstrate significant reductions in noradrenaline in the frontal cortex in samples taken after open field exposure (Roche *et al.,* 2012). OB rat hyperactivity in the open field approx. a week prior to sample collection also

demonstrate a significant decrease in noradrenaline in the frontal cortex, with no significant alterations in dopamine or DOPAC levels (Redmond *et al.*, 1999). The role of dopamine in OB locomotor activity has been assessed via acute amphetamine treatment (Eisenstein *et al.*, 2009). When assessed in a locomotor activity monitor chamber, OB rats have heightened locomotor activity response to acute amphetamine (Eisenstein *et al.*, 2009). In contrast to sham rats, further sensitisation to amphetamine following repeated amphetamine administration was absent in OB rats, suggesting that OB rats may to 'presensitised' to indirect dopaminergic agonists (Eisenstein *et al.*, 2009). This could therefore suggest that altered dopaminergic signalling could also play a role in OB hyperactivity. Assessment of cholinergic parameters (2 weeks after open field assessment) in female OB rats, reveal that the hyperactivity is not accompanied by a considerable reduction in the number of choline acetyltransferase positive neurons, as well as no substantial difference in cholinergic indices in the septum, hippocampus and neocortex (Stepanichev *et al.*, 2016).

As already mentioned, ketamine has been shown to have rapid antidepressant effects. Modulation of the glutamatergic system is believed to be an important in the fast onset of antidepressant effects (reviewed in Du et al., 2006). During the first 10 min of open field exposure, hyperactivity and therefore the novelty of the area results in a significant increase in striatal glutamate release, with no effect found in shams (Ho *et al.*, 2000). This could therefore suggest that the striatal glutamate release in response to novelty exposure could cause behavioural hyperactivity (Ho *et al.*, 2000). Additionally, dizocilpine resulted in a hyperactive response in the homecage, which was enhanced in OB rats (Redmond *et al.*, 1997). Suggesting readaptation in NMDA receptor complex functioning in OB rats following chronic treatment with a NMDA receptor antagonist (Redmond *et al.*, 1997). This possible altered receptor complex functioning may not be due to structural alterations in the receptors as NMDA and AMPA receptor subunit levels does not differ between sham and OB rats in the prefrontal cortex, hippocampus and amygdala (Pochwat *et al.*, 2015). Therefore, the authors concluded that there is a lack of association between the levels of glutamate receptors and the depressive phenotype of OB, acknowledging that this is dependent on the regions assessed (Pochwat *et al.*, 2015).

Nocturnal homecage activity, an additional parameter that was assessed, could be monitored on a continuous basis and was easily incorporated into the study design, as animals remained in their homecage for the duration of the study and thus were not subject to repeated exposure to novel arenas. Although hyperactivity in OB rats was detected consistently in the 14 day period following lesion, during antidepressant treatment the hyperactivity waned. This suggests that as time progresses, the behaviour may not be as robust as during the initial stages of syndrome establishment. Hence it remains to be further investigated as to whether there is a behavioural paradigm that can be assessed, with easy incorporation into a study design, which will be continuously and reliably detected in the OB rat. Although others have demonstrated nocturnal hyperactivity in OB rats (Vinkers *et al.,* 2009) the novel design of this parameter allows the behaviour to be monitored for the duration of a study, rather than a selected period of time.

In addition to the results found, numerous parameters of the serotonergic system were assessed, with many of the same parameters being measured in multiple different studies. This provided the opportunity to examine the consistency of the parameters from one study to the next. In assessing the results of Chapter 3 and 5, it was important to determine how the baseline values between studies compared both to each other and to the literature. Given that the same serotonergic components were, for the most part, assessed in both chapters gives valuable insight into changes that may exist between different cohorts of rats. Assessing those parameters, that have not been normalised relative to a control group, TPH, 5-HT, 5-HIAA, 5-HIAA/5-HT and SERT it can be seen that the baseline values of the sham-lesion rats do not change substantially between cohorts of rats.

Assessing the number of TPH cells in the DRN in sham-lesion rats across all studies demonstrates the consistency across studies, with average number of all three cohorts being 339, with all other values being within the standard range of 2 standard deviations. In the terminal regions, within the antidepressant studies the values are approximately similar but they are slightly higher compared to those assessed in Chapter 3. This is more than likely due to methodological differences, whereby a higher concentration of antibody was used in order to obtain more robust staining pattern. This, together with the values being in line with the literature values of 250-

400 (Shin *et al.,* 2017; Roh *et al.,* 2016; Kim *et al.,* 2015; Saitoh *et al.,* 2008) demonstrates consistency in the methodologies used and in the assessment methods.

With regards to 5-HT, 5-HIAA and 5-HIAA/5-HT the average for each measurement in sham-lesion rats across all studies are as follows 374 ng/g, 2600 ng/g and 7 respectively, with all other values falling within the accepted range of 2 standard deviations. Thus demonstrating that the consistency across all three studies. Comparison the literature reveals that the results found in this thesis are lower for 5-HT and higher for 5-HIAA. While samples were measured in a different laboratory, the system used is the same as has been used previously in our laboratory. Comparing the values obtained in this thesis with Roche *et al.,* (2012) (5-HT – 1021 ng/g and 5-HIAA – 1040ng/g), Burke *et al.,* (2010) (5-HT – 659 ng/g; 5-HIAA – 238 ng/g and 5-HIAA/5-HT – 0.36) and Harkin *et al.,* (1999) (5-HIAA – 430 ng/g) reveal that the results obtained for 5-HT are lower, 5-HIAA and turnover are higher. In addition in surgery naïve Sprague-Dawley males, where although the values are expressed at lower tissue concentrations the values obtained remain substantially higher (Scholl *et al.,* 2010). This demonstrates that although there is a degree of variation between the results presented in this thesis and the literature, those in the literature are themselves inconsistent. Differing time following lesion as well as how the tissue was obtained either from gross dissection (Burke *et al.,* 2010; Roche *et al.,* 2012) or via tissue punching (Scholl *et al.,* 2010) could also account for the discrepancies. As serotonin is susceptible to degradation, the length of time after sacrifice the tissue is collected, as well as if the tissue is frozen and then thawed for dissection could also have an impact on the results obtained.

SERT demonstrated slightly more variability between studies in this thesis. Expression in the PFC remained largely similar (5.49 vs 6.55 OD% area), however the values in the increased between the two cohorts of animals. The reason for this discrepancy could again be methodological, whereby the primary antibody may have been left to incubate in the sections for a slightly longer period of time, which resulted in a higher intensity of staining. In comparing the values obtained with those in the literature, many of the papers assessing SERT staining do so with respect to tissue where an internal control is present (Brown and Gerfen, 2006), therefore the results are expressed as a percentage of the control side and as such direct comparison is not possible. However, analysis carried out in wistar rats (Casu *et al.,* 2004) would indicate

the values are obtained in the hippocampus samples in this thesis are lower. The discrepancy between the values obtained in this thesis and those in the literature could be due to differences in the strain of rat and antibody used.

7.1 Conclusion

The work undertaken in this thesis was carried out with the aim of investigating the central serotonergic circuitry in the OB rat, including assessment behavioural alterations reflective of receptor functionality. Although there were limited alterations found in both the central serotonergic system and antidepressant response, this work has contributed to our knowledge regarding time course of changes in the OB rat, behavioural and serotonergic. Furthermore, the novel approach taken to assess receptor functionality in the homecage not only addressed a gap in the literature regarding this model but also provides an important additional dimension to the behaviours that can be assessed. Although limited OB-induced serotonergic changes and antidepressant responses were found, the ability to assess the consistency of the parameters across all studies has been very useful. The addition of nocturnal HCA and evidence of hyperactivity earlier than 2 weeks following lesion, suggests that the window of antidepressant treatment may be at an earlier time point than previously thought.

7.2 Future work

This undertaking of analysing behavioural changes as well as multiple aspects of central serotonergic circuitry proved to be extensive and challenging. However, it remains a valuable study design and as such, it is thought that the following recommendations for future work would be valuable additions.

1. Further characterisation of open field and nocturnal homecage activity to identify a parameter that is robust and is responsive to antidepressant treatment. It is suggested that the automated behaviours such as time spent moving or not moving, which are automatically calculated by EthoVision® be focused on initially.
2. Further assessment of 5-HT, 5-HIAA and 5-HIAA/5-HT in additional regions also be evaluated, as only the levels in the amygdala were measured in this

thesis. Microdialysis investigations would also complement these investigations.

3. Serotonergic receptor mRNA levels, 5-HT_{1A} and 5-HT_{2A}, were measured in this thesis, however protein expression via western blot should also be assessed.
4. It was not possible to assess the firing rates of the serotonergic system within this body of work, therefore future work should include an assessment of serotonergic neuronal activity via electrophysiological experiments.

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