STUDIES OF EMOTIONALITY IN GENETIC MOUSE MODELS OF ALTERED GLUTAMATE OR 5-HT FUNCTION

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Abstract

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Studies of emotionality in genetic mouse models of

altered glutamate or 5-HT function

The role of 5-HT in depression and its treatment is well established but there is also growing evidence for the role of glutamate in emotionality. Several transgenic mouse lines were used to investigate how alterations in the glutamate or 5-HT systems impact upon emotional behaviour and relevant neuronal systems.

Transgenic mice with direct manipulations of the glutamate system did not provide a selective model with which to further investigate changes in emotionality. GluA1 knockout mice showed only transient effects on antidepressant-sensitive tests which may alternatively be explained by deficits in habituation. GluN1 hypomorph mice displayed altered behaviours in all tests performed, including global cognitive impairments, making further interpretation of the results difficult. Vesicular glutamate transporter 1 heterozygote mice, which have been implicated as a model depression, were found to have only minor alterations in behaviour, none relating to emotionality.

In contrast, the 5-HT transporter overexpressor mice have previously been shown to reliably display reduced anxiety and a fear conditioning deficit in line with results in humans with differing expression levels of the 5-HT transporter. Constant potential amperometry was used to measure local tissue oxygen content as an equivalent to the BOLD response in fMRI, and local field potential recordings of population electrical activity were also recorded in the basolateral amygdala of 5-HT transporter overexpressor and wildtype mice during a differential fear conditioning paradigm. This revealed that 5-HT transporter overexpressor mice have reduced basolateral amygdala oxygen responses to a conditioned stimulus associated with footshock, correlating with fMRI responses to fearful faces, and a reduced theta:delta ratio in this brain area that is most prominent following conditioning, implying a less active amygdala in these mice under these conditions.

In summary, gross manipulations of glutamate function may not be useful in investigating the role of this neurotransmitter system in emotionality. Manipulation of the expression levels of the 5-HT transporter that mimics the variation seen across the human population alters neurochemical responses to conditioned fear stimuli, providing a translational model for future investigations into emotional processing.

Foreword

Predictions can be very difficult, especially about the future.

-Niels Bohr

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The work presented in this thesis would not have been possible without the support and input of my two academic supervisors, Drs David Bannerman and Trevor Sharp, who have been a large factor in making my time as a D.Phil student both challenging and enjoyable. I'm also very grateful they gave me this chance in the first place. Their equivalents at GSK, Drs Lee Dawson and Marie Woolley, also played a part in guiding this project and securing the GluN1 hypomorph mice used in Chapter 3. I am also very grateful to GSK and the BBSRC for the financial input that supported this project. I would also like to thank Dr Rosa Tordera for supplying the vesicular glutamate transporter 1 heterozygote mice used in Chapter 4.

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Long abstract

This thesis investigated the role of two neurotransmitter systems in emotionality, glutamate and 5-HT, with the overall aim of further our understanding of the role of these systems in conditions such as depression. The role of glutamate in depression and antidepressant treatment is of growing interest, but not fully understood. The role of 5-HT in mood is more established, although the mechanisms by which alterations in the 5-HT system lead to changes in emotionality still warrants further investigation. Genetically modified mice were used to investigate the consequences of altered glutamate- or 5-HT-mediated neurotransmission on emotionality. These mice were tested in a number of emotion-related behavioural paradigms.

Chapter 1 introduces the clinical definition of the major depressive disorders and the current treatments for these conditions. The way in which the variety of symptoms that constitute a depressive episode can be modelled in rodents is then presented. The validity of these models in recapitulating the symptoms seen in humans is vital to our ability to investigate this disease preclinically. The roles of glutamate and 5-HT in depression and antidepressant treatments are also broadly covered and finally the aims of the thesis are given.

Chapters 2-4 detail the behavioural phenotyping of three lines of mutant mice with targeted alterations to the glutamate system. Chapter 2 presents data from GluA1 knockout (KO) mice. These mice lack one of the possible subunits of AMPA-class glutamate receptors. The possible role of the GluA1 protein in emotionality is discussed before results from the GluA1 KO mice in several tests of emotionality are presented. These mice and their wildtype (WT) littermates were tested on the

elevated plus maze (EPM) and exposed to several versions of a social interaction task to assess their levels of anxiety. No overall change was seen in the performance of the GluA1 KO mice in the EPM. GluA1 KO mice also showed a reduction in social and aggressive encounters only when tested in a novel environment. This novel context-dependent social deficit may reflect another facet of the altered habitation-processing reported previously in the GluA1 KO mice. Several tests were also used to investigate mood in the GluA1 KO mice; sucrose preference, a measure of anhedonia, locomotor activity across the diurnal cycle to investigate alteration in circadian rhythm, and the forced swim test (FST), an assessment of behavioural despair. The GluA1 KO mice showed no change in sucrose preference and initial hyperactivity in the test of locomotion but no alteration in their diurnal cycle. In the FST, the GluA1 KO mice showed decreased immobility, indicative of an anti-depressive phenotype, on the first day of testing. However, on re-exposure to the test, GluA1 KO mice did not show this reduced immobility. This again implies the phenotype seen in this mouse is dependent on novelty. How these data fit with the hypothesis of altered habituation processing in the GluA1 KO mice is also discussed.

Chapter 3 deals with the GluN1 hypomorph (HYPO) mouse line in tests of emotionality as well as cognition and naturalistic behaviours. The GluN1 HYPO mice express 5-10% of WT levels of the constitutive subunit of NMDA-class glutamate receptors, GluN1. This results in the GluN1 HYPO mice having only 5-10% normal levels of functional NMDA receptors. The role of NMDA receptor-mediated neurotransmission in emotionality, specifically the possible clinical application of NMDA receptor antagonists as novel antidepressants, is presented in this chapter. In line with this evidence for the antidepressant effects of NMDA receptor antagonists, the GluN1 HYPO mice displayed a robust anti-depressive-like phenotype in the FST

and sucrose preference tests. However, the results obtained from tests in other domains of behaviour suggest the GluN1 HYPO mice have wide-ranging alterations and global cognitive impairments. The implications of these findings on previous work using these mice are then discussed.

Chapter 4 presents the behavioural phenotyping of the vesicular glutamate transporter (VGluT) 1 heterozygote (HET) mice in tests of emotionality, cognition and naturalistic behaviour. These mice have previously been presented as a model of depression. However, the work presented here does not support this. Possible reasons for this failure to replicate previous findings are then discussed, the most prominent hypothesis being that these mice are of a subtly different genetic background to those used in previously published studies.

Chapter 5 describes a study performed combining the 5-HT transporter (5-HTT) overexpressor (OE) mice and techniques for the measurement of neuronal activity. These measures were constant potential amperometry (CPA) as a method for measuring local tissue oxygen, and local field potential (LFP) assessments of local neuronal electrical activity. The use of CPA as a method for measuring tissue oxygen *in vivo* has previously been validated and shown to correlate highly with the fMRI BOLD signal. However, CPA can be used in freely-moving and behaving rodents. 5-HTT OE mice express approximately three times normal levels of the 5-HTT protein, the key regulator of extracellular 5-HT. This mimics the variation in expression levels seen across the human population. Alterations in the expression levels of the 5-HTT protein have also been shown to correlate with several measures of emotionality, including amygdala reactivity to negative emotional cues. This is further discussed in the chapter before presenting the method by which these findings from human

neuroimaging studies were recapitulated in the 5-HTT OE mice. Amygdala responses were elicited in these mice through use of a differential fear conditioning paradigm. While no behavioural differences were seen between the 5-HTT OE mice and their WT littermates, alterations to the two measures of neuronal activity were seen. This mimics the results seen in a human neuroimaging studies that demonstrate a relationship between the 5-HTT and amygdala reactivity to fearful faces with no alterations in behavioural responding. This altered reactivity may be a direct consequence of alterations to the 5-HT input to the amygdala or modulation of amygdala activity by another structure such as the frontal cortex. The implications of this study, as well as the multitude of future experiments that present themselves using CPA as a measure of regional brain oxygen, are then discussed.

Chapter 6 summarises all of the findings of this thesis and further discusses the validity of the methods employed. This includes the tests of emotionality used here and their validity as models of human depression, the mouse lines used, and the use of CPA as a method of indirectly assessing neuronal activity. What these findings reveal about the role of glutamate and 5-HT in emotionality and its implications are then discussed before a final summary is given.

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Papers and abstracts arising from this thesis

Papers:

P.J. Fitzgerald, C. Barkus, M. Feyder, L.M. Wiedholz, Y.C. Chen, R.M. Karlsson, R. Machado-Vieira, C. Graybeal, T. Sharp, C. Zarate, J. Harvey-White, J. Du, R. Sprengel, P. Gass, D.M. Bannerman, A. Holmes (2010) Does gene deletion of AMPA GluA1 phenocopy features of schizoaffective disorder? Neurobiology of Disease

The review "Hippocampal NMDA receptors and anxiety: at the interface between cognition and emotion." (Eur J Pharmacol 626:49-56) also contains some material that appears in Chapter 3.

Abstracts:

EMBL Workshop on Translating Behaviour, 2009: C. Barkus, L.A. Dawson, T. Sharp, D.M. Bannerman. A mouse model of NMDA hypofunction in tests of cognition and emotionality.

Neuroscience, 2008: C. Barkus, R. Sprengel, P.H. Seeburg, L.A. Dawson, J.N.P. Rawlins, T. Sharp, D.M. Bannerman. Investigating the role of GluRA AMPA receptor subunits in emotionality.

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List of frequently used abbreviations

5-HT	5-hydroxytryptamine (serotonin)
5-HTT	5-hydroxytryptamine transporter
5-HTTLPR	5- hydroxytryptamine transporter gene linked polymorphic region
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
ANOVA	analysis of variance
BLA	basolateral complex of the amygdala
BOLD	blood-oxygen level dependent
CeN	central nucleus of the amygdala
CPA	constant potential amperometry
CPE	carbon paste electrode
CS	conditioned stimulus
DSM	diagnostic statistical manual of mental disorders
EEG	electroencephalogram
EPM	elevated plus maze
fMRI	function magnetic resonance imaging
FST	forced swim test
HET	heterozygote
HYPO	hypomorph
i.p.	intraperitoneal
ITI	inter-trial interval
KO	knockout
LA	lateral nucleus of the amygdala
LFP	local field potential
LTP	long-term potentiation

MRS	magnetic resonance spectroscopy
NMDA	N-methyl-D-aspartic acid
OE	overexpressor
PET	positron emission tomography
SEM	standard error of the mean
SSRI	selective serotonin reuptake inhibitor
s. c.	subcutaneous
VGluT	vesicular glutamate transporter
WT	wildtype

CHAPTER 1: General Introduction

Our understanding of psychiatric disease remains, at best, poor. This has led to both social stigma and a very limited choice of treatments for highly debilitating disorders. A lack of successful treatment makes these disorders life-long conditions for a great number of patients, exacerbating the degree to which psychiatric illness reduces the quality of life for a large number of people. Major depressive disorders are estimated to be the leading cause of disability in the Western World and third most disabling globally (The Global Burden of Disease: 2004 update, 2008). This statistic is, in part, due to the prevalence of this condition, with a lifetime risk of approximately 15% (DSM IV-TR, 2000, Kessler *et al.*, 2003, Hasin *et al.*, 2005), as well the limited efficacy of existing treatments. Depression therefore compromises the everyday lives of a huge number of people.

This thesis aims to better understand the neurobiology of emotionality in the hope of providing novel targets for therapy and furthering our knowledge about the role of human genetic variation as a risk factor for depression. The term "emotionality" is used in this thesis as an overarching principle that encompasses the measurable outcome to stimuli or contexts intended to evoke an emotional response. This term is therefore used to cover the behavioural and physiological components of mood, anxiety and fear equally. As such, this is a broad term that is limited in its specificity but is intended as an umbrella term for the differing forms of emotional responses discussed and investigated. The work presented is contextualised in terms of mood disorders, as these are the major set of pathological conditions associated with altered emotionality in humans. However, components of this work could equally be applied to other conditions such as anxiety disorders.

The work presented here investigates the role of two neurotransmitter systems, glutamate and 5-HT, in emotionality by assessing depression-related behaviours in mutant mouse models. The tests used encompass several domains of behaviour in the hope of providing a more complete phenotypic assessment of these mice than has been sought previously. The effect that variation in the level of expression of the 5-HT transporter (5-HTT) protein has on emotional processing is also assessed. This is done through measures of neuronal activity in the amygdala, an area of the brain associated with emotionality, in response to an emotionally-salient cue. This takes advantage of constant potential amperometry as a method of assessing tissue oxygen levels. This is a relatively novel method for assessing neuronal activity and is potentially a highly translatable tool.

Treatments for depression were discovered serendipitously when an early anti-tuberculosis drug was noted to improve the mood of melancholic patients (Crane, 1956, Kline, 1958). Subsequent research and development of antidepressants led to the isolation of monoamines as having a prominent role in the treatment of depression (Zeller *et al.*, 1955, Zeller and Sarkar, 1962). Eventually, selective serotonin reuptake inhibitors (SSRIs) were developed and continue to be the first line pharmacological treatment for depression. The role of 5-HT in the treatment of depression has resulted in it also being considered important in the pathophysiology of the disease. However, it is becoming increasingly apparent that other neurotransmitter systems also have a role to play in depression. Amongst these is glutamate, the major excitatory neurotransmitter in the central nervous system.

An important form of investigation into clinical disease is producing a model of the condition in experimental animals. Such models allow for more in-depth analysis of the underpinnings of the disease and can also provide a platform in which to test novel therapies not yet suitable for human trials. Using experimental animals allows for investigation of the consequences of individual interventions to be made in isolation from other factors. This is due to the genetic and environmental homology of experimental rodents as compared to the huge variance in these factors seen across the human population. Clinical depression involves a variety of symptoms, not just the lowered mood that is commonly thought to be the only characteristic of the disease. A good preclinical model should encompass as many of the alterations seen in humans as possible. This thesis therefore employs a variety of behavioural tests thought to be analogous to aspects of clinical depression.

This introduction will first present the psychiatric definition of depression and the approaches taken to model depression in rodents will be put forward. The neurobiology of depression and current treatments will also be covered in the context of the possible roles played by glutamate and 5-HT, and the overall aims of this thesis given.

1.1. Depression

The clinical diagnosis of psychiatric disease is carried out using a diagnostic manual, commonly either the Diagnostic and Statistical Manual (DSM), produced by the American Psychiatric Association (DSM IV-TR, 2000), or the appropriate section of the International Classification of Diseases and Related Health Problems (ICD), published by the World Health Organisation (ICD-10, 1992). These two guideline

systems have developed in parallel and so are largely similar, although minor differences remain in their definitions of some disorders[1]. Given the prominence and relative specificity of the DSM, it is the criterion from this publication that will be focussed on here. The DSM is currently in its fourth edition, although proposed changes to be incorporated into the new edition have been released and so will also be discussed.

1.1.1. Major depressive disorders

The diagnosis of major depression, as defined by the DSM, is currently split into two related conditions; major depressive disorder, single episode and major depressive disorder, recurrent (DSM IV-TR, 2000). Both of these conditions first rely on the absence of manic or manic-like symptoms from the patient's history as well as their symptoms not being better described by disorders such as schizophrenia or a related psychotic disorder. The main defining feature of major depression is the presence of major depressive episodes, either in the singular for major depressive disorder, single episode or the plural for major depressive disorder, recurrent. Further qualifiers relating to severity or if the patient fits one of the sub-categories of depression, with catatonic features, with melancholic features or with atypical features, can also be given.

[1] Depression is treated similarly in both although the way in which specifiers and severity are considered does differ.

1.1.2. A major depressive episode

A major depressive episode is defined as a period of two weeks or more during which at least five of the following symptoms of depression are present, leading to a reduction in functionality of the patient. At least one of these five symptoms must be either depressed mood or anhedonia, and be present most of the time. In the case of depressed mood, this is what is commonly and over-simplistically thought to be depression. Anhedonia is characterised as a reduced interest or enjoyment in daily activities. This often encompasses a reduction in social and sexual activity, as well as other pursuits. There are a further seven possible features of a major depressive episode; i) noticeable changes in weight and/or appetite, most commonly a reduction in both, ii) alterations in sleep, most commonly insomnia, iii) fatigue[2], iv) psychomotor changes, either agitation or retardation, that are noticeable to others, v) feelings of guilt or worthlessness[3], vi) reduced concentration and vii) suicidal ideation or recurrent thoughts of death[4]. These symptoms must not be accounted for by substance abuse and must not feature alongside manic symptoms (which may indicate bipolar disorder or a related condition). The current criterion also expects these symptoms to not be accounted for by bereavement, although this exclusion may be removed in DSM V (see http://www.dsm5.org/ProposedRevisions/Pages/proposedrevision.aspx?rid=427#) as depression resulting from such events has not been found to differ clinically from other forms of depression (Zisook and Kendler, 2007).

[2] "Decreased energy" is put on a par with depressed mood and anhedonia in the ICD criteria, with at least two out of three needing to be present for a depressive episode (ICD-10, 1992).
[3] The ICD further separates this into two possible symptoms; loss of self-esteem and inappropriate guilt (ICD-10, 1992).
[4] Suicide completion occurs in about 15% of depressed patients (Coryell *et al.*, 1982)

The presence of five of these nine symptoms, therefore, is the backbone of any diagnosis of major depression, whilst further distinctions deal with the time course or selection of symptoms present. When discussing the treatment and modelling of depression, it is these phenotypes that should concern us.

1.1.3. Additional symptoms and associated disorders

Other symptoms have also been found to be common in depression that are difficult to assess reliably in the clinical setting but nonetheless may enrich our understanding of the disease. Cognitive deficits associated with reduced concentration, for instance, have been investigated but are time consuming to assess fully in the context of a diagnostic interview (Porter *et al.*, 2003, Airaksinen *et al.*, 2004). Behaviourally, patients often report physical pain and/or excessive worry over their health. They may also report phobias and appear very anxious. Anxiety disorders comprise a diagnostic family of their own, so this does not make up part of the criteria for depression[5], although the two are often co-morbid (DSM IV-TR, 2000, Zimmerman *et al.*, 2000). Other common co-morbid conditions include alcohol or other substance abuse and panic disorders (DSM IV-TR, 2000).

Up to a quarter of patients with major depression will initially present with dysthymic disorder. This disease is currently defined as a period of at least two years during which the patient exhibits depressed mood most of the time as well as two of the following; i) elevated or reduced appetite, ii) elevated or reduced levels of sleep, iii) low levels of energy, iv) low self-esteem, v) poor concentration and vi) feelings of worthlessness. Periods of symptomatic remission must not be longer than 2 months

[5] The inclusion of heightened anxiety in the definition of depression is a common misconception, possibly due to psychomotor agitation being conflated with anxiety.

during this time and, importantly, the patient must not have experienced a full major depressive episode during the first two years of this condition. Dysthymia is therefore akin to major depression but involves prolonged periods of reduced function due to relatively few of the symptoms required for a major depressive episode. This final exclusion criterion, that those suffering from dysthymic disorder must have never reached the full quotient of symptoms for a major depressive episode, is currently being considered for revision, however. Some involved in the revision of the DSM have questioned the likelihood of those having suffered from this condition for two years being able to accurately report whether, at any time for a period of two weeks, they have met the criteria for a major depressive episode (http://www.dsm5.org/ProposedRevisions/Pages/proposedrevision.aspx?rid=46#) . Additionally, this board report that the clinical population found to suffer from dysthymic disorder and those with chronic major depression (in which depressive symptoms have persisted without remission for two years or more) do not differ (McCullough *et al.*, 2000, Yang and Dunner, 2001, McCullough *et al.*, 2003). How chronic depression and the progression from dysthymia to major depression are considered may therefore soon be altered with the creation of a separate disorder, chronic depressive disorder, based largely on dysthymia with this divisive contraindicator removed.

In the laboratory, the most common finding associated with a depressive episode is alterations to sleep architecture (Kupfer, 1976, Steiger and Kimura, 2010). This involves an increase in REM sleep and a reduction in the latency to the first bout of REM with an associated reduction in slow-wave sleep. This is found in approximately half of outpatient individuals and up to 90% of inpatients, and may persist beyond the depressive episode proper (DSM IV-TR, 2000). This finding is not

unique to major depressive disorders, however, and is also seen during depressive episodes seen as part of bipolar disorders. Limiting REM sleep selectively in depressed patients has been found to be an effective, if impractical, antidepressant intervention (e.g. Vogel, 1975, Vogel *et al.*, 1975, Vogel, 1983). This therapy suggests that these changes in sleep architecture are more than just curious observations. Interestingly, alterations to other body cycles such as cortisol release and temperature also appear to be disrupted in individuals with depression.

1.1.4. Risk factors and the demographics of depression

The greatest risk factor for experiencing a depressive episode is having had a prior episode. About half of patients who experience one episode will go on to experience a second (Thase, 1990, DSM IV-TR, 2000). The subsequent risk of a third episode is then 70%, and those who experience this third episode have a 90% risk of experiencing a fourth (NIMH Consensus Development Conference statement, 1985, DSM IV-TR, 2000). This increasing likelihood of relapse may indicate some form of ”kindling” resulting in damage to the brain brought about by a depressive episode, or progressive neurodegeneration that continues throughout life. Evidence for this is difficult to assess in humans, however, as alterations seen *post mortem* or using neuroimaging techniques in patients may be due to disease, treatment or other confounds.

Clinically, it has been noted that life stressors are a strong precipitating factor for the first, but not subsequent, bouts of depression (DSM IV-TR, 2000). Neurobiological vulnerability may therefore become more important in these latter episodes (Post, 1992). There is also evidence that some individuals are genetically predisposed

towards developing depression before their first depressive episode, termed "prekindling" (Kendler *et al.*, 2001). These individuals were defined by being closely related to other individuals that had developed depression and compared to those with no family history of the disease. In familial patients, stressful life events were seen to be a poorer predictor for onset of depression than for those not predisposed. In non-predisposed subjects, stress is a good predictor in the early stages but less and less so with each relapse. It is worth noting, however, that Kendler and colleagues show stress as a precipitating factor is still of reasonable predictive value, even in those in the highest familial risk group for depression (2001). This is lower than its predictive value in the less at-risk groups, however. That stress becomes less important with each relapse may support the notion of an incremental shift in neurobiology, possibly as kindling events during depressive episodes. That the importance of stress as a precipitating factor is mediated by family history may also show these life experiences interact with a baseline level of genetic predisposition towards depression.

While the genetic factors involved in depression are not fully understood, there is a clear familial link, with first-degree relatives being up to three times more likely to develop major depression than those in the general population. Gene*environment interactions must also be considered in this case, however, as early-life stressors such as neglect and childhood abuse are likely risk factors for depression (Harkness and Monroe, 2002). Adoption studies do not give a clear delineation between the influence of genetic and environmental factors at present (Cadoret *et al.*, 1985, Wender *et al.*, 1986, Kendler *et al.*, 1992). Depression is twice as common amongst women as in men, although this is not true in pre-pubertal populations. This implies

sex hormones may have a considerable impact on the disease, post-natal depression offering further support for this.

Depression is most commonly found in 25-45 year olds, with an average age of onset in mid-20s, and a significant decline in rates of depression in those over 65 (Weissman *et al.*, 1988, DSM IV-TR, 2000). The mean age of onset may be decreasing in more recent generations, with depression becoming more common generally (Klerman and Weissman, 1989, Joyce *et al.*, 1990, DSM IV-TR, 2000). This observation may, of course, be confounded by other factors such as the stigma of mental illness declining somewhat, therefore encouraging more people to seek help when it is needed. The estimated lifetime prevalence of depression is between 5-12% for men and 10-25% for women, the overall prevalence being about 15% (DSM IV-TR, 2000, Kessler *et al.*, 2003, Hasin *et al.*, 2005). Depression is found to be even more common in those with other chronic health problems. No difference in incidence is reported in the DSM across ethnicities, levels of education or levels of income (DSM IV-TR, 2000), although some studies do find members of low socioeconomic groups to be more at risk of depression (Weissman *et al.*, 1988, Blazer *et al.*, 1994). Nonetheless, depression is clearly a ubiquitous disorder that is common across a wide range of ages and population groups. That it may also be becoming more common adds to the reasons for seeking a better understanding of this disease.

1.1.5. Current treatments

If untreated, depressive episodes last from 6 to at least 24 months (DSM IV-TR, 2000), a long period of time in which to leave an individual unable to function. The aim of treatment is therefore to initially ameliorate the symptoms of depression to a level at which the individual can return to their normal life, leading ideally to full remission. Commonly, pharmacological interventions are paired with some form of behavioural therapy initially, with the pharmacological treatment often being extended for several months after remission to prevent relapse. This treatment may become life-long in individuals prone to depressive episodes.

The most commonly used antidepressant drugs currently are selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine (better known by its trade name Prozac), sertraline (Zoloft) and citalopram (Celexa/Cipramil). Whilst seemingly no more efficacious than their predecessors, the tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors (MAOIs), SSRIs appear to be better tolerated and have fewer side effects (Anderson, 2000, Cipriani *et al.*, 2009). These older compounds have a wider pharmacological profile, affecting not only the 5-HT system but also other amines such as noradrenaline. That all of these compounds affect amine neurotransmitters, coupled with the depressogenic nature of reserpine-induced amine depletion, contributed to these systems being the focus for neurobiological theories of depression (Schildkraut, 1965).

More recently, there has been a move back towards drugs that affect several neurotransmitter systems. This is in the form of drugs that act as selective serotonin and noradrenaline reuptake inhibitors such as venlafaxine (Efexor). These drugs still act at discrete sites, the reuptake proteins at the synapses for these transmitters, and so still result in much fewer side effects than the earlier compounds. Having multiple sites of action, however, is thought to increase efficacy, although this has not been seen to be the case clinically (Cipriani *et al.*, 2009). Indeed, it appears as though an individual's response to a particular drug is highly variable; some will respond well to SSRIs whilst others will fare better on TCAs. The underlying basis for this phenomenon is not well understood and is relatively unresearched. While some have suggested that different treatments are more effective in particular subtypes of depression than others, this is more based on experience and anecdotal reports than on empirical investigation. Which drug is used and which drug provides relief in individual cases is therefore highly variable, and is guided mostly by resulting efficacy and professional instincts. An increase in the number of treatments available, even of equivocal clinical efficacy, may therefore benefit some.

An estimated 20% of depressed individuals are resistant to all known forms of treatment including all classes of antidepressant drugs, as well as electroconvulsive therapy (ECT, Thase and Howland, 1994). ECT is still used in those that do not respond to pharmacological intervention; despite being seen as a highly unpleasant form of treatment, some individuals that do not respond to any known antidepressant drugs do benefit from ECT. The existence of treatment-resistant depression highlights our need for more research into, not only potential new drugs that act on aminergic systems, but also potential novel targets. A better

understanding of the disease itself is paramount in this discovery of novel treatments.

1.2. Modelling depression and antidepressant treatment

This thesis aims to investigate rodent analogues of human emotional behaviour. The approach taken was to use tests of phenotypes that are relevant to depression. While symptoms of human depression such as guilt and suicidal tendencies would be difficult to assess in rodents, several other symptoms are more easily measured.

1.2.1. Cardinal symptoms of major depression

1.2.1.1. Depressed mood

Depressed mood, as it is understood in the clinical setting, is impossible to measure in rodents owing to the emotions of these animals not being accessible for our evaluation. Depressed mood is therefore more abstractly measured through levels of behavioural despair in tests such as the forced swim test (FST, Porsolt *et al.*, 1977a, Porsolt *et al.*, 1977b, Porsolt *et al.*, 1978a, Porsolt *et al.*, 1978b, Hansen *et al.*, 1997) and the tail suspension test (TST, Steru *et al.*, 1985, Vaugeois *et al.*, 1996, Caldarone *et al.*, 2003, El Yacoubi *et al.*, 2003, Cryan and Mombereau, 2004).

The FST was first developed using rats in the late 1970s as a test sensitive to antidepressant activity (Porsolt *et al.*, 1977b, Porsolt *et al.*, 1978a) and was quickly modified for use with mice (Porsolt *et al.*, 1977a, 1978b). At its simplest, this test involves placing the rodent in a cylinder of water and monitoring its behaviour. As both mice and rats naturally float, they do not necessarily need to continue

swimming when put in this environment. However, natural instinct dictates they attempt to escape. Measuring the time spent actively moving in an attempt to escape and the time spent passively immobile therefore provides a way of quantifying the rodent's propensity towards helplessness; finding the situation inescapable, they shift from attempted escape to energy preservation. Time immobile is therefore used as the main measure in this test and is reduced by antidepressants which shift behaviour away from helplessness. Conversely, an increase in immobility is seen as an indicator of behavioural despair, analogous to depressed mood, although this inverse interpretation remains controversial (Gardier and Bourin, 2001).

The sensitivity of this test to antidepressants is well established. However, its selectivity must also be considered, particularly given its locomotor component. In line with this, assessments of drug effects on general locomotor activity are generally also assessed. For instance, psychomotor stimulants reduce levels of immobility but are not generally used as antidepressants clinically. This is complicated by the historical use of amphetamines as antidepressants, a practice not entirely abolished in palliative care. Anxiolytics such as benzodiazepines, however, do not generally show any effect in this test, dissociating anti-anxiety drugs from anti-depressants.

1.2.1.2. Anhedonia

In humans, we think of anhedonia as a loss of interest in everyday activities, including those once found highly enjoyable. Given the resulting loss of reward found in daily life, this is in many ways the most debilitating and pathological of the possible symptoms of depression. In rodents, assessment of normal behaviour such as burrowing and nesting can easily been made (Deacon, 2006b, a). Social interaction

may also be considered to be relevant both to normal behaviour generally and anhedonia specifically, owing to the specific mention of reduced social activity in the description of anhedonia in the DSM. However, disruption to normal, everyday life is common to all psychiatric diseases. These tests are therefore important in establishing that the phenotype in the experimental animals is sufficient to be considered pathological, but would be expected in all psychiatric models, and so are not selective for depression.

Anhedonia is usually assessed in rodents by measuring their preference for a naturally rewarding substance, typically a sucrose solution (El Yacoubi *et al.*, 2003, Strekalova *et al.*, 2004). Experimental animals and their controls are given the option to drink this solution or water, and their preference ratio for the rewarding solution is taken as a measure of hedonic behaviour. As this relies on a natural behaviour for rodents, the baseline ratio is typically very high. This ratio can be decreased by stress and so this two-bottle preference measure is often paired with longitudinal stress paradigms. The reduction in sucrose preference over this time is ameliorated by an antidepressant intervention (Willner *et al.*, 1987). Antidepressant treatments may also further increase sucrose preference in control animals. In this thesis, this measure is used in transgenic lines to investigate basal levels of hedonic behaviour in comparison to wildtype (WT) mice.

1.2.2. Other symptoms

Of the remaining symptoms of a depressive episode, some are difficult to model in rodents. Feelings of fatigue, guilt and suicidal ideation will always remain beyond the reach of a rodent model of depression. Measures of anxiety in rodents are also commonly performed as anxiety disorders are highly co-morbid with clinical depression (DSM IV-TR, 2000, Zimmerman *et al.*, 2000). Reduced concentration could be investigated through cognitive tasks, although the precise nature of the cognitive deficits expected would be difficult to predict based solely on a reduction in attention. A variety of anxiety and cognitive tests have therefore been used in this thesis on transgenic lines of interest, both to cover these possible symptoms but also to act as control measures for the other tests performed.

Many of the other symptoms relevant to a major depressive episode can, however, be measured in a highly translational way. Alterations in weight are a prime example of a somatic marker that can easily be measured in rodents in the same way they would be investigated clinically. Food intake, as well as the above-mentioned sucrose preference test, can be taken as a measure of appetite. Alterations in sleep/wake cycles can also be measured in a manner similar to studies in humans by monitoring active and inactive periods over a prolonged period of time. Additionally, alterations in sleep architecture can be measured through the use of electrodes placed on the skull surface of rodents for an analogous signal to that taken in humans using EEG caps. Here, assessments are made in some of the transgenic lines used of weight, food and fluid intake, as well as investigations of locomotor activity over a prolonged period of time to investigate sleep/wake cycles in order to give a complete picture of the phenotype presented by these mice.

1.2.3. Emotional learning

Other tests are also used to investigate emotionality in rodents that bear less similarity to the clinical symptoms of depression but which are still informative. A disruption of emotional learning is likely to underpin part of the psychopathology of depression, and can be investigated in rodents in such tests as learned helplessness (Anisman *et al.*, 1979, Sherman *et al.*, 1982, Shanks and Anisman, 1989, 1993) and fear conditioning (Fanselow and Tighe, 1988, Chen *et al.*, 1996, Caldarone *et al.*, 1997, Wehner *et al.*, 1997, Fanselow and Poulos, 2005). Both involve cognitive elements (Fanselow and Gale, 2003) as they rely on associations being made between aversive stimuli, usually foot shocks, and cues or contexts. Learned helplessness typically involves the delivery of uncontrolled shocks within a confined space which offers no escape. When reintroduced to this area on a separate, test occasion, an escape route is now offered. The latency of the rodent to take advantage of this escape is taken as a measure of how helpless the rodent has become through the previous exposure to this environment which had established it as an inescapable and unpleasant place. A decrease in latency to escape shows a shift away from helpless behaviour and occurs following antidepressant treatment (Petty and Sherman, 1979, Sherman and Petty, 1980). An increase in latency to escape compared to a control group would indicate greater learned helplessness.

Fear conditioning is utilised in many areas of research. As this is an experimental way of investigating emotional learning, it is highly relevant to research into mood disorders. Further advantages of using fear conditioning to investigate emotional learning include its translational nature, as it is used both in humans (eg Buchel *et al.*, 1998, LaBar *et al.*, 1998, Morris *et al.*, 2001) and in rodents (eg Kim *et al.*, 1991, Kim *et*

al., 1993, Fanselow and Kim, 1994), as well as a well understood neurobiology (LeDoux, 2000, Fanselow and Gale, 2003, Maren, 2003). Cued fear conditioning in rodents involves the pairing of a discrete cue, such as a tone or light, with an aversive stimulus, usually a foot shock. Pairing this neutral cue, which will become the conditioned stimulus (CS), with an unconditioned negative stimulus, elicits a well-established conditioned response in rodents, namely freezing. The amount of freezing in response to the CS seen when tested later in the absence of the shock gives a measure of the strength of this association. While this may relate to many types of learning and behaviour, the rate of acquisition of this association and its extinction is of increasing interest to researchers interested in psychiatric disease. For instance, a more tenacious response to the CS, one that persists following extensive extinction, may provide a preclinical model of post-traumatic stress disorder (Wellman *et al.*, 2007). More generally, it may indicate a propensity towards preserving negative associations, something that may occur in many mood disorders in line with Beck's cognitive models of depression (Beck, 1976, Beck, 2008). Fear conditioning and its underlying neurobiology are discussed further in Section 5.1.1.

As mentioned above, fear conditioning and related tests are also used in human participants. Some of this work has looked at variation in 5-HTT expression levels and its consequences on neuronal activity (Garpenstrand *et al.*, 2001, Hariri *et al.*, 2002, Hariri *et al.*, 2005, Armbruster *et al.*, 2009). This is explored in Chapter 5 translationally by assessing regional tissue oxygen levels in 5-HTT OE mice (Jennings *et al.*, 2006) and their WT counterparts. This experiment was done using these animals as they have already been assessed on many of the behavioural tasks detailed above (Jennings *et al.*, 2006, Line *et al.*, 2010).

Rodents can therefore be used to model many of the behavioural aspects of a major depressive episode. A reliable preclinical model of depression would provide a platform on which to investigate future treatments and the underlying neuropathology of the disease. The lines used in this thesis were chosen on the basis of existing knowledge of the neurobiology of depression and antidepressant treatments. Of particular interest was the emerging role of elements of the glutamate system in emotionality. The possible link between the uptake protein involved in regulating 5-HT neurotransmission and amygdala reactivity was also tested. This was done in light of the conflicting data surrounding this link from work done in humans, discussed below.

1.3. The neurobiology of depression

The biological changes that result in depression are not well understood. Gross changes in neuroanatomy in depressed patients have been reported, such as a reduction in grey and white matter in occipital lobe (Sanacora *et al.*, 2004) and reduced hippocampal volume (McKinnon *et al.*, 2009). A large body of literature has focussed on changes to the 5-HT system owing to the efficacy of drugs that affect this system as antidepressants. However, other neurotransmitter systems such as the glutamatergic system are increasingly being investigated.

1.3.1. 5-HT

1.3.1.1. The 5-HT system

The neurotransmitter 5-hydroxytryptamine (5-HT, also known as serotonin) is heavily implicated in the regulation and dysregulation of mood (Owens and Nemeroff, 1994, Handley, 1995, Nemeroff and Owens, 2009). The cell bodies of 5-HT neurons are found in midbrain and brain stem structures such as the raphe nuclei and project diffusely to many areas of the brain including the frontal cortex, amygdala and hippocampus (Azmitia and Segal, 1978). The 5-HT system contains 14 known receptors grouped into seven families. These have a variety of roles across the brain and are predominantly metabotropic (Barnes and Sharp, 1999). Following the release of 5-HT into the synapse, neurotransmission is terminated by the reuptake of the neurotransmitter into the presynaptic neurone via the 5-HT transporter (SERT, Blakely *et al.*, 1994).

Depletion of 5-HT through *para*-chlorophenylalanine (PCPA)-mediated blockade of 5-HT production has been shown to reverse the effects of antidepressants in depressed patients (Shopsin *et al.*, 1975). Administration of 5-HT precursors tryptophan and 5-hydroxytryptophan (5-HTP) have also been shown to have some antidepressant effect (Coppen *et al.*, 1967, Sano, 1972). This suggests that overall levels of 5-HT may be altered in depression and may account for the disease in part. There is also evidence for a reduction in the levels of 5-HT and its main metabolite, 5-hydroxyindoleacetic acid (5-HIAA), in patient cerebral spinofluid (Gibbons and Davis, 1986). This is only seen in a sub-population of patients with depression, however. Interestingly, which patients show this change in 5-HIAA levels may coincide with a genetic marker for depression, a polymorphism in the gene encoding

the 5-HTT protein (Barton *et al.*, 2008). Changes such as reduced 5-HT_2 and 5-HT_{1A} receptor binding have also been noted in depressed patients (Staley *et al.*, 1998, Drevets *et al.*, 1999, Stockmeier, 2003, Bhagwagar *et al.*, 2004b, Meltzer *et al.*, 2004). However, the role of the 5-HTT protein in emotionality is of growing interest and is investigated further in this thesis.

1.3.1.2. The 5-HT transporter and emotionality

The 5-HTT is a key component in regulating 5-HT neurotransmission and turnover. It is also heavily implicated in mood as it is the primary target of many antidepressant drugs (Owens and Nemeroff, 1994, Owens *et al.*, 1997). There is some evidence for a reduction in 5-HTT expression in *post mortem* tissue from depressed patients (Perry *et al.*, 1983, Leake *et al.*, 1991). However, while these decreases in 5-HTT have been confirmed by some *in vivo* neuroimaging studies of depressed patients (Malison *et al.*, 1998, Staley *et al.*, 1998, Dhaenen, 2001, Reimold *et al.*, 2008), both increases (Reivich *et al.*, 2004) and no change in 5-HTT binding in unmedicated depressed patients relative to controls have also been reported (Meyer *et al.*, 2001). These discrepancies may in part be explained by influences such as age and sex on 5-HTT expression (Staley *et al.*, 2006).

5-HTT expression levels vary across the human population and this may correlate with measures of emotionality. This variation may, in part, be genetically driven by a number of polymorphisms that exist within the gene. The most widely investigated polymorphism is a 44 base pair insertion/deletion in the 5-HT transporter gene linked polymorphic region (5-HTTLPR), a GC-rich repetitive sequence located within the regulatory region of the 5-HTT gene promoter (Heils *et al.*, 1996, Lesch *et al.*,

1996). This insertion/deletion gives rise to long (*l*) and short (*s*) alleles, respectively, with an approximate distribution within the population of 57% and 43%, respectively, resulting in an approximate Hardy-Weinberg distribution of 32% *l*/*l*, 49% *l*/*s* and 19% *s*/*s* individuals (Lesch *et al.*, 1996). Studies introducing these different alleles into cultured cells show that the *l*/*l* genotype results in approximately three times more transcription of the 5-HTT gene (Heils *et al.*, 1996) and the resulting protein displays a two-fold increase in 5-HT reuptake compared to the heterozygote or *s*/*s* genotypes (Lesch *et al.*, 1993). This striking difference in functionality between the two genotypes may be expected to have a great impact on emotionality given the role of 5-HT in these behaviours and the key role of 5-HTT in regulating 5-HT neurotransmission.

The evidence for the *in vivo* consequences of this allelic variation in the 5-HTT gene is, however, disputed. While some studies report increased 5-HTT expression in *l*/*l* individuals as compared to *s* carriers (Little *et al.*, 1998, Heinz *et al.*, 2000), no correlation between the 5-HTTLPR allele and 5-HTT levels has been seen by others (Mann *et al.*, 2000, Willeit *et al.*, 2001). Many studies have also found correlations between this polymorphism and measures of personality traits thought to be predisposing towards depression, particularly in women (Lesch *et al.*, 1996, Du *et al.*, 2000, Greenberg *et al.*, 2000, Melke *et al.*, 2001). Neuroticism is the most common trait investigated, although other traits such as harm avoidance have also been measured. There are also several studies linking this polymorphism directly to mood disorders (Furlong *et al.*, 1998, Lotrich and Pollock, 2004, Lasky-Su *et al.*, 2005) and suicide (Anguelova *et al.*, 2003b, Roy *et al.*, 2007). These studies find that the *s* allele, thought to result in lowered 5-HTT expression, results in higher scores on these personality trait questionnaires and is therefore acting as a genetic risk factor for depression.

There is also evidence to suggest that an individual's 5-HTTLRP allele correlates with amygdala reactivity (Hariri *et al.*, 2002, Hariri *et al.*, 2005). The *s* allele is implicated as being a risk factor; individuals carrying this allele display a hyper-reactive amygdala in response to negative stimuli such as fearful and angry faces. However, this reactivity did not correlate with the results of personality questionnaires taken of depressive-related traits (Hariri *et al.*, 2005). Indeed, an association between the 5-HTTLPR polymorphism and measures of traits such as neuroticism is refuted by many studies (Ball *et al.*, 1997, Hoehe *et al.*, 1998, Jorm *et al.*, 1998, Deary *et al.*, 1999, Gustavsson *et al.*, 1999, Seretti *et al.*, 1999), as is any direct correlation with depression (Anguelova *et al.*, 2003a), although interactions with stressful life events may account for some of this variability (Caspi *et al.*, 2003, Roy *et al.*, 2007, Caspi *et al.*, 2010). The trait investigated and the measure used by individual studies also appears to have a significant impact upon the result (Sen *et al.*, 2004).

Additionally, discrepancies between single polymorphisms of the 5-HTT gene and actual protein expression may exist due to the influence of other polymorphisms in the 5-HTT gene as well as by other genes and environmental factors. This may in part explain the controversies surrounding these correlations between the 5-HTTLPR polymorphism and emotionality. A more direct measure of protein expression may therefore remove these confounding factors.

An elegant study by Rhodes and colleagues (2007) used a combination of PET imaging and fMRI to correlate individuals' 5-HTT binding levels with amygdala activity in an emotionally driven task. The task involved the matching the emotion of a sample face to a selection of others and was used as a way of eliciting implicit emotional processing. The authors demonstrated a strong inverse correlation

between 5-HTT binding within the left amygdala and activation of this area during this emotionally-driven task. It is worth noting that in a small subgroup of their sample group that were genotyped, Rhodes and colleagues observed no correlation between the 5-HTTLPR polymorphism present and either 5-HTT binding or fMRI signal in response to emotional cues (Rhodes *et al.*, 2007). While this analysis was only performed on 13 of the 20 participants and may therefore be underpowered, it adds further evidence to the argument that although protein expression levels of 5-HTT may affect emotionally-driven brain activity, this is not solely determined by a single genetic polymorphism.

The study presented in Chapter 5 of this thesis attempts to recapitulate this fMRI study by combining transgenic mice with altered 5-HTT expression and measures of neuronal activity. Constant potential amperometry, a method for measuring tissue oxygen, is used in combination with measures of local field potentials in the amygdala of transgenic and wildtype (WT) mice. This expands on the work already done using 5-HTT mutant mice as a means of investigating the consequences of 5-HTT variation in isolation from other genetic and environmental factors.

1.3.1.3. 5-HT transporter mutant mice

5-HTT knockout (KO) mice (Bengel *et al.*, 1998, Holmes *et al.*, 2003c, Lira *et al.*, 2003) and overexpressor (OE) mice (Jennings *et al.*, 2006) have been used to investigate the influence of the 5-HTT on emotionality. The 5-HTT KO mice show a reduction in tissue 5-HT (Bengel *et al.*, 1998, Kim *et al.*, 2005), a gross loss of 5-HT neurones within the raphe nuclei (Lira *et al.*, 2003), and a huge increase in extracellular 5-HT as measured by *in vivo* microdialysis (Mathews *et al.*, 2004, Shen *et al.*, 2004).

Behaviourally, the 5-HTT KO mice have been found to show a robust increase in anxiety across several tests (Holmes *et al.*, 2003a, Holmes *et al.*, 2003b, Holmes *et al.*, 2003c, Lira *et al.*, 2003, Fox *et al.*, 2007, Line *et al.*, 2010). Interestingly, although these mice show an increase in immobility in the FST, they show decreased immobility on the closely related TST (Holmes *et al.*, 2002, Lira *et al.*, 2003). This result appears to be background-strain dependent, as 5-HTT KO mice bred on a 129S6 background strain show this contradictory set of results whilst no effects in either the FST or TST were seen in 5-HTT KO mice bred on a C57Bl/6 background (Holmes *et al.*, 2002).

5-HTT OE mice have been constructed through insertion of the human 5-HTT gene (carrying the *s* allele of the 5-HTTLPR variation) inserted via a yeast artificial chromosome (Jennings *et al.*, 2006). These mice demonstrated a 2.9-fold increase in 5-HTT expression as measured by levels of 5-HTT mRNA in the raphe nuclei (Jennings *et al.*, 2006), a similar effect size to some of the reported effects of the *l*/*l* genotype in humans (Little *et al.*, 1998). 5-HTT OE mice have reduced levels of 5-HT both in whole tissue samples and extracellularly as measured by *in vivo* microdialysis (Jennings *et al.*, 2006, Jennings *et al.*, 2008). Behaviourally, the 5-HTT OE mice showed reduced anxiety (Jennings *et al.*, 2006, Line *et al.*, 2010). The results from these experiments are consistent with human data showing a reduction in anxiety-related traits in *l*/*l* individuals when compared to *s* carriers (Lesch *et al.*, 1996, Du *et al.*, 2000, Greenberg *et al.*, 2000, Melke *et al.*, 2001). These results also demonstrate a complementary pattern of changes in anxiety in the 5-HTT KO and 5-HTT OE mice (Line *et al.*, 2010).

The study presented in Chapter 5 makes use of the 5-HTT OE mice. The reason for selecting these mice over the 5-HTT KO mice is that there have been no reported cases of complete 5-HTT expression loss in humans. Furthermore, the increase seen in 5-HTT expression in the 5-HTT OE mice relative to WT mice is similar in magnitude to that seen across the human population. This makes the 5-HTT OE mice a more attractive option for use in a study attempting to model variation seen naturally within the human population.

1.3.2. Glutamate

1.3.2.1. The glutamate system

Glutamate is packaged into vesicles ready for release by one of three vesicular glutamate transporter (VGluT) proteins (see Section 4.1.1). Following release, glutamate is taken up by neurones and astrocytes by sodium-driven amino acid transporter proteins and is recycled (Seal and Amara, 1999). The glutamate system, ubiquitous as it is, consists of several different classes of receptor. These can broadly be split into ionotropic and metabotropic receptors by their mechanism of action, but can be further characterised into distinct families within these designations based on receptor pharmacology. Manipulations of metabotropic glutamate receptors do not feature in this thesis, but the effects of alterations to two of the ionotropic classes of receptor on emotionality are assessed.

Ionotropic glutamate receptors are tetrameric structures composed of subunits with three transmembrane domains (TM1-3) and a re-entry loop between TM1 and 2 (Figure 1.1). It is this loop that forms the ion pore which is permeable only to cations. Glutamate ionotropic receptors were initially split into three families based on their pharmacology, each named after the selective agonist acting at those receptors; AMPA (α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate), kainate and NMDA (N-methyl-D-aspartic acid). Work done later on the molecular construction of these receptors showed them to also be made of distinct proteins from separate gene families. In the case of AMPA receptors, these proteins are now known as GluA1-4 (the gene names being gria1-4), for kainate GluK1-5 (genes grik1-5) and for NMDA receptors GluN1, GluN2A-D, and GluN3A and B (genes grin1, grin2a-d and grin3a and b, respectively, Collingridge *et al.*, 2009)[6]. The contribution of different subunit composition to receptor pharmacology is discussed for each of the receptor types investigated here in the appropriate chapter (for AMPA receptors, see Chapter 2, for NMDA receptors, see Chapter 3). A more general overview of each of the receptor families is given below.

[6] This is the most recent nomenclature system proposed for the systematic naming of all ionotropic subunits and will be used consistently throughout this thesis. The equivalent protein names in previous systems can be found in the reference given.

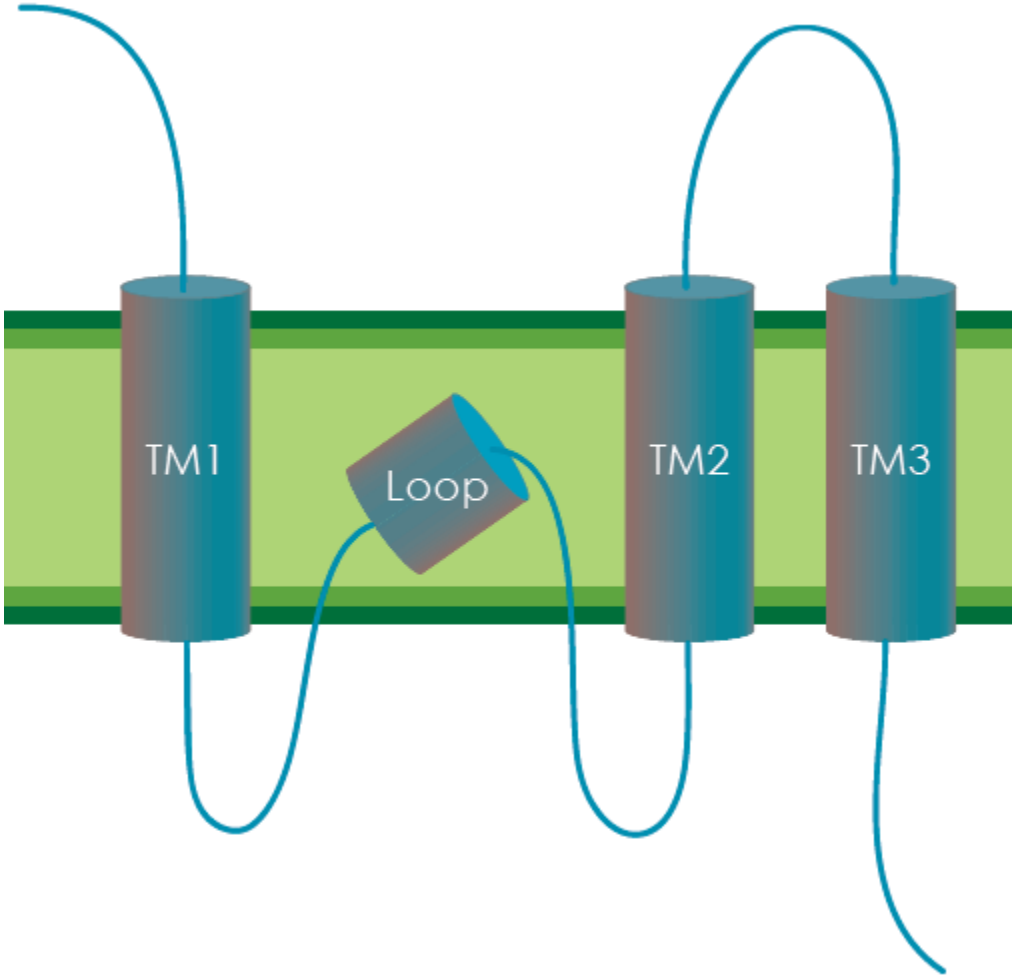


Figure 1.1: Diagrammatic representation of a glutamate receptor subunit

Glutamate receptor subunits consist of three transmembrane (TM) domains and one intramembrane loop. This loop forms the channel pore when four of these subunits collect together as a functional glutamate receptor.

AMPA receptors are thought to be the predominant receptor type involved in fast synaptic glutamate transmission (eg Davies and Collingridge, 1989). Their up-regulation may also be one of many processes that leads to the maintenance of plastic changes, such as long-term potentiation (LTP, eg Hayashi *et al.*, 2000). This neuroplastic event may be initiated by NMDA receptors (Collingridge *et al.*, 1983). NMDA receptors differ greatly from the other classes of ionotropic glutamate receptor, as well as from other families of ligand-gated receptors. The characteristic property of the NMDA receptor is its role as a coincidence detector, as it is both ligand- and voltage-gated (Seeburg *et al.*, 1995). The latter of these gating mechanisms is due to the Mg^{2+} block of the ion pore of the channel that is only removed when the neurone expressing this receptor is already depolarised. Activation of the receptor also requires the already-depolarised neurone to be further stimulated by the release of glutamate. The NMDA receptor also has many sites of allosteric modulation, as well as a co-agonist site. Binding of glycine or D-serine to this site is required for NMDA receptor function (Dingledine *et al.*, 1990, Wolosker, 2006).

The work presented in Chapters 2-4 of this thesis makes use of mutant mice with alterations to the glutamate system. These mice, with their WT controls, are subjected to several tests relating to emotionality. The role of glutamate in mood is not yet fully understood. However, the mice used were chosen on the basis of the growing literature on the role that this neurotransmitter has in depression and antidepressant activity in the hope of expanding this knowledge.

1.3.2.2. Glutamate in depression

Glutamate, as the major excitatory neurotransmitter in the central nervous system, is likely to play a pivotal role in most, if not all, physiological and pathological conditions concerning the brain. In depression, gross alterations in the levels of glutamate in the brains of depressed individuals have been noted (e.g. Auer *et al.*, 2000, Sanacora *et al.*, 2004). The most recent research in this area is based on magnetic resonance spectroscopy (MRS) which can be used to measure neurotransmitter levels *in vivo*. MRS is a form of analytical chemistry that allows for the levels of substances to be determined in an unknown sample based on their nuclear magnetic resonance signature. Two measures are commonly used to investigate glutamate levels; glutamate proper ("glu"), or "glx", which corresponds to combined glutamate and glutamine, the inactivated form of this neurotransmitter. The reason for this combined measure, and its popularity in the literature, is because the spectroscopic signatures given by the two compounds in MRS are very similar and thus difficult to dissociate. Further to this, MRS is currently very limited in its spatial resolution and so the region-specificity reported in many studies should be interpreted cautiously.

Nonetheless, increases in glu and glx have been shown in the occipital lobe of depressed patients (Sanacora *et al.*, 2004, Bhagwagar *et al.*, 2007). Others have found a reduction in glu and glx in prefrontal regions (Hasler *et al.*, 2007) and the anterior cingulate gyrus (Auer *et al.*, 2000), while one study found no change in either the occipital or cingulate cortices (Price *et al.*, 2009). Reductions in glx in the hippocampus have also been noted (Block *et al.*, 2009). Several studies find reduced glx is reversed following clinically effective electroconvulsive therapy in areas such as the dorsolateral prefrontal cortex (Michael *et al.*, 2003a), amygdala (Michael *et al.*,

2003b) and anterior cingulate (Pfleiderer *et al.*, 2003). This implies that these changes may be state dependent, i.e., only present during a depressive episode and not during remission. This may, in part, explain the discrepancies seen in the studies done to date. What seems clear is that changes in glu and glx levels are detectable in depressed populations, although the direction of these changes and in which areas they are seen needs further systematic investigation.

The elevation in glutamate reported by some studies may be due to molecular changes in components of the glutamate system, such as the enzyme responsible for its inactivation and the transporters responsible for its uptake (Choudary *et al.*, 2005). There is also evidence implicating alterations in glutamate receptor expression in depression (Freed *et al.*, 1993, Nowak *et al.*, 1995, Noga *et al.*, 1997). The evidence for each specific class of glutamate receptor having a role in emotionality is presented later in this thesis (for AMPA receptors, see Section 2.1.1.2, and for NMDA receptors, see Sections 3.1.1.2 and 3.1.1.3).

Conversely, glutamate neurotransmission may also have a part to play in antidepressant activity. Compounds that increase AMPA glutamate receptor mediated neurotransmission, so called "AMPAkines", have shown some antidepressant activity preclinically in tests such as the FST (Bai *et al.*, 2001, Li *et al.*, 2001a, Li *et al.*, 2003). However, perhaps the most striking evidence for a role for glutamate in antidepressant treatment, and consequently in depression, is the antidepressant actions of NMDA receptor antagonists, seen both clinically (Berman *et al.*, 2000, Zarate *et al.*, 2006a) and preclinically (Trullas and Skolnick, 1990, Trullas *et al.*, 1991, Maj *et al.*, 1992b, a, Panconi *et al.*, 1993, Layer *et al.*, 1995, Przegalinski *et al.*, 1997). In the case of human trials, one of these studies demonstrated an improvement

in mood in (otherwise) treatment-resistant patients that lasted for several days following a single infusion of the NMDA receptor antagonist ketamine (Zarate *et al.*, 2006a). These results are discussed more in Section 3.1.1.3.2. That a clinically tolerable novel antidepressant could be derived from these findings provides hope for those unresponsive to existing treatments for depression, as well as great impetus for searching for a better understanding of the role glutamate plays in mood.

This thesis makes use of mutant mice with alterations to either their AMPA or NMDA receptors. In the former case, GluA1 KO mice (Zamanillo *et al.*, 1999) are used and the results presented in Chapter 2. These mice lack the GluA1 AMPA subunit, implicated in neuronal plasticity as well as in depression and antidepressant activity (discussed further in Section 2.1.1.2). The GluN1 hypomorph (HYPO) mice (Mohn *et al.*, 1999) are the subject of Chapter 3. These mice express very low levels of the obligatory GluN1 subunit for NMDA receptors and consequently have very low levels of functional NMDA receptors.

The role of the VGluT in emotionality, specifically VGluT1, the predominant isoform found in the cortex and hippocampus, is also investigated here. Chapter 4 presents the results from tests of emotionality using the VGluT1 heterozygote (HET) mice (Tordera *et al.*, 2007). These mice express only 50% of normal levels of VGluT1 and have previously been reported as a model of depression (Tordera *et al.*, 2007, Garcia-Garcia *et al.*, 2009).

The use of genetically modified mice allows for the investigation of the consequences of life-long changes that are present from throughout development. In the case of the use of the 5-HTT OE mice, this allows for the modelling of the life-long variation in expression levels seen in humans. The use of constitutive knockout mice in the case of the glutamate system allows for the investigation of other alterations that may contribute to the genetic predisposition mooted by the familial component of depression (DSM IV-TR, 2000, Kendler *et al.*, 2001).

1.4. Aims of this thesis

This introduction covers some of the general evidence for the roles of the glutamate and 5-HT systems in emotionality, as well as the clinical features of depression and the possible ways in which this can be modelled in rodents. The overall aim of this thesis is to further our knowledge of the role of these two systems in emotionality. Genetically modified mice were used as a means to do this in order to provide a preclinical model in which to explore emotionality. The first set of investigations involved the exposure of several lines of mutant mice that have alterations in their glutamate system to tests of emotionality. Tests of naturalistic behaviour and cognition were also performed, both as they are of interest to the model and to account for possible confounding factors. To cover several aspects of the glutamate system, models of altered AMPA receptor- and NMDA receptor-mediated neurotransmission were used, as well as altered presynaptic functionality. Therefore, extensive behavioural investigations were done using the GluA1 KO mice (Zamanillo *et al.*, 1999, Chapter 2), the GluN1 HYPO mice (Mohn *et al.*, 1999, Chapter 3) and the VGluT1 HET mice (Tordera *et al.*, 2007, Chapter 4). These chapters aimed to provide information on the role of these proteins in behaviour generally and in

emotionality specifically. Each of these chapters also further discusses the evidence for particular aspects of the glutamate system in depression and antidepressant activity, in addition to the validity of these mouse lines as models of altered emotionality.

Alterations in the expression levels of the 5-HTT protein have been shown to correlate with several measures of emotionality (Lesch *et al.*, 1996, Hariri *et al.*, 2002, Hariri *et al.*, 2005, Rhodes *et al.*, 2007). Here, 5-HTT OE mice (Jennings *et al.*, 2006) were used in combination with the relatively-recent development of constant potential amperometry as a method for recording tissue oxygen levels *in vivo* in freely moving, behaving animals. The homology of this signal to the BOLD response that underlies human fMRI makes this approach highly translatable. Results from this and measures of local field potentials in the amygdala, an area heavily implicated in emotionality, in these transgenic mice during an emotionally-driven task are presented in Chapter 5.

In summary, this thesis investigates the role of the glutamate and 5-HT systems in emotionality. Transgenic mice offer a means by which to study the consequences of long-term changes to neurotransmitter systems. How this work impinges upon the clinical situation, the broad validity of the methods employed and the overall conclusions of this thesis will be considered in Chapter 6.

CHAPTER 2: Behavioural study of emotionality in the GluA1 knockout mice

2.1. Introduction

2.1.1. AMPA receptors

AMPA receptors are tetrameric protein complexes that have a ligand site for glutamate which leads to the opening of a cation-selective ion pore. The channel properties of AMPA receptors depend on the construction of the final functional receptor from subunits that show some spatial- and developmental-specific expression patterns (Monyer *et al.*, 1991).

2.1.1.1. AMPA receptor subunits

As with all ionotropic glutamate receptor subunits, the proteins that make up an AMPA receptor contain three transmembrane domains (TM1-3) with a re-entry loop between TM1 and 2 (see Figure 1.1 in Section 1.3.2.1, page 28). This loop results in an extracellular N-terminus region and an intracellular C-terminus region. Interactions between a sequence towards the N-terminus region (S1) and part of the extracellular loop between TM2 and TM3 (S2) form the binding regions for glutamate. Four possible subunits (GluA1-4) have been identified that can form AMPA-class glutamate receptors (Collingridge *et al.*, 2009). GluA2 has been noted to markedly alter the rectification of AMPA receptors from inward to linear (Verdoorn *et al.*, 1991), but otherwise these subunits appear equivocal in their effects on receptor pharmacology when constructed into homomeric protein complexes *in vitro*. The diversity of AMPA receptors is instead ensured by a number of splice variants and

RNA editing sites within these genes. The flip/flop region is an extracellular stretch of 115 base pairs close to the final transmembrane domain that undergoes splice variation in all four AMPA receptor subunits (Sommer *et al.*, 1990). Subunits expressing the flip variant desensitize much slower than flop variants (Partin *et al.*, 1995), are more sensitive to glutamate (Sommer *et al.*, 1990) and are highly expressed before birth. During adulthood, the relative expression of these variants for all subunits is approximately equal, with flop variants increasing from near-absent levels at P1 to adult expression at P15 (Monyer *et al.*, 1991). GluA2 and 4, furthermore, have short and long forms due to splice variants at the C-terminus of the protein. The short form of the GluA4 subunit is often denoted as GluA4c as this splice variant is predominantly and selectively expressed in the cerebellum (Gallo *et al.*, 1992) [7]. The long and short forms of GluA2 do not show this spatial selectivity, but the short form is much more common than the long. The functional consequences of these mutations are likely to relate to the protein-protein interactions of these subunits with structural and downstream-processing proteins.

All subunits except the GluA1 protein have a RNA editing site just prior to the flip/flop site, the R/G (arginine/glycine) site, where a single nucleotide change results in glycine rather than arginine being present in the final protein. This editing leads to less desensitisation in receptors containing these subunits and in the adult brain is found in 80-90% of GluA2-4 subunits (Lomeli *et al.*, 1994). Finally, an RNA editing site is present in the re-entry loop region of the GluA2 subunit, the Q/R (glutamine/arginine) site. This site is found to be edited in almost all GluA2 subunits

[7] The nomenclature of splice variants was not covered by the Collingridge *et al*., 2009 report, so this name uses the modern root name of the protein plus the previously used suffix to maintain consistent protein names within this thesis.

in the adult brain (i.e., almost all GluA2 subunits have an arginine residue at this site) and results in a large reduction in Ca^{2+} permeability in any receptors expressing this subunit as well as decreasing channel conductance and removing the usual inward rectification (Hume *et al.*, 1991, Verdoorn *et al.*, 1991, Washburn *et al.*, 1997). *In vivo*, the consequence of this is that non-GluA2 subunit-containing AMPA receptors are permeable to both Ca^{2+} and monovalent cations whereas those containing GluA2 are almost all impermeable to Ca^{2+}. This has important consequences due to the role of Ca^{2+} entry into the cell in neuroplasticity as well as excitotoxicity. This latter point may also explain the very high level of expression of GluA2 subunits in development (Monyer *et al.*, 1991); a reduction in Ca^{2+} permeability will limit the number of possible excitotoxic events, thus limiting cell death in the brain's infancy when the number of neurones present is much fewer than in adulthood. The specific molecular construction of AMPA receptors therefore has a profound effect on neuronal excitability and plasticity.

In addition to this, these proteins, like many others, have sites for post-translational modulation via methylation and phosphorylation. Of particular note, the GluA1 subunit contains two phosphorylation sites at serine residues 831 and 845 that have been shown to be important in regulating channel conductance (Roche *et al.*, 1996, Barria *et al.*, 1997) and have been used as markers of plasticity (Lee *et al.*, 2000). GluA1 also has a role to play in the trafficking of AMPA receptors to the synapse during the establishment of long-term potentiation (LTP, see for instance Shi *et al.*, 2001, Makino and Malinow, 2009) as well as altering the channel properties of AMPA receptors in this potentiated state. These GluA1-specific mechanisms implicate this subunit particularly in plasticity.

2.1.1.2. GluA1 in depression and antidepressant activity

There is growing evidence for a role for AMPA-class ionotropic glutamate receptors in depression and antidepressant activity (for a review, see Alt *et al.*, 2006). Very few human *post mortem* studies investigating alterations in AMPA receptors have been performed, although there is evidence for decreased expression of GluA1 in the perirhinal cortices of depressed patients (Beneyto *et al.*, 2007). Work on AMPA receptors as targets for antidepressants, however, has been growing due to evidence that "AMPAkine" drugs have antidepressant properties in rodents (Bai *et al.*, 2001, Li *et al.*, 2001a, Li *et al.*, 2003). These are drugs that positively modulate AMPA receptor-mediated neurotransmission through various mechanisms such as slowing deactivation or desensitisation of AMPA receptors (Partin *et al.*, 1996, Baumbarger *et al.*, 2001). These drugs therefore mimic some of the allosteric modulation accounted for by post-translational modifications mentioned above. These studies also demonstrate that the subunits present in the AMPA receptors, as well as the splice variants of these subunits, play a part in determining the efficacy of these compounds (Partin *et al.*, 1994, Partin *et al.*, 1995, Partin *et al.*, 1996, Sekiguchi *et al.*, 1997, Quirk and Nisenbaum, 2003, O'Neill *et al.*, 2005). This selectivity for AMPA receptors of a specific molecular construction could be used as a way of targeting these drugs towards specific areas of the brain or differing cell types.

The introduction of these positive allosteric modulators of AMPA receptors as novel putative antidepressants is a logical progression from the observations made of the effects of current antidepressants on AMPA receptors. Chronic injection of SSRIs such as paroxetine and imipramine into rats has been shown to increase expression of AMPA receptor subunits, especially GluA1 in the hippocampus (Martinez-

Turrillas *et al.*, 2002, Du *et al.*, 2004). It has also been shown that both acute and chronic treatment with fluoxetine can differentially modulate the phosphorylation of GluA1 in mice in areas involved in emotionality such as the hippocampus and the frontal cortex (Svenningsson *et al.*, 2002). These findings implicate the GluA1 subunit as particularly important in the antidepressant properties of drugs acting at AMPA receptors. That these alterations are seen following administration of SSRIs also provides some evidence for a functional link between the 5-HT system, canonically associated with depression, and the glutamate system. Without drugs to specifically target GluA1-containing AMPA receptors, however, work on its precise contribution to mood have been hampered.

2.1.2. The GluA1 knockout mouse

2.1.2.1. Construct

The function of this specific subunit can now be explored following the creation of a GluA1 knockout (KO) mouse (Zamanillo *et al.*, 1999). This subunit, which is usually highly expressed in the amygdala and hippocampus (Monyer *et al.*, 1991), is entirely absent in these mice due to deletion of a portion of the gene containing exon 11. Initial studies found that GluA1 KO mice demonstrate a reduction in AMPA receptor-mediated currents and a profound deficit in LTP induction, although subsequent studies have shown these mice can exhibit LTP when using the theta-burst induction paradigm (Hoffman *et al.*, 2002, Romberg *et al.*, 2009). The implication of these and other studies is that GluA1 may have a role to play in a form of short-term potentiation that precedes LTP (Erickson *et al.*, 2009, Sanderson *et al.*, 2010). This further implicates the GluA1 AMPA subunit specifically in neural plasticity. GluA1

KO mice showed only subtle alterations in the spatial organisation of other AMPA subunit expression and no overall changes in AMPA receptor or NMDA receptor subunit expression levels were seen in the hippocampus of these mice (Zamanillo *et al.*, 1999). The GluA1 KO mice develop normally and, despite initially having a lower body weight than their wildtype (WT) littermates, were indistinguishable as adults from other mice in this original study.

The gross behavioural phenotype of the GluA1 KO mouse has since been further investigated and shows some alteration from that of WT mice. Male GluA1 KO mice showing a nesting deficit, decreased body weight as adults and increased food intake whereas female mice show a reduction in glucose intake which the male mice do not (Bannerman *et al.*, 2004). In the rotarod and static rods tests of motor coordination, the GluA1 KO mice show a deficit (Bannerman *et al.*, 2004) and show increased locomotor activity (Bannerman *et al.*, 2004, Wiedholz *et al.*, 2008), although this was not seen in a familiar environment (Wiedholz *et al.*, 2008). Indeed, the GluA1 KO mice are highly hyperactive in response to an unfamiliar object being introduced into a testing environment (ibid.), highlighting that this hyperlocomotion may only be seen in response to novelty. The GluA1 KO mice may also be more anxious as shown by an increased latency to eat in the hyponeophagia test, female GluA1 KO mice also spending reduced amounts of time in the white part of a black/white alley (Bannerman *et al.*, 2004) and GluA1 KO mice spending less time in the open arms of an elevated plus maze (Mead *et al.*, 2006). They also display subtle alterations in their social interactions (Wiedholz *et al.*, 2008). While hyperactivity may act as a confound to these tests, this cannot account for all differences seen; simple hyperactivity that was not modulated by the environment would lead to mice spending approximately equal amounts of time in the white or open areas of the approach/avoidance anxiety

tasks. Compared to control mice, this would result in the GluA1 KO mice appearing much less anxious rather than more anxious as is generally seen in these studies.

The majority of work with these mice, however, has focussed on their cognitive abilities due to the association between the glutamate system and neuroplasticity and the perceived association between this and learning and memory. The original paper describing these mice demonstrated that, despite their deficit in LTP induction, they could acquire the spatial Morris Water Maze task as well as WT mice (Zamanillo *et al.*, 1999). This finding has been replicated and expanded on to include other spatial and non-spatial tasks (Reisel *et al.*, 2002, Bannerman *et al.*, 2003) including object recognition (Wiedholz *et al.*, 2008) and certain operant-based learning tasks (Mead and Stephens, 2003, Mead *et al.*, 2006). However, GluA1 KO mice do show deficits in tasks such as spontaneous (Bannerman *et al.*, 2004, Sanderson *et al.*, 2007) and rewarded alternation (Reisel *et al.*, 2002, Bannerman *et al.*, 2003) and reversal learning (Bannerman *et al.*, 2003). These findings have led to a theory that, rather than a spatial versus non-spatial dissociation, GluA1 KO mice are selectively impaired at tasks requiring working memory but not those reliant on reference memory. This is to say that these GluA1 KO mice can acquire tasks that involve incremental learning over several trials but cannot retain and/or utilise within-trial information to guide their behaviour.

This is most elegantly demonstrated in a study employing a radial maze task in which three of six arms are baited with a reward. Mice are initially trained to these target arms by being allowed to enter each arm only once, a door being used to block further entries from being made. Entries into arms that are never baited are considered reference memory errors and reduce over time as mice acquire this task.

GluA1 KO mice acquire this part of the task as quickly as WT mice. However, if mice are then allowed to make repeated entries to the same arm, working memory becomes part of this task. This can be assessed by considering re-entry into a rewarded arm after the reward has already been received as a working memory error. The distinction between these two types of error is reliant on the distinction between animals remembering which arms are never baited (requiring reference memory) and those that have already been visited (which requires intra-trial working memory). The GluA1 KO mice are impaired at the working memory component of the task, demonstrating a dissociation between these two forms of memory within the same task and using the same subject animals (Schmitt *et al.*, 2003).

2.1.2.2. Previous findings from the GluA1 knockout mice relevant to emotionality

In addition to the anxiety and social interaction changes mentioned above and female GluA1 KO mice having reduced glucose intake, a naturally rewarding substance for mice (Bannerman *et al.*, 2004), there are further behaviours these mice display that may be relevant to affect. Principal amongst these is a study exposing GluA1 KO mice to a learned helplessness paradigm. This task uses a "shuttle-box"; an arena of two sections, one in which the mice receive a foot shock and another, safe compartment to which the mice can escape through a small opening. In the case of this study, mice first received 360 foot shocks over two days in a separate arena to stress the animals and induce "helpless" behaviour. The following day, mice were placed into a shuttle box in which a red light predicted a shock within the compartment the mouse was in. The mouse's latency to move to the other

compartment was then taken as a measure of helpless behaviour over several trials. GluA1 KO mice displayed a greater tendency to not escape at all ("escape failures") as well as an increase in escape latency (Chourbaji *et al.*, 2008). This is in contrast to their noted hyperactivity. The authors conclude that this is therefore a robust demonstration of increased helplessness in GluA1 KO mice.

A further phenotype of interest, however, may confound the above experiment. Fear conditioning paradigms involve the pairing of a cue such as a tone or light with an aversive stimulus such as a footshock. This aversive stimulus also becomes associated with the context in which this training occurs. The degree to which these associations have been learned can then be assessed by either presenting the cue in a novel context or by re-exposing the animals to the training context to dissociate cued and contextual fear conditioning, respectively. Typically, freezing is then used as a behavioural measure of fear in these tasks. GluA1 KO mice have been shown to have a deficit in both these forms of fear conditioning (Feyder *et al.*, 2007, Humeau *et al.*, 2007). This reduced expression of conditioned fear may represent an alteration in acquisition of a task that is emotionally driven, although it would be expected for a depressed animal to show a greater degree of conditioning rather than a reduced response. This seeming anti-depressive result is therefore in disagreement with the learned helplessness result. It could be, however, that both of these results may simply be a further demonstration of cognitive deficits in these mice, in this case in associative learning. This would also account for the deficits seen in these mice in other conditioning tasks (Mead and Stephens, 2003, Johnson *et al.*, 2005, Mead *et al.*, 2006). Therefore, despite most previous results pointing towards these mice as having lowered mood such as by displaying increased anxiety and learned

helplessness, their position as a model of depression cannot be taken as read given these alternative, cognitive explanations for some of their behavioural changes.

2.1.3. Aims of this chapter

Previous results suggest that GluA1 KO mice show some behavioural changes consistent with a depressive-like phenotype (Bannerman *et al.*, 2004, Chourbaji *et al.*, 2008). However, other results suggest this may not be the case (Feyder *et al.*, 2007, Humeau *et al.*, 2007), necessitating further testing. This chapter aims to expand upon the number of tests performed relating to emotionality, both in terms of anxiety and affect.

2.2. Methods

2.2.1. Animals

Three cohorts of male and female GluA1 knockout mice (KO, Zamanillo *et al.*, 1999) and their wildtype (WT) littermates were tested on a number of behavioural tests and the results pooled. Mice were bred from male and female GluA1 heterozygote crosses and maintained in The University of Oxford on a mixed background of 129Sv and C57Bl/6 in holding rooms on a 12/12hr light/dark cycle (lights on at 7am and off at 7pm). Mice were housed in groups of 2-4 with *ad libitum* food and water and were typically of 6 months of age at the start of testing which lasted approximately 2 months. All testing was done during the light phase unless otherwise noted.

2.2.2. Behavioural protocols

The first cohort (22 WT and 26 GluA1 KO mice) were tested, in order, on the elevated plus maze, sucrose preference, locomotor activity across the diurnal cycle, social interaction and the forced swim test (Figure 2.1A). The second cohort (24 WT and 21 GluA1 KO mice) were put through these same tests in this order excluding the forced swim test (Figure 2.1B). The final cohort consisted of 32 male mice (16 WT and 16 GluA1 KO mice) and was only used in an experiment further investigating the effects of habituation on the social behaviour of GluA1 KO mice.

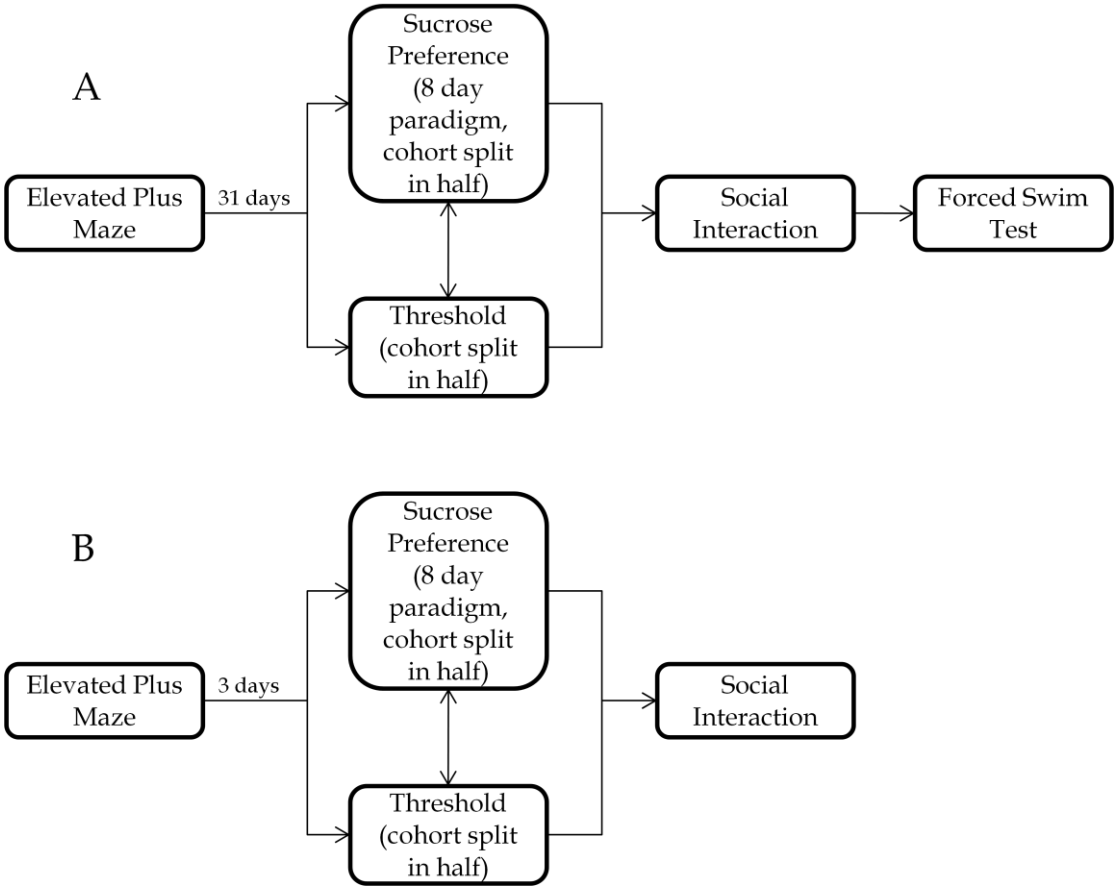


Figure 2.1: Timeline outlining the behavioural testing of cohort one (A) and two (B) of the GluA1 knockout mice

GluA1 knockout mice and their wildtype counterparts performed a number of behavioural tests with at least a two day gap between tests. Arrows between tests represent a two day gap unless otherwise stated.

2.2.2.1. Tests of anxiety

2.2.2.1.1. Elevated plus maze

Mice find open areas more aversive than closed environments. The elevated plus maze forms a conflict for the mice between their need to stay in safe areas with their innate curiosity (Handley and Mithani, 1984, Pellow *et al*., 1985, Lister, 1987). A plus-shaped maze with arms of 35x6cm leading from a 6x6cm centre square was used with two closed arms with 20cm walls and two open arms with 0.5cm high walls. The maze was elevated by 70cm from the ground and Ethovision XT (Noldus) used to measure latency, frequency and time spent in the open arms as measures of anxious behaviour. Total distance travelled was also taken to account for any locomotor differences.

2.2.2.1.2. Social interaction

Social interaction is a naturalistic test for anxiety, specifically social anxiety (File and Hyde, 1978, Dunn and File, 1987, File, 1996, 1997). Two mice of the same genotype that had previously been housed in separate cages and therefore naïve to one another were placed in a clean cage of 41.5x25.5x11.5cm for 10min. Bedding sufficient to cover the bottom of the cage was present and changed for each trial. This protocol was repeated on two consecutive days, novel pairings being used on each day. These encounters were scored to give a measure of social interaction in a novel environment. Mice were then extensively habituated to the testing arena by individual exposure for 10mins per day for a period of 5 days. Litter was once again present and changed for every trial. Social interaction was then conducted on the sixth and seventh days using the protocol above. These encounters were scored to

give a measure of social behaviour in a familiar environment. Trials were scored blind with respect to genotype for duration of social and aggressive interactions, social behaviour being defined as sniffing, grooming or following and aggressive behaviour as biting, scratching and chasing.

To further investigate the effects of habituation on the performance of GluA1 KO mice in this task, a third cohort of male mice was used. Two social arenas were placed in distinct testing rooms with differing spatial cues to create two separate testing contexts. Mice were then habituated to one of the two environments for 10min per day for 7 days, half of each genotype in each context. Pairs of mice were then tested as described above over 3 days for 5min, novel pairings each day. For half of each genotype, this testing occurred in the same context in which the given mice were habituated. For the other half, the testing was done in the novel context. This resulted in mice from each genotype being tested in either a familiar environment or a novel context without prior exposure to the test period of the social interaction test. Test bouts were recorded and scored manually blind with respect to genotype. Unfortunately, a technical fault in the recording equipment resulted in three test periods being lost and so had to be excluded from analysis. All three of these periods were GluA1 KO bouts.

2.2.2.2. Tests of affect

2.2.2.2.1. Sucrose preference

Preference for a naturally rewarding substance such as sucrose has been used to investigate pleasure-seeking and a reduction in this behaviour is taken to be indicative of anhedonia (El Yacoubi *et al.*, 2003, Strekalova *et al.*, 2004). Mice were individually housed in large cages (41.5x25.5x11.5cm) with enrichment for 8 days with *ad libitum* food and access to two bottles. On the first four days, both bottles contained water. On the second four day period one of these bottles contained water, the other an 8% (weight/volume) sucrose solution. The relative positions of the bottles were counter-balanced and switched after 24hrs, 72hrs, 120hrs and 168hrs to prevent place-preference from developing. The experimenter was blind to both the genotype of individual animals as well as which solution was in each bottle.

The weight of each bottle was taken at 8am and 6pm each day and sucrose consumption calculated by first dividing the weights of the bottles by 1.08, the weight in grams of 1ml of 8% sucrose w/v solution. Values were then averaged across this time and a preference ratio calculated by dividing sucrose consumption by total fluid consumption (sucrose/(sucrose+water)).

2.2.2.2.2. Locomotor activity across the diurnal cycle

Threshold pressure pads were used to measure the activity of mice over a 23.5hr period to assess activity across the diurnal cycle and investigate any changes in the circadian cycle. The Threshold system (Version 3, Med Associates) converts changes in pressure on pads into changes in voltage. Dedicated software records these changes in voltage and allows threshold values to be set to give sensitive readings of locomotor activity. In the present experiments, the lower detection threshold was set to 20V and the upper threshold to 50V. Mice were placed in cages of 22.5x12.5x13cm at 9.30am, 2.5hrs after lights on, and left with *ad libitum* food and water throughout the remains of the light period, the whole of the dark period (7pm-7am), then removed from the cages at 9am the following morning. The time spent between the two threshold values was taken to represent locomotor activity in the mice and was collected in 30min time bins.

2.2.2.2.3. Forced swim test

Time spent immobile in the forced swim test is used as a measure of behavioural despair (Porsolt *et al.*, 1977a, Porsolt *et al.*, 1977b, Porsolt *et al.*, 1978a, Porsolt *et al.*, 1978b, Drugan *et al.*, 1989, Detke *et al.*, 1995, Hansen *et al.*, 1997, Redrobe and Bourin, 1998, Lucki *et al.*, 2001). The protocol chosen here consisted of two exposures on consecutive days to 15cm of 25±1°C water in a cylinder of 20cm diameter, 26cm in height. The first exposure lasted 15min, the second 6min, and on each occasion the amount of climbing, swimming and immobility was scored during minutes 3-6 to give a comparable window of activity across both days and to discount the overall high levels of activity seen during the first two minutes of this test.

2.2.3. Statistical analyses

Univariate ANOVAs with genotype and sex as between-subject factors were used with habituation being used as an additional between-subject factor in the case of the social interaction experiments. This was done rather than considering it as a within subject factor as, in the first experiment, although the same mice were used, the pairings on which the data are based were not the same on these two separate occasions, and in the second experiment different mice were used in the same and different context test conditions. When an interaction was significant, simple main effects analyses were performed with Least Significant Difference (LSD) comparisons. Because measurements of rodent behaviour often display a non-normal distribution results sometimes violated assumptions of normality and/or equal variance. Many of these violations could not be corrected for using transformations and non-parametric tests would not detect interactions of between subject factors and would involve multiple testing to investigate the given factors. Thus, in accordance with Glass and colleagues, ANOVAs were performed in some cases despite the lack of normality and/or equal variance (Glass *et al.*, 1972). $p<0.05$ was considered statistically significant and all statistics were done using SPSS version 15.

2.3. Results

2.3.1. Tests of anxiety

The results for the tests of anxiety in the GluA1 knockout (KO) and their wildtype (WT) counterparts are summarised in Table 2.1 and Figures 2.1-2.4. Univariate ANOVAs using genotype and sex as between-subject factors were used to compare time, latency and number of entries into the open arms in the elevated plus maze (EPM) and the distance moved during this test. Univariate ANOVAs were used for duration of social and aggressive encounters during the social interaction tests using habituation as an additional between-subject factor to those detailed above. Sex was excluded from the analysis of aggression as this behaviour was only observed in male mice as well as in the second social interaction test in which only male mice were used.

2.3.1.1. Elevated plus maze

In the EPM (Table 2.1), time spent in the open arms is the main measure of anxiety which showed no effect of genotype ($F_{(1,88)}$=3.071, p=0.083) but with a trend for GluA1 KO mice to spend more time in the open arms than WT mice (see Table 2.1). Latency to enter the open arm also showed no effect of genotype ($F_{(1,88)}$=0.016, p=0.900), but an effect of genotype was seen in the number of entries made into the open arms ($F_{(1,88)}$=11.028, p=0.001), GluA1 KO mice making significantly more open arm entries than WT mice. This may be related to the increase in distance covered by GluA1 KO mice ($F_{(1,88)}$=47.921, p<0.001). Indeed, if distance covered is

Table 2.1: Results for GluA1 knockout (KO) and wildtype (WT) mice in the elevated plus maze anxiety test

Measure	**WT, n=45**	**KO, n=47**	**Statistics**
Time spent in open arms (s)	15.68 ± 2.63	29.69 ± 6.76	Geno $F_{(1,88)}$=3.071, p=0.083
			Sex $F_{(1,88)}$=4.335, p=0.040
			Int $F_{(1,88)}$=6.308, p=0.014
Latency to open arm (s)	90.93 ± 15.97	82.70 ± 14.20	Geno $F_{(1,88)}$=0.016, p=0.900
			Sex $F_{(1,88)}$=7.531, p=0.007
			Int $F_{(1,88)}$=4.303, p=0.041
No. of open arm entries	14.58 ± 2.34	36.09 ± 5.75	**Geno $F_{(1,88)}$=11.028, p=0.001**
			Sex $F_{(1,88)}$=10.137, p=0.002
			Int $F_{(1,88)}$=4.856, p=0.030
Distance moved (cm)	1224.11 ± 59.54	1916.48 ± 95.34	**Geno $F_{(1,88)}$=47.921, p<0.001**
			Sex $F_{(1,88)}$=39.274, p<0.001
			Int $F_{(1,88)}$=10.214, p=0.002

Presented as mean ± SEM and analysed with univariate ANOVA.
Geno = effect of genotype, sex = effect of sex, int = genotype*sex interaction.

GluA1 knockout mice show an increased number of open arm entries on the elevated plus maze as well as increased locomotion but do not spend any more or less time in the open arms compared to wildtype mice.

taken as a covariate in the analysis, the effect of genotype does not remain ($F_{(1,87)}$=1.543, p=0.218) and distance accounted for at least a proportion of the variance in number of open arm entries ($F_{(1,87)}$=6.483, p=0.013).

An effect of sex was seen in the time spent in the open arm ($F_{(1,88)}$=4.335, p=0.040) with female mice (30.74s±6.68) spending significantly more time in the open arm than male mice (14.59s±2.72; $F_{(1,88)}$=4.335, p=0.040). This sex effect interacts with genotype ($F_{(1,88)}$=6.308, p=0.014) as the sex effect is driven by the GluA1 KO mice (female 44.20s±11.08, male 11.73s±4.03; simple main effect of sex $F_{(1,88)}$=10.747, p=0.001) rather than WT mice (female 14.06s±3.82, male 17.10s±3.69; simple main effect of sex $F_{(1,88)}$=0.091, p=0.764), and an effect of genotype only seen in female (simple main effect of genotype $F_{(1,88)}$=9.260, p=0.003) and not male mice ($F_{(1,88)}$=0.283, p=0.596). As may be expected, a complimentary pattern of results was seen in the latency to first enter the open arm, male mice being slower to make this first entry (male 115.73s±16.49, female, 58.94s±12.36; $F_{(1,88)}$=7.531, p=0.007), this difference being present in the GluA1 KO mice (male 136.83s±25.51, female 38.97s±8.91; simple main effect of genotype $F_{(1,88)}$=11.826, p=0.001) but not in WT mice (male 97.27s±21.18, female 83.66s±24.66; $F_{(1,88)}$=0.220, p=0.640), but a main effect of genotype was not detected in either sex (simple main effect of genotype; female $F_{(1,88)}$=2.467, p=0.120; male $F_{(1,88)}$=1.864, p=0.176). Frequency of open arm entries also matched these results, with female mice (35.72±5.08) entering the open arm more than male mice (14.96±3.70; $F_{(1,88)}$=10.137, p=0.002), this difference being present in GluA1 KO mice (female 50.31±7.62, male 18.48±7.23; simple main effect of genotype $F_{(1,88)}$=14.782, p<0.001) but not in WT mice (female 17.67±3.70, male 11.88±2.89; simple main effect of genotype $F_{(1,88)}$=0.472, p=0.494).

Again, these effects did not remain when distance was taken as a covariate (effect of sex $F_{(1,87)}$=1.543, p=0.218; genotype*sex interaction $F_{(1,87)}$=1.766, p=0.187), which was also the case for time spent in the open arm (effect of distance $F_{(1,87)}$=3.722, p=0.057; effect of genotype $F_{(1,87)}$=0.082, p=0.775; effect of sex $F_{(1,87)}$=0.471, p=0.494; genotype*sex interaction $F_{(1,87)}$=3.210, p=0.077) and latency (effect of distance $F_{(1,87)}$=1.702, p=0.195; effect of genotype $F_{(1,87)}$=0.453, p=0.503; effect of sex $F_{(1,87)}$=2.453, p=0.121; genotype*sex interaction $F_{(1,87)}$=2.405, p=0.125).

Distance travelled itself also showed an effect of sex ($F_{(1,88)}$=39.274, p<0.001), with female mice (1889.54cm±100.95) moving more than male mice (1252.24cm±56.51) which interacted with genotype ($F_{(1,88)}$=10.214, p=0.002), with the difference being exaggerated in GluA1 KO (female 2305.34cm±106.44, male 1435.03cm±91.14; $F_{(1,88)}$=45.607, p<0.001) compared to WT mice (female 1374.74cm±104.73, male 1092.30cm±52.46; $F_{(1,88)}$=4.631, p=0.034) but with a significant effect in both cases. There was also a main effect of genotype in each sex (female $F_{(1,88)}$=52.145, p<0.001; male $F_{(1,88)}$=6.819, p=0.011). Again, this pattern of data coincided with the results on measures of anxiety, implying distance travelled may better explain these differences rather than genotype.

2.3.1.2. Social interaction

In the first set of social interaction tests, social (Figure 2.2) and aggressive encounters (Figure 2.3) were treated separately. GluA1 KO mice spent less time socially interacting than their WT counterparts ($F_{(1,172)}$=8.335, p=0.004, Figure 2.2) and habituation increased levels of social interacting ($F_{(1,172)}$=18.647, p<0.001). This effect interacted with genotype ($F_{(1,172)}$=22.169, p<0.001) and was mostly driven by the greater increase in social interaction seen in GluA1 KO mice compared to WT mice as revealed in simple main effects analysis which shows a difference between the groups prior to habituation ($F_{(1,172)}$=29.481, p<0.001) but not after ($F_{(1,172)}$=1.624, p=0.204) and an effect of habituation on GluA1 KO ($F_{(1,172)}$=40.588, p<0.001) but not WT mice ($F_{(1,172)}$=0.076, p=0.783).

Male mice interacted more than female mice ($F_{(1,172)}$=50.703, p<0.001), but this did not interact with genotype ($F_{(1,172)}$=0.088, p=0.767) or habituation ($F_{(1,172)}$=1.775, p=0.184). However, these three factors did interact ($F_{(1,172)}$=7.454, p=0.007).

Aggression was seen only in males (Figure 2.3). This was reduced in the GluA1 KO mice ($F_{(1,84)}$=4.748, p=0.032) and was increased by habituation to the testing environment ($F_{(1,84)}$=4.605, p=0.035), but these two factors did not interact ($F_{(1,84)}$=1.035, p=0.312).

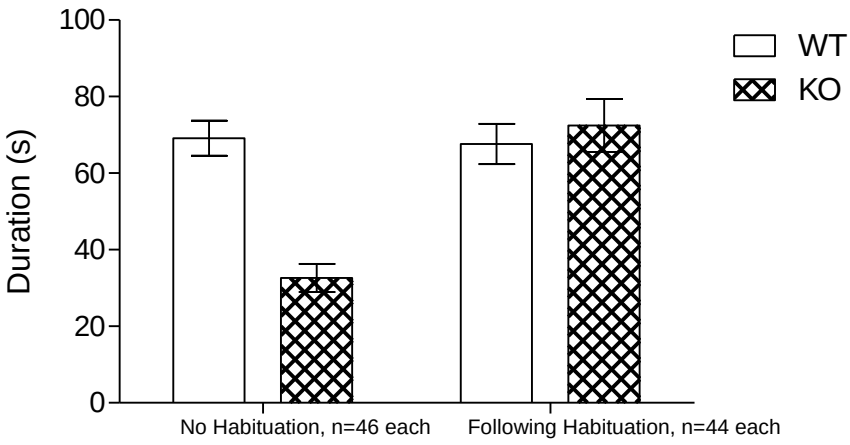


Figure 2.2: Time spent socially interacting for GluA1 knockout (KO) and wildtype (WT) mice prior to and following habituation to the testing environment

GluA1 knockout mice show an initial decrease in social interaction but this is rectified by extensive habituation to the testing environment. Presented as mean ± SEM.

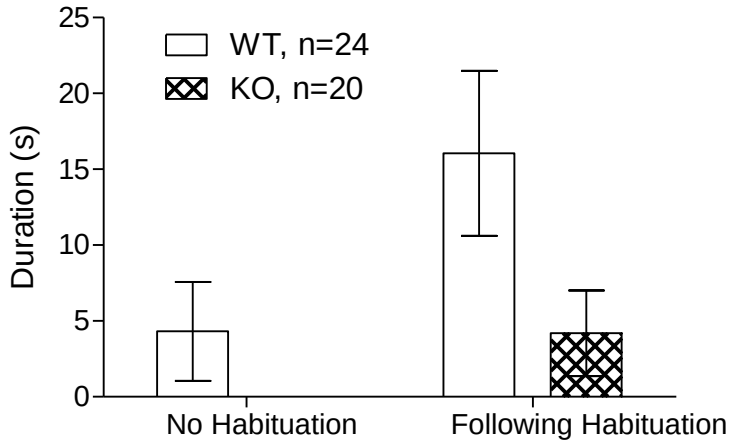


Figure 2.3: Time spent in aggressive encounters for male GluA1 knockout (KO) and wildtype (WT) mice prior to and following habituation to the testing environment

Aggression was seen only in male mice and was reduced in the GluA1 knockout mice relative to their wildtype counterparts. Habituation to the testing environment increased aggression equally in both genotypes.

The second social interaction test also treated social (Figure 2.4) and aggressive (Figure 2.5) encounters separately. Unfortunately, three test periods were lost due to a technical fault in the recording equipment, so these had to be excluded from analysis. While there was no main effect of genotype detected for time spent socially interacting ($F_{(1,41)}$=0.015, p=0.902, Figure 2.4), there was an effect of context condition ($F_{(1,41)}$=15.366, p<0.001) with mice overall interacting more in the habituated environment and an interaction between these two factors (genotype*context interaction $F_{(1,41)}$=7.872, p=0.008). Simple main effects revealed that there was a difference between the genotypes when tested in the habituated environment ($F_{(1,41)}$=4.649, p=0.037) but not when tested in a novel environment ($F_{(1,41)}$=3.339, p=0.075), although there is a trend for a reduction in social interaction in the GluA1 KO mice compared to WT mice. There was also an effect of testing environment in the GluA1 KO mice ($F_{(1,41)}$=21.002, p<0.001) but not in WT mice ($F_{(1,41)}$=0.673, p=0.417). This indicates the GluA1 KO mice show an increase in social behaviour in the habituated context versus the novel context to the extent that they interact more than the WT mice in this condition but tend towards interacting less in the novel context.

Aggression was not affected by genotype ($F_{(1,41)}$=1.024, p=0.317, Figure 2.5) or testing condition ($F_{(1,41)}$=1.761, p=0.192) and no interaction was seen between these two factors ($F_{(1,41)}$=1.024, p=0.317) in this social interaction experiment. This is likely to be due to the overall low levels of aggression seen.

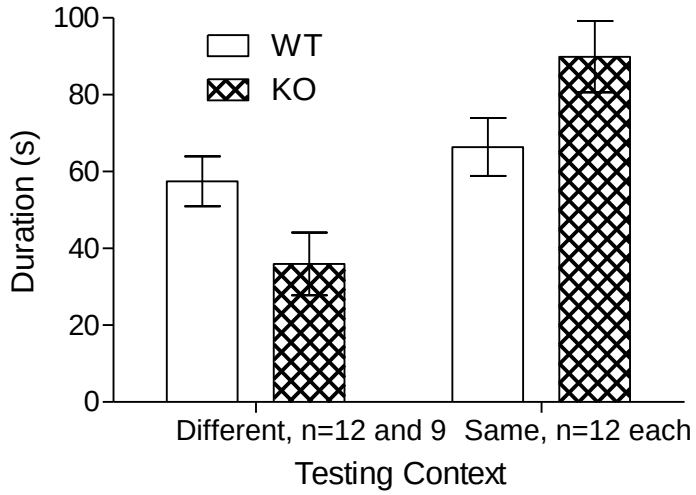


Figure 2.4: Time spent socially interacting for GluA1 knockout (KO) and wildtype (WT) mice when tested in either the same context as habituation or a different testing environment.

GluA1 knockout mice trend towards interacting less in a novel testing environment but interact more when tested in the same environment as they were habituated. Presented as mean ± SEM.

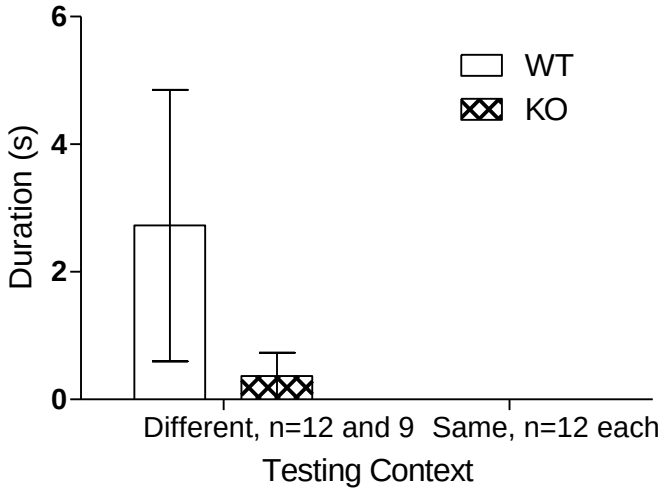


Figure 2.5: Time spent in aggressive encounters for GluA1 knockout (KO) and wildtype (WT) mice when tested in either the same context as habituation or a different testing environment.

Aggression was not affected by genotype or testing environment in this social interaction paradigm, possibly because overall levels of aggression were very low. Presented as mean ± SEM.

2.3.2. Tests of affect

The results for the tests of affect in the GluA1 knockout (KO) and their wildtype (WT) counterparts are summarised in Tables 2.2-4 and Figures 2.5-2.8. Univariate ANOVAs using genotype and sex as between-subject factors were used to compare sucrose preference ratio, water intake and sucrose intake in the sucrose preference test and immobility, swimming and climbing in the forced swim test (FST). Repeated measures ANOVAs were then used to compare the time spent immobile in the FST across the two days of the test using day as the within-subject factor and the same between-subject factors as above. A repeated measures ANOVA was also used to analyse locomotor activity across the diurnal cycle with time as the within-subject factor.

2.3.2.1. Sucrose preference

Sucrose preference (Table 2.2) was unaffected by genotype ($F_{(1,88)}$=0.175, p=0.677) or sex ($F_{(1,88)}$=1.982, p=0.163) and no interaction between these two factors was seen ($F_{(1,88)}$=1.530, p=0.219) which was also true for the raw intake of both water (effect of genotype $F_{(1,88)}$=0.016, p=0.900; effect of sex $F_{(1,88)}$=0.001, p=0.974; genotype*sex interaction $F_{(1,88)}$=1.089, p=0.299) and the sucrose solution (effect of genotype $F_{(1,88)}$=0.696, p=0.406; effect of sex $F_{(1,88)}$=0.227, p=0.635; genotype*sex interaction $F_{(1,88)}$=0.306, p=0.581).

Table 2.2: Results for GluA1 knockout (KO) and wildtype (WT) mice in the sucrose preference test

Test & Measure	**WT, n=45**	**KO, n=47**	**Statistics**
Sucrose preference	0.72 ± 0.02	0.73 ± 0.02	Geno $F_{(1,88)}$=0.175, p=0.677
			Sex $F_{(1,88)}$=1.982, p=0.163
			Int $F_{(1,88)}$=1.530, p=0.219
Water Intake (ml)	10.45 ± 0.87	10.31 ± 0.89	Geno $F_{(1,88)}$=0.016, p=0.900
			Sex $F_{(1,88)}$=0.001, p=0.974
			Int $F_{(1,88)}$=1.089, p=0.299
Sucrose Intake (ml)	26.28 ± 1.24	27.84 ± 1.27	Geno $F_{(1,88)}$=0.696, p=0.406
			Sex $F_{(1,88)}$=0.227, p=0.635
			Int $F_{(1,88)}$=0.306, p=0.581

Presented as mean ± SEM and analysed with univariate ANOVA.
Geno = effect of genotype, sex = effect of sex, int = genotype*sex interaction.

GluA1 knockout mice show no change in their preference for sucrose solution when compared to wildtype mice.

2.3.2.2. Locomotor activity across the diurnal cycle

Across a whole 23.5hr diurnal cycle (Figure 2.6) both genotypes were seen to alter their levels of activity (effect of time $F_{(46,4048)}$=49.273, p<0.001) and there was no overall difference between GluA1 KO and WT mice (effect of genotype $F_{(1,88)}$=0.930, p=0.338). These two factors, however, did interact ($F_{(46,4048)}$=2.012, p<0.001) due to the initial hyperactivity of the GluA1 KO mice (p<0.05 between 10am and 2pm, p>0.05 from 2.30pm onwards, see Table 2.3).

Sex also affected locomotor levels ($F_{(1,88)}$=11.041, p=0.001) with male mice typically moving more than female mice, this difference interacting with time ($F_{(46,4048)}$=3.284, p<0.001). Simple main effects reveals this to be due to this difference between the sexes only being seen during the first two hours and around the period of lights on (5am-8am, with lights on at 7am, see Table 2.4). Sex did not interact with genotype ($F_{(1,88)}$=2.621, p=0.338) and no three-way interaction was seen ($F_{(46,4048)}$=0.892, p=0.680).

2.3.2.3. Forced swim test

On day 1 of the FST (Figure 2.7) GluA1 KO mice spent less time immobile than their WT counterparts ($F_{(1,44)}$=42.751, p<0.001) which was driven by changes in swimming ($F_{(1,44)}$=40.282, p<0.001) but not climbing ($F_{(1,44)}$=1.340, p=0.253). Sex also altered the levels of immobility ($F_{(1,44)}$=8.377, p=0.006) with male mice (105.80s±11.50) spending more time immobile than female mice (65.97s±11.58). Again, this was driven by changes in swimming ($F_{(1,44)}$=5.264, p=0.027) but not climbing ($F_{(1,44)}$=3.500, p=0.068). This did not interact with genotype (immobility $F_{(1,44)}$=1.100, p=0.300; swimming $F_{(1,44)}$=2.178, p=0.147; climbing $F_{(1,44)}$=1.223, p=0.275).

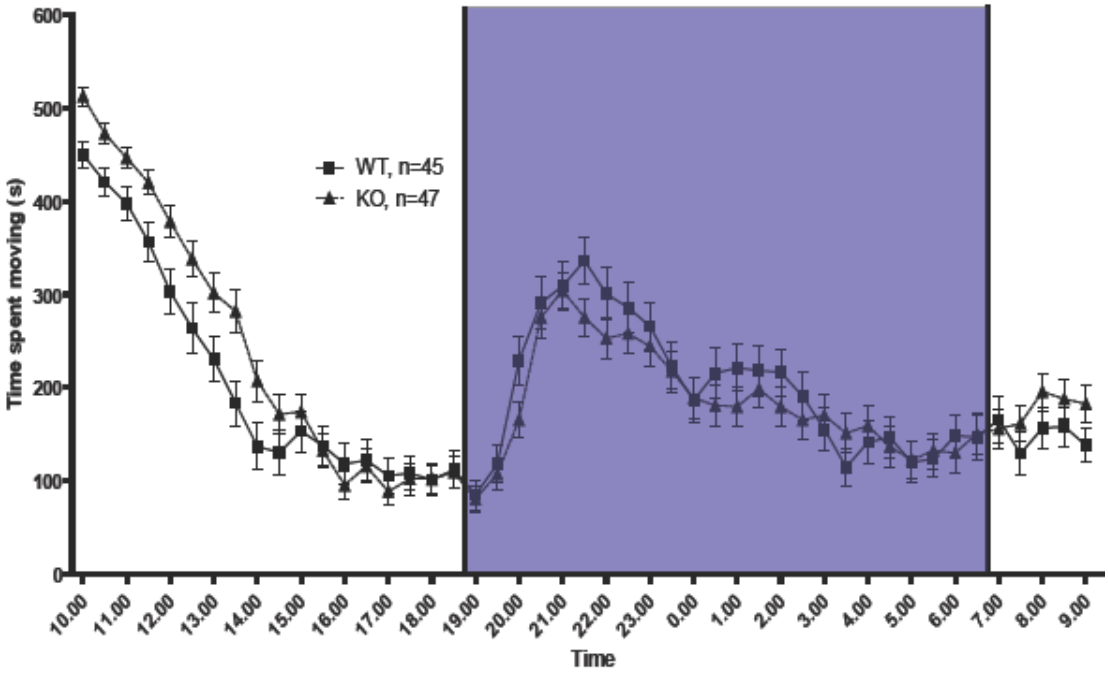


Figure 2.6: Locomotor activity for GluA1 knockout (KO) and wildtype (WT) mice over a 23.5 hour period

Overall levels of activity across the diurnal cycle did not differ between the GluA1 and wildtype mice, although the GluA1 mice did show initial hyperactivity when exposed to the novel testing environment. Shaded area = lights off period, 7pm-7am, presented as mean ± SEM in 47 30min bins.

Table 2.3: Simple main effect of genotype at each time point during the test of locomotor activity over the diurnal cycle in GluA1 knockout and wildtype mice

Time	$F_{(1,88)}$	p	Time	$F_{(1,88)}$	p
10am	**21.543**	**<0.001**	10pm	1.397	0.240
10.30am	**12.687**	**0.001**	10.30pm	0.554	0.459
11am	**7.363**	**0.008**	11pm	0.357	0.551
11.30am	**8.211**	**0.005**	11.30pm	0.031	0.860
12pm	**7.119**	**0.009**	12am	0.027	0.869
12.30pm	**5.597**	**0.020**	12.30am	0.771	0.382
1pm	**4.976**	**0.028**	1am	1.268	0.263
1.30pm	**9.159**	**0.003**	1.30am	0.176	0.676
2pm	**4.283**	**0.041**	2am	1.128	0.291
2.30pm	1.475	0.228	2.30am	0.480	0.490
3pm	0.399	0.529	3am	0.309	0.580
3.30pm	0.086	0.770	3.30am	2.361	0.128
4pm	0.987	0.323	4am	0.469	0.495
4.30pm	0.170	0.681	4.30am	0.056	0.813
5pm	0.747	0.390	5am	0.064	0.801
5.30pm	0.095	0.759	5.30am	0.257	0.613
6pm	0.002	0.962	6am	0.085	0.772
6.30pm	0.011	0.917	6.30am	0.200	0.656
7pm	0.093	0.761	7am	0.007	0.935
7.30pm	0.164	0.686	7.30am	2.310	0.132
8pm	3.190	0.078	8am	2.842	0.095
8.30pm	0.153	0.696	8.30am	1.331	0.252
9pm	0.010	0.919	9am	2.244	0.138
9.30pm	3.406	0.068			

GluA1 knockout mice are initially hyperactive but reach wildtype levels of activity at approximately 2.30pm.

Table 2.4: Simple main effect of sex at each time point during the test of locomotor activity over the diurnal cycle in GluA1 knockout and wildtype mice

Time	$F_{(1,88)}$	p	Time	$F_{(1,88)}$	p
10am	**35.338**	**<0.001**	10pm	**4.112**	**0.046**
10.30am	**28.946**	**<0.001**	10.30pm	2.147	0.146
11am	**10.850**	**0.001**	11pm	0.274	0.602
11.30am	**7.238**	**0.009**	11.30pm	0.023	0.880
12pm	**4.533**	**0.036**	12am	1.193	0.278
12.30pm	2.512	0.117	12.30am	2.408	0.124
1pm	0.162	0.688	1am	3.342	0.071
1.30pm	0.455	0.502	1.30am	**4.131**	**0.045**
2pm	0.002	0.965	2am	2.855	0.095
2.30pm	0.318	0.574	2.30am	1.970	0.164
3pm	0.397	0.530	3am	1.973	0.164
3.30pm	1.311	0.255	3.30am	8.668	0.004
4pm	2.564	0.113	4am	3.347	0.071
4.30pm	3.286	0.073	4.30am	1.198	0.277
5pm	3.320	0.072	5am	**4.760**	**0.032**
5.30pm	0.595	0.442	5.30am	**5.134**	**0.026**
6pm	0.797	0.375	6am	**12.321**	**0.001**
6.30pm	0.066	0.798	6.30am	**15.897**	**<0.001**
7pm	1.372	0.245	7am	**23.513**	**<0.001**
7.30pm	0.193	0.661	7.30am	**17.704**	**<0.001**
8pm	**24.760**	**<0.001**	8am	**12.429**	**0.001**
8.30pm	0.799	0.374	8.30am	3.739	0.056
9pm	0.513	0.476	9am	0.955	0.331
9.30pm	0.052	0.820			

Male mice are initially hyperactive and become more active than female mice again towards the end of the dark cycle.

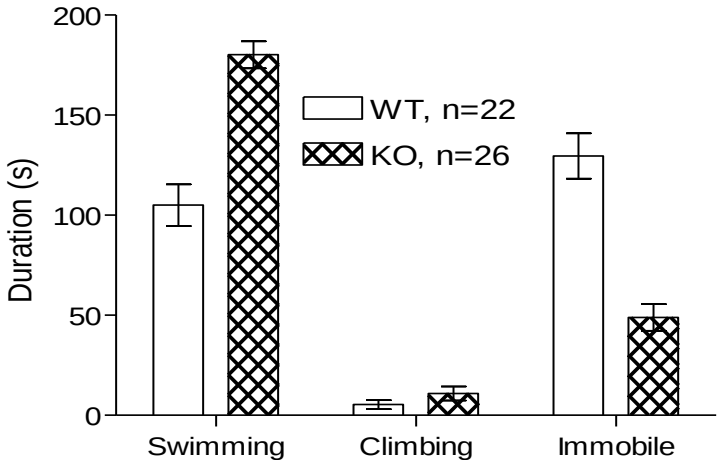


Figure 2.7: Time spent swimming, climbing and immobile in minutes 3-6 on day 1 of the forced swim test by GluA1 knockout (KO) and wildtype (WT) mice

GluA1 knockout mice showed a decrease in immobility on day 1 of the forced swim test relative to their wildtype counterparts. This was due to an increase in time spent swimming but not climbing. Presented as mean ± SEM.

On day 2 of the FST (Figure 2.8), no effect of genotype was seen on immobility ($F_{(1,44)}$=1.200, p=0.279). Swimming and climbing were also unaffected (swimming $F_{(1,44)}$=0.706, p=0.405; climbing $F_{(1,44)}$=3.006, p=0.090). Sex had no effect on these measures, either (immobility $F_{(1,44)}$=0.029, p=0.866; swimming $F_{(1,44)}$=0.027, p=0.870; climbing $F_{(1,44)}$=0.808, p=0.373), and no genotype*sex interactions were seen (immobility $F_{(1,44)}$=0.748, p=0.392; swimming $F_{(1,44)}$=1.617, p=0.210; climbing $F_{(1,44)}$=2.838, p=0.099).

Due to an effect of genotype being present on day 1 but not on day 2, levels of immobility were compared across these days using a repeated measures ANOVA (Figure 2.9). Across the two days, levels of immobility increased (effect of day $F_{(1,44)}$=64.266, p<0.001). GluA1 KO mice also showed less immobility (effect of genotype $F_{(1,44)}$=17.725, p<0.001) and an interaction between day and genotype was seen ($F_{(1,44)}$=18.388, p<0.001). Simple main effects revealed an effect of day in both genotypes (WT $F_{(1,44)}$=6.409, p=0.015; GluA1 KO $F_{(1,44)}$=82.690, p<0.001) but, as shown in the previous analysis, a difference in the genotypes was only seen on day 1 ($F_{(1,44)}$=42.751, p<0.001) not on day 2 ($F_{(1,44)}$=1.200, p=0.279). Sex did not have an overall effect (effect of sex $F_{(1,44)}$=2.749, p=0.104) and did not interact with genotype ($F_{(1,44)}$=1.264, p=0.267) but did interact with day ($F_{(1,44)}$=4.907, p=0.032). No three way interaction was seen ($F_{(1,44)}$<0.001, p=0.996).

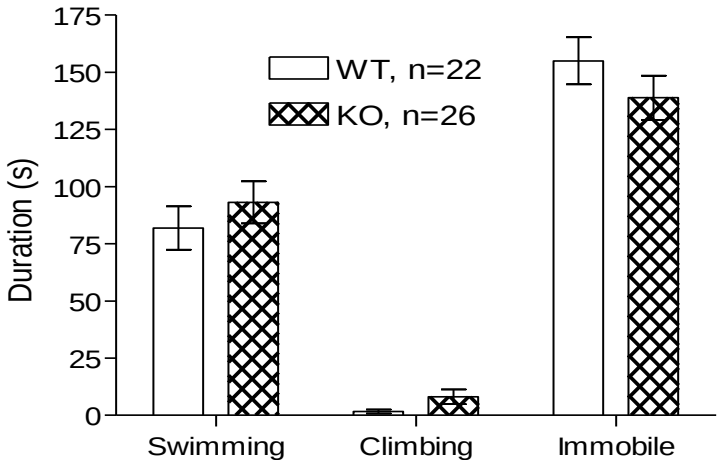


Figure 2.8: Time spent swimming, climbing and immobile in minutes 3-6 on day 2 of the forced swim test by GluA1 knockout (KO) and wildtype (WT) mice

GluA1 knockout mice do not show any changes in the amount of time spent swimming, climbing or immobile on day 2 of the forced swim test relative to their wildtype counterparts. Presented as mean ± SEM.

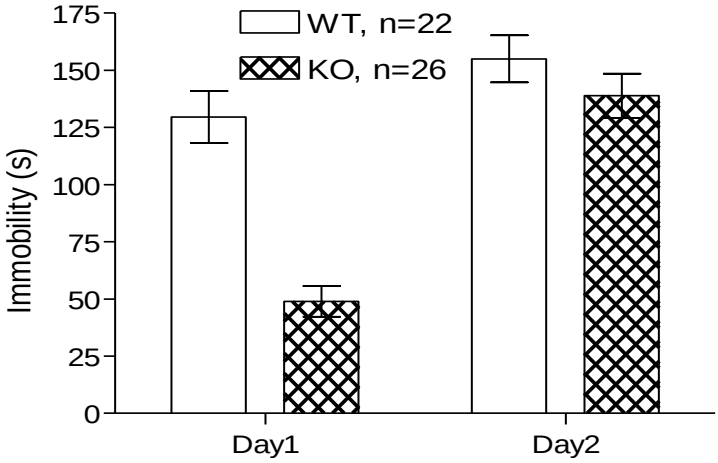


Figure 2.9: Time spent immobile in minutes 3-6 on days 1 and 2 of the forced swim test by GluA1 knockout (KO) and wildtype (WT) mice

GluA1 knockout mice show a decrease in time spent immobile on day 1 of the forced swim test relative to their wildtype counterparts but not on day 2. This is due to a preferential increase in levels of immobility from day 1 to 2 in the knockout mice. Presented as mean ± SEM.

2.4. Discussion

2.4.1. Summary of results

This chapter aimed to assess the GluA1 knockout (KO) mice on tests of emotionality. Male and female GluA1 KO mice were compared to their wildtype (WT) littermates in several behavioural tests. This revealed subtle changes in the behaviour of the GluA1 KO mice in these tests that may further reflect the alterations in habituation observed previously in these mice (Sanderson *et al.*, 2007, Sanderson *et al.*, 2009).

2.4.1.1. Anxiety

GluA1 KO mice showed no overall change in the elevated plus maze (EPM), although a genotype*sex interaction showed that female GluA1 KO mice spent more time in the open arm than female WT mice indicating a possible reduction in anxiety. This is in contrast to previous results showing GluA1 KO mice to have increased anxiety in this test (Mead *et al.*, 2006). However, the increased time spent in the open arms seen here may be explained by an overall increase in exploration given that the same type of interaction is seen in distance travelled during this task, with female GluA1 KO mice moving more than other groups.

GluA1 KO mice also showed a reduction in social and aggressive encounters only when tested in a novel environment but not after extensive habituation to the testing arena. Both this finding and the increased locomotion in the EPM may reflect a reduced ability in the GluA1 KO mice to habituate to spatial environments. This would lead to increased exploration of the given area as it would remain relatively novel for a longer period of time as compared to WT mice. This is in keeping with

previous observations of an elevated response to novelty but a normalisation of behaviour of locomotor activity following habituation by the GluA1 KO mice (Wiedholz *et al.*, 2008) and directly explains the increased movement in the EPM. It may also explain the reduced social interaction in a relatively-novel environment if spatial- and social-exploration can only occur exclusively from one another, in competition; the extra time the GluA1 KO mice spend exploring the environment is time they do not spend socially interacting. If then extensively habituated, however, this balance is restored to being similar to WT mice. That the second social interaction experiment showed that GluA1 KO mice interact more when in an environment in which they have been habituated may represent a failure to habituate to the novel mouse, further demonstrating this habituation deficit. This reduced habituation processing has been observed previously in these mice by employing tasks reliant on exploration of novel spatial environments (Sanderson *et al.*, 2007, Sanderson *et al.*, 2009). Alternatively, the long-term habituation to the context may be enhanced in the GluA1 KO mice as compared to WT mice and in contrast to the GluA1 KO mice's impaired short-term habituation. This dichotomy of short- and long-term habituation processing in the GluA1 KO mice has been noted previously in spatial memory tasks (Sanderson *et al.*, 2009). This would also result in reduced spatial exploration and increased social interaction in the GluA1 KO mice in the familiar environment.

In summary, while GluA1 KO mice did appear to show alterations in these tests of anxiety under certain conditions, these changes may be the result of habituation problems and not an indication of anxiety levels in these mice *per se*.

2.4.1.2. Affect

GluA1 KO mice showed no difference compared to WT mice in their preference for sucrose and appear hyperactive initially in the assessment of circadian activity but this is otherwise unchanged. This initial hyperactivity, again, may be accounted for by the GluA1 KO mice's exaggerated response to novelty due to impaired habituation processing (Sanderson *et al.*, 2007, Sanderson *et al.*, 2009) and is in agreement with previous findings that the GluA1 KO mice are not hyperactive in familiar environments (Wiedholz *et al.*, 2008).

In the forced swim test (FST), similar to the results in social interaction, GluA1 KO mice showed altered behaviour on first exposure but not when re-exposed. In this case, the GluA1 KO mice show reduced immobility on day 1 but not on day 2. This would usually be interpreted as indicative of a transient anti-depressive phenotype. While this may be confounded by the hyperlocomotion seen in these mice, general levels of activity and levels of immobility in the FST are not necessarily correlated (eg Trullas and Skolnick, 1990, Maj *et al.*, 1992a, Layer *et al.*, 1995, Przegalinski *et al.*, 1997). The increase in immobility over the two days may imply learning across the two days and that these mice can learn something about aversive stimuli. However, whether this is altered as compared to WT mice is difficult to ascertain in this task due to the differing behaviour between these groups on day 1. This may have otherwise informed upon the previous finding that GluA1 KO mice have exaggerated learned helplessness (Chourbaji *et al.*, 2008). Indeed, Chourbaji and colleagues imply the GluA1 KO mice show a more depressive-like phenotype, the opposite of the results reported here using the FST.

These previous results, however, may be confounded due to the associative learning component of the learned helplessness task which pairs footshocks with a specific compartment of a shuttle box. The GluA1 KO mice were observed to take longer to exit this compartment on subsequent testing, taken as an indicator of helpless behaviour. However, this could alternatively be explained by a weaker association being formed between the aversive stimulus and the punctate and contextual cues for this shock. This idea is supported by the fear conditioning deficit observed in the GluA1 KO mice in a number of different paradigms (Feyder *et al.*, 2007, Humeau *et al.*, 2007) as well as deficits in other conditioning paradigms (Mead and Stephens, 2003, Johnson *et al.*, 2005, Mead *et al.*, 2006). The results in the learned helplessness task, therefore, do not necessarily conflict with those in the FST despite these tests seemingly showing an increase and decrease in helplessness, respectively. This contrast may, in fact, highlight that it is an alteration in associative learning that underlies the changes in learned helplessness and are not, in this case, a reliable measure of mood.

2.4.1.3. The GluA1 knockout mouse phenotype

The GluA1 KO mice show some altered phenotypes that relate to emotionality such as reduced social interaction and decreased immobility in the FST. However, performing the social interaction in a more familiar environment and re-exposure to the FST removes these differences. These results may therefore add to the growing literature that GluA1 KO mice require a longer period of time to habituate to novel stimuli and environments as compared to WT mice. They provide some evidence for an acute antidepressant phenotype although the novel-context-specificity of these effects makes it unlikely that this is truly the explanation for the alterations seen.

2.4.2. Limitations and future directions

The hyperactivity in response to novelty noted previously in these animals and observed in the threshold system may act as a confound in the tests performed here. Indeed, it could explain both a decrease in social interaction as well as a reduction in immobility in the FST. That these deficits are not seen following habituation to the tasks also fits with the hyperactivity of these mice only being seen transiently following exposure to a novel stimulus or environment which can again be seen in the data from the threshold system. This hyperactivity may therefore be representative of a more general problem with habituation processing, while the phenotypes seen here may represent other facets of this altered psychological process. This possible confound may therefore provide an explanation of the transient nature of the deficits seen as well as the nature of the alterations seen.

2.4.3. Implications for previous findings

As discussed in Section 2.4.1.2, the apparent anti-depressive phenotype seen here in the FST appears to be at odds with previous reports of a depressive phenotype in the GluA1 KO mice in the learned helplessness paradigm (Chourbaji *et al.*, 2008). The increased latency to escape in the learned helplessness task does have the advantage over the FST of opposing the possible confound of hyperactivity in these mice. However, observations that the GluA1 KO mice have a deficit in both cue and contextual fear conditioning (Feyder *et al.*, 2007, Humeau *et al.*, 2007) may give an alternative explanation to this alteration in learned helplessness. Both of these tasks rely upon an association being established between an aversive stimulus (i.e. a footshock) and a cue and/or spatial location. GluA1 KO mice appear to be impaired at this form of associate learning in both learned helplessness and fear conditioning.

This may in turn be the result of altered long-term potentiation (LTP) in these mice (Zamanillo *et al.*, 1999, Humeau *et al.*, 2007), thought to underlie many forms of learning and memory. The GluA1 KO mice have provided much information on the subtleties of this association between neuroplasticity and learning. The previous result using learned helplessness may therefore be seen as a part of this literature and represent weakened associative learning in the GluA1 KO mice rather than an alteration in emotionality *per se*.

The antidepressant effect seen in the FST is also contrary to the increase in GluA1 expression (Martinez-Turrillas *et al.*, 2002, Du *et al.*, 2004) and functionality (Svenningsson *et al.*, 2002) following antidepressant treatment as well as drugs that increase AMPA receptor activity ("AMPAkines", Partin *et al.*, 1996, Baumbarger *et al.*, 2001) giving antidepressant effects (Bai *et al.*, 2001, Li *et al.*, 2001a, Li *et al.*, 2003). There is also evidence for decreased expression of GluA1 in the perirhinal cortices of depressed patients (Beneyto *et al.*, 2007). This apparent correlation between AMPA receptor-mediated neurotransmission and mood makes the present result surprising, although the lack of GluA1-specific drugs does not allow for further investigation. It may be that developmental compensation has occurred in the GluA1 KO mice and is responsible for the contradiction of these results with those produced following acute pharmacological interventions. However, there is little evidence for changes in other glutamate receptor subunits in these mice (Zamanillo *et al.*, 1999). This idea of developmental alterations also does not take into account the changes seen *post mortem* in patients which are in contrast to the results obtained in these mice (Beneyto *et al.*, 2007). Beneyto and colleagues found a reduction in GluA1 in the brains of those with depression, whereas the present results imply a reduction in GluA1 results in an antidepressive phenotype. The changes seen in the *post mortem*

human study may have been life-long and, indeed, present from inception as is the reduction in these mice that would allow for neurodevelopmental shifts to occur and compensate for this change. If a reduction in GluA1 did result in compensatory changes, they would be expected to give the same behavioural results in both mice and humans, not what is seen here. It is difficult, therefore, to reconcile the results obtained on day 1 of the FST with histological studies on the effects of depression and antidepressants on the GluA1 subunit.

Further to this, if the expected depressive phenotype had been observed, it would have been of interest to investigate the importance of GluA1 phosphorylation in antidepressant activity (Svenningsson *et al.*, 2002). This enhancement of AMPA receptor-mediated neurotransmission is integral to the idea of AMPAkines as potential novel antidepressants and therefore an important cornerstone in the literature on the role of glutamate in emotionality. The current results, however, do not offer a baseline from which to investigate antidepressant effects.

2.4.4. Conclusions

The GluA1 mice may offer some alterations in behaviour representative of altered emotionality. However, these changes are ameliorated by extensive habituation or re-exposure to the tests. These altered behaviours may therefore represent other aspects of a reduced ability for these mice to habituate to novel stimuli reported previously. This alternative explanation, coupled with the transient nature of these changes, precludes more in-depth investigation into emotionality in these mice.

CHAPTER 3: Study of naturalistic behaviour, cognition and emotionality in the GluN1 hypomorph mice

3.1. Introduction

3.1.1. NMDA receptors

NMDA receptors are tetrameric[8] protein complexes that have a number of binding sites for agonists, antagonists and modulators of the receptor's activity. The major sites are the binding site for glutamate, the co-agonist "glycine" site, the modulatory polyamine site and three intra-channel sites that predominately bind Mg^{2+}, Zn^{2+} and non-competitive antagonists (the "PCP" site), respectively. There are further sites that respond to, for instance, protons and redox agents. The channel properties and pharmacology of NMDA receptors depend on the heteromeric construction of the final functional receptor. The current theory of NMDA receptor construction is that they consist of a dimer of homodimers, one dimer consisting of two GluN1 subunits, the other of two GluN2 or 3 subunits (Schorge and Colquhoun, 2003).

3.1.1.1. NMDA receptor subunits

As with all ionotropic glutamate receptor subunits, the proteins that make up an NMDA receptor contain three transmembrane domains (TM1-3) with a re-entry loop also present between TM1 and 2 (see Figure 1.1 in Section 1.3.2.1, page 28). This loop results in an extracellular N-terminus region and an intracellular C-terminus region. Interactions between a sequence towards the N-terminus region (S1) and part of the

[8] Some older papers refer to NMDA receptors as being pentameric as there was a long period of time when theories of glutamate receptors being composed of four or five subunits were both quite prevalent. Current thinking favours a tetrameric structure, although this remains contentious for some.

extracellular loop between TM2 and TM3 (S2) form the binding regions for the agonist and co-agonist sites. These two sites are found to be present on the GluN2 and GluN1 sub-types of subunit, respectively. Unlike AMPA receptor subunits, all NMDA receptor subunit proteins have an asparagine (N) residue at the Q/R site of RNA editing (which contributes to the NMDA receptor's permeability to Ca^{2+}) and no flip/flop variants. Nonetheless, diversity within the system is achieved through multiple subunits forming heteromeric receptor complexes and splice variants, predominantly within the GluN1 subunit.

3.1.1.1.1. GluN1

The GluN1 subunit is considered the constitutive subunit for NMDA receptor construction; without it, no functional NMDA receptor can be formed due to its role in the trafficking of the receptor complex and the co-agonist binding site formed by the S1 and S2 loops. Additionally, functional homomeric GluN1 channels can be formed *in vitro* (channels which only respond to glycine, not glutamate) which is not the case for other NMDA receptor subunit families. The N-terminus region is important in forming GluN1 homodimers (Meddows *et al.*, 2001) and contains modulatory sites for polyamines. Only one gene exists in this family (grin1) of 22 exons that results in the GluN1 protein. However, eight splice variants of the GluN1 subunit exist due to alternate splicing of exons 5, 21 and 22 of the gene. Exon 5 gives rise to the N1 cassette within the protein which is either present or absent, exon 21 the C1 cassette, again either present or absent. Exon 22 gives rise to the C2 cassette. Splice variants that do not translate this exon express a novel 66 base pair cassette at the C-terminus often indicated as C2'. The nomenclature for these various splice

variants is yet to be standardised[9] but is often given by a number and letter suffix. For instance, GluN1-1 is used for clones with both C1 and C2, -2 for those with C2 but not C1, -3 for those with C1 and C2′ and -4 for those with no C1 and C2′. An a or b is then added to indicate the absence or presence of the N1 cassette, respectively, resulting in, for instance, GluN1-1b being the full-length splice variant and GluN1-4a being the variant with only the truncated C2′ cassette and no N1 or C1 cassette present (Holemans *et al.*, 1993, Dingledine *et al.*, 1999)[10]. Despite this, GluN1-1a is often referred to as the "full length" form of the gene as it is the most abundant, expression of the exon 5-mediated N1 cassette being seen as an insertion into this protein.[11] Table 3.1 gives further examples of common nomenclature systems for these splice variants. These splice variants have an important role in determining NMDA receptor properties. This includes the role of the N1 cassette in determining agonist sensitivity and reactivity to polyamine modulation (Durand *et al.*, 1993) and the role of C1 in initiating downstream processing following activation of the NMDA receptor (Bradley *et al.*, 2006).

[9] This was sadly outside the remit of the Collingridge *et al*., 2009 report referenced above.
[10] These references use the noted suffixes with the older NR1 protein name as opposed to the more recent GluN1. GluN1 is used in the text here with these suffixes to maintain consistent protein names within this thesis but to give an example of splice variant naming. This does not suppose that these suffixes should become the standardised nomenclature for these splice variants. It does, hopefully, highlight the need for such a standardised nomenclature to exist, however.
[11] This also leads to confusing inconsistencies even within papers. For instance, compare the textual and diagrammatic explanations of these splice variants on page 16 of Dingledine *et al*., 1999, an authoritative review on this area as a whole.

Table 3.1: A summary of three prominent nomenclature systems for indicating GluN1 splice variants based on cassette presence (+) or absence (-)

Cassette			Suffix		
N1	**C1**	**C2**	**Hollmann *et al*, 1993**	**Sugihara *et al*, 1992**	**Durand *et al*, 1993**
-	+	+	-1a	A	$_{011}$
+	+	+	-1b	B	$_{111}$
-	-	+	-2a	C	$_{001}$
+	-	+	-2b	F	$_{101}$
-	+	-	-3a	D	$_{010}$
+	+	-	-3b	H	$_{110}$
-	-	-	-4a	E	$_{000}$
+	-	-	-4b	G	$_{100}$

This table demonstrates the inconsistent naming across three different systems for indicating GluN1 splice variants which would give examples such as GluN1-1a, GluN1A and GluN1$_{011}$ for the same protein, respectively. Adapted from Yamakura and Shimoji, 1999.

3.1.1.1.2. GluN2

The binding site for glutamate is found in the S1-S2 complex of the GluN2 subunit of the NMDA receptor that co-assembles with the GluN1 subunits. Four different GluN2 subunits exist, GluN2A-D, from four separate genes (grin2a-d, respectively). Which of these subunits is present can drastically alter the sensitivity of the receptor and the transmission properties of the channel pore. For instance, the binding of drugs such as ifenprodil, an antagonist to the polyamine binding site, are found to bind predominantly to the GluN2B subunit (Perin-Dureau *et al.*, 2002) whereas the extracellular binding site for Zn^{2+} appears to lie on the GluN2A subunit (Paoletti *et al.*, 2000). This illustrates how the presence of different GluN2 subunits can alter the NMDA receptor's sensitivity to certain forms of modulation. More striking, the final receptor's sensitivity to glutamate can also differ drastically based on which GluN2 subunit is present, with GluN1/N2A receptors showing an EC_{50} of approximately 1.7μM (Kutsuwada *et al.*, 1992), GluN2B- and GluN2C-containing receptors being around 0.7-0.8μM (ibid.) but GluN2D-containing receptors having an EC_{50} of approximately 0.4μM (Ikeda *et al.*, 1992), four-fold more sensitive than GluN2A-containing receptors. These GluN2D-containing receptors also appear to show little to no desensitisation to the continued presence of glutamate.

This form of variability appears to be important in the spatial and temporal specificity of NMDA receptor function. While NMDA receptors are widely expressed across the whole brain, particularly in cortical and cerebellar regions, the expression of specific GluN2 subunits alters depending on the area of the brain being investigated. For instance, GluN2C is the predominant subunit seen in the cerebellum whereas GluN2A is more abundant in neocortical regions. More striking,

however, is the developmental switch from high expression of GluN2B and D subunits during development to the more adult forms of A and C (Akazawa *et al.*, 1994, Monyer *et al.*, 1994). These studies also show specificity between cell types such as between pyramidal and interneurones within the hippocampus. There even appears to be evidence for specific expression at an ultrastructure level, with GluN2D-containing receptors only being expressed extrasynaptically (Misra *et al.*, 2000). These examples illustrate how the diverse roles played by the NMDA receptor can be fulfilled through the molecular multiplicity possible through differential expression of differing subunits.

3.1.1.1.3. GluN3

The GluN3 family of subunits is a relatively recent discovery and appear to be heavily expressed during development but are only expressed in very low levels in the adult rodent brain (Ciabarra *et al.*, 1995, Sucher *et al.*, 1995, Wong *et al.*, 2002). Its presence during development in humans has also been shown (Eriksson *et al.*, 2002). They appear to form glycine-sensitive receptors, not glutamate, their glycine binding sites being distinct from those on the GluN1 subunits. GluN1-GluN3 channels, however, are still cation-selective, resulting in excitatory glycine receptors (Chatterton *et al.*, 2002). *In vivo*, GluN3 subunits also co-assemble with a GluN2A subunit and the obligatory GluN1 homodimer. (It should be noted that triheteromeic structures have also been noted containing only GluN1 and two forms of GluN2, although these appear to be in the vast minority). The pharmacology of such receptors, as well as their functional role, is yet to be elucidated. Indeed, very little is known about the GluN3 family of subunits to date. However, they do appear to reduce the overall activity of the NMDA receptors that contain them which acts to

protect against excitotoxicity (Nakanishi *et al.*, 2009). This may account for their heavy expression during development.

3.1.1.2. GluN1 in emotionality

3.1.1.2.1. GluN1 in anxiety

There is strong evidence for a role for NMDA receptors in anxiety, most of which comes from studies using NMDA receptor antagonists as possible anxiolytic agents. Several competitive antagonists have been shown to be effective in rodents. 3-(2-carboxy piperazine-4yl)-propyl-1-phosphonic-acid (CPP) has been shown to be anxiolytic in the EPM and to increase social interaction in rats at doses of 1-5mg/kg with no overall reduction in locomotor activity (Dunn *et al.*, 1989, Corbett and Dunn, 1993). At 10mg/kg, generalised sedative effects begin to confound such tests (Corbett and Dunn, 1991, 1993). CGP 37849 has been shown to be anxiolytic in the EPM at doses of 0.1 and 1mg/kg (i.p) with no general motor changes (Plaznik *et al.*, 1994). 3mg/kg of the related compound CGP 39551 is anxiolytic in the EPM without producing generalized locomotor effects (Karcz-Kubicha *et al.*, 1997, but see also the study by Plaznik and colleagues, 1994, where 5mg/kg did not produce an effect in the EPM using Wistar rather than Sprague-Dawley rats). Both AP5 and AP7, also competitive antagonists, showed anxiolytic effects in rats on the EPM at 30 and 60mg/kg and 20 and 40mg/kg i.p, respectively. They also both increase social interactions at 40 and 60mg/kg and 20 and 40mg/kg, respectively, although 160mg/kg ip of AP7 appears to be required to produce an anxiolytic effect in mice (Stephens *et al.*, 1986). No generalized locomotor activity is presented in this study to account for possible confounds at this high dose. An increase in social interaction

following AP5 has also been seen when infused locally into the basolateral amygdala (Sajdyk and Shekhar, 1997). Other studies have shown intracerebral (icv) injections of AP7 into rats to be effective in the EPM, although 5µg produces profound ataxia (Plaznik *et al.*, 1994). AP5 has also been infused locally into the dorsal and ventral hippocampus in a two-trial version of the EPM in rats (Nascimento Hackl and Carobrez, 2007). This revealed an anxiolytic effect of 6 and 24nmol only when infused into the ventral hippocampus prior to the first exposure. No effect of drug in the EPM was seen with AP7 when locally infused into the dorsal hippocampus (Padovan *et al.*, 2000). This provides evidence for a role for NMDA receptors in the ventral but not dorsal hippocampus in anxiety.

Antagonists acting at the glycine site of the NMDA receptor complex have also been shown to have anxiolytic effects in rodents including 7-chlorokynurenic acid (7 CKA, Trullas *et al.*, 1989, Anthony and Nevins, 1993), 5,7-dichlorokynurenic acid (5,7 DCKA, Kehne *et al.*, 1991, Corbett and Dunn, 1993, Plaznik *et al.*, 1994) and L-701,324 (Karcz-Kubicha *et al.*, 1997, Kotlinska and Liljequist, 1998, Przegalinski *et al.*, 1998) as well as other experimental compounds (Kehne *et al.*, 1995, Wiley *et al.*, 1995). Additionally, ifenprodil, an antagonist that acts at the polyamine site of the NR2B subunit of the NMDA receptor complex, has also been shown to be effective in mice in the EPM (Fraser *et al.*, 1996). Memantine, too, shows anxiolytic effects in mice (Minkeviciene *et al.*, 2008). These results suggest that it is not only direct antagonism that can be effective in producing the desired anxiolytic effects.

MK-801, an uncompetitive antagonist to NMDA receptors, has been shown to be an effective anxiolytic in the EPM in both rats (Dunn *et al.*, 1989, Corbett and Dunn, 1991, 1993, Criswell *et al.*, 1994, Plaznik *et al.*, 1994, Karcz-Kubicha *et al.*, 1997, Bertoglio and Carobrez, 2003, Wieronska *et al.*, 2003, Engin *et al.*, 2009) and mice (Fraser *et al.*, 1996) and increases rat social interaction (Dunn *et al.*, 1989, Corbett and Dunn, 1991, 1993) at doses that do not effect locomotor activity. Other non-competitive antagonists of the NMDA receptor have also been shown to be effective anxiolytics in rodents, such as phencyclidine (PCP) in mouse EPM (Wiley *et al.*, 1995). Ketamine, however, shows anxiogenic effects in rats by both decreasing time spent in the open arms of the EPM and decreasing social interaction (Silvestre *et al.*, 1997, but see also Engin *et al.*, 2009). This effect in the EPM has also been shown following chronic (35 day) oral voluntary consumption (Silvestre *et al.*, 2002), although no effect was seen when testing weeks after five daily injections of 30mg/kg i.p (Becker *et al.*, 2003), suggesting the effects may not be long-lasting. Ketamine did appear to act as an anxiolytic in mice on the EPM, however (Hayase *et al.*, 2006). Interestingly, ketamine in healthy volunteers at 0.1mg/kg i.v appears to decrease levels of anxiety but at 0.5mg/kg increases it transiently. This may, of course, be confounded by the wide-ranging effects ketamine has, including the induction of dissociative feelings (Krystal *et al.*, 1994). These results do, however, illustrate that the role NMDA receptors play in anxiety may not be simple and that effects at this receptor may always be confounded.

3.1.1.2.2. GluN1 in depression

There is also some evidence for a role for NMDA glutamate receptors more directly in depression and antidepressant treatment (for a review, see Petrie *et al.*, 2000). Binding and expression studies performed on *post mortem* human prefrontal cortex suggest there is no overall change in the expression of functional NMDA receptors in suicide victims (Palmer *et al.*, 1994, Nowak *et al.*, 1995) or confirmed cases of depression (Holemans *et al.*, 1993, Toro and Deakin, 2005, Feyissa *et al.*, 2009). More subtle alterations in the molecular makeup of NMDA receptors in depression may occur, however (Nowak *et al.*, 1995, Feyissa *et al.*, 2009). This is based on the lack of evidence for any alteration in binding of labelled MK-801 which binds to the channel pore of the receptor at the PCP site (Holemans *et al.*, 1993, Palmer *et al.*, 1994, Nowak *et al.*, 1995, Feyissa *et al.*, 2009), but, at the same time, changes in binding to the glycine site (Nowak *et al.*, 1995) and expression of GluN2 subunits, as well as post synaptic density (PSD) protein 95, an anchor protein for the NMDA receptor (Feyissa *et al.*, 2009), are seen. This suggests the functionality and channel properties of NMDA receptors may be altered but without any overall change in the number of these receptors.

Interestingly, one study did find reductions in GluN1 expression levels, as well as reduced GluN2A, in the dorsolateral prefrontal cortex of depressed samples, but no change in NMDA receptor binding properties (Beneyto and Meador-Woodruff, 2008). This indicates a discrepancy between overall expression levels and the formation of functional receptor complexes on the surface of neurones. Alterations in GluN2 subunit composition are also seen elsewhere in the brains of depressed patients such as the locus coeruleus (Karolewicz *et al.*, 2005), the lateral amygdala

(Karolewicz *et al.*, 2009) and perirhinal cortex (Beneyto *et al.*, 2007). While these changes will influence the transmission properties of NMDA receptors, they offer little indication for the role of GluN1 specifically and gross changes in NMDA receptor levels generally in the pathogenesis of depression. However, there is stronger evidence for a role for NMDA receptors in antidepressant treatment.

Several studies have shown that tricyclic antidepressants bind to NMDA receptors and act as antagonists, although the site to which they bind is debated (Reynolds and Miller, 1988, Sernagor *et al.*, 1989, Sills and Loo, 1989, White *et al.*, 1990, Cai and McCaslin, 1992). Chronic treatment with several antidepressants has also been shown to alter the NMDA receptor's binding properties for the endogenous ligands at both the glutamate and glycine sites (Kitamura *et al.*, 1991, Nowak *et al.*, 1993, Paul *et al.*, 1993, Paul *et al.*, 1994). That these alterations are seen following almost all treatments, including all classes of pharmacological antidepressants, as well as following electroconvulsive shock, suggests this is an important common mechanism of successful treatment (Skolnick *et al.*, 1996). These findings have led to the investigation of direct NMDA receptor antagonism as a novel antidepressant mechanism.

3.1.1.3. NMDA receptor antagonists as antidepressants

3.1.1.3.1. Preclinical evidence

As detailed above, NMDA receptor antagonists have been shown to be potential anxiolytics. The blockade of NMDA receptors has also been investigated as a possible target for novel antidepressant treatment using several different compounds. Several experimental NMDA receptor antagonists have been shown to have effects in rodent models of antidepressant activity acutely following a single injection. These tests typically rely upon measuring locomotion in stressful situations and have therefore been plagued with the issue of false positives due to general increases in activity (Trullas and Skolnick, 1990, Maj *et al.*, 1992b, Panconi *et al.*, 1993, Engin *et al.*, 2009). There are, however, several studies that have found effects in emotionality tests without these confounding changes.

A decrease in immobility in the forced swim test (FST) without a generalised increase in locomotor activity was seen using the competitive antagonist dl-(E)-2-amino-4-methyl-5-phosphono-3-pentenoic acid (CGP 37849) in Wistar rats at doses ranging from 0.31-5.0mg/kg intraperitoneal (i.p.) and 0.03 and 0.1µg intrahippocampal (i.hp.) infusion (Przegalinski *et al.*, 1997). 10mg/kg i.p. CGP 37849 has also been shown to reduce immobility in the FST while decreasing general locomotor activity at the lower dose of 5 mg/kg in Wistar rats, 2.5mg/kg being sufficient to reduce immobility in Swiss mice with no overall change in locomotor activity (Maj *et al.*, 1992a). These results again show a dissociation between doses able to lower immobility levels in the FST and those that lead to overall hyperactivity. Maj and colleagues (1992a) also found the related compound, CGP 39551, had similar selective effects in Wistar rats, but failed to see an antidepressive effect at 20mg/kg

in Swiss mice. Yet another experimental compound, L-701,324 which acts as an antagonist at the glycine site, has also been shown to decrease immobility time in the FST without effecting overall locomotor activity in rats (Przegalinski *et al.*, 1998). Trullas and Skolnick (1990) found 100mg/kg i.p. of another competitive antagonist, amino-7-phosphonoheptanoic acid (AP7) gave an antidepressive effect in the FST using NIH-Swiss mice, again with no effect of locomotion in an open field. There is also some suggestion that injection of AP7 into the dorsal hippocampus can attenuate stress-induced increases in immobility in the FST (Padovan and Guimaraes, 2004).

Eliprodil, an antagonist to the ifenprodil/polyamine site of the NMDA receptor complex, has also been shown to have antidepressive effects in mice in the FST at 20mg/kg while not increasing overall activity (Layer *et al.*, 1995). However, when given to C57Bl/6 mice tested in the tail suspension test of behavioural despair it was found to have no effect (ibid.).

The chronic mild stress (CMS) paradigm has been used by Papp and Moryl to investigate the effect of chronic treatment with NMDA receptor antagonists on a rodent model thought to have more face validity than the tests mentioned above (Willner *et al.*, 1987, Willner *et al.*, 1992, but see also Forbes *et al.*, 1996). Work by this group has found antidepressive effects specifically in stressed animals with chronic MK-801, a non-competitive antagonist that binds at the PCP site (Papp and Moryl, 1993, 1994), 1-aminocyclopropanecarboxylic acid (ACPC, Papp and Moryl, 1996), which binds at the glycine site, and CGP 37849 and 40116 (Papp and Moryl, 1994), both competitive antagonists at the glutamate site, in the sucrose consumption measure associated with CMS.

Ketamine, a non-competitive antagonist that binds at the PCP site, has been found to have an effect in the FST at doses that do not effect overall locomotor activity (Hayase *et al.*, 2006, Engin *et al.*, 2009). Other studies have also found this antidepressive effect using lower doses of 5 (Rosa *et al.*, 2003) and 1mg/kg (Mantovani *et al.*, 2003) i.p ketamine, but do not explicitly measure overall locomotor activity. Larger, anaesthetic doses have also been shown to have a lasting, subsequent antidepressive effect in the FST (Yilmaz *et al.*, 2002). A striking antidepressant effect with ketamine has now also been demonstrated in patients with (otherwise) treatment resistant depression.

3.1.1.3.2. Potential clinical application

Work has now started in investigating the effects of ketamine in humans as an antidepressant. Principle amongst this literature are two studies using a single intravenous (i.v.) infusion of ketamine in patients with major depressive disorder (Berman *et al.*, 2000, Zarate *et al.*, 2006a). The study performed by Zarate and colleagues (2006a) showed the potential use of this non-competitive NMDA receptor antagonist in patients previously unresponsive to conventional treatment and that these positive effects can last for at least 7 days following a single dose. A case study also exists demonstrating that a 5 day infusion of ketamine beginning at a rate of 15-20mg/h had very long lasting effects in two treatment-resistant patients (Correll and Futter, 2006).

Evidence for the clinical tolerability of other NMDA receptor antagonists has been questioned, however, due to the possibility of acute psychomimetic effects. For instance, antidepressive effects have also been seen in patients with an antagonist selective for those NMDA receptors expressing the GluN2B subunit (CP101,606, Preskorn *et al.*, 2008). The authors do, however, question the use of these drugs chronically given the acute psychomimetic side-effects seen after injection with both ketamine and CP101,606. However, "open-label" trials of drugs currently used for other disorders have shown the potential clinical tolerability of NMDA receptor antagonists[12].

Memantine, a voltage-dependent, low-affinity uncompetitive antagonist of NMDA receptors used as a treatment for Alzheimer's disease (Parsons *et al.*, 1999, Rogawski and Wenk, 2003) has shown an antidepressive effect in the FST using Wistar rats (Moryl *et al.*, 1993). It has now also been shown to have an effect in depressed patients in a small-scale study (Ferguson and Shingleton, 2007) as well as in a larger study in patients with co-morbid alcoholism (Muhonen *et al.*, 2008). However, a study by Zarate and colleagues (2006b) failed to see an effect in another population of patients with depression.

Riluzole, currently used in amyotrophic lateral sclerosis (ALS), is thought to affect several targets, primarily sodium channels, but may also show some NMDA receptor antagonism (Doble, 1996, De Sarro *et al.*, 2000, Lamanauskas and Nistri, 2008, but see also the study by Debono and colleagues, 1993). It has been shown to be an effective monotherapy to treatment-resistant depression (Zarate *et al.*, 2004) as well as a useful

[12] It should also be noted that, in many cases, the alternative to these issues of drug tolerability will be the patient's suicide.

adjunct to conventional antidepressant treatment (Sanacora *et al.*, 2007). These studies indicate the possible translation of preclinical work on NMDA receptor antagonists in emotionality into the patient population as well as the possible clinical tolerability of these drugs.

3.1.2. The GluN1 hypomorph mouse

3.1.2.1. Construct

Investigations into the effect of alterations in the NMDA receptor on behaviour in transgenic mice have been hampered by some of the gross receptor subunit deletions being perinatally lethal (Forrest *et al.*, 1994, Kutsuwada *et al.*, 1996). However, a mouse line is now available that expresses approximately 10% wildtype levels of the constitutive GluN1 subunit, so-called GluN1 hypomorph (HYPO) mice (Mohn *et al.*, 1999). Consequently, these mice have only 10% normal levels of NMDA receptors. This was achieved by the insertion of a neomycin cassette into intron 20 of the gene encoding GluN1, Northern blots being used to detect this new allele and confirm the absence of any unexpected novel transcripts. Western blots, as well as MK-801 binding, were used to further validate this reduction of GluN1 expression and subsequent NMDA receptor formation. The GluN1 HYPO mice survive to adulthood, offering an opportunity to study what effect an overall reduction in NMDA receptor levels from conception will have on behaviour. So far, work on these mice has focused on the glutamatergic hypothesis of schizophrenia (Mohn *et al.*, 1999, Duncan *et al.*, 2004, Fradley *et al.*, 2005, Duncan *et al.*, 2006b, a, Bickel *et al.*, 2007, 2008), but they may also be useful in investigating the role of NMDA receptors in depression and antidepressant treatment.

The original paper describing the GluN1 hypomorph mice focussed on this line's hyperlocomotion and increased stereotypy that mimicked wildtype behaviour following administration of 3mg/kg phencyclidine (PCP) or 0.2mg/kg MK-801 (Mohn *et al.*, 1999). These behaviours were reversed in the GluN1 HYPO mice by 0.5mg/kg haloperidol, a typical antipsychotic, and 0.5mg/kg clozapine, an atypical antipsychotic. The GluN1 HYPO mice are insensitive to the psychomimetic effects of NMDA receptor antagonists. They show social withdrawal in the resident-intruder paradigm and reduced mating frequency, both recognised as negative symptoms of schizophrenia. These behaviours were, again, reversed by 0.5mg/kg clozapine. These schizophrenia-related phenotypes were present despite the release and metabolism of dopamine being seemingly normal in these mice.

A deficit in pre-pulse inhibition of auditory startle (PPI), seen by many as the "gold standard" test of a model of schizophrenia, was reported in these mice by the same group (Duncan *et al.*, 2004), who also demonstrated alterations in brain activity in areas thought to be involved in schizophrenia through measures of blood glucose consumption (Duncan *et al.*, 2002). The 2004 paper also confirmed the reduced social behaviour in these mice (Duncan *et al.*, 2004), also seen by others (Halene *et al.*, 2009). 1mg/kg clozapine was not sufficient to reverse the PPI deficit (Fradley *et al.*, 2005, Duncan *et al.*, 2006a), nor was 2mg/kg but 3mg/kg clozapine was sufficient to have an effect in the sensorimotor gating of the GluN1 HYPO mice (Duncan *et al.*, 2006a). However, this dose also affected the wildtype mice, as well as reducing the startle amplitude in both groups. This was also true of 20mg/kg quetiapine and, as shown in another paper, 0.3mg/kg risperidone (Duncan *et al.*, 2006b). 0.5 and 1mg/kg haloperidol has also been shown to improve PPI performance in the GluN1 HYPO mice, but, once again, these doses also showed effects in the wildtype controls

(Duncan *et al.*, 2006a). Differential effects can be seen using 0.625mg/kg olanzapine, which partially rescues the hyperactivity of the GluN1 HYPO mice without having any sedative effects on the wildtype mice (Duncan *et al.*, 2006b). The GluN1 HYPO mice have also been shown to be more sensitive to amphetamine-induced disruption of PPI (Moy *et al.*, 2006).

The large doses of antipsychotics required to produce the desired effect in the PPI test in these mice may indicate sedation as a possible confound to the antipsychotics effects on PPI. While pharmacokinetics differ greatly between species, it is worthy of note that high acute daily doses of antipsychotics given in humans are, for instance, 10mg haloperidol (approximately 0.15mg/kg based on a 70kg body weight), 50mg quetiapine (0.70mg/kg), 4mg risperidone (0.06mg/kg) and 15mg olanzapine (0.20mg/kg). This gives some indication that the doses used in these rodent studies could be much greater than those that are clinically relevant and may lead to sedation. Sedation itself would also reduce reactivity to any stimulus and so would act as a confound to this measure of sensorimotor gating. With no clear demonstration of true drug activity in this test, many would question the validity of this mouse as a model of schizophrenia. A recent paper has suggested these mice also display behaviours related to autism (Moy *et al.*, 2008) raising questions over the selectivity of these mice as a model for schizophrenia. The use of these mice in other fields, such as in investigating emotionality, is therefore warranted, although these previous findings must be taken into consideration during interpretation of any results found. Indeed, the phenotype of this mouse needs to be further defined to rule out the possibility of global impairments.

3.1.2.2. Previous findings from the GluN1 hypomorph mice relevant to affect

Due to the focus on schizophrenia, little work has been done on emotionality in these mice. However, the reduced social behaviours shown in these mice (Mohn *et al.*, 1999, Duncan *et al.*, 2004, Halene *et al.*, 2009) may have relevance both in schizophrenia as well as in investigating alterations in affect. Social withdrawal can be seen as a symptom in itself or as indicative of an increase in anxiety, often associated with depression (DSM IV-TR, 2000), although exacerbation of anxiety is also seen acutely with antidepressant treatment both in humans (den Boer *et al.*, 1987, Gorman *et al.*, 1987, Den Boer and Westenberg, 1990, Ramos *et al.*, 1993, Jick *et al.*, 2004, Grillon *et al.*, 2007) and in rodents (Liu *et al.*, Griebel *et al.*, 1994, File *et al.*, 1999, Kurt *et al.*, 2000, Bagdy *et al.*, 2001, Belzung *et al.*, 2001, Burghardt *et al.*, 2004, Drapier *et al.*, 2007). The GluN1 HYPO mice could be used to elucidate the effects of chronic, and indeed developmental, reduction in NMDA receptor function on anxiety, including social behaviour, as well as other emotional behaviours.

3.1.3. Aims of this chapter

Given the evidence for a role of NMDA receptor-mediated neurotransmission in antidepressant activity and the possible use of NMDA receptor antagonists as novel antidepressants, this chapter aims to assess the behaviour of the GluN1 HYPO mice in tests relating to emotionality. A robust anti-depressive phenotype would be expected in light of previous results from pharmacological studies of reduced NMDA receptor-mediated neurotransmission. Results in these tests, however, must be considered in relation to the overall phenotype of these mice, particularly given the global nature of the manipulation, the pervasiveness of glutamate in the central

nervous system as well as the GluN1 HYPO mice's status as a model of schizophrenia. As such, this chapter has the secondary aim to comprehensively phenotype these mice in tests of other domains of behaviour including cognition and naturalistic behaviour.

3.2. Methods

3.2.1. Animals

The majority of work presented in the chapter is concerned with the behaviour of the GluN1 hypomorph (HYPO) mice. However, a cohort of hippocampal lesioned animals were also used to assess the hippocampal dependency of the visual discrimination task.

3.2.1.1. Transgenic cohorts

Two cohorts of male and female GluN1 HYPO mice (Mohn *et al.*, 1999) and their wildtype (WT) littermates on a 129Sv background were tested on a number of behavioural tests. These mice were obtained from GlaxoSmithKline (LAS Department, Hertfordshire, UK) and maintained at The University of Oxford in holding rooms on a 12/12hr light/dark cycle (lights on at 7am and off at 7pm). Mice were housed in groups of 3 with *ad libitum* food and water unless otherwise stated and were typically of 3 months of age at the start of testing which lasted approximately 3 months. All testing was done during the light phase unless otherwise noted. Hypomorph mice were noted to persistently scratch, usually in the neck/flank region, which has also been seen by other laboratories (Moy *et al.*, 2008). In some cases this behaviour led to an open wound and the animal needing to be culled. This accounts for the slow decline in numbers across the behavioural experiments.

3.2.1.2. Hippocampal lesion cohort

To assess the importance of the hippocampus on the non-spatial visual discrimination task, a group of hippocampal lesioned animals were used. These lesions were performed by Dr Robert Deacon on female C57Bl/6 mice at approximately 3 months of age at the time of surgery.

3.2.1.2.1. Surgery

Briefly, mice were anaesthetised with isoflurane and administered meloxicam (1mg/kg intraperitoneal (i.p.) injection), chlordiazepoxide (10mg/kg i.p.) and atropine (10mg/kg i.p.) prior to mounting in a stereotaxic frame. A scalp incision was made and four holes drilled per hemisphere to allow for injection of N-methyl-D-aspartic acid (NMDA, 10mg/ml) in phosphate buffered saline (pH 7.4) with a 5µl Hamilton syringe adapted with a 34 gauge stainless steel needle. The stereotaxic coordinates and volumes of each injection are listed in Table 3.2. Following these injections, the scalp was sutured, the animal given a second injection of chlordiazepoxide (10mg/kg i.p.) as well as 1ml saline subcutaneously (s. c.) and then placed in a heated recovery chamber (30°C) until it was observed to be freely moving. Sham-operated animals received all drugs and anaesthesia, were placed in the stereotaxic frame and the scalp incision made but were then sutured and allowed to recover as above. Animals were given a minimum of one week to recover from surgery before being tested.

Table 3.2: Stereotaxic coordinates for complete, bilateral hippocampal lesions

Site	AP	ML	DV	Volume (µl)
1	+ 0.70	± 3.00	- 4.00	0.20
2	+ 1.00	± 2.20	- 2.00	0.10
3	+ 1.50	± 1.70	- 1.90	0.15
4	+ 2.10	± 1.20	- 1.90	0.10

The values are given as mm from stereotaxic zero except for dorsal/ventral, given in mm from skull surface at bregma.

3.2.1.2.2. Histology

Lesions were confirmed behaviourally using spontaneous alternation (data not shown) and histologically. Briefly, mice were overdosed with 0.3ml Euthatal (200mg/ml pentobarbital sodium, Merial Animal Health Ltd) and transcardially perfused with 10% formalin (diluted with saline), their brains removed and stored in 10% formalin until sectioning. 1:5 40μm sections were cut through the whole length of the hippocampus and mounted on glycerine-coated slides. These were then stained with 0.5% cresyl violet stain (diluted in deionised water) by initially washing the slides in histoclear before being dehydrated in descending concentrations of alcohol (100%, 96%, 80% and 70%). Slides were then washed in deionised water before being left in the stain for 6min, washed again in water, then taken through ascending concentrations of alcohol (70%, 80%, 96% and 100%) to wash and set.

Slides were viewed on a Leitz Dialux 22EB microscope at x10 zoom and images taken using a Polaroid SprintScan 35Plus digital imager. Figure 3.1 shows a layered image of all of the lesions, Figure 3.2 shows the maximum and minimum lesion sizes seen at each site and Figure 3.3 shows representative micrographs taken from one lesioned animal. Briefly, five of the eight lesioned animals showed some superior ventral sparing with two other animals having unilateral sparing of the most ventral portions of the hippocampus. One animal showed sparing of the hippocampus in several slides across the length of the hippocampus (resulting in the "minimum lesion size" often being absent in Figure 3.2). However, this animal still displayed significant tissue loss in many slides and behaviourally did not differ from other lesioned animals and so was included in analysis. No significant extra-hippocampal damaged was noted in any of the animals.

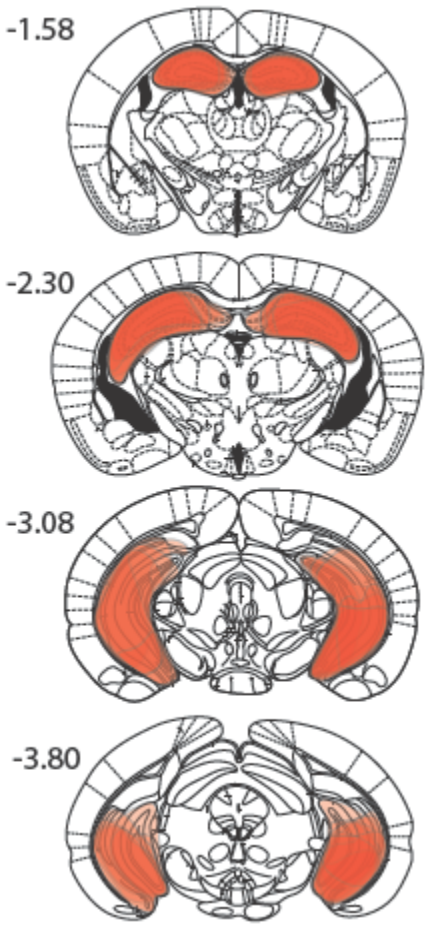


Figure 3.1: Layered representation of the extent of tissue damage in the eight hippocampal lesion animals used in the appetitive visual discrimination task

Many of the mice showed sparing of the superior aspects of the ventral hippocampus but little extra-hippocampal damage was noted.

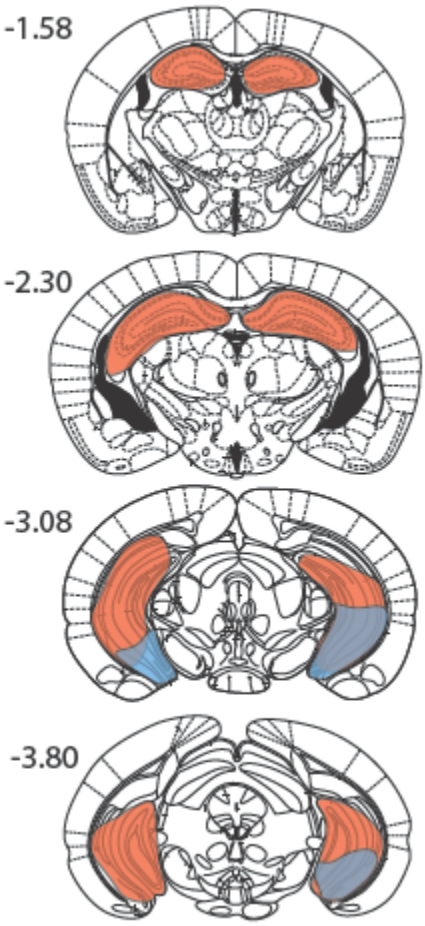


Figure 3.2: Maximum (red) and minimum (blue) lesion size seen at each site in the eight hippocampal lesion mice used in the appetitive visual discrimination task

Unilateral sparing of the ventral hippocampus was seen in two mice and dorsal sparing in one mouse, noted by the absence of a minimum lesion marker at these points. Even the largest lesion sites did not extend out of the hippocampus.

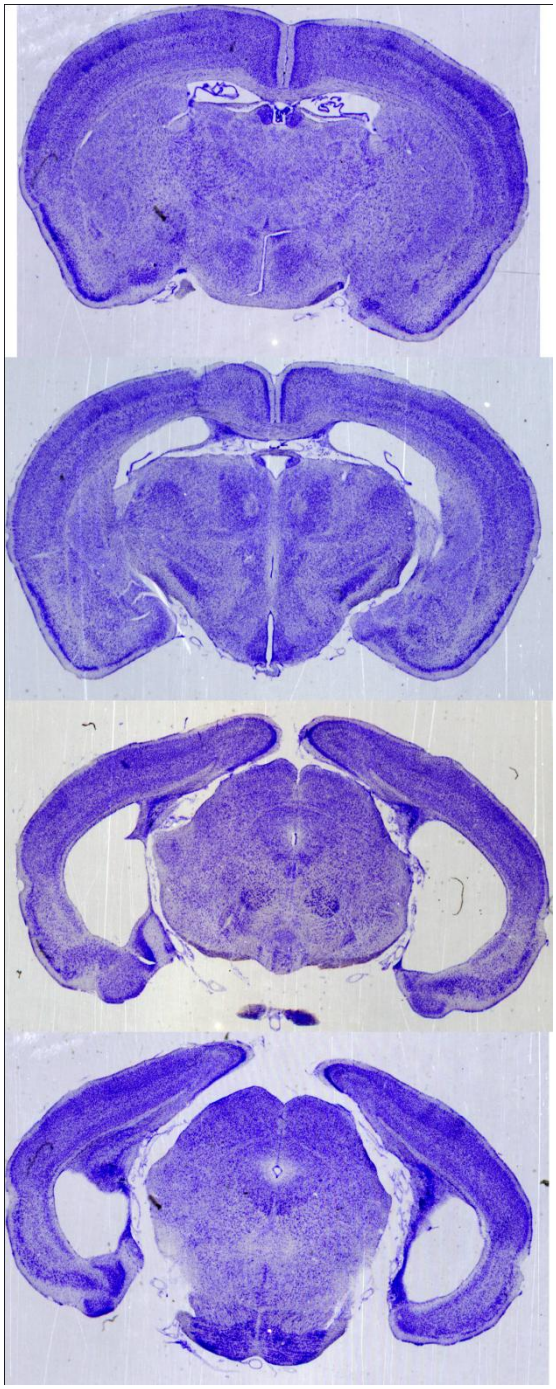


Figure 3.3: Representative micrographs of a hippocampal lesion taken from one of the lesion mice used in the appetitive visual discrimination task

The typical lesion seen in this cohort of mice showed some sparing of the superior aspect of the ventral hippocampus but otherwise complete loss of this structure with negligible damage to other areas of the brain.

3.2.2. Behavioural protocols

The first cohort of GluN1 hypomorph mice (29 WT and 30 GluN1 HYPO mice) were tested, in order, on the elevated plus maze (EPM), the black/white alley, the light/dark box, the successive alleys, open field, hyponeophagia, spontaneous alternation, spatial novelty preference Y-maze test, nesting, food/fluid consumption, burrowing, the non-anxious open field, locomotor activity over 2hr, sucrose preference, locomotor activity across the diurnal cycle, social interaction following habituation, the appetitively motivated spatial reference memory Y-maze test and the forced swim test (FST, Figure 3.4A). The second cohort (30 WT and 30 GluN1 HYPO mice) were tested on, in order, social interaction without habituation, object recognition and the appetitive visual discrimination task (Figure 3.4B). This final task was also assessed for hippocampal dependency with a cohort of hippocampal lesioned animals (11 sham-operated animals, 8 lesioned animals).

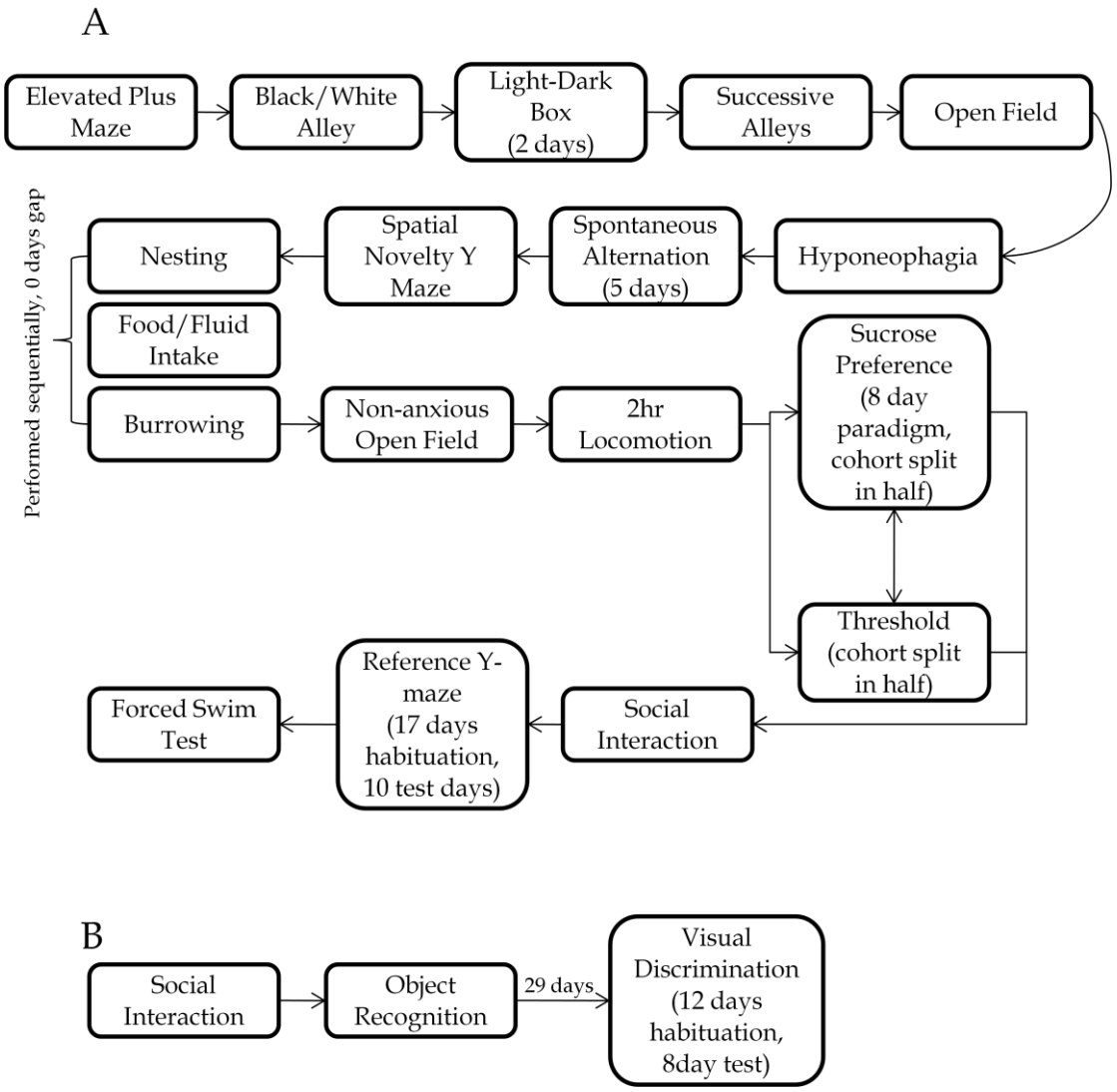


Figure 3.4: Timeline outlining the behavioural testing of cohort one (A) and two (B) of the GluN1 hypomorph mice

GluN1 hypomorph mice and their wildtype counterparts performed a number of behavioural tests. Arrows between tests represent a two day gap unless otherwise stated.

3.2.2.1. Tests of naturalistic behaviour

3.2.2.1.1. Nesting

Nesting is a naturalistic behaviour (Deacon, 2006a) that has been shown to be disrupted by induction of disease and synaptic deficits (Cunningham *et al.*, 2003). Mice were individually housed 1 hour before the onset of the dark phase with no enrichment aside from 3g of pressed cotton (a "nestlet" that makes up part of normal cage enrichment). Nests were assessed the following morning on the 1-5 rating scale below:

1: Nestlet not noticeably touched (>90% intact).

2: Nestlet partially torn up (50-90% remaining intact).

3: Mostly shredded but often no identifiable nest site.

4: An identifiable, but flat nest.

5: A (near) perfect nest.

3.2.2.1.2. Food/fluid consumption

Food consumption was measured by placing animals into individual, clean cages complete with enrichment and 6g of food pellets 1 hour before the onset of the dark cycle. The food remaining the next morning was then weighed. This was repeated twice on consecutive days and the values averaged.

Water consumption was assessed similarly by weighing the water bottle given to individual animals 1 hour before the onset of the dark cycle and weighed again the following morning. This was repeated twice on consecutive days and the values averaged.

The consumption of glucose solution, a naturally rewarding substance for mice, was assessed in a similar way by replacing the water bottle for individually housed mice with a 8% (w/v) glucose solution one hour before the onset of the dark cycle and then again the following morning. This was repeated twice on consecutive days and the values averaged.

The weight of the animals was also taken each day and the values when water and pellets (but not glucose) were present were used to give baseline readings.

3.2.2.1.3. Burrowing

Burrowing is a naturalistic behaviour (Deacon, 2006b) that can be disrupted by induction of disease and synaptic deficits (Deacon *et al.*, 2001, Guenther *et al.*, 2001, Cunningham *et al.*, 2003). A cylinder of dark grey plastic, 6.8cm diameter, 20cm in length, with the open end raised by 3cm was filled with 200g food pellets and placed in a clean cage with a thin layer of bedding. Mice were placed in the cage 3 hours before the onset of the dark cycle. Pellets remaining in the tube were measured i) after 2 hours and the tubes refilled to be measures again ii) the following morning to assess burrowing. This refilling of the tubes after 2 hours, not part of the original protocol, was done due to the generally very high levels of burrowing seen in many animals after the 2hr interval.

3.2.2.1.4. Non-anxious open field

A grey enclosed arena of 50x30cm was used to assess locomotor behaviour. Mice were placed into the open field facing a corner and observed for 3 min. Ethovision XT (Noldus) was used to measure the distance covered in this time and the number of rears was manually scored.

3.2.2.1.5. Locomotor activity

An automated beam-break system was used to assess locomotor activity over a 2 hour period. Mice were placed in transparent plastic cages 26x16x17cm fitted with ventilated lids. Two infra-red beams crossed the width of the cage floor, each 7cm from the centre of the cage, 1.5 cm above the floor. Beam crossings were recorded in 5min time bins, total beam-breaks being used as a measure of all movement and "crossovers" (breaking the two beams consecutively) were used as a measure of larger locomotor movements that excluded small, stereotyped movements.

3.2.2.2. Tests of cognition

3.2.2.2.1. Spontaneous alternation

A T-shaped maze made of wood, painted dark grey, with 30x10x29cm arms and a central partition extending 7cm into the start arm from the back of the maze was used to assess spontaneous alternation. Mice were placed in the start arm facing the back wall and allowed to make a free choice of arm. The mouse was then restricted to that arm for 30s by use of a guillotine door. The central partition was then removed and all doors re-opened. The mouse was again placed at the end of start arm facing the wall and allowed to make a further free choice. Choosing the novel arm

("alternating") is indicative of intact working memory (Dember and Fowler, 1958, Lalonde, 2002, Deacon and Rawlins, 2006) and the percentage of these correct choices made over 5 days, 2trial/day, was recorded.

3.2.2.2.2. Spatial novelty preference Y-maze test

This task is based on a spatial working memory task developed in this lab (Sanderson *et al.*, 2007). A Perspex Y-maze with arms of 30x8x20cm was placed into a room containing a variety of extramaze cues. A thin layer of wood chips covered the floor. This was not changed between mice, but was redistributed between phases for individual mice and between mice to eliminate meaningful intramaze cues.

Mice were assigned two arms (a start arm and one other arm) to which they were exposed during the first trial, the exposure phase, for 5min. This selection of arms was counterbalanced with respect to genotype, and timing of the 5min period began only once the mouse had left the start arm. An entry into an arm was defined by a mouse placing all four paws inside an arm. Similarly, a mouse was considered to have left an arm if all four paws were placed outside the arm.

The test phase began 1min after the exposure phase, during which the mouse had been returned to its home cage. During the test phase, mice were allowed free access to all three arms. Mice were placed at the end of the start arm and allowed to explore all three arms for 2min once they had left the start arm. The time that mice spent in each arm was recorded manually and a novelty preference ratio was calculated as time in novel arm/(time in novel arm+time in other arm). As a control measure, the preference for the non-start arm during the sample phase was also calculated as time

in other arm/(time in start arm+time in other arm). Mice with in-tact memory would be expected to show a strong novelty preference during the test phase.

3.2.2.2.3. Appetitively motivated spatial reference memory Y-maze test

A Y-shaped maze consisting of a central polygonal area, 14cm in diameter, and three arms of 50x9cm with 0.5 cm high walls and elevated 80cm from the ground was used in this spatial reference memory task. A small food well 5cm from the distal end of each arm was used to contain a 50% condensed milk reward (Nestlé Carnation condensed milk, diluted with water) which was available if the mouse located the correct arm.

The mice were initially extensively habituated to drinking the rewards from the maze, first in groups and then individually. When testing began, each mouse was assigned a target arm in which a milk reward could be obtained, defined by allocentric spatial cues in a counterbalanced manner and either one of the other two arms could be used as a start arm. Each mouse received 10trials/day for 10 days with five trials started from each of the non-target arms in a pseudorandom sequence with a maximum number of three consecutive identical arm starts. Mice were allowed to make a single arm entry, drink the reward if the correct choice was made, before being removed from the maze. If an incorrect choice was made, the mice were removed once they had reached the end of the arm and an error was recorded. Mice were run in a "round robin" fashion in groups of approximately 10 to keep arousal levels high. The maze was periodically rotated to prevent the use of meaningful intramaze cues. Mice were maintained at a level of food deprivation sufficient to run

readily and never below 85% of free-feeding weight. To ensure the reward was not providing an olfactory cue, the rewarded arm was only baited after the arm choice had been made by the mice on the final day of testing ("post-choice baiting"). Percentage of correct arm choices was recorded as a measure of spatial reference memory.

3.2.2.2.4. Object recognition

Object recognition was performed in a grey arena of 40x40x40cm in low light levels. Mice were habituated to the empty test arena for 10min/day on the three days preceding the test.

For the training phase, T1, two identical objects (A_1 and A_2) were placed in the arena 10cm away from two adjacent corners and mice allowed to freely explore for 10min. The mice were placed facing the centre of the wall opposite the objects. After an intertrial interval (ITI) of 1 hour, mice were reintroduced to the testing arena for the test, T2, lasting 5min. For T2, the arena contained one object identical to those used in T1 (A_3), plus one novel object (B_1, see Figure 3.5). The positions of the novel and familiar object were counterbalanced and the arena and objects wiped down with 70% ethanol between trials to minimise olfactory cues.

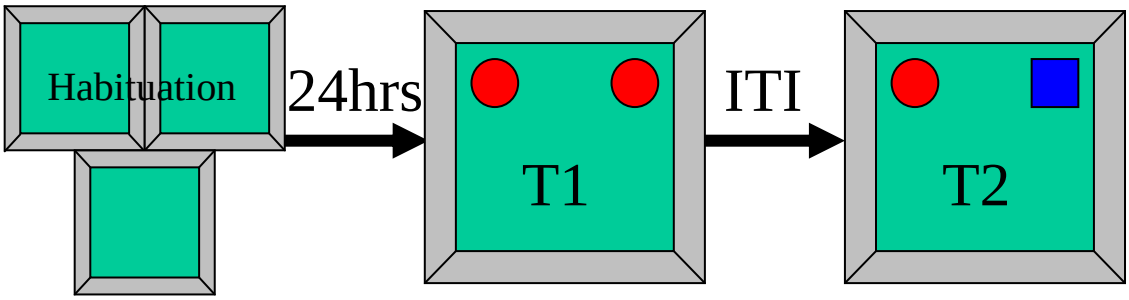


Figure 3.5: Object recognition method

Mice were habituated to an open arena for 10mins daily for 3 day prior to testing. Following this, they were placed in the arena with two identical objects and allowed to freely explore for 10mins (T1). After the inter-trial interval (ITI) of 1 hour, they were then put back in the arena with an object identical to those used previously, plus a novel one and allowed to freely explore for 5mins (T2). The time spent exploring each object is recorded to give a measure for preference for novelty.

Object exploration was scored manually and defined as the animal being within 1cm of the object and orientated towards it. Rearing against the object was counted but not sitting on the object (rarely observed). The objects used were a cylindrical tin can 13.2cm high and 7.5cm in diameter with corrugated sides (container for Homepride Curry Sauce, Premier Foods) and a stippled glass bottle 18cm high and 8cm in diameter at its widest point (container for Orangina, A. G. Barr). The ratio between the time spent exploring the novel object versus total time spent exploring both of the objects (N/(N+F)) was taken as a measure of object memory, a non-spatial form of memory. Total exploration during T1 was also calculated to ensure equivocal exposure to the familiar object between groups during the exposure phase.

3.2.2.2.5. Appetitive visual discrimination task

Using a wooden T-maze with arms of 10x30x30cm, mice were assigned to either a grey or black-and-white striped arm as a goal arm. They were initially habituated to this maze with plain wooden arms, initially by being closed into one arm with the 50% condensed milk reward present and then allowed to run throughout all three arms with rewards being available in all arms. This was continued until mice were running and feeding freely in the maze. They were trained with one striped and one grey arm to associate a reward (50% condensed milk) with one of the visually cued arms. The right/left position of these arms was altered in a pseudorandom order to remove any spatial component to the task with a maximum of three consecutive identical arm positions and an equal number of each position within a block of 10 trials. Mice were placed in the start arm and allowed to make one arm choice. If this was the correct choice, they were allowed to eat the reward before being removed from the maze. If they made an incorrect choice, they were removed from the maze

once they had reached the end of the arm. Mice were tested for 10trials/day for 8 days. To ensure the reward was not providing an olfactory cue, post-choice baiting was employed on the final day of testing.

3.2.2.3. Tests of anxiety

3.2.2.3.1. Elevated plus maze

The elevated plus maze (EPM) was performed as previously described in Section 2.2.2.1.1.

3.2.2.3.2. Black/white alley

This test for anxiety uses a simplified version of the light/dark box (McHugh *et al.*, 2004). It consists of an alley formed by two sections, one white, one black, each 60x9x29cm. Mice were placed at the closed end of the black alley, facing the end wall, and latency to enter (with all four paws) into the white half, time spent in the white and number of crossings were scored during a 2min test.

3.2.2.3.3. Light/dark box

The light/dark box is a test for anxiety (Crawley and Goodwin, 1980, Crawley, 1981, Crawley and Davis, 1982, Blumstein and Crawley, 1983, Mathis *et al.*, 1994, Mathis *et al.*, 1995) and consists of an open white compartment 30x20x20 cm joined by a 3x3cm opening to a dark box (painted black and with a lid), 15x20x20cm. The anxiogenic nature of the white compartment is increased by additional illumination with a 60W angle poise lamp placed 45 cm above the centre of the floor.

On separate occasions, the mice were started in both sections of the apparatus to look at both emergence into the anxiogenic section as well as escape latency from it. These two forms of the test were done on consecutive days, the start position used first (light or dark) being counterbalanced across experimental groups. The mice were placed in the middle of the section facing away from the opening and behaviour scored for 5min. The latency to cross (defined by all four feet entering the box), time spent in each section (again defined by all four feet being in that area) and the number of transitions though the opening were measured.

3.2.2.3.4. Successive alleys

This anxiety test is similar to the EPM but eliminates the central square which has been associated with interpretational problems (Contet *et al.*, 2001, McHugh *et al.*, 2004). The apparatus consists of a sequence of four increasingly anxiogenic alleys in a row, each 25cm long, elevated 50cm from the ground. Alley one is black, 8.5cm wide with 25cm high walls. A 0.5cm step down led to alley two, which is grey, 8.5cm wide with 1.3cm high walls. A 1.0cm step down led to alley three which was white, 3.5cm wide with 0.8cm high walls. A 0.4cm step led to alley four, also white, 1.2cm wide with 0.2cm high walls (Figure 3.6). During testing, mice were placed at the closed end of alley one facing the wall. Latencies, frequencies and time spent in each of the alleys were recorded over a 5min test.

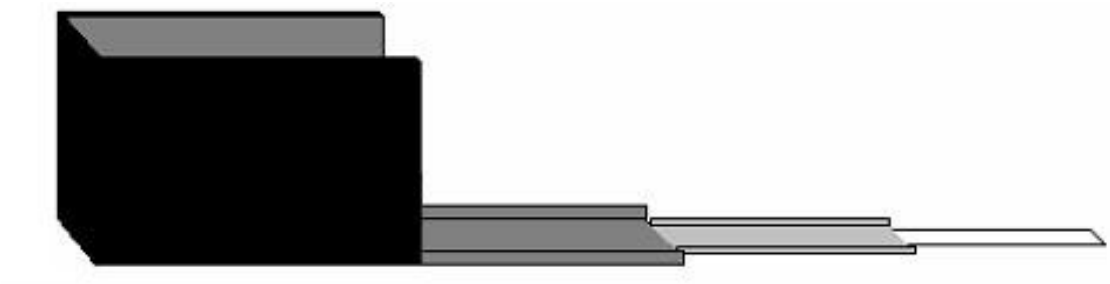


Figure 3.6: The successive alleys

The successive alleys is comprised of four sections of increasingly anxiogenic quality.

3.2.2.3.5. Open field

Mice find open areas more anxiogenic than keeping close to high walls and so open fields have been used as measures of anxiety in rodents (Hall, 1936, Treit and Fundytus, 1988). A white open field of 60cm diameter was used to assess this behaviour. Ethovision XT (Noldus) was used to split this arena into an open, central area of 10cm radius and an outer area of 20cm radius. Mice were placed into the maze at the edge and latency, frequency and time spent in the open area was taken as measures of anxiety. Total distance travelled was also taken to account for any locomotor differences.

3.2.2.3.6. Hyponeophagia

Mice are reluctant to try new food stuffs in a novel environment (Burns *et al.*, 1996). 50% condensed milk was used in this test in an upturned translucent plastic jug, 15cm in diameter with a spout protruding a further 2cm in which a food well was located. This restricted environment reduces locomotor/orientation confounds.

Mice, naïve to milk, were food restricted overnight to 1g/mouse and tested the following morning. When tested, they were placed facing away from the well and the jug gently lowered into position. The latency to drink was taken with a cut-off time of 2min; mice that did not drink in this time were individually housed for approximately 3min before being retested. Mice were tested a maximum of 3 times, the cumulative latency being used if necessary. Following testing, mice were held in a separate cage from their homecage to prevent the social transmission of the novel taste. Time to first contact, usually recorded, was not taken for the GluN1

hypomorph mouse cohort tested as this was routinely too short to be measured accurately.

3.2.2.3.7. Social interaction

Social interaction was performed as previously described in section 2.2.2.1.2 but with different cohorts of animals being used for the habituated and non-habituated forms of the test used in the first social interaction experiment. So, the first cohort of GluN1 hypomorph mice were habituated to the test arena for 10min/day for five days then tested on days six and seven, whereas the second cohort were tested with no habituation.

3.2.2.4. Tests of affect

Sucrose preference, locomotor activity across the diurnal cycle and the forced swim test were all performed as previously described in section 2.2.2.2.

3.2.3. Statistical analyses

Univariate ANOVAs with genotype and sex as between-subject factors, repeated measures ANOVAs and t-tests were used when appropriate. When an interaction was found to be significant in an ANOVA, simple main effects analyses were also done with Least Significant Difference (LSD) comparisons. As nesting and spontaneous alternation use non-continuous values, these were analysed using two-tailed Mann-Whitney U tests with the data collapsed across sex. Otherwise, statistical analyses were conducted as described in Section 2.2.3.

3.3. Results

3.3.1. Tests of naturalistic behaviour

The results for the tests of naturalistic behaviour in the GluN1 hypomorph (HYPO) and their wildtype (WT) counterparts are summarised in Tables 3.3-7 and Figures 3.6 and 3.7. Nesting was analysed using two-tailed Mann-Whitney U test with the data collapsed across sex. Univariate ANOVAs using genotype and sex as between-subject factors were used to compare food pellet, water and glucose consumption and body weight during the food/fluid intake study, the amount burrowed after two hours and overnight and the distance travelled and bouts of rearing in the non-anxious open field. Repeated measures ANOVAs were used for the measures of locomotor activity using time as the within-subject factor and the same between-subject factors as above.

3.3.1.1. Nesting

GluN1 hypomorph mice showed a deficit in nesting behaviour ($U_{(56)}$=190.500, $p<0.001$) with no influence of sex (see Table 3.3).

Table 3.3: Results for GluN1 hypomorph (HYPO) and wildtype (WT) mice in several tests of naturalistic behaviour

Test & Measure	WT, n=29	HYPO, n=27	Statistics
Nesting	5 (4.5-5)	3.5 (1.5-5)	**$U_{(56)}$=190.500, p<0.001**
Food/Fluid Consumption			
Pellets consumption (g)	1.86 ± 0.13	2.61 ± 0.09	**Geno $F_{(1,52)}$=22.943, p<0.001**
			Sex $F_{(1,52)}$=2.292, p=0.136
			Int $F_{(1,52)}$=0.006, p=0.938
Water consumption (ml)	3.64 ± 0.14	3.73 ± 0.42	Geno $F_{(1,52)}$=0.006, p=0.940
			Sex $F_{(1,52)}$=0.932, p=0.339
			Int $F_{(1,52)}$=1.625, p=0.208
Glucose (8%) (ml)	11.49 ± 0.60	9.40 ± 0.52	**Geno $F_{(1,52)}$=6.382, p=0.015**
			Sex $F_{(1,52)}$=0.282, p=0.598
			Int $F_{(1,52)}$=4.149, p=0.047
Body Weight (g)	26.73 ± 0.78	23.07 ± 0.49	**Geno $F_{(1,52)}$=42.164, p<0.001**
			Sex $F_{(1,52)}$=86.570, p<0.001
			Int $F_{(1,52)}$=12.042, p=0.001
Burrowing			
2 hours (g)	140.32 ± 14.00	6.29 ± 3.52	**Geno $F_{(1,52)}$=87.685, p<0.001**
			Sex $F_{(1,52)}$=4.810, p=0.033
			Int $F_{(1,52)}$=1.511, p=0.225
Overnight (g)	139.95 ± 6.32	10.60 ± 5.65	**Geno $F_{(1,52)}$=229.585, p<0.001**
			Sex $F_{(1,52)}$=2.406, p=0.127
			Int $F_{(1,52)}$=0.769, p=0.385
Non-anxious open field			
Distance travelled (cm)	1091.78 ± 48.79	2001.94 ± 152.89	**Geno $F_{(1,52)}$=33.113, p<0.001**
			Sex $F_{(1,52)}$=0.275, p=0.602
			Int $F_{(1,52)}$=1.994, p=0.164
Rearing	5.21 ± 0.63	21.33 ± 1.63	**Geno $F_{(1,52)}$=87.877, p<0.001**
			Sex $F_{(1,52)}$=0.021, p=0.886
			Int $F_{(1,52)}$=2.352, p=0.131
Locomotion over 2 hours			
Total beam-breaks	725.21 ± 68.73	3775.04 ± 261.40	**Geno $F_{(1,52)}$=132.247, p<0.001**
			Sex $F_{(1,52)}$=0.126, p=0.724
			Int $F_{(1,52)}$=0.393, p=0.534
Crossovers	106.38 ± 9.74	764.04 ± 51.79	**Geno $F_{(1,52)}$=164.115, p<0.001**
			Sex $F_{(1,52)}$=0.717, p=0.401
			Int $F_{(1,52)}$=0.283, p=0.597

Parametric data presented as mean ± SEM and analysed with univariate ANOVA. Non-parametric data presented as median (IQR) and analysed with Mann-Whitney U-tests with the data collapsed across the sexes.
Geno = effect of genotype, sex = effect of sex, int = genotype*sex interaction.

With the exception of water consumption, GluN1 hypomorph mice show altered behaviour in all of the tests of naturalistic behaviour performed.

3.3.1.2. Food/fluid consumption

The GluN1 HYPO mice showed an increase in pellet consumption ($F_{(1,52)}$=22.943, p<0.001), a decrease in glucose consumption ($F_{(1,52)}$=6.382, p=0.015) and decreased body weight ($F_{(1,52)}$=42.164, p<0.001) but showed normal water consumption ($F_{(1,52)}$=0.006, p=0.940, Table 3.3). Effects of sex were seen in body weight ($F_{(1,52)}$=86.570, p<0.001) with male mice (27.84g±0.75) being heavier than female mice (22.47g±0.29). A genotype*sex interaction was seen in body weight ($F_{(1,52)}$=12.042, p=0.001) due to the differences in weight being greater in male mice (GluN1 HYPO 24.87g±0.68, WT 30.39g±0.77, simple main effect of genotype $F_{(1,52)}$=46.200, p<0.001) than female mice (GluN1 HYPO 21.63g±0.41, WT 23.31g±0.30, simple main effect of genotype $F_{(1,52)}$=4.937, p=0.031), although a significant effect of genotype is seen in both cases, and a simple main effect of sex was seen in both genotypes (GluN1 HYPO $F_{(1,52)}$=16.343, p<0.001, WT $F_{(1,52)}$=85.111, p<0.001). A genotype*sex interaction was also seen in glucose consumption ($F_{(1,52)}$=4.149, p=0.047) owing to the effect of genotype only being present in female (WT 12.47ml±0.72, GluN1 HYPO 8.87ml±0.77, $F_{(1,52)}$=11.248, p=0.001) but not male mice (WT 10.44ml±0.93, GluN1 HYPO 10.06ml±0.66, $F_{(1,52)}$=0.111, p=0.740). Simple main effects failed to find an effect of sex in either genotype (GluN1 HYPO $F_{(1,52)}$=1.089, p=0.302; WT $F_{(1,52)}$=3.440, p=0.069). No other interactions were seen (see Table 3.3)

3.3.1.3. Burrowing

GluN1 HYPO mice displayed a burrowing deficit both at 2hr ($F_{(1,52)}$=87.685, p<0.001) and overnight ($F_{(1,52)}$=229.585, p<0.001, Table 3.3). Effects of sex were seen after 2hr ($F_{(1,52)}$=4.810, p=0.033) with male mice (95.72g±17.57) burrowing more than female mice (58.36g±15.13). Sex did not interact with genotype at either time point (see Table 3.3).

3.3.1.4. Non-anxious open field

The GluN1 HYPO mice showed marked hyperactivity in the open field (distance covered $F_{(1,52)}$=33.113, p<0.001; rearing $F_{(1,52)}$=87.877, p<0.001) with no influence of sex (Table 3.3).

3.3.1.5. Locomotor activity

GluN1 HYPO mice also displayed hyperactivity in the photobeam arena (total beam-breaks $F_{(1,52)}$=132.247, p<0.001; total crossovers $F_{(1,52)}$=164.115, p<0.001, Table 3.3). Across the two hours of the photobeam activity test (Figure 3.7), GluN1 HYPO mice consistently showed increased total beam-breaks (effect of genotype $F_{(1,52)}$=132.247, p<0.001). All mice show a decrease in beam-breaks over time (effect of time $F_{(23,1196)}$=38.548, p<0.001) and this habituation differs between the genotypes (genotype*time interaction $F_{(23,1196)}$=9.855, p<0.001) as well as between the sexes (time*sex interaction $F_{(23,1196)}$=1.882, p=0.007), although sex shows no other influence (effect of sex $F_{(1,52)}$=0.126, p=0.724; genotype*sex interaction $F_{(1,52)}$=0.393, p=0.534; genotype*time*sex interaction $F_{(23,1196)}$=1.218, p=0.218).

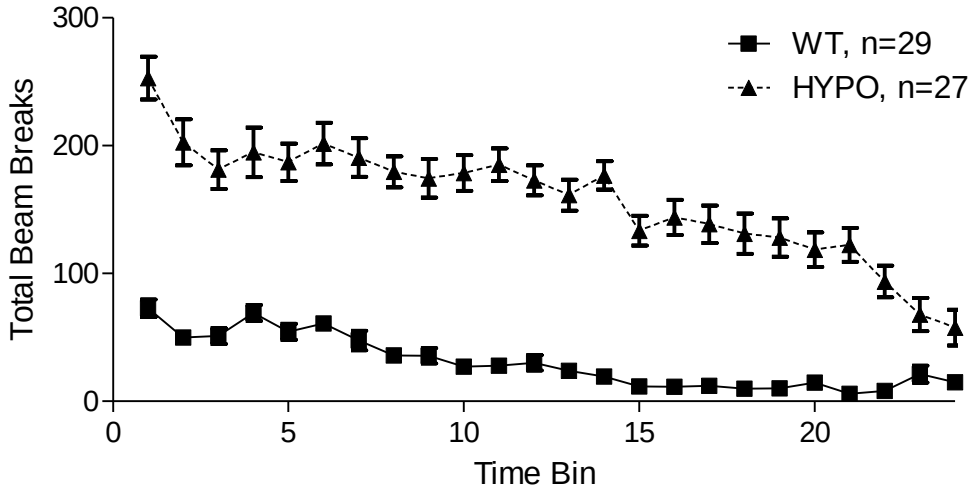


Figure 3.7: Mean number of beam-breaks made by GluN1 hypomorph (HYPO) and wildtype (WT) mice in 2 hours

GluN1 hypomorph mice appear hyperactive across the whole two hour period and habituate to the environment at a different rate to their wildtype counterparts. Presented as mean ± SEM in 24 5min bins.

Simple main effects show both genotype groups habituate (simple main effects of time WT $F_{(23,644)}$=21.338, p<0.001; GluN1 HYPO $F_{(23,598)}$=22.460, p<0.001) with an effect of genotype at every time point (Table 3.4) and that both sexes show this habituation (male $F_{(23,575)}$=18.615, p<0.001; female $F_{(23,667)}$=14.080, p<0.001) with no effect of sex at any individual time point (Table 3.5).

The number of crossover beam-breaks (Figure 3.8) shows a similar pattern of results with GluN1 hypomorph mice again showing consistent hyperactivity (effect of genotype $F_{(1,52)}$=164.115, p<0.001). Mice show habituation to the environment (effect of time $F_{(23,1196)}$=41.836, p<0.001) and this habituation differs between the genotypes (genotype*time interaction $F_{(23,1196)}$=9.855, p<0.001) as well as between the sexes (time*sex interaction $F_{(23,1196)}$=13.487, p<0.001) which also interacts with genotype to give a three-way interaction ($F_{(23,1196)}$=1.813, p=0.011), although sex has no main effect ($F_{(1,52)}$=0.717, p=0.401) and does not interact with genotype ($F_{(1,52)}$=0.283, p=0.597).

Simple main effects shows both genotype groups habituate (simple main effects of time: WT $F_{(23,644)}$=19.390, p<0.001; GluN1 HYPO $F_{(23,598)}$=25.082, p<0.001) with effects of genotype at every time point (Table 3.6) and both sexes also habituate (female $F_{(23,30)}$=14.098, p<0.001; male $F_{(23,30)}$=18.507, p<0.001) with male mice moving more than female mice at the start of testing (Table 3.7). Exploring the three-way interaction shows that sex interacts with time only in the hypomorph mice (WT female $F_{(23,30)}$=1.282, p=0.259; male $F_{(23,30)}$=1.319, p=0.236; GluN1 HYPO female $F_{(23,30)}$=6.747, p<0.001; male $F_{(23,30)}$=13.334, p<0.001).

Table 3.4: Simple main effects of genotype at each time bin of the total beam breaks made by GluN1 hypomorph and wildtype mice over two hours

Time Bin	$F_{(1,52)}$	p
1	**114.134**	**<0.001**
2	**75.211**	**<0.001**
3	**70.976**	**<0.001**
4	**42.119**	**<0.001**
5	**76.392**	**<0.001**
6	**71.262**	**<0.001**
7	**74.703**	**<0.001**
8	**118.672**	**<0.001**
9	**72.370**	**<0.001**
10	**102.727**	**<0.001**
11	**130.204**	**<0.001**
12	**122.512**	**<0.001**
13	**109.383**	**<0.001**
14	**168.761**	**<0.001**
15	**109.451**	**<0.001**
16	**88.335**	**<0.001**
17	**71.778**	**<0.001**
18	**57.762**	**<0.001**
19	**61.259**	**<0.001**
20	**53.951**	**<0.001**
21	**77.173**	**<0.001**
22	**47.230**	**<0.001**
23	**11.251**	**0.001**
24	**8.915**	**0.004**

GluN1 hypomorph mice are hyperactive at every time point compared to wildtype mice.

Table 3.5: Simple main effects of sex at each time bin of the total beam breaks made by GluN1 hypomorph and wildtype mice over two hours

Time Bin	$F_{(1,52)}$	p
1	2.817	0.099
2	2.127	0.151
3	1.441	0.235
4	1.783	0.188
5	2.716	0.105
6	1.044	0.312
7	0.313	0.578
8	0.083	0.775
9	0.173	0.679
10	0.005	0.945
11	0.001	0.979
12	0.074	0.787
13	1.664	0.203
14	0.051	0.822
15	0.279	0.599
16	0.202	0.655
17	0.528	0.471
18	0.210	0.649
19	0.049	0.825
20	1.716	0.196
21	0.217	0.644
22	1.046	0.311
23	0.668	0.417
24	0.119	0.732

There is no difference between the sexes at any individual time point.

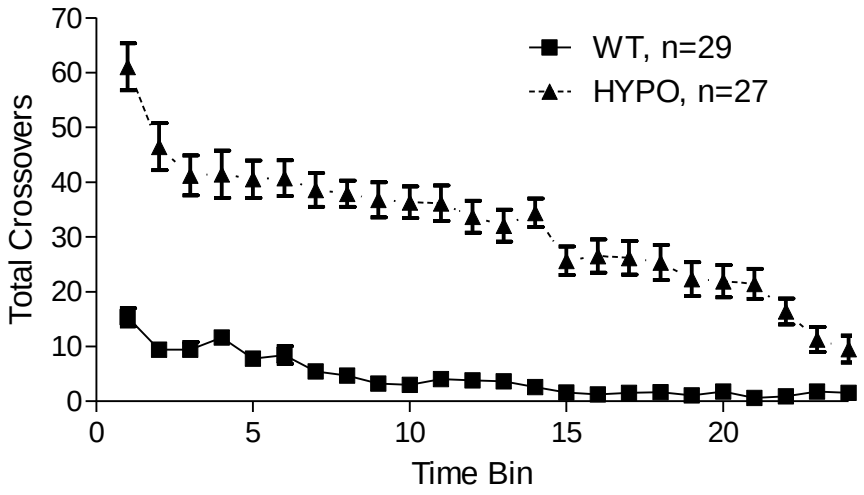


Figure 3.8: Mean number of crossover beam-breaks made by GluN1 hypomorph (HYPO) and wildtype (WT) mice in 2 hours

GluN1 hypomorph mice appear hyperactive across the whole two hour period and habituate to the environment at a different rate to their wildtype counterparts. Presented as mean ± SEM in 24 5min bins.

Table 3.6: Simple main effects of genotype at each time bin of the crossover beam breaks made by GluN1 hypomorph and wildtype mice over two hours

Time Bin	$F_{(1,52)}$	p
1	**135.347**	**<0.001**
2	**80.806**	**<0.001**
3	**81.663**	**<0.001**
4	**50.179**	**<0.001**
5	**86.090**	**<0.001**
6	**83.697**	**<0.001**
7	**108.664**	**<0.001**
8	**172.392**	**<0.001**
9	**104.351**	**<0.001**
10	**123.717**	**<0.001**
11	**90.042**	**<0.001**
12	**98.971**	**<0.001**
13	**90.167**	**<0.001**
14	**141.384**	**<0.001**
15	**81.332**	**<0.001**
16	**68.939**	**<0.001**
17	**64.674**	**<0.001**
18	**52.189**	**<0.001**
19	**48.094**	**<0.001**
20	**44.358**	**<0.001**
21	**57.589**	**<0.001**
22	**42.244**	**<0.001**
23	**17.486**	**<0.001**
24	**10.837**	**0.002**

GluN1 hypomorph mice are hyperactive at every time point compared to wildtype mice.

Table 3.7: Simple main effects of sex at each time bin of the crossover beam breaks made by GluN1 hypomorph and wildtype mice over two hours

Time Bin	$F_{(1,52)}$	p
1	**8.382**	**0.006**
2	**4.613**	**0.036**
3	**5.250**	**0.026**
4	3.410	0.071
5	1.178	0.283
6	1.268	0.265
7	0.628	0.432
8	0.051	0.822
9	0.057	0.812
10	0.012	0.913
11	0.705	0.405
12	0.665	0.419
13	1.058	0.308
14	0.045	0.832
15	0.008	0.930
16	0.006	0.940
17	0.145	0.705
18	0.085	0.772
19	0.320	0.574
20	0.351	0.556
21	0.000	0.992
22	0.757	0.388
23	0.722	0.399
24	0.380	0.540

Male mice are initially more active than female mice.

3.3.2. Tests of cognition

The results for the tests of cognition in the GluN1 HYPO mice and their WT counterparts are summarised in Table 3.8 and Figures 3.8-10. The percentage of correct responses in spontaneous alternation was analysed using a Mann-Whitney U test with the data collapsed across sex. Univariate ANOVAs using genotype and sex as between-subject factors were used to compare novelty preference in the spatial novelty preference Y-maze test and object recognition, as well as total levels of exploration during the test and training periods of the object recognition test. Repeated measures ANOVAs were used for the spatial reference Y-maze and visual discrimination task using day as the within-subject factor and the same between-subject factors as above.

3.3.2.1. Spontaneous alternation

GluN1 HYPO mice showed a deficit in spontaneous alternation ($U_{(57)}$=63.500, $p<0.001$, Table 3.8).

3.3.2.2. Spatial novelty preference Y-maze test

GluN1 HYPO mice displayed a deficit in spatial novelty preference Y-maze test ($F_{(1,52)}$=7.067, p=0.010) with no effects of sex during testing or any effects of genotype or sex during the sample phase (Table 3.8).

Table 3.8: Performance of GluN1 hypomorph (HYPO) and wildtype (WT) mice in several tests of cognition

Test & Measure	WT, n=29	HYPO, n=26-28	Statistics
Spontaneous Alternation	90% (80-95)	55% (40-70)	**$U_{(57)}$=63.500, p<0.001**
Spatial Novelty Y-maze	0.71 ± 0.04	0.57 ± 0.04	**Geno $F_{(1,52)}$=7.067, p=0.010**
			Sex $F_{(1,52)}$=0.383, p=0.539
			Int $F_{(1,52)}$=1.415, p=0.240
Sample phase ratio	0.51 ± 0.02	0.50 ± 0.02	Geno $F_{(1,52)}$=0.116, p=0.735
			Sex $F_{(1,52)}$=0.165, p=0.687
			Int $F_{(1,52)}$=1.265, p=0.266
Object Recognition			
Preference ratio	0.68 ± 0.03	0.55 ± 0.02	**Geno $F_{(1,51)}$=6.660, p=0.013**
			Sex $F_{(1,51)}$=6.771, p=0.012
			Int $F_{(1,51)}$=0.764, p=0.386
Total exploration in test (s)	23.09 ± 2.02	38.97 ± 4.05	**Geno $F_{(1,51)}$=15.674, p<0.001**
			Sex $F_{(1,51)}$=2.108, p=0.158
			Int $F_{(1,51)}$=5.763, p=0.020
Total exploration in training (s)	56.67 ± 5.03	69.65 ± 6.80	Geno $F_{(1,51)}$=2.581, p=0.114
			Sex $F_{(1,51)}$=0.645, p=0.426
			Int $F_{(1,51)}$=1.421, p=0.239

Parametric data presented as mean ± SEM and analysed with univariate ANOVA. Non-parametric data presented as median (IQR) and analysed with Mann-Whitney U-tests with the data collapsed across the sexes.
Geno = effect of genotype, sex = effect of sex, int = genotype*sex interaction.

GluN1 hypomorph mice show deficits in all tests performed.

3.3.2.3. Appetitively motivated spatial reference memory Y-maze test

GluN1 HYPO mice displayed a deficit in the spatial reference Y-maze test (effect of genotype $F_{(1,47)}$=59.805, p<0.001; effect of day $F_{(9,423)}$=17.789, p<0.001; genotype*day interaction $F_{(9,423)}$=19.617, p<0.001; simple main effects of day; WT $F_{(9,39)}$=17.914, p<0.001; GluN1 HYPO $F_{(9,39)}$=0.885, p=0.547, Figure 3.9). Sex did not affect the spatial reference memory Y-maze test (effect of sex $F_{(1,47)}$=3.277, p=0.077; genotype*sex interaction $F_{(1,47)}$=0.150, p=0.700; day*sex interaction $F_{(9,423)}$=1.673, p=0.093; genotype*day*sex interaction $F_{(9,423)}$=0.515, p=0.863).

3.3.2.4. Object recognition

GluN1 HYPO mice showed a deficit in object recognition ($F_{(1,51)}$=6.660, p=0.013) despite the GluN1 HYPO mice's overall exploration of the objects being greater than in WT mice in the test session ($F_{(1,51)}$=15.674, p<0.001) and being equivalent in the training session ($F_{(1,51)}$=2.581, p=0.114, Table 3.8). Sex affected object recognition performance ($F_{(1,54)}$=6.771, p=0.012) with female mice (0.63±0.02) performing better than male mice (0.54±0.02). Sex and genotype interact on total exploration during the test phase of object recognition ($F_{(1,51)}$=5.763, p=0.020) as a main effect of genotype is seen in female (WT 21.04s±3.31, GluN1 HYPO 47.65±7.17, $F_{(1,51)}$=19.134, p<0.001) but not male mice (WT 25.01s±2.42, GluN1 HYPO 31.53s±3.48, $F_{(1,51)}$=1.288, p=0.262), an effect of sex being seen in GluN1 HYPO mice ($F_{(1,51)}$=7.021, p=0.011) but not WT mice ($F_{(1,51)}$=0.477, p=0.493). No other interactions were seen (see Table 3.8).

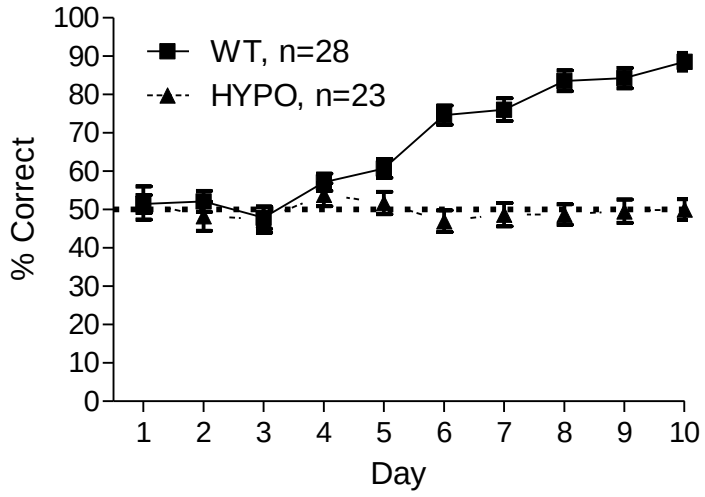


Figure 3.9: Percentage of correct arm choices of GluN1 hypomorph (HYPO) and wildtype (WT) mice in the appetitive spatial reference memory Y-maze test

GluN1 hypomorph mice fail to acquire this spatial reference memory task. Presented as mean ± SEM . Dashed line at 50%, chance level performance.

3.3.2.5. Appetitive visual discrimination task

GluN1 HYPO mice displayed a deficit in the simple visual discrimination task (effect of genotype $F_{(1,46)}$=98.630, p<0.001; effect of day $F_{(7,322)}$=23.386, p<0.001; genotype*day interaction $F_{(7,322)}$=22.328, p<0.001; simple main effects of day; WT $F_{(7,40)}$=51.353, p<0.001; GluN1 HYPO $F_{(7,40)}$=0.918, p=0.503, see Figure 3.10). Sex had no effect on this task (effect of sex F(1,46)=0.294, p=0.590; genotype*sex interaction F(1,46)=0.007, p=0.932; day*sex interaction F(7,322)=1.334, p=0.233; genotype*day*sex interaction F(7,322)=1.026, p=0.412).

The neuroanatomical sensitivity of the visual discrimination task was also explored by performing the same test using hippocampal lesion and sham female C57Bl/6 mice (Figure 3.11). Mice from both groups were seen to acquire this task over time ($F_{(7,119)}$=30.019, p<0.001) but no effect of lesion was seen ($F_{(1,17)}$=0.081, p=0.780) and this did not interact with day ($F_{(7,119)}$=1.447, p=0.193).

3.3.3. Tests of anxiety

The results for the tests of anxiety in the GluN1 HYPO mice and their WT counterparts are summarised in Table 3.9 and Figures 3.11-13. Univariate ANOVAs using genotype and sex as between-subject factors were used to compare several measures in the elevated plus maze (EPM; duration, latency to and frequency of visits to the open arm as well as total distance moved), black/white alley (time spent in and latency to enter the white alley and the number of transitions), the light-dark box (time spent in the light area, latency to the first transition and the total

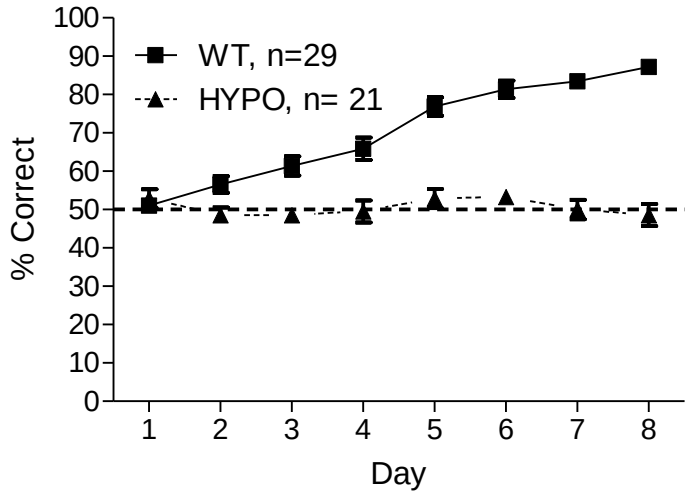


Figure 3.10: Percentage correct arm choices of GluN1 hypomorph (HYPO) and wildtype (WT) mice in a simple visual discrimination task

GluN1 hypomorph mice fail to acquire this non-spatial reference memory task. Presented as mean ± SEM . Dashed line at 50%, chance level performance.

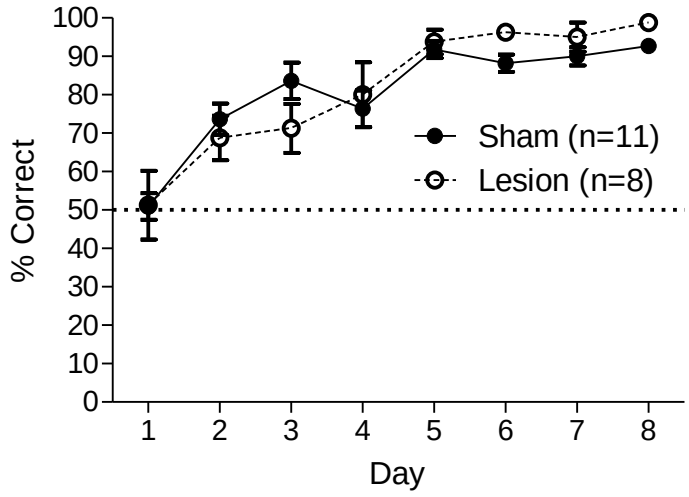


Figure 3.11: Percentage correct arm choices of sham and hippocampal lesion mice in a simple visual discrimination task

Both groups acquired this non-spatial reference memory task. Presented as mean ± SEM . Dashed line at 50%, chance level performance.

number of transitions) both when the mice were started in the light and dark areas, the anxiogenic open field (duration, frequency and latency to the centre as well as total distance moved), the latency to drink in the hyponeophagia test and social and aggressive encounters in the social interaction test. Habituation was used as an additional between-subjects factor in these final two measures. Sex was excluded from the analysis of aggression as this was only observed in male mice. For the successive alleys test, due to the interdependency of duration and latency in each alley on the other alleys, a repeated measures ANOVA was performed for duration with the numerator degrees of freedom reduced by one. Genotype and sex were used as between subject factors and alley as the within-subject factor. p values were then calculated using an online p-value calculator (http://www.danielsoper.com/statcalc/calc07.aspx) based on the F value delivered by SPSS. Latency to enter each alley was analysed by performing separate univariate ANOVAs on each alley. Frequency was treated as independent in each alley and so analysed with an uncorrected repeated measures ANOVA. Alley 4 was excluded from all analyses as no animals entered this area of the apparatus.

3.3.3.1. Elevated plus maze

GluN1 HYPO mice showed no changes in the time spent in ($F_{(1,55)}$=0.459, p=0.501), latency to ($F_{(1,55)}$=0.005, p=0.946), or number of entries into ($F_{(1,55)}$=0.923, p=0.341, Table 3.9) the open arms of the EPM. However, overall time spent in the open arms was low. Genotype did affect the total distance moved during the test ($F_{(1,55)}$=21.704, p<0.001) with GluN1 HYPO mice being hyperactive. An effect of sex was seen

Table 3.9: Results for GluN1 hypomorph (HYPO) and wildtype (WT) mice in several tests of anxiety

Test & Measure	WT, n=24-29	HYPO, n=26-27	Statistics
Elevated Plus Maze			
Time spent in open arms (s)	8.33 ± 2.30	12.46 ± 5.41	Geno $F_{(1,55)}$=0.459, p=0.501 Sex $F_{(1,55)}$=0.003, p=0.959 Int $F_{(1,55)}$=0.085, p=0.771
Latency to open arm (s)	143.24 ± 21.23	139.84 ± 24.83	Geno $F_{(1,55)}$=0.005, p=0.946 **Sex $F_{(1,55)}$=4.215, p=0.045** Int $F_{(1,55)}$=0.021, p=0.886
No. of open arm entries	5.1 ± 0.99	7.7 ± 2.42	Geno $F_{(1,55)}$=0.923, p=0.341 Sex $F_{(1,55)}$=1.190, p=0.280 Int $F_{(1,55)}$=0.081, p=0.777
Distance moved (cm)	1373.34 ± 48.41	1815.46 ± 78.83	**Geno $F_{(1,55)}$=21.704, p<0.001** Sex $F_{(1,55)}$=0.024, p=0.877 Int $F_{(1,55)}$=0.002, p=0.967
Black/White Alley			
Time spent in white alley (s)	14.49 ± 1.80	3.33 ± 1.68	**Geno $F_{(1,55)}$=21.874, p<0.001** **Sex $F_{(1,55)}$=4.214, p=0.045** Int $F_{(1,55)}$=0.849, p=0.361
Latency to white alley (s)	47.23 ± 7.38	104.53 ± 5.34	**Geno $F_{(1,55)}$=44.434, p<0.001** **Sex $F_{(1,55)}$=6.522, p=0.013** Int $F_{(1,55)}$=0.143, p=0.707
Transitions	2.66 ± 0.33	0.63 ± 0.23	**Geno $F_{(1,55)}$=29.731, p<0.001** **Sex $F_{(1,55)}$=7.800, p=0.007** Int $F_{(1,55)}$=2.407, p=0.127
Light-Dark Box: Started in light area			
Time spent in light area (s)	27.26 ± 5.07	74.68 ± 16.45	**Geno $F_{(1,55)}$=7.086, p=0.010** Sex $F_{(1,55)}$=0.139, p=0.711 Int $F_{(1,55)}$=0.025, p=0.876
Latency to dark area (s)	11.49 ± 2.99	61.65 ± 15.41	**Geno $F_{(1,55)}$=9.647, p=0.003** Sex $F_{(1,55)}$=0.243, p=0.624 Int $F_{(1,55)}$=0.588, p=0.446
Transitions	2.79 ± 0.42	2.17 ± 0.43	Geno $F_{(1,55)}$=1.201, p=0.278 **Sex $F_{(1,55)}$=5.040, p=0.029** Int $F_{(1,55)}$=1.736, p=0.193
Light-Dark Box: Started in dark area			
Time spent in light area (s)	21.73 ± 5.91	22.81 ± 9.65	Geno $F_{(1,55)}$=0.611, p=0.438 Sex $F_{(1,55)}$=0.611, p=0.438 Int $F_{(1,55)}$=3.652, p=0.061
Latency to light area (s)	183.66 ± 19.98	208.47 ± 20.20	Geno $F_{(1,55)}$=0.728, p=0.397 Sex $F_{(1,55)}$=0.012, p=0.914 Int $F_{(1,55)}$=3.020, p=0.088
Transitions	1.86 ± 0.38	1.83 ± 0.54	Geno $F_{(1,55)}$=0.001, p=0.972 Sex $F_{(1,55)}$=0.188, p=0.666 Int $F_{(1,55)}$=0.741, p=0.393

Test & Measure	WT, n=24-29	HYPO, n=26-27	Statistics
Open Field			
Time spent in centre (s)	3.36 ± 0.38	5.94 ± 1.00	**Geno $F_{(1,54)}$=7.159, p=0.010**
			Sex $F_{(1,54)}$=9.225, p=0.004
			Int $F_{(1,54)}$=2.665, p=0.108
No. of centre entries	3.93 ± 0.43	6.48 ± 0.95	**Geno $F_{(1,54)}$=7.074, p=0.010**
			Sex $F_{(1,54)}$=7.257, p=0.009
			Int $F_{(1,54)}$=2.467, p=0.122
Latency to enter centre (s)	85.00 ± 14.25	51.60 ± 11.58	Geno $F_{(1,54)}$=3.362, p=0.072
			Sex $F_{(1,54)}$=1.748, p=0.192
			Int $F_{(1,54)}$=0.242, p=0.625
Distance moved (cm)	2442.15 ± 68.69	3279.41 ± 167.71	**Geno $F_{(1,54)}$=20.585, p<0.001**
			Sex $F_{(1,54)}$=0.153, p=0.697
			Int $F_{(1,54)}$=0.521, p=0.473
Hyponeophagia			
Latency to drink (s)	81.17 ± 18.45	266 ± 22.13	**Geno $F_{(1,54)}$=40.152, p<0.001**
			Sex $F_{(1,54)}$=0.654, p=0.422
			Int $F_{(1,54)}$=0.006, p=0.941
Social Interaction (s) (no habituation)	78.34 ± 10.42	43.67 ± 8.73	**Geno $F_{(1,50)}$=9.961, p=0.003**
			Sex $F_{(1,50)}$=20.220, p<0.001
			Int $F_{(1,50)}$=5.952, p=0.018
Aggression (s) (males only, n=14 each)	2.19 ± 2.19	0.84 ± 0.81	$t_{(26)}$=0.580, p=0.567
Social Interaction (s) (following habituation)	98.80 ± 11.61	71.30 ± 6.99	**Geno $F_{(1,46)}$=6.073, p=0.018**
			Sex $F_{(1,46)}$=30.293, p<0.001
			Int $F_{(1,46)}$=2.119, p=0.152
Aggression (s) (males only, n=12 and 10)	62.49 ± 18.04	2.28 ± 0.90	**$t_{(20)}$=3.334, p=0.003**

Presented as mean ± SEM and analysed with univariate ANOVA or t-test.
Geno = effect of genotype, sex = effect of sex, int = genotype*sex interaction.

GluN1 hypomorph mice show mixed results in the battery of anxiety tests performed.

on latency to the open arm ($F_{(1,55)}$=4.215, p=0.045) with female mice (173.93s±23.37) taking a longer time to make their first entry into the open arm compared to male mice (107.97s±21.15). Sex did not interact with genotype on any measure in this test (see Table 3.9).

3.3.3.2. Black/white alley

GluN1 HYPO mice spent less time in the white alley ($F_{(1,55)}$=21.874, p<0.001) of the black/white alley and took more time to first enter this area ($F_{(1,55)}$=44.434, p<0.001, Table 3.9), implying they are more anxious in this test. GluN1 HYPO mice also made fewer transitions between the black and white areas ($F_{(1,55)}$=29.731, p<0.001). Sex also affected these measures with male mice (11.22s±2.26) spending more time in the white area than female mice (6.49s±1.67, $F_{(1,52)}$=4.214, p=0.045), making their first entry into this area sooner (male 65.68s±8.78, female 86.69s±7.46, $F_{(1,52)}$=6.522, p=0.013) and making more transitions between areas, also (male 2.14±0.39, female 1.13±0.25, $F_{(1,55)}$=7.800, p=0.007). Sex did not, however, interact with genotype on any of these measures in this test (see Table 3.9).

3.3.3.3. Light/dark box

In the light/dark box, GluN1 HYPO mice were seen to spend more time in the light area than WT mice ($F_{(1,55)}$=7.086, p=0.010) and take a longer period of time to move into the dark area ($F_{(1,55)}$=9.647, p=0.003, see Table 3.9) when started in the light, suggesting they are more anxious in this test. These effects were not seen when started in the dark (time spent in light area $F_{(1,55)}$=0.611, p=0.438; latency to light area $F_{(1,55)}$=0.728, p=0.397). No effect of genotype was seen in the number of transitions

made between different areas either when started in the light ($F_{(1,55)}$=1.201, p=0.278) or dark area ($F_{(1,55)}$=0.001, p=0.972). However, sex did affect the number of transitions made when started in the light ($F_{(1,55)}$=5.040, p=0.029) with male mice (3.14±0.53) making more transitions than female mice (1.83±0.26), but sex did not interact with genotype in any measure across the two versions of this test (see Table 3.9).

3.3.3.4. Successive alleys

In the successive alleys test, there is a strong trend for a genotype*alley interaction on duration in each section ($F_{(1,108)}$=3.837, p=0.053, Figure 3.12). Simple main effects were analysed nonetheless and revealed differences between the genotypes in alley 2 (alley 1 $F_{(1,54)}$=3.239, p=0.077; alley 2 $F_{(1,54)}$=5.690, p=0.021; alley 3 $F_{(1,54)}$=0.051, p=0.822) with WT mice (31.33s±4.67) spending more time than GluN1 HYPO mice (15.60s±4.67) in this section, again implying the GluN1 HYPO mice are more anxious. This genotype*alley interaction was not seen in frequency of arm entries ($F_{(2,108)}$=0.266, p=0.767, Figure 3.13) nor was any main effect of genotype ($F_{(1,54)}$=0.062, p=0.804), implying both genotypes showed a similar level of activity in this test.

Latency to the second alley was affected by genotype ($F_{(1,54)}$=10.951, p=0.002) with GluN1 HYPO mice (183.35s±21.27) taking longer to make their first entry to alley 2 than WT mice (93.24s±16.15; Figure 3.14). This was not seen for alley 3 ($F_{(1,54)}$=1.755, p=0.191).

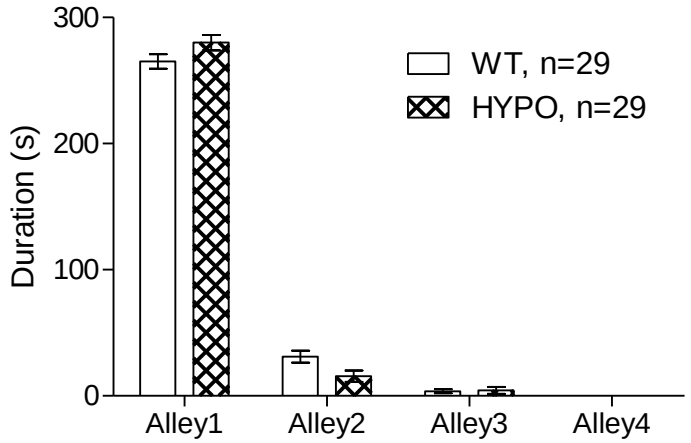


Figure 3.12: The time spent in each alley of the successive alleys by GluN1 hypomorph (HYPO) and wildtype (WT) mice

The GluN1 hypomorph mice spend less time in alley 2 of the successive alleys than their wildtype counterparts. NB: no mice entered alley 4. Presented as mean ± SEM.

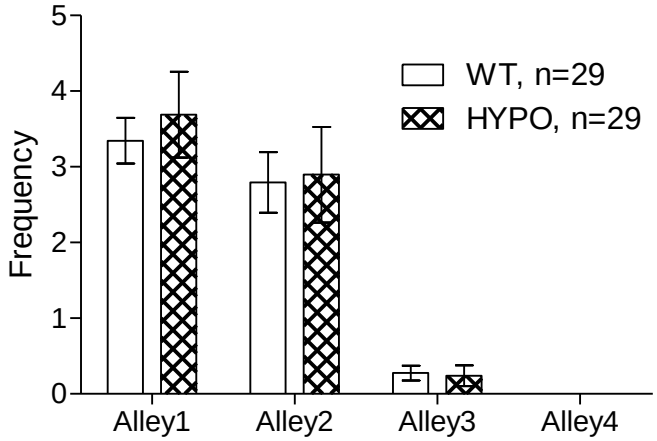


Figure 3.13: The number of entries into each alley of the successive alleys by GluN1 hypomorph (HYPO) and wildtype (WT) mice

The behaviour of the GluN1 hypomorph mice does not differ from that of their wildtype counterparts in the number of alley entries made in the successive alleys. NB: no mice entered alley 4. Presented as mean ± SEM.

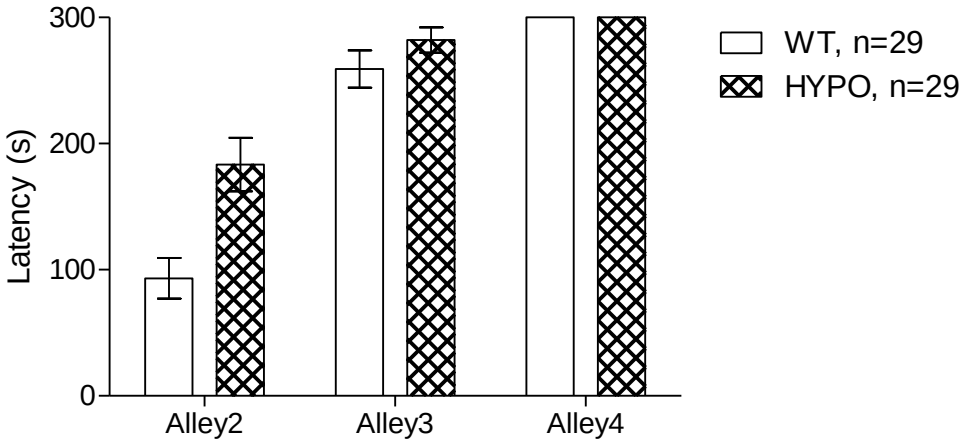


Figure 3.14: The latency to first entry into each alley of the successive alleys by GluN1 hypomorph (HYPO) and wildtype (WT) mice

GluN1 hypomorph mice are slower to cross into alley 2 of the successive alleys than their wildtype counterparts. NB: no mice entered alley 4. Presented as mean ± SEM.

There were no interactions with sex in any of these three measures in the successive alleys (duration: alley*sex interaction $F_{(1,108)}$=2.202, p=0.141; genotype*alley*sex interaction $F_{(1,108)}$=0.343, p=0.559; number of entries: main effect of sex $F_{(1,54)}$=1.757, p=0.191; genotype*sex interaction $F_{(1,54)}$=0.748, p=0.391; alley*sex interaction $F_{(2,108)}$=1.069, p=0.337; genotype*alley*sex interaction $F_{(2,108)}$=0.842, p=0.434; latency: alley 2: sex $F_{(1,54)}$=0.130, p=0.719; sex*genotype interaction $F_{(1,54)}$=0.050, p=0.825; alley 3: sex $F_{(1,54)}$=2.771, p=0.102; sex*genotype interaction $F_{(1,54)}$=0.404, p=0.528).

3.3.3.5. Open field

In the open field, GluN1 HYPO mice spent more time in the centre area than WT mice (effect of genotype $F_{(1,54)}$=7.159, p=0.010) and also made more entries into this area ($F_{(1,54)}$=7.074, p=0.010, see Table 3.9) implying they are less anxious in this test. GluN1 HYPO mice were also observed to cover a greater distance during the test than WT mice ($F_{(1,54)}$=20.585, p<0.001). When this is taken as a covariate, these genotype effects no longer remain (effects of genotype: time spent in centre $F_{(1,53)}$=1.728, p=0.194; number of entries to centre $F_{(1,53)}$=0.309, p=0.581) and the overall effect of distance is much greater in the number of entries made (effect of distance $F_{(1,53)}$=48.486, p<0.001) than in the time spent there ($F_{(1,53)}$=3.769, p=0.058). An effect of sex is also seen in both duration in ($F_{(1,54)}$=9.225, p=0.004) and number of entries to the centre ($F_{(1,54)}$=7.257, p=0.009) with male mice (6.20s±0.91) spending more time in the centre than female mice (3.20s±0.60), as well as making more entries into the centre (male 6.57±0.86, female 3.93±0.60). These effects remain with distance taken as a covariate (effects of sex: time spent in centre $F_{(1,53)}$=9.040, p=0.004; number of entries to centre $F_{(1,53)}$=11.006, p=0.002) and also pulls out an interaction between genotype and sex in number of entries ($F_{(1,53)}$=7.973, p=0.007) but not in duration ($F_{(1,53)}$=3.443,

p=0.069). Simple main effects reveal this to be driven by effects of genotype being seen in female (GluN1 HYPO 4.47±0.98, WT 3.40±0.70; $F_{(1,53)}$=4.986, p=0.030) but not male mice (GluN1 HYPO 8.64±1.50, WT 4.50±1.50; $F_{(1,53)}$=2.040, p=0.159), effects of sex being present in GluN1 HYPO ($F_{(1,53)}$=18.950, p<0.001) but not WT mice ($F_{(1,53)}$=0.116, p=0.734). No genotype*sex interactions are seen when distance in not taken as a covariate (see Table 3.9).

3.3.3.6. Hyponeophagia

GluN1 HYPO mice were seen to take longer before drinking in the hyponeophagia test ($F_{(1,54)}$=40.152, p<0.001, Table 3.9) implying they are more anxious in this test. Sex had no effect on this test (see Table 3.9).

3.3.3.7. Social interaction

With no habituation to the testing arena, GluN1 HYPO mice showed a decrease in social behaviour (effect of genotype $F_{(1,50)}$=9.961, p=0.003) but no alteration in aggression (males only, $t_{(20)}$=3.334, p=0.003, Table 3.9). Sex affected time spent socially interacting ($F_{(1,50)}$=20.220, p<0.001) with male mice (85.89s±11.91) interacting more than female (35.53s±3.14) mice. Sex also interacted with genotype ($F_{(1,50)}$=5.952, p=0.018) due to a simple main effect of genotype only being present in male ($F_{(1,50)}$=16.308, p<0.001) and not female mice ($F_{(1,50)}$=0.247, p=0.662) and an effect of sex only being present in WT ($F_{(1,50)}$=25.059, p<0.001) and not GluN1 ($F_{(1,50)}$=2.034, p=0.160) mice. This is due to a surprisingly low level of social behaviour in WT female mice (39.25s±4.22) which does not differ from the levels in female GluN1 HYPO mice (31.18s±4.55), but more usual levels of social behaviour being seen in the

male WT mice (117.42s±14.07) which is much greater than that seen in male GluN1 mice (54.36s±15.43).

GluN1 HYPO mice also displayed a reduction in social behaviour even with habituation to the testing arena ($F_{(1,46)}$=6.073, p=0.018) as well as in aggressive behaviour (males only, $t_{(20)}$=3.334, p=0.003, Table 3.9). This difference may be present here and not in the animals tested with no habituation due to the overall higher levels of aggression seen in this version of the test. Social interaction following habituation was also affected by sex ($F_{(1,46)}$=30.293, p<0.001) with male mice (120.14s±11.34) spending more time interacting than female mice (58.46s±4.85) but no genotype*sex interaction was seen following habituation ($F_{(1,46)}$=2.119, p=0.152). Again, this may be due to higher levels of social behaviour in the female mice in this version of the test.

3.3.4. Tests of affect

The results for the tests of affect in the GluN1 HYPO mice and their WT counterparts are summarised in Tables 3.10-12 and Figures 3.14-17. Univariate ANOVAs using genotype and sex as between-subject factors were used to compare sucrose preference ratio, water intake and sucrose intake in the sucrose preference test and immobility, swimming and climbing in the forced swim test (FST). Repeated measures ANOVAs were then used to compare the time spent immobile in the FST across the two days of the test using day as the within-subject factor and the same between-subject factors as above. A repeated measures ANOVA was also used to analyse locomotor activity across the diurnal cycle with time as the within-subject factor.

3.3.4.1. Sucrose preference

Sucrose preference was increased in GluN1 HYPO mice ($F_{(1,50)}$=6.386, p=0.015, Table 3.10). This was driven by an increase in sucrose consumption ($F_{(1,50)}$=6.680, p=0.013) and no change in water intake ($F_{(1,50)}$= 2.355, p=0.131). A genotype*sex interaction was seen in sucrose intake ($F_{(1,50)}$=4.842, p=0.032) as the increase is seen in male mice (WT 24.09ml±1.01; GluN1 HYPO 32.12ml±2.58; $F_{(1,50)}$=10.594, p=0.002) but not female mice (WT 27.10ml±1.47; GluN1 HYPO 27.74ml±1.66; $F_{(1,50)}$=0.080, p=0.778) with sex effects not seen in either genotype (WT $F_{(1,50)}$=1.745, p=0.193; GluN1 HYPO $F_{(1,50)}$=3.151, p=0.082). Interactions were not seen in water intake or sucrose preference ratio (see Table 3.10).

3.3.4.2. Locomotor activity across the diurnal cycle

Locomotor activity differed across the diurnal cycle for all mice (effect of time $F_{(46,2346)}$=33.570, p<0.001, Figure 3.15) and the two genotypes differed over this time (effect of genotype $F_{(1,51)}$=164.438, p<0.001) with GluN1 HYPO mice generally looking hypoactive. The changes over time also differed between the genotypes (genotype*time interaction $F_{(46,2346)}$=13.835, p<0.001) with both genotypes showing simple main effects of time (WT $F_{(46,1288)}$=25.467, p<0.001; GluN1 HYPO $F_{(46,1150)}$=25.910, p<0.001) and differences between the genotypes being present in most of the time bins except towards the end of the dark period (4am-7.30am, see Figure 3.15 and Table 3.11). Seemingly, at this point the WT mice have reduced their activity across the dark period to a level that matches the constantly low activity of the GluN1 HYPO mice over this time. There was no main effect of sex ($F_{(1,51)}$=3.425,

Table 3.10: Results for GluN1 hypomorph (HYPO) and wildtype (WT) mice in the sucrose preference test

Measure	WT, n=29	HYPO, n=25	Statistics
Sucrose preference	0.68 ± 0.01	0.72 ± 0.01	**Geno $F_{(1,50)}$=6.386, p=0.015**
			Sex $F_{(1,50)}$=0.046, p=0.832
			Int $F_{(1,50)}$=1.589, p=0.213
Water Intake (ml)	10.94 ± 0.33	10.19 ± 0.31	Geno $F_{(1,50)}$= 2.355, p=0.131
			Sex $F_{(1,50)}$=0.403, p=0.528
			Int $F_{(1,50)}$=0.128, p=0.722
Sucrose Intake (ml)	25.65 ± 0.93	29.67 ± 1.50	**Geno $F_{(1,50)}$=6.680, p=0.013**
			Sex $F_{(1,50)}$=0.168, p=0.684
			Int $F_{(1,50)}$=4.842, p=0.032

Presented as mean ± SEM and analysed with univariate ANOVA.
Geno = effect of genotype, sex = effect of sex, int = genotype*sex interaction.

GluN1 hypomorph mice show an increase in sucrose preference driven by an increase in sucrose intake.

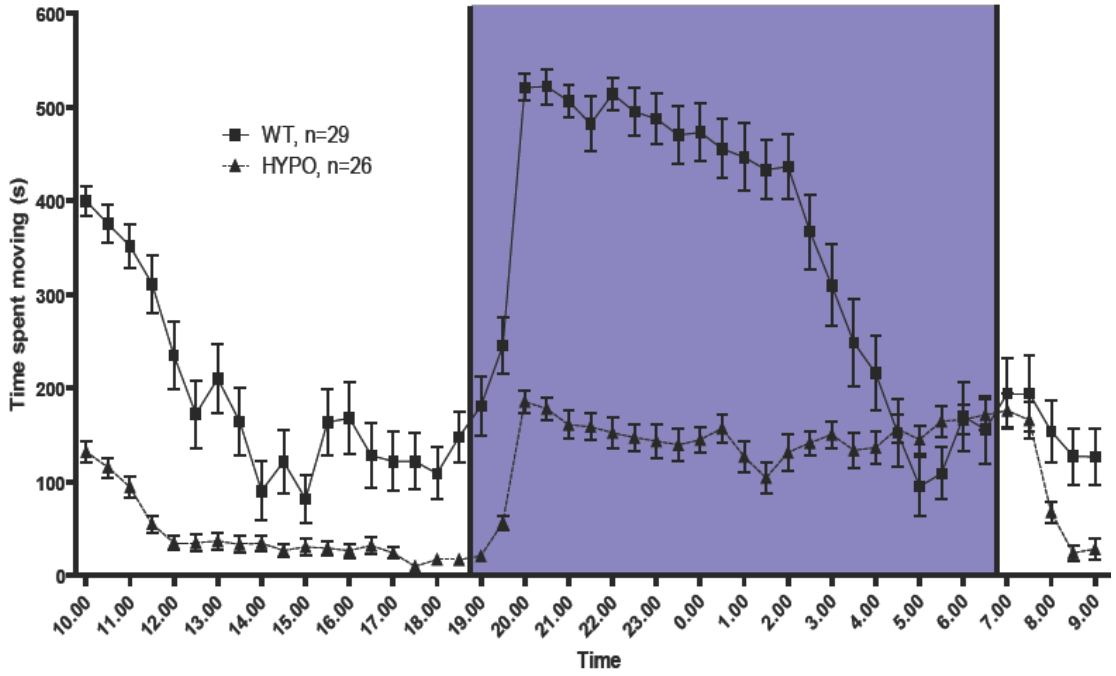


Figure 3.15: Locomotor activity for GluN1 hypomorph (HYPO) and wildtype (WT) mice over a 23.5 hour period

The level of movement of the GluN1 hypomorph mice differed greatly from that of their wildtype counterparts over the diurnal cycle. Shaded area = lights off period, 7pm-7am, presented as mean ± SEM in 47 30min bins.

Table 3.11: Simple main effect of genotype at each time point during the test of locomotor activity over the diurnal cycle in GluN1 hypomorph and wildtype mice

Time	$F_{(1,51)}$	p	Time	$F_{(1,51)}$	p
10am	**200.282**	**<0.001**	**10pm**	**243.468**	**<0.001**
10.30am	**138.071**	**<0.001**	**10.30pm**	**130.788**	**<0.001**
11am	**109.306**	**<0.001**	**11pm**	**113.348**	**<0.001**
11.30am	**58.444**	**<0.001**	**11.30pm**	**85.857**	**<0.001**
12pm	**26.587**	**<0.001**	**12am**	**97.315**	**<0.001**
12.30pm	**12.165**	**0.001**	**12.30am**	**70.223**	**<0.001**
1pm	**18.199**	**<0.001**	**1am**	**60.850**	**<0.001**
1.30pm	**10.986**	**0.002**	**1.30am**	**80.229**	**<0.001**
2pm	2.490	0.121	**2am**	**54.280**	**<0.001**
2.30pm	**6.889**	**0.011**	**2.30am**	**27.086**	**<0.001**
3pm	3.277	0.076	**3am**	**11.182**	**0.002**
3.30pm	**14.528**	**<0.001**	**3.30am**	**4.987**	**0.030**
4pm	**13.345**	**0.001**	4am	3.192	0.080
4.30pm	**6.587**	**0.013**	4.30am	0.005	0.945
5pm	**8.839**	**0.004**	5am	1.940	0.170
5.30pm	**11.952**	**0.001**	5.30am	2.697	0.107
6pm	**9.310**	**0.004**	6am	0.003	0.956
6.30pm	**19.774**	**<0.001**	6.30am	0.151	0.699
7pm	**23.700**	**<0.001**	7am	0.229	0.635
7.30pm	**31.007**	**<0.001**	7.30am	0.506	0.480
8pm	**304.471**	**<0.001**	**8am**	**5.865**	**0.019**
8.30pm	**224.833**	**<0.001**	**8.30am**	**9.417**	**0.003**
9pm	**216.911**	**<0.001**	**9am**	**8.237**	**0.006**
9.30pm	**97.125**	**<0.001**			

GluN1 hypomorph mice show differing locomotor activity to wildtype mice at most points of the diurnal cycle except towards the end of the dark period.

p=0.070), but sex, too, interacted with time ($F_{(46,2346)}$=1.720, p=0.002) with both sexes showing an effect of time (male $F_{(46,1150)}$=13.245, p<0.001; female $F_{(46,1288)}$=17.079, p<0.001) but the differences between the sexes being present only transiently across the cycle (Table 3.12). Sex did not interact with genotype in this test ($F_{(1,51)}$=1.116, p=0.296) and there was no three-way interaction ($F_{(46,2346)}$=1.159, p=0.216).

3.3.4.3. Forced swim test

On day 1 of the FST (Figure 3.16), GluN1 HYPO mice spent less time immobile than WT mice (effect of genotype $F_{(1,47)}$=27.596, p<0.001), so appear less helpless than their WT counterparts. Sex also effected time spent immobile ($F_{(1,47)}$=5.411, p=0.024) with male mice (123.03s±11.27) spending more time immobile than female mice (87.14s±9.24). Genotype and sex did not interact ($F_{(1,47)}$=1.648, p=0.206). The differences in time spent immobile between the genotypes and the sexes were driven by differences in time spent swimming (effect of genotype $F_{(1,47)}$=30.835, p<0.001; effect of sex $F_{(1,47)}$=6.649, p=0.013; genotype*sex interaction $F_{(1,47)}$=2.109, p=0.153) and not by changes in climbing (effect of genotype $F_{(1,47)}$=0.155, p=0.696; effect of sex $F_{(1,47)}$=0.053, p=0.819; genotype*sex interaction $F_{(1,47)}$=1.230, p=0.273).

On day 2 of the FST (Figure 3.17), GluN1 HYPO mice again spent less time immobile than WT mice (effect of genotype $F_{(1,47)}$=12.756, p=0.001), driven this time by changes both in time spent swimming ($F_{(1,47)}$=8.066, p=0.007) and climbing ($F_{(1,47)}$=13.296, p=0.001).

Table 3.12: Simple main effect of sex at each time point during the test of locomotor activity over the diurnal cycle in GluN1 hypomorph and wildtype mice

Time	$F_{(1,51)}$	p	Time	$F_{(1,51)}$	p
10am	**8.580**	**0.005**	10pm	**4.953**	**0.030**
10.30am	**7.411**	**0.009**	10.30pm	2.415	0.126
11am	**4.935**	**0.031**	11pm	**4.810**	**0.033**
11.30am	2.177	0.146	11.30pm	4.014	0.050
12pm	1.193	0.280	12am	**7.195**	**0.010**
12.30pm	0.475	0.494	12.30am	**4.234**	**0.045**
1pm	0.328	0.570	1am	2.787	0.101
1.30pm	0.177	0.676	1.30am	2.762	0.103
2pm	0.042	0.838	2am	1.007	0.320
2.30pm	0.613	0.437	2.30am	2.481	0.121
3pm	0.589	0.446	3am	2.583	0.114
3.30pm	**5.248**	**0.026**	3.30am	2.095	0.154
4pm	**5.260**	**0.026**	4am	0.712	0.403
4.30pm	0.742	0.393	4.30am	0.552	0.461
5pm	3.610	0.063	5am	0.465	0.498
5.30pm	0.961	0.332	5.30am	0.276	0.602
6pm	0.562	0.457	6am	0.075	0.785
6.30pm	0.019	0.892	6.30am	2.207	0.144
7pm	2.959	0.091	7am	3.079	0.085
7.30pm	0.000	0.989	7.30am	0.054	0.817
8pm	0.250	0.620	8am	1.246	0.270
8.30pm	1.311	0.257	8.30am	1.221	0.274
9pm	2.008	0.163	9am	0.002	0.966
9.30pm	**4.301**	**0.043**			

Effects of sex are only present transiently in the locomotor activity of GluN1 hypomorph and wildtype mice over the diurnal cycle.

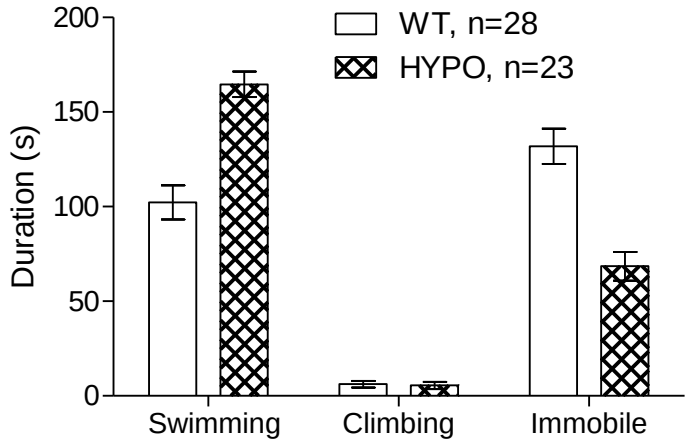


Figure 3.16: Time spent swimming, climbing and immobile in minutes 3-6 on day 1 of the forced swim test by GluN1 hypomorph (HYPO) and wildtype (WT) mice

GluN1 hypomorph mice spend less time immobile than their wildtype counterparts on day 1 of the forced swim test. This is driven by an increase in swimming but not climbing. Presented as mean ± SEM.

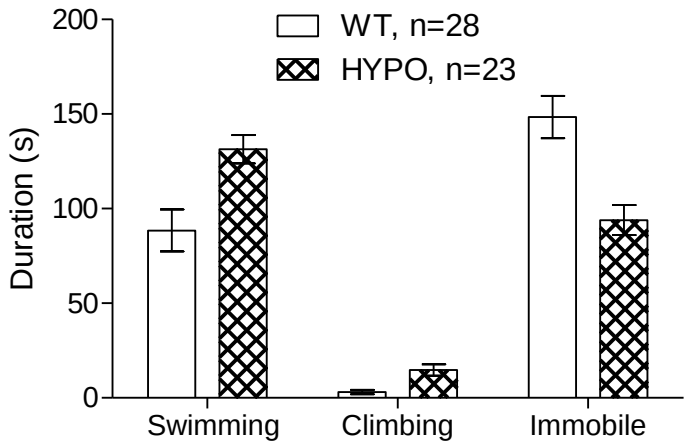


Figure 3.17: Time spent swimming, climbing and immobile in minutes 3-6 on day 2 of the forced swim test by GluN1 hypomorph (HYPO) and wildtype (WT) mice

GluN1 hypomorph mice spend less time immobile than their wildtype counterparts on day 2 of the forced swim test. This is driven by an increase in swimming as well as climbing. Presented as mean ± SEM.

There was no main effect of sex or interaction involving sex in any of these measures on day 2 of the FST (effects of sex: immobility $F_{(1,47)}$=3.740, p=0.059; swimming $F_{(1,47)}$=3.068, p=0.086; climbing $F_{(1,47)}$=0.944, p=0.336; genotype*sex interactions: immobility $F_{(1,47)}$=0.200, p=0.657; swimming $F_{(1,47)}$=0.048, p=0.827; climbing $F_{(1,47)}$=1.273, p=0.265).

To investigate if any changes occurred across the two days of the test, a repeated measures ANOVA was performed on the time spent immobile on both days. Time spent immobile increased over the two days of the forced swim test (Day1: WT 131.93s±9.26, GluN1 HYPO 68.50s±7.62; Day2: WT 148.38s±11.17, GluN1 HYPO 93.94s±7.93; effect of day $F_{(1,47)}$=6.042, p=0.018, Figure 3.18) and GluN1 HYPO mice overall spent less time immobile than WT mice (effect of genotype $F_{(1,47)}$=35.414, p<0.001) but genotype did not interact with day ($F_{(1,47)}$=0.396, p=0.532). Time spent immobile differed between the sexes ($F_{(1,47)}$=8.406, p=0.006) but this, too, did not interact with other factors (genotype*sex interaction $F_{(1,47)}$=0.216, p=0.644; day*sex interaction $F_{(1,47)}$<0.001, p=1.000; genotype*day*sex interaction $F_{(1,47)}$=1.430, p=0.238). This shows the effect of genotype to be robust across days.

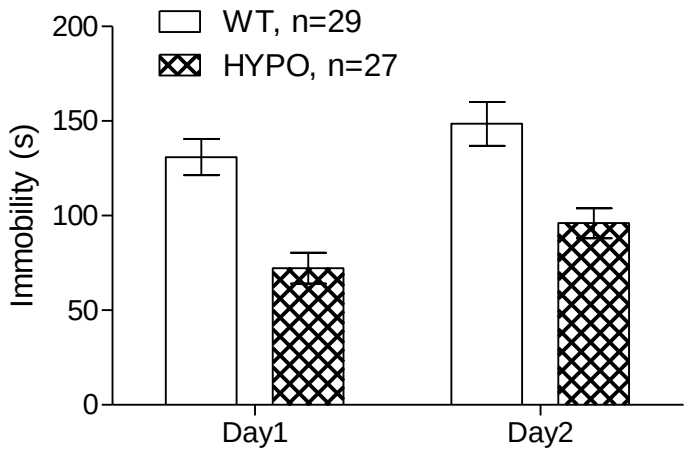


Figure 3.18: Time spent immobile in minutes 3-6 on days 1 and 2 of the forced swim test by GluN1 hypomorph (HYPO) and wildtype (WT) mice

GluN1 hypomorph mice show decreased levels of immobility on both days of the forced swim test as compared to their wildtype counterparts. Presented as mean ± SEM.

3.4. Discussion

3.4.1. Summary of results

This chapter aimed to behaviourally phenotype the GluN1 hypomorph (HYPO) mice with particular focus on tests of emotionality. Male and female GluN1 HYPO mice were compared to their wildtype (WT) littermates in several behavioural tests. This revealed a global impairment in the GluN1 HYPO mice across all domains of behaviour tested including naturalistic behaviour, cognition, anxiety and affect.

3.4.1.1. Naturalistic behaviour

GluN1 HYPO mice showed alterations in all naturalistic behaviours with the only exception being water consumption. This included deficits in nesting behaviour and burrowing, two tests of "species-typical behaviour". Deficits in these, therefore, illustrate the global and great magnitude of the changes in behaviour seen in these mice. GluN1 HYPO mice show an increase in pellet consumption, a decrease in body weight and increases in locomotion. This increase in locomotion and weight loss are also seen following NMDA receptor antagonist treatment (eg Tonkiss *et al.*, 1988, Trullas and Skolnick, 1990, Maj *et al.*, 1992b, Panconi *et al.*, 1993, Engin *et al.*, 2009). All of these measures may be related; the great increase in locomotor activity would likely result in an increase in calorie use, reducing body weight and increasing calorific intake through increased pellet consumption. Contrary to this, the GluN1 HYPO mice show a decrease in glucose consumption compared to WT mice. This, however, may be confounded by changes in responses to novelty (see, for instance, the findings in the hyponeophagia test, Sections 3.3.3 and 3.4.1.3); the GluN1 HYPO mice may consume less glucose as they are less willing to consume a novel substance

than the WT mice. The massive increase in locomotion may, in itself, confound many other measures.

The enormous difference in short-term exposure to a testing environment, as would be common in other behavioural tests, is well demonstrated in the non-anxious open field in which the GluN1 HYPO mice show an approximately two-fold increase in the distance covered. This short exposure is more akin to the testing context in other behavioural paradigms rather than the long-term exposure in the beam-break locomotor test. As to whether the GluN1 HYPO mice ever reach WT levels, the 2 hour assessment of locomotor activity suggests this would take a long time, if it was ever achieved; while the GluN1 HYPO mice do show some habituation to the environment, implying this hyperactivity may be a response to novelty rather than a generalised increase in motion, it still does not appear to be normalised by the end of this period of time. A habituation deficit is therefore not likely to completely explain the hyperactivity seen. Other behavioural testing will not involve the hours of exposure seemingly needed to normalise this behaviour in the GluN1 HYPO mice. This hyperactivity is therefore a possible confound that must be considered in other tests.

3.4.1.2. Cognition

GluN1 HYPO mice showed deficits in all of the cognitive tasks performed. This included spontaneous alternation and the spatial novelty preference Y-maze test, both tests of spatial working memory, the appetitively motivated spatial reference memory Y-maze, a test of spatial reference memory, object recognition and a simple visual discrimination task, both being non-spatial memory tasks. WT performance in

all of these tasks was above chance, validating the tests themselves. That hippocampectomised mice can perform the simple visual discrimination task confirms this test to be hippocampal-independent. The impairments seen in the GluN1 HYPO mice, in contrast to the good performance of the WT mice in these tests, implies the GluN1 HYPO mice show a generalised impairment across all domains of cognition. This includes both hippocampal-dependent and –independent tasks, both working-memory tasks as well as those involving incremental learning. Having been unable to demonstrate any task in which the GluN1 HYPO mice perform normally also precludes any dissection of these mice's ability to learn versus their performance or demonstration of learning. This makes them unsuitable to testing on more complex tasks. This severe cognitive impairment may also act as a confound to other tests performed that involve any learning element.

The analogous pharmacological studies using NMDA receptor antagonists focus, again, on the use of NMDA receptor antagonism in modelling schizophrenia as well as the possible use of some antagonists in improving cognition in dementia as discussed below. There is also a great deal of interest in the role of NMDA receptors in the establishment of LTP, thought to be the molecular correlate of learning, and how antagonism of NMDA receptors may effect this (Collingridge *et al.*, 1983). This field therefore assumes NMDA receptor antagonism will always result in a cognitive deficit in any task requiring this form of plasticity. This demonstrates the contrasting results found with differing pharmacological tools; some produce cognitive deficits, particularly in tasks requiring LTP-induced changes, while some improve learning and memory. However, confounding factors such as hyperactivity and increased impulsivity found with a lot of NMDA receptor antagonists limit the amount of complex cognitive work done using these drugs.

The competitive NMDA receptor antagonist AP5 may impair acquisition of a spatial task in the Morris Water Maze (MWM, Morris *et al.*, 1986, Morris, 1989. For an in-depth discussion of these results, see Bannerman and colleagues, 2006) but not a visual task in the water maze. Infusion of 30mM AP5 also impairs the spatial rewarded alternation task (Tonkiss and Rawlins, 1991). Interestingly, this study also shows intraperitoneal (i.p.) injection of MK-801 does not affect learning on this task, whereas others have shown it only produces a mild deficit as compared to those produced by ketamine or phencyclidine (PCP, Wiley *et al.*, 2003). PCP treatment early in life, which may better recapitulate the neurodevelopmental aspects of the GluN1 HYPO mice, also produced deficits in the MWM task, although only in male and not female rats (Andersen and Pouzet, 2004). Similar dosing protocols also show a deficit in rewarded alternation (Wiley *et al.*, 2003) as well as the intradimensional/extradimensional shift test of executive function (Wang *et al.*, 2001).

In contrast, chronic memantine has been shown to increase performance on the MWM in C57Bl/6 mice (Minkeviciene *et al.*, 2004, 2008). This discrepancy is thought to be due to the relatively low affinity of memantine for the NMDA receptor when compared to other NMDA receptor antagonists. This has also made it an attractive candidate for a "cognitive enhancer" for use in Alzheimer's disease. It further indicates that the role of the NMDA receptor in cognition is more complex than previously thought and antagonism at this receptor cannot be thought to universally result in deficits in learning and memory tasks.

In the case of the GluN1 HYPO mice, the sparing of performance in a visual platform form of the MWM following AP5 treatment is in contrast to the failure of the GluN1 HYPO mice to acquire a simple visually-guided discrimination task in a T-maze. That hippocampal lesioned animals were able to acquire this task also implies this does not rely upon NMDA receptor-dependent hippocampal LTP, thought to underlie spatial tasks. This implies, therefore, a global cognitive deficit that goes beyond that seen in postnatally-induced reductions of NMDA receptor-mediated neurotransmission.

3.4.1.3. Anxiety

GluN1 HYPO mice show mixed performance on the tests of anxiety performed (see Table 3.13). They appear less anxious than WT mice in the light/dark box when started in the light area and the open field test, although the group difference seen in the latter test is no longer present when the hyperactivity of the GluN1 HYPO mice is taken into consideration. They appear more anxious in the black/white alley and the hyponeophagia test, as well as possibly in the successive alleys, and show decreased social interactions. GluN1 HYPO mice show no altered anxiety compared to WT mice in the elevated plus maze (EPM) or the light-dark box when started in the dark area. This makes any assertions on the overall levels of anxiety in these mice difficult. What is clear, however, is that simple hyperactivity cannot explain all of these results; if this was all that was being observed, the GluN1 HYPO mice would be expected to look less anxious in tests such as the hyponeophagia test that use latency measures. The same pattern of decreased movement or increased latency to the

Table 3.13: Summary of anxiety results for GluN1 hypomorph (HYPO) mice compared to their wildtype (WT) counterparts

Test & Measure	HYPO mice anxiety levels compared to WT
Elevated Plus Maze	
Time spent in open arms (s)	↔
Latency to open arm (s)	↔
No. of open arm entries	↔
Black/White Alley	
Time spent in white alley (s)	↑
Latency to white alley (s)	↑
Light-Dark Box: Started in light area	
Time spent in light area (s)	↓
Latency to dark area (s)	↓
Light-Dark Box: Started in dark area	
Time spent in light area (s)	↔
Latency to light area (s)	↔
Open Field	
Time spent in centre (s)	↓
No. of centre entries	↓
Latency to enter centre (s)	↔
Hyponeophagia	
Latency to drink (s)	↑
Social Interaction (s)	
No habituation	↑
Following habituation	↑

↑ = increased anxiety, ↓ = decreased anxiety, ↔ = no change.

GluN1 hypomorph mice show mixed results on tests of anxiety, with no change being seen in the elevated plus maze and light-dark box when started in the dark, an increase in anxiety being seen in the black/white alley, hyponeophagia and social interaction tests and an apparent decrease in anxiety being seen in the light-dark box when started in the light area and in the open field.

anxiogenic area of the testing environment is also seen in the black/white alley. GluN1 HYPO mice would also be expected to look more anxious in the light-dark box when started in the light as, if simple hyperactivity were being observed, their latency to the less-anxiogenic dark area would be decreased compared to WT mice, not increased.

The idea of locomotor activity as a confound may also be inappropriate when discussing anxiety tests, as it may be that alterations in the level of movement in a given environment result from levels of anxiety and not *vice versa*. Furthermore, if the increase in locomotion does represent a failure to habituate, this may offer a more cognitive account for any apparent alterations in anxiety. Measures of anxiety that do not rely upon movement would dissociate these factors. Examples of this would be using telemetric systems to measure blood pressure or heart rate or more simply by measuring defecation during these anxiety tests (Hall, 1921). This latter measure was taken during anxiety tests but did not prove any more consistent than other measures and so has not been presented. There is a further anecdotal piece of evidence to suggest a complex anxiety phenotype in these mice; while the GluN1 HYPO mice appeared less anxious in the light-dark box when started in the light, this was largely due to the GluN1 HYPO mice freezing when initially introduced to the testing arena. This, in fact, would imply an increase in anxiety and is due to an unusual reduction in locomotion in the GluN1 HYPO mice, again illustrating that simple hyperactivity cannot explain in the entirety the results seen here. It may also represent a competing factor to the usual hyperactivity and therefore a polyphenotypic condition.

The generally low levels of time spent in the centre of the open field and on the open arms of the EPM may account for the weak/absence of a genotype effect seen in these tests. However, given the conflicting results seen elsewhere, these potential floor effects do not fully explain the results in tests of anxiety presented here.

These conflicting results in many ways reflect what is seen in the literature dealing with the effects of NMDA receptor antagonists in some of these rodent tests of anxiety, with the majority of literature supports NMDA receptor antagonism as being anxiolytic but much of the ketamine literature contradicting this (see Section 3.1.1.2.1). NMDA receptors therefore have a complex, unclear role in anxiety which may account for the inconsistent results seen in the GluN1 HYPO mice. Alternatively, these ambiguous results may be due to competing behaviours.

3.4.1.4. Affect

GluN1 HYPO mice show an overall anti-depressive phenotype as exhibited by their increase in sucrose preference and decrease in immobility in the forced swim test (FST) on both days. They also show hypoactivity in the assessment of activity across the diurnal cycle. This is in contrast to the marked hyperactivity they display in all other tests. While hyperactivity may act as a confound in some of these procedures, it is not the sole behaviour being observed in these tests.

While the general hyperactivity of the GluN1 HYPO mice may offer a non-affect explanation for their reduced immobility in the FST, general activity in mice and performance in this test are not always correlated, including when NMDA receptor antagonists are used (Trullas and Skolnick, 1990, Maj *et al.*, 1992a, Layer *et al.*, 1995).

However, this evidence comes from competitive antagonists. Non-competitive NMDA receptor antagonists which target the channel pore such as MK-801 and ketamine may more closely mimic the GluN1 HYPO mice rather than antagonists that target the ligand binding sites of the receptors due to the gross reduction in NMDA receptors in the GluN1 HYPO mice rather than alterations to their binding properties. MK-801 increases both general locomotor activity as well as reducing immobility in the FST (Trullas and Skolnick, 1990) whereas ketamine has been shown to reduce immobility in the FST at doses that do not effect overall locomotor activity (Hayase *et al.*, 2006, Engin *et al.*, 2009). The importance of this distinction between general locomotor activity and seeming antidepressant activity in tests such as the FST in the case of NMDA receptor antagonists may be now less important given the demonstration of antidepressant activity of ketamine in humans (Berman *et al.*, 2000, Correll and Futter, 2006, Zarate *et al.*, 2006a).

Further to these concerns over locomotion as a confound to the FST, the hypoactivity seen in the threshold activity system is at odds with the general hyperactivity seen in all other tests. Given this odd result, mice were manually observed in both the photobeam and threshold systems for two hours to ensure both were giving accurate results, which proved to be the case (data not shown). While no definitive answer can otherwise be found for this discrepancy, the greatest difference between the threshold set-up and other tests is the presence of food and water and a generally more homecage-like environment. It may be, therefore, that the hyperactivity in these mice in a novel environment is partly driven by their seeking out of available food. This would preferentially affect the GluN1 HYPO mice over WT mice due to their increase in food intake shown in Section 3.3.1. Hypoactivity in a homecage-like environment may then compensate for the massive hyperactivity seen in these novel

arenas, helping to conserve the energy expended following this explosion of activity. This raises the question of whether the increase in locomotor activity generally seen, the increase in food intake and the reduced body weight are all interrelated as posited in Section 3.4.1.1 or if there are also metabolic differences. In this case, the reduced body weight would drive the increase in food intake and an increase in the importance in expending energy to quickly locate a source of food rather than the reduced body weight being a consequence of these behaviours. More in-depth study of the metabolism of these mice would need to be done to fully answer this question.

These data also reveal a possible alteration in the GluN1 HYPO mice's sensitivity to circadian shifts as shown by their constant level of activity across the dark period which only ceases at lights-on. This is in contrast to the change in locomotor activity seen in the WT mice during the dark period, leading them to be at a resting level of activity before the lights are switched on again. This, however, is confounded by the great discrepancy in the overall levels of activity between these two genotypes. Again, further work on the entrainment of the GluN1 HYPO mice may reveal a longer active period in these mice, but would have to be performed in more specialised equipment than the locomotor boxes used here to remove locomotor confounds.

In summary, the GluN1 HYPO mice show an antidepressive phenotype in the FST and sucrose preference tests consistent with the antidepressant properties of NMDA receptor antagonists. These results, however, must be viewed in the context of the other altered behaviours seen in these mice.

3.4.1.5. The GluN1 hypomorph mouse phenotype

The GluN1 HYPO mice show greatly altered naturalistic behaviours, including massive hyperactivity, as well as profound cognitive deficits, but appear to show an antidepressant-like phenotype in most tests of affect. Their anxiety phenotype is not clear due to the GluN1 HYPO mice not giving a consistent phenotype across the tests performed. Overall, GluN1 HYPO mice show a global alteration in behaviour even in very basic ethological and cognitive tests, limiting the validity of more complex tasks performed with these mice.

3.4.2. Limitations and future directions

Many of the behaviours measured may be confounded by the profound hyperactivity shown in the GluN1 HYPO mice, although this cannot account for all of the behaviours seen as discussed above. That the GluN1 HYPO mice do not normalise their locomotor activity after 2 hours in the beam-break system implies that it would be difficult to attempt to remove this possible confound. Further to this, the global impairment seen in the cognitive tests performed may also confound any more complex assessments of behaviour. As such, while there remain some unanswered questions with these mice, these issues will always play a part in any assessments done. The apparent global nature of the alterations in their behaviour also does not make them an attractive model of any specific complex psychiatric disorder such as depression or of antidepressant activity.

3.4.3. Implications for previous findings

Most of the work on these mice has so far has focussed on the GluN1 HYPO mice as a model of schizophrenia (Mohn *et al.*, 1999, Duncan *et al.*, 2004, Fradley *et al.*, 2005, Duncan *et al.*, 2006b, a, Bickel *et al.*, 2007, 2008). Given the global nature of the deficits seen in the more wide-ranging behavioural phenotyping reported here, their usefulness as a model of schizophrenia or of any specific disease state should be questioned. More recent models with more discrete manipulations to the NMDA receptor may prove more useful in schizophrenia research, such as a recent report on mice with a postnatal reduction of NMDA receptors via GluN1 knockdown specifically in cortical and hippocampal interneurones (Belforte *et al.*, 2010).

Despite this, the results on tests of affect are what would be predicted for a genetic homologue of NMDA receptor antagonist treatment, as is the general increase in locomotor activity. The cognitive deficits seen are profound but arguably not dissimilar from those seen following pharmacological inhibition of NMDA receptors across the whole brain when administered at high doses.

Overall, the work here lends some support to the role of NMDA receptor antagonism in antidepressant treatment. However, the most striking outcome of this work is the global nature of the GluN1 HYPO mice's altered behaviour. This brings into question the validity of considering these mice as a model of schizophrenia as many have done to date.

3.4.4. Conclusions

Given the global impairments seen in the GluN1 hypomorph mice, their usefulness for investigating the implications of manipulating NMDA receptors in emotionality are limited. Indeed, their appropriateness in modelling any specific disease, including the previous suggestions of schizophrenia (Mohn *et al.*, 1999, Duncan *et al.*, 2004, Fradley *et al.*, 2005, Duncan *et al.*, 2006b, a, Bickel *et al.*, 2007, 2008) and autism (Moy *et al.*, 2008), is questionable. While further investigations could be performed to better elucidate, for instance, the possibility of an altered circadian rhythm in these animals, these global impairments will always be a hindrance to future study. No further experiments are therefore to be done using the GluN1 HYPO mice despite the results in the FST and sucrose preference test confirming what would be expected given the antidepressant activity of NMDA receptor antagonists.

CHAPTER 4: Study of naturalistic behaviour, cognition and emotionality in the vesicular glutamate transporter 1 heterozygote mice

4.1. Introduction

4.1.1. The vesicular glutamate transporters

Vesicular glutamate transporters (VGluTs) are the proteins responsible for loading glutamate into vesicles ready for release. Three isoforms have been identified which are structurally similar and preferentially load L- rather than D-glutamate (Moriyama and Yamamoto, 1995) and show differing and complimentary patterns of expression. VGluT1 is the predominant VGluT expressed in the cortex and hippocampus (Fremeau *et al.*, 2001, Herzog *et al.*, 2001), VGluT2 is found predominantly in sub-cortical regions but also layer IV of the neocortex (Fujiyama *et al.*, 2001, Herzog *et al.*, 2001, Sakata-Haga *et al.*, 2001, Takamori *et al.*, 2001) and VGluT3 is expressed diffusely in, surprisingly, non-glutamatergic neurones (Fremeau *et al.*, 2002, Gras *et al.*, 2002, Schafer *et al.*, 2002, Herzog *et al.*, 2004).

The expression pattern of the first two isoforms has been interpreted to mean that VGluT1 is expressed in neurones predisposed towards LTP while VGluT2 is in LTD-prone neurones (Fremeau *et al.*, 2001, Varoqui *et al.*, 2002, Liguz-Lecznar and Skangiel-Kramska, 2007). Alternatively, these two isoforms can be seen as being expressed in presynaptic boutons of neurones of cortical and thalamic origins, respectively (Fujiyama *et al.*, 2004, Hur and Zaborszky, 2005, Fujiyama *et al.*, 2006). However, absolute categorisation of glutamatergic neurones based on the isoform they express is problematic by the demonstration of co-expression of VGluT1 and 2 in the hippocampus (Herzog *et al.*, 2006) and cortex (Nakamura *et al.*, 2005) as well as

in the cerebellum (Hisano *et al.*, 2002). That VGluT3 is expressed by non-glutamatergic neurones means it does not co-express with others (Boulland *et al.*, 2004). Additionally, some neuronal populations display a developmental switch from VGluT2 to VGluT1 (Miyazaki *et al.*, 2003, Fremeau *et al.*, 2004, Nakamura *et al.*, 2005) with VGluT1 expression rising overall in the cortex during postnatal development (Minelli *et al.*, 2003), further complicating the categorisation of glutamatergic neurones based solely on VGluT isoform.

As VGluT1 is expressed in areas likely to be involved in emotional processing such as the cortex, hippocampus and amygdala, this isoform has been concentrated on in investigating the role of VGluT proteins in emotionality. However, there is still little evidence for its role in depression and antidepressant activity. Its expression has been shown to be reduced in the entorhinal cortex of depressed patients (Uezato *et al.*, 2009) and can be up-regulated by chronic antidepressant treatment in both mice (Moutsimilli *et al.*, 2005) and rats (Tordera *et al.*, 2005). However, it is worth noting that the Moutsimilli study also found increases in response to the atypical antipsychotic clozapine (Moutsimilli *et al.*, 2005), so these changes in VGluT levels are not a selective response to antidepressants. Furthermore, *post mortem* patient studies have revealed reduced expression of VGluT1 in the prefrontal cortex and hippocampus of schizophrenic patients (Eastwood and Harrison, 2005). Again, this may indicate VGluT1 alterations are not specific to depression and have a more general role in psychiatric diseases in which glutamate is implicated.

VGluT1 knockout (KO) mice have been produced by removing a large section of the gene, extending from the start codon of the coding region to a site within the fifth exon (Wojcik *et al.*, 2004). The complete VGluT1 KO mice are perinatally lethal. However, hippocampal neurones cultured from the brains of these mice have been used to show reduced quantal release size (Wojcik *et al.*, 2004). This is the expected consequence of a reduction in the loading of glutamate into vesicles. That it is not reduced to nothing is further evidence for the co-localisation of several VGluT isomers at individual synapses. Indeed, co-expression of VGluT1 and 2 has been demonstrated even on the same vesicles (Herzog *et al.*, 2006).

The heterozygote (HET) mice from the VGluT1 KO line show a 50% reduction in expression levels of VGluT1 with no alteration in VGluT2 expression and a small increase in VGluT3 expression (Tordera *et al.*, 2007) with no obvious reduction in quantal size (Wojcik *et al.*, 2004). This may be due to compensation by VGluT3 and other alterations, discussed below, or may indicate that there is no electrophysiological consequence to a 50% reduction in VGluT1 expression. A reduction in the size of the vesicular reserve pool in the VGluT1 HET mice has been noted (Tordera *et al.*, 2007). This may imply that the electrophysiological consequences would be observed following large stimulations or following the establishment of long-term potentiation. Plasticity is yet to be investigated in these mice, however. Unlike the complete VGluT1 KO mice, the VGluT1 HET mice flourish and so have been used to investigate the behavioural consequences of reduced VGluT1 expression.

4.1.2. The vesicular glutamate transporter 1 heterozygote mice

The first study to detail the behaviour of the VGluT1 HET mice focussed on tests of emotionality as well as some tests of cognition. Tordera and colleagues (2007) demonstrated these mice display phenotypes related to depression such as increased immobility in the forced swim test (FST) as well as an increase in anxiety behaviour in a test of dark-light exploration. However, they showed no change in behaviour compared to their wildtype (WT) counterparts in the elevated plus maze anxiety test. No change was seen in the Morris Water Maze test of spatial reference memory or in general locomotion. In the novel object recognition test of non-spatial memory, the VGluT1 HET mice were not impaired at a one hour interval between sample and test but did show a deficit in object memory at a 24hr intertrial interval. This shows these animals have only subtle alterations in cognition and a robust depressive-like phenotype in the FST as well as a possible increase in anxiety. This marks them out as a potentially quite specific model of depression. Importantly, this paper also showed no change in monoamine levels in the frontal cortex or hippocampus of the VGluT1 HET mice; both dopamine and 5-HT are unchanged (Tordera *et al.*, 2007), giving a model in which there are changes in emotionality with no changes in these monoamine systems but differences in the glutamate system.

A further study from this same group investigated the interaction between genotype and environmental stressors, namely a chronic mild stress (CMS) paradigm in these VGluT1 HET mice (Garcia-Garcia *et al.*, 2009). The claim of this paper is that a reduction in VGluT1 expression, such as is seen in the VGluT1 HET mice, exacerbates the detrimental effects of stress. The implication of this work is that individuals with low VGluT1 expression are at risk of developing depression following stressful life

events and this may account for the reduced expression seen *post mortem* in depressed patients. In this study on the VGluT1 HET mice, however, such a genotype*stress interaction was only seen statistically in i) locomotor activity, with VGluT1 HET mice becoming hyperactive following stress in comparison to all other groups, and ii) the sucrose preference measure taken throughout the CMS paradigm, with VGluT1 HET mice having a much greater decrease in sucrose intake in response to stress than WT mice. That non-stressed VGluT1 HET mice also show a decrease in sucrose intake as compared to control WT mice is an important validation of the VGluT1 HET mice as a complete model of depression, not just in terms of behavioural despair as measured by FST. Indeed, non-stressed VGluT1 HET mice showed a similar sucrose intake to stressed WT mice, this intake decreasing further in the stressed VGluT1 HET mice. This demonstrates both that the VGluT1 HET mice display anhedonia-like behaviour without any further environmental stressor but that this altered baseline does not preclude a genotype*stress interaction being detected, thus showing them to also be more sensitive to adverse life events.

In addition to the behavioural results, this study also demonstrated an increase in the neuronal production of glutamate in the VGluT1 HET mice that may be indicative of downstream compensation for reduced VGluT1 expression in these mice. By injecting ^{13}C glucose and 1,2-^{13}C acetate, the authors were able to use magnetic resonance spectroscopy (MRS) to investigate products formed by neurones and glia, respectively. This revealed a specific increase in neuronal glutamate production with no alteration in the production of GABA or glutamine and no change in neurotransmitter production by glia. As this MRS study used whole brain samples, it cannot be said that these alterations are seen specifically in areas that predominantly express VGluT1. However, it does indicate a potential compensatory change to the

reduction in VGluT1 expression and, more generally, a wider change to the glutamatergic system in these mice. This is also supported by the previous finding of a modest (approximately 15%) increase in VGluT3 expression levels in the VGluT1 HET mice (Tordera *et al.*, 2007) as well as the reduction in the excitatory amino acid transporter (EAAT) 1 in the VGluT1 HET mice (Garcia-Garcia *et al.*, 2009). EAAT1 is responsible for the reuptake of glutamate from the synapse and is predominantly expressed on glia (Storck *et al.*, 1992, Chaudhry *et al.*, 1995) but may also be expressed by neurones (Rothstein *et al.*, 1994). These alterations would increase the amount of glutamate available within a neurone and diminish its clearance from the synapse. The increased expression of VGluT3 may also directly compensate for the reduction in VGluT1 expression levels in the VGluT1 HET mice. This final point would rely on VGluT3 being detectable in neuronal populations that normally express VGluT1, however, which is yet to be demonstrated. These potential compensatory alterations may in turn account for the lack of any altered electrophysiology in the VGluT1 HET mice (Wojcik *et al.*, 2004) despite a broadly altered glutamate system. Again, however, the reported reduction in vesicular reserve pools in the VGluT1 HET mice (Tordera *et al.*, 2007) may result in alterations to neurotransmission in potentiated states but not during normal transmission, but this is yet to be investigated.

Given the relatively selective alterations in behaviour in the VGluT1 HET mice in the field of emotionality with no reported changes in the monoamine systems, this mouse line offers important evidence for a role for glutamate in mood disorders as well as a great opportunity to further explore the area.

4.1.3. Aims of this chapter

Previous results with the VGluT1 HET mice suggest they show several changes relating to depression and so provide evidence for a role for glutamate in the pathogenesis of this disease. This chapter aims to confirm these results and expand upon the number of behavioural tests performed relating to emotionality. Results in these tests, however, must be considered in relation to the overall phenotype of these mice, particularly given the global nature of the manipulation, the pervasiveness of glutamate in the central nervous system and alterations seen in the levels of VGluT1 in studies focussing on schizophrenia and its treatments. As such, this chapter has the secondary aim to comprehensively phenotype these mice in tests of other domains of behaviour including cognition and naturalistic behaviour.

4.2. Methods

4.2.1. Animals

A cohort of male and female vesicular glutamate transporter 1 (VGluT1) heterozygote mice (HET, Tordera *et al*., 2007) and their wildtype (WT) littermates on a C57Bl/6 background were tested on a number of behavioural tests. These mice were obtained from Dr Rosa Tordera (Department of Pharmacology, University of Navarra, Pamplona, Spain) and maintained at The University of Oxford in holding rooms on a 12/12hr light/dark cycle (lights on at 7am and off at 7pm). Male mice were individually housed owing to high levels of aggression and female mice were housed in groups of 4-5, both sexes having access to *ad libitum* food and water unless otherwise noted. Mice were typically 4 months of age at the start of testing which lasted approximately 3 months. All testing was done during the light phase unless otherwise noted.

4.2.2. Behavioural protocols

Mice (21 WT and 23 VGluT1 HET mice) were tested, in order, on the elevated plus maze, the black/white alley, the light/dark box, the successive alleys, open field, hyponeophagia, spontaneous alternation, spatial novelty preference Y-maze test, nesting, food/fluid consumption, burrowing, the non-anxious open field, locomotor activity over 2hrs, sucrose preference, locomotor activity across the diurnal cycle, social interaction following habituation, the appetitively motivated spatial reference memory Y-maze test and the forced swim test (Figure 4.1).

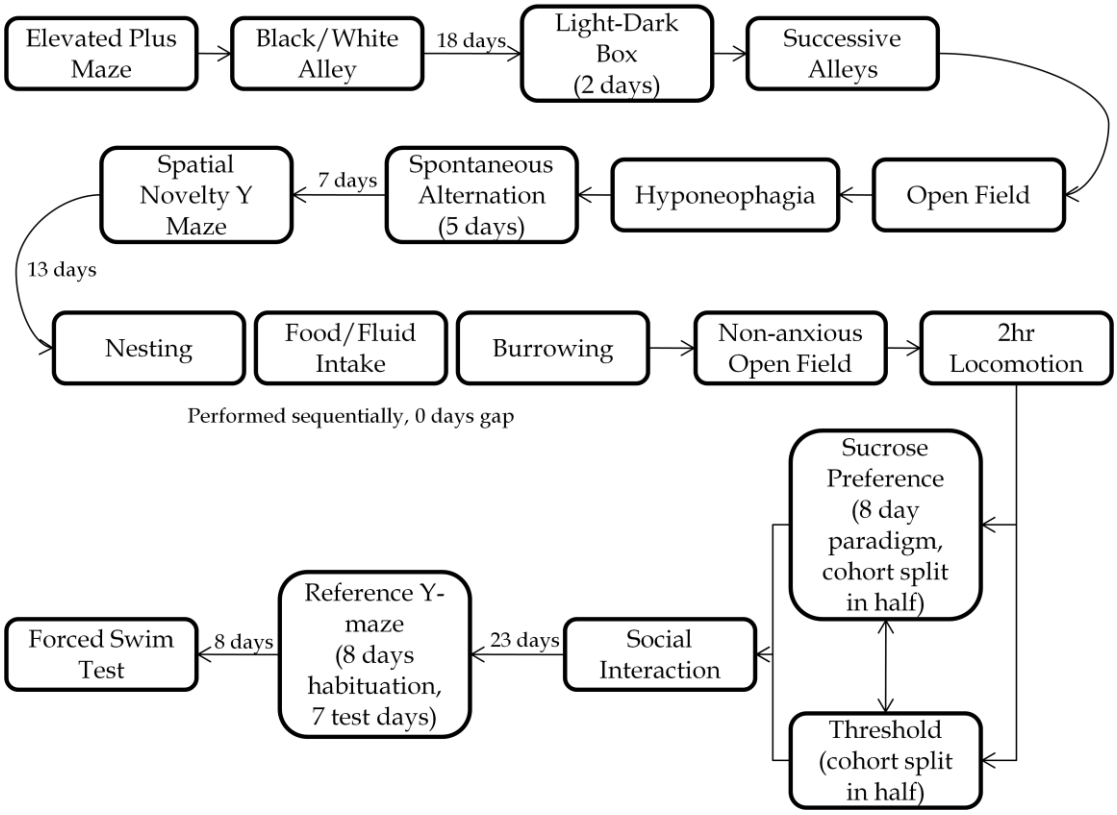


Figure 4.1: Timeline outlining the behavioural testing of the vesicular glutamate transporter 1 heterozygote mice

Vesicular glutamate transporter 1 heterozygote mice and their wildtype counterparts performed a number of behavioural tests. Arrows between tests represent a two day gap unless otherwise stated.

4.2.2.1. Tests of naturalistic behaviour

Nesting, food/fluid consumption, burrowing, the non-anxious open field and locomotor activity were all performed on the VGluT1 HET mice and their WT counterparts as described in section 3.2.2.1.

4.2.2.2. Tests of cognition

Spontaneous alternation, spatial novelty preference Y-maze test and the appetitively motivated spatial reference memory Y-maze test were all performed on the VGluT1 HET mice and their WT counterparts as described in Section 3.2.2.2 except that the spatial reference Y-maze was stopped after 7 days due to generally high levels of performance. Post-choice baiting was still employed on the final day of testing.

4.2.2.3. Tests of anxiety

The elevated plus maze was performed on the VGluT1 HET mice and their WT counterparts as described in Section 2.2.2.1.1. Social interaction following habituation was performed as described in Section 3.2.2.3.7 with mice habituated individually to the test arena for 5 days and then novel pairings used to assess social interaction on days 6 and 7. The black/white alley, light/dark box, successive alleys, open field and hyponeophagia were all performed on the VGluT1 HET mice and their WT counterparts as described in section 3.2.2.3. In the hyponeophagia test, time to contact was recorded in addition to latency to begin eating. The main measure used in this test was latency to eat – latency to first contact to account for any differences in attention/orienting to the food.

4.2.2.4. Tests of affect

Sucrose preference, locomotor activity across the diurnal cycle and the forced swim test were all performed on the VGluT1 HET mice and their WT counterparts as described in section 2.2.2.2.

4.2.3. Statistical analyses

Statistical analyses were performed as described in Section 3.2.3.

4.3. Results

4.3.1. Tests of naturalistic behaviour

The results for the tests of naturalistic behaviour in the VGluT1 heterozygote (HET) mice and their wildtype (WT) counterparts are summarised in Tables 4.1-2 and Figures 4.1-2. Nesting was analysed using a two-tailed Mann-Whitney U test with the data collapsed across sex. Univariate ANOVAs using genotype and sex as between-subject factors were used to compare food pellet, water and glucose consumption and body weight during the food/fluid intake study, the amount burrowed after two hours and overnight and the distance travelled and bouts of rearing in the non-anxious open field. Repeated measures ANOVAs were used for the measures of locomotor activity using time as the within-subject factor and the same between-subject factors as above.

4.3.1.1. Nesting

VGluT1 HET mice showed no change in nesting behaviour ($U_{(44)}$=199.000, p=0.309, Table 4.1).

Table 4.1: Results for vesicular glutamate transporter 1 heterozygote (HET) and wildtype (WT) mice in several tests of naturalistic behaviour

Test & Measure	WT, n=21	HET, n=23	Statistics
Nesting	3 (2.5-4.75)	4.5 (3-4.5)	$U_{(44)}$=199.000, p=0.309
Food/Fluid Consumption			
Pellets consumption (g)	4.48 ± 0.22	4.36 ± 0.18	Geno $F_{(1,40)}$=0.670, p=0.418
			Sex $F_{(1,40)}$=14.131, p=0.001
			Int $F_{(1,40)}$=0.061, p=0.807
Water consumption (ml)	9.49 ± 0.86	8.52 ± 0.46	Geno $F_{(1,40)}$=0.999, p=0.324
			Sex $F_{(1,40)}$=1.990, p=0.166
			Int $F_{(1,40)}$=0.252, p=0.618
Glucose (8%) (ml)	22.58 ± 2.47	18.73 ± 1.50	Geno $F_{(1,40)}$=2.674, p=0.110
			Sex $F_{(1,40)}$=7.992, p=0.007
			Int $F_{(1,40)}$<0.001, p=0.983
Body Weight (g)	27.53 ± 0.80	29.92 ± 1.13	**Geno $F_{(1,40)}$=18.570, p<0.001**
			Sex $F_{(1,40)}$=83.692, p<0.001
			Int $F_{(1,40)}$=8.557, p=0.006
Burrowing			
2 hours (g)	107.49 ± 17.51	131.49 ± 14.17	Geno $F_{(1,40)}$=1.976, p=0.168
			Sex $F_{(1,40)}$=12.675, p=0.001
			Int $F_{(1,40)}$=0.051, p=0.823
Overnight (g)	152.07 ± 13.64	163.69 ± 9.62	Geno $F_{(1,40)}$=0.578, p=0.452
			Sex $F_{(1,40)}$=10.410, p=0.003
			Int $F_{(1,40)}$=1.363, p=0.250
Non-anxious open field			
Distance travelled (cm)	1759.30 ± 98.10	1590.54 ± 58.91	Geno $F_{(1,40)}$=1.389, p=0.245
			Sex $F_{(1,40)}$=0.045, p=0.833
			Int $F_{(1,40)}$=1.297, p=0.262
Rearing	26.12 ± 1.74	22.00 ± 1.83	Geno $F_{(1,40)}$=1.546, p=0.221
			Sex $F_{(1,40)}$=1.974, p=0.168
			Int $F_{(1,40)}$=1.265, p=0.267
Locomotion over 2 hours			
Total beam-breaks	2461.38 ± 165.54	1695.22 ± 146.29	**Geno $F_{(1,40)}$=10.380, p=0.003**
			Sex $F_{(1,40)}$<0.001, p=0.990
			Int $F_{(1,40)}$=0.137, p=0.714
Crossovers	309.38 ± 25.38	217.65 ± 17.36	**Geno $F_{(1,40)}$=6.969, p=0.012**
			Sex $F_{(1,40)}$=1.271, p=0.266
			Int $F_{(1,40)}$=0.973, p=0.330

Parametric data presented as mean ± SEM and analysed with univariate ANOVA. Non-parametric data presented as median (IQR) and analysed with Mann-Whitney U-tests with the data collapsed across the sexes.
Geno = effect of genotype, sex = effect of sex, int = genotype*sex interaction.

Vesicular glutamate transporter 1 heterozygote mice are heavier than wildtype mice and hypoactive but otherwise show normal naturalistic behaviours

4.3.1.2.Food/fluid consumption

VGluT1 HET mice did not show any change in any intake measures (pellet consumption $F_{(1,40)}$=0.670, p=0.418; water $F_{(1,40)}$=0.999, p=0.324; glucose $F_{(1,40)}$=2.674, p=0.110, Table 4.1). VGluT1 HET mice were, however, heavier than WT mice ($F_{(1,40)}$=18.570, p<0.001). Effects of sex were seen in pellet consumption ($F_{(1,40)}$=14.131, p=0.001) with female mice (4.79g±0.18) eating more than male mice (3.84g±0.12;), and glucose intake ($F_{(1,40)}$=7.992, p=0.007), again with female mice (23.40ml±2.04) consuming more than male mice (16.07ml±1.17). Sex also affected body weight ($F_{(1,40)}$=83.692, p<0.001) with male mice (33.22g±0.96) being heavier than female mice (25.98g±0.51). A genotype*sex interaction was seen for body weight ($F_{(1,40)}$=8.557, p=0.006) which simple main effects revealed was due to an effect of genotype being present in male mice (WT 30.44g±0.81, VGluT1 HET 36.35g±0.98; $F_{(1,40)}$=21.397, p<0.001) but not in female mice (WT 25.35g±0.81, VGluT1 HET 26.49g±0.65; $F_{(1,40)}$=1.233, p=0.274). Male mice were significantly heavier than female mice in both genotypes (simple main effect of sex WT $F_{(1,40)}$=19.225, p<0.001; VGluT1 HET $F_{(1,40)}$=73.414, p<0.001). No other interactions were seen (see Table 4.1).

4.3.1.3. Burrowing

VGluT1 HET mice showed no change in burrowing behaviour (2 hours $F_{(1,40)}$=1.979, p=0.167; overnight $F_{(1,40)}$=0.572, p=0.454, Table 4.1). Sex, however, did effect burrowing (2 hours $F_{(1,40)}$=12.675, p=0.001; overnight $F_{(1,40)}$=10.410, p=0.003) with male mice burrowing more at both time points (2 hours: male 163.32g±14.22, female 92.78g±13.59; overnight: male 187.49g±6.06, female 139.67g±11.49). Sex did not interact with genotype at either time point (see Table 4.1).

4.3.1.4. Non-anxious open field

VGluT1 HET mice showed no alteration in activity in the non-anxious open field (distance covered $F_{(1,40)}$=1.389, p=0.245; rearing $F_{(1,40)}$=1.546, p=0.221, Table 4.1) and sex showed no effect, either (see Table 4.1).

4.3.1.5. Locomotor activity

VGluT1 HET mice moved less over the 2 hour beam break assessment of locomotor activity (total beam-breaks $F_{(1,40)}$=10.380, p=0.003; crossovers $F_{(1,40)}$=6.969, p=0.012, Table 4.1).

Across the 2 hours of the beam break assessment, total beam breaks showed a main effect of genotype ($F_{(1,40)}$=10.380, p=0.003, Figure 4.2) as well as a main effect of time bin ($F_{(23,920)}$=38.210, p<0.001) due to the reduction in activity seen in both genotypes across time. This habituation did not differ between genotypes (genotype*time interaction $F_{(23,920)}$=0.874, p=0.635) and sex showed no main effect or interactions (effect of sex $F_{(1,40)}$<0.001, p=0.990; genotype*sex interaction $F_{(1,40)}$=0.137, p=0.714; time*sex interaction $F_{(23,920)}$=0.931, p=0.556; time*genotype*sex interaction $F_{(23,920)}$=1.074, p=0.368). These results are mimicked in the crossover measure of larger locomotor events (effect of genotype $F_{(1,40)}$=6.969, p=0.012; effect of sex $F_{(1,40)}$=1.271, p=0.266; genotype*sex interaction $F_{(1,40)}$=0.973, p=0.330; effect of time $F_{(23,920)}$=39.572, p<0.001; time*genotype interaction $F_{(23,920)}$=0.915, p=0.579; genotype*time*sex interaction $F_{(23,920)}$=0.617, p=0.920, Figure 4.3) except that a time*sex interaction was seen ($F_{(23,920)}$=2.671, p<0.001) due to male mice initially being more active than female

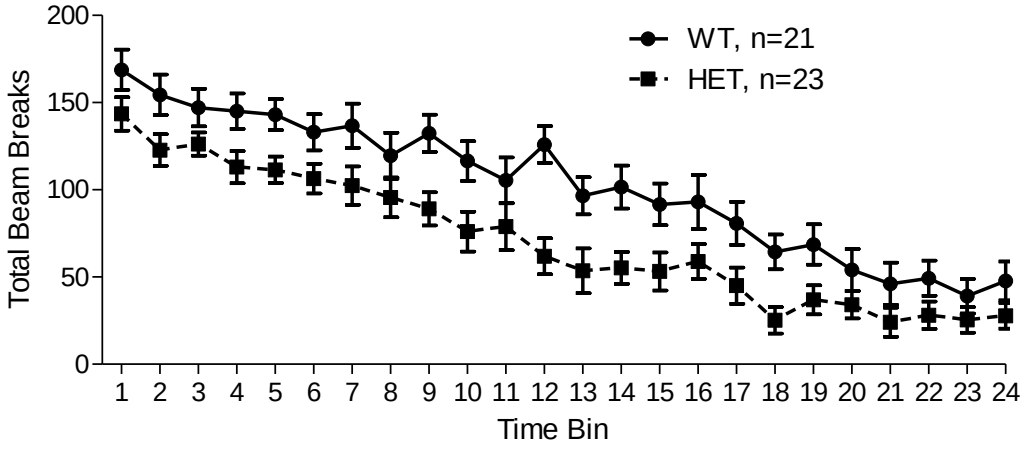


Figure 4.2: Mean number of beam-breaks made by vesicular glutamate transporter 1 heterozygote (HET) and wildtype (WT) mice in 2 hours

Vesicular glutamate transporter 1 heterozygote mice are persistently hypoactive during the 2 hours but habituate normally. Presented as mean ± SEM in 24 5min bins.

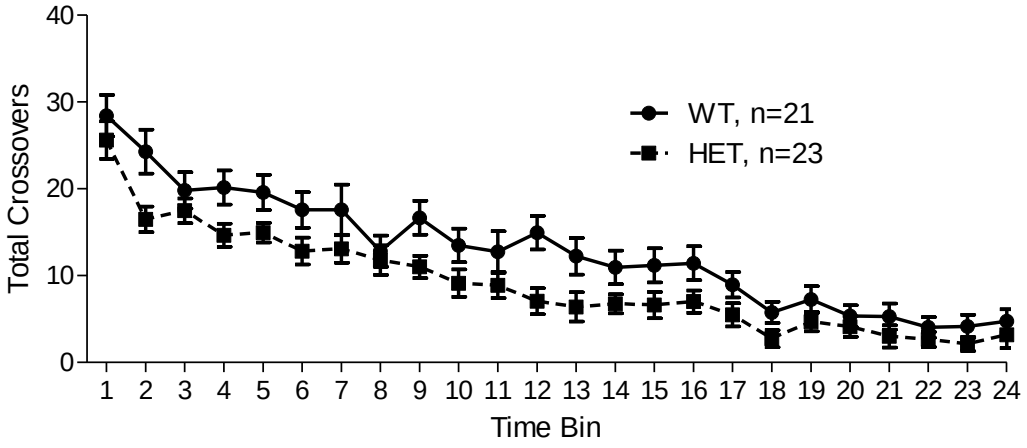


Figure 4.3: Mean number of crossover beam-breaks made by vesicular glutamate transporter 1 heterozygote (HET) and wildtype (WT) mice in 2 hours

Vesicular glutamate transporter 1 heterozygote mice are persistently hypoactive during the 2 hours but habituate normally. Presented as mean ± SEM in 24 5min bins.

mice (time bins 1, 3 and 5, see Table 4.2) but quickly normalise, with both sexes showing a simple main effect of time (male $F_{(23,18)}$=9.509, p<0.001; female $F_{(23,18)}$=4.554, p=0.001).

4.3.2. Tests of cognition

The results for the tests of cognition in the VGluT1 HET mice and their WT counterparts are summarised in Tables 4.3-4 and Figures 4.3-5. The percentage of correct responses in spontaneous alternation was analysed using a Mann-Whitney U test with the data collapsed across sex. A univariate ANOVA using genotype and sex as between-subject factors was used to compare novelty preference in the spatial novelty preference Y-maze test and a repeated measures ANOVA was used for the spatial reference Y-maze using day as the within-subject factor and the same between-subject factors as above.

4.3.2.1. Spontaneous alternation

VGluT1 HET mice showed no change in performance in spontaneous alternation ($U_{(44)}$=230.500, p=0.780, Table 4.3).

Table 4.2: Simple main effect of sex at each time point during the beam break assessment of locomotor activity in vesicular glutamate transporter 1 heterozygote and wildtype mice

Time Bin	$F_{(1,40)}$	p
1	**13.980**	**0.001**
2	1.942	0.171
3	**4.927**	**0.032**
4	3.597	0.065
5	**7.360**	**0.010**
6	2.432	0.127
7	0.181	0.673
8	0.788	0.380
9	0.028	0.869
10	0.028	0.867
11	2.463	0.124
12	1.118	0.297
13	2.543	0.119
14	0.053	0.818
15	1.325	0.257
16	0.062	0.805
17	0.029	0.865
18	0.828	0.368
19	0.152	0.698
20	0.010	0.922
21	1.497	0.228
22	0.220	0.642
23	0.020	0.887
24	0.404	0.528

Male mice are initially more active than female mice but quickly normalise

Table 4.3: Performance of vesicular glutamate transporter 1 heterozygote (HET) and wildtype (WT) mice in two tests of spatial working memory

Test & Measure	WT, n=21	HET, n=23	Statistics
Spontaneous Alternation	90% (80-90)	90% (80-90)	$U_{(44)}$=230.500, p=0.780
Spatial Novelty Y-maze	0.68 ± 0.02	0.72 ± 0.03	Geno $F_{(1,40)}$=0.102, p=0.751
			Sex $F_{(1,40)}$=5.885, p=0.020
			Int $F_{(1,40)}$=7.695, p=0.008
Sample phase ratio	0.48 ± 0.02	0.47 ± 0.02	Geno $F_{(1,40)}$=0.455, p=0.504
			Sex $F_{(1,40)}$=0.013, p=0.911
			Int $F_{(1,40)}$=0.174, p=0.679

Parametric data presented as mean ± SEM and analysed with univariate ANOVA. Non-parametric data presented as median (IQR) and analysed with Mann-Whitney U-tests with the data collapsed across the sexes.
Geno = effect of genotype, sex = effect of sex, int = genotype*sex interaction.

Vesicular glutamate transporter 1 heterozygote mice show no change in spatial working memory

4.3.2.2.Spatial novelty preference Y-maze test

No main effect of genotype was seen in performance in the spatial novelty preference Y-maze test ($F_{(1,40)}$=0.102, p=0.751, Table 4.3). Sex did affect performance ($F_{(1,40)}$=5.885, p=0.020), with female mice (0.74±0.02) having a greater novelty preference than male mice (0.64±0.04). This also interacted with genotype ($F_{(1,40)}$=7.695, p=0.008) with simple main effects revealing an effect of genotype in female mice (WT 0.67±0.03, VGluT1 HET 0.79±0.02; $F_{(1,40)}$=6.157, p=0.017) but not in male mice (WT 0.69±0.03, VGluT1 HET 0.60±0.07; $F_{(1,40)}$=2.464, p=0.124), the simple main effect of sex being present in VGluT1 HET mice ($F_{(1,40)}$=13.617, p=0.001) but not WT mice ($F_{(1,40)}$=0.060, p=0.807), showing female VGluT1 HET mice out-perform female WT mice but male mice perform similarly in both genotype groups. This may imply an increase in performance on this task in the female VGluT1 HET mice. No effects were seen during the sample phase (see Table 4.3).

4.3.2.3. Appetitively motivated spatial reference memory Y-maze test

No main effect of genotype was seen in performance in the appetitively motivated spatial reference memory Y-maze test ($F_{(1,40)}$=0.631, p=0.432, Figure 4.4). An effect of day was seen ($F_{(6,240)}$=77.414, p<0.001) that interacted with genotype ($F_{(6,240)}$=2.178, p=0.046), but simple main effects failed to pull out any further differences between genotypes (simple main effects of day WT $F_{(6,35)}$=34.820, p<0.001; VGluT1 HET $F_{(6,35)}$=43.785, p<0.001; simple main effects of day, see Table 4.4). These effects imply all mice show a gradual improvement over the days of the test and at a similar rate in both genotypes.

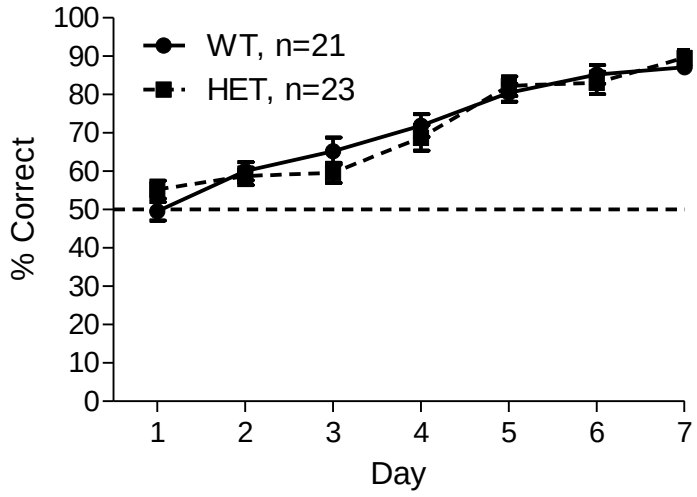


Figure 4.4: Percentage of correct arm choices of vesicular glutamate transporter 1 heterozygote (HET) and wildtype (WT) mice in the appetitive spatial reference memory Y-maze test

Vesicular glutamate transporter 1 heterozygote mice show no change in performance in the spatial reference Y-maze. Presented as mean ± SEM. Dashed line at 50%, chance level performance.

Table 4.4: Simple main effect of genotype on each day of the appetitively motivated spatial reference memory Y-maze test in vesicular glutamate transporter 1 heterozygote and wildtype mice

Day	**$F_{(1,40)}$**	**p**
1	2.116	0.154
2	1.061	0.309
3	3.308	0.076
4	1.438	0.238
5	0.071	0.791
6	1.312	0.259
7	0.357	0.553

No simple main effect of genotype is seen between vesicular glutamate transporter 1 heterozygote and wildtype mice during performance of the spatial reference Y-maze.

Sex showed no main effect ($F_{(1,40)}$=1.639, p=0.208) and did not interact with day ($F_{(6,240)}$=0.662, p=0.681) but did interact with genotype ($F_{(1,40)}$=5.730, p=0.021). Simple main effects revealed male mice show a just-significant effect of genotype (WT 0.73±0.03, VGluT1 HET 0.65±0.03; $F_{(1,40)}$=4.156, p=0.048, Figure 4.5) but female mice showed no simple main effect of genotype (WT 0.70±0.02, VGluT1 HET 0.74±0.02; $F_{(1,40)}$=1.646, p=0.207, Figure 4.6) and an effect of sex was seen only in the VGluT1 HET ($F_{(1,40)}$=6.799, p=0.013) and not WT mice ($F_{(1,40)}$=0.615, p=0.437). These effects show male WT mice out-performing male VGluT1 HET mice but female mice of both genotypes performing similarly. No three-way interaction was seen ($F_{(6,240)}$=1.363, p=0.230), implying this is not due to differences in the rate of acquisition of this task.

4.3.3. Tests of anxiety

The results for the tests of anxiety in the VGluT1 HET mice and their WT counterparts are summarised in Table 4.5 and Figures 4.6-8. Univariate ANOVAs using genotype and sex as between-subject factors were used to compare several measures in the elevated plus maze (duration, latency to and frequency of visits to the open arm as well as total distance moved), black/white alley (time spent in and latency to enter the white alley and the number of transitions), the light-dark box (time spent in the light area, latency to the first transition and the total number of transitions) both when the mice were started in the light and dark areas, the anxiogenic open field (duration, frequency and latency to the centre as well as total distance moved), latency to drink, to first contact and the difference between these two measures in the hyponeophagia test and duration of social and aggressive

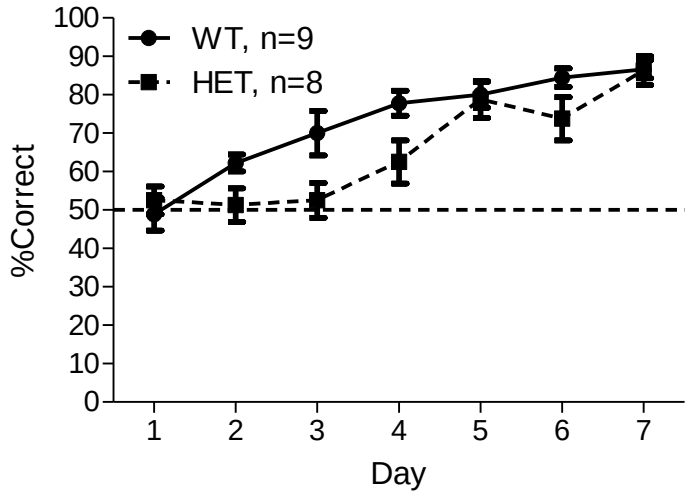


Figure 4.5: Percentage of correct arm choices of male vesicular glutamate transporter 1 heterozygote (HET) and wildtype (WT) mice in the appetitive spatial reference memory Y-maze test

Male wildtype mice out-perform male vesicular glutamate transporter 1 heterozygote mice in the appetitive spatial reference memory Y-maze test. Presented as mean ± SEM.

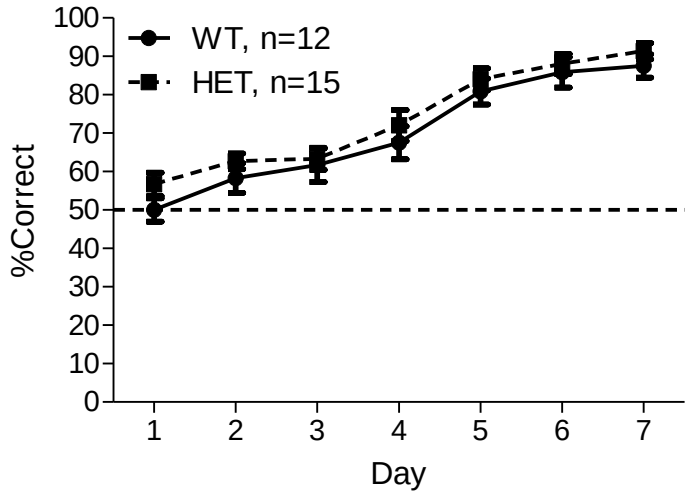


Figure 4.6: Percentage of correct arm choices of female vesicular glutamate transporter 1 heterozygote (HET) and wildtype (WT) mice in the appetitive spatial reference memory Y-maze test

No difference is seen in performance on the appetitive spatial reference memory Y-maze test between female vesicular glutamate transporter 1 heterozygote and wildtype mice. Presented as mean ± SEM.

encounters in the social interaction test. Sex was excluded from the analysis of aggression as this was only observed in male mice. For the successive alleys test, due to the interdependency of duration and latency in each alley on the other alleys, a repeated measures ANOVA was performed for duration with the numerator degrees of freedom reduced by one. Genotype and sex were used as between subject factors and alley as the within-subject factor. p values were then calculated using an online p-value calculator (http://www.danielsoper.com/statcalc/calc07.aspx) based on the F value delivered by SPSS. Latency to enter each alley was analysed by performing separate univariate ANOVAs on each alley. Frequency was treated as independent in each alley and so analysed with an uncorrected repeated measures ANOVA. Alley 4 was excluded from all analyses as no animals entered this area of the apparatus.

4.3.3.1. Elevated plus maze

VGluT1 HET mice showed no change in any measures of the EPM (see Table 4.5). Sex affected the distance moved in the elevated plus maze ($F_{(1,39)}$=10.646, p=0.002) with female mice (1595.55cm±59.68) moving more than male mice (1307.35cm±60.13) but had no effects on the other measures and did not interact with genotype (see Table 4.5).

Table 4.5: Results for vesicular glutamate transporter 1 heterozygote (HET) and wildtype (WT) mice in several tests of anxiety

Test & Measure	WT, n=20-21	HET, n=22-23	Statistics
Elevated Plus Maze			
Time spent in open arms (s)	9.71 ± 2.67	7.75 ± 1.92	Geno $F_{(1,39)}$=0.192, p=0.664 Sex $F_{(1,39)}$=1.852, p=0.181 Int $F_{(1,39)}$=1.400, p=0.244
Latency to open arm (s)	127.65 ± 29.38	131.22 ± 29.72	Geno $F_{(1,39)}$=0.001, p=0.970 Sex $F_{(1,39)}$=0.147, p=0.704 Int $F_{(1,39)}$=0.008, p=0.928
No. of open arm entries	8.19 ± 2.39	5.86 ± 1.41	Geno $F_{(1,39)}$=0.338, p=0.564 Sex $F_{(1,39)}$=0.737, p=0.396 Int $F_{(1,39)}$=2.254, p=0.141
Distance moved (cm)	1474.66 ± 80.30	1488.24 ± 55.87	Geno $F_{(1,39)}$=0.070, p=0.793 **Sex $F_{(1,39)}$=10.646, p=0.002** Int $F_{(1,39)}$=2.406, p=0.129
Black/White Alley			
Time spent in white alley (s)	27.01 ± 2.19	29.23 ± 1.59	Geno $F_{(1,39)}$=0.295, p=0.590 Sex $F_{(1,39)}$=0.061, p=0.806 Int $F_{(1,39)}$=1.362, p=0.250
Latency to white alley (s)	32.66 ± 4.14	22.89 ± 2.23	**Geno $F_{(1,39)}$=4.587, p=0.039** Sex $F_{(1,39)}$=1.482, p=0.231 Int $F_{(1,39)}$=0.635, p=0.430
Transitions	4.38 ± 0.30	4.82 ± 0.33	Geno $F_{(1,39)}$=0.870, p=0.357 Sex $F_{(1,39)}$=3.827, p=0.058 Int $F_{(1,39)}$=0.161, p=0.691
Light-Dark Box: Started in light area			
Time spent in light area (s)	42.48 ± 6.47	46.00 ± 6.23	Geno $F_{(1,40)}$=0.038, p=0.846 **Sex $F_{(1,40)}$=5.074, p=0.030** Int $F_{(1,40)}$=0.006, p=0.967
Latency to dark area (s)	11.44 ± 2.00	14.79 ± 4.57	Geno $F_{(1,40)}$=0.161, p=0.691 Sex $F_{(1,40)}$=0.203, p=0.654 Int $F_{(1,40)}$=0.678, p=0.415
Transitions	5.24 ± 0.81	4.70 ± 0.46	Geno $F_{(1,40)}$=0.333, p=0.567 **Sex $F_{(1,40)}$=6.187, p=0.017** Int $F_{(1,40)}$=1.132, p=0.294
Light-Dark Box: Started in dark area			
Time spent in light area (s)	53.85 ± 8.93	49.45 ± 6.77	Geno $F_{(1,40)}$=0.270, p=0.606 **Sex $F_{(1,40)}$=11.717, p=0.001** Int $F_{(1,40)}$=0.700, p=0.408
Latency to light area (s)	109.46 ± 23.22	76.03 ± 18.71	Geno $F_{(1,40)}$=1.685, p=0.202 **Sex $F_{(1,40)}$=38.689, p<0.001** Int $F_{(1,40)}$=1.613, p=0.211
Transitions	5.48 ± 0.84	5.61 ± 0.68	Geno $F_{(1,40)}$=0.001, p=0.982 **Sex $F_{(1,40)}$=18.321, p<0.001** Int $F_{(1,40)}$=1.017, p=0.319

Test & Measure	WT, n=20-21	HET, n=22-23	Statistics
Open Field			
Time spent in centre (s)	5.81 ± 0.86	6.80 ± 0.87	Geno $F_{(1,40)}$=0.765, p=0.387
			Sex $F_{(1,40)}$=10.183, p=0.003
			Int $F_{(1,40)}$=1.377, p=0.248
No. of centre entries	9.38 ± 1.32	10.13 ± 1.18	Geno $F_{(1,40)}$=0.071, p=0.791
			Sex $F_{(1,40)}$=14.857, p<0.001
			Int $F_{(1,40)}$=0.226, p=0.637
Latency to enter centre (s)	57.49 ± 10.29	55.77 ± 10.07	Geno $F_{(1,40)}$=0.017, p=0.898
			Sex $F_{(1,40)}$=5.242, p=0.027
			Int $F_{(1,40)}$=0.748, p=0.392
Distance moved (cm)	3915.06 ± 233.98	3664.62 ± 181.77	Geno $F_{(1,40)}$=1.134, p=0.293
			Sex $F_{(1,40)}$=6.352, p=0.016
			Int $F_{(1,40)}$=0.002, p=0.966
Hyponeophagia			
Drink – contact latency (s)	31.03 ± 12.56	29.45 ± 7.17	Geno $F_{(1,40)}$=0.003, p=0.954
			Sex $F_{(1,40)}$=5.943, p=0.019
			Int $F_{(1,40)}$=0.019, p=0.911
Latency to drink (s)	49.20 ± 19.86	42.81 ± 8.97	Geno $F_{(1,40)}$=0.027, p=0.869
			Sex $F_{(1,40)}$=1.075, p=0.306
			Int $F_{(1,40)}$=0.034, p=0.856
Latency to contact (s)	18.17 ± 11.34	13.37 ± 5.53	Geno $F_{(1,40)}$=0.121, p=0.730
			Sex $F_{(1,40)}$=0.754, p=0.390
			Int $F_{(1,40)}$=0.191, p=0.665
Social Interaction (s) (following habituation)	73.72 ± 11.30	52.22 ± 7.26	Geno $F_{(1,38)}$=3.479, p=0.070
			Sex $F_{(1,38)}$=10.181, p=0.003
			Int $F_{(1,38)}$=0.944, p=0.337
Aggression (s) (males only, n=8 each)	17.13 ± 5.30	16.80 ± 9.17	$t_{(14)}$=0.031, p=0.976

Presented as mean ± SEM and analysed with univariate ANOVA or t-test.
Geno = effect of genotype, sex = effect of sex, int = genotype*sex interaction.

Vesicular glutamate transporter 1 heterozygote mice show decreased latency to the white alley in the black/white alley test but otherwise show no changes in tests of anxiety.

4.3.3.2.Black/white alley

VGluT1 HET mice showed no change in time spent in the white area of the black/white alley ($F_{(1,39)}$=0.295, p=0.590) but were quicker to enter this more anxiogenic compartment ($F_{(1,39)}$=4.587, p=0.039, Table 4.5) implying they may be less anxious in this test. Transitions were not altered between genotypes ($F_{(1,39)}$=0.870, p=0.357) suggesting alterations in latency to cross were not due to a generalised alteration in locomotion. Sex had no effect in this test (see Table 4.5).

4.3.3.3. Light/dark box

VGluT1 HET mice showed no alteration in their behaviour in any of the measures in the light/dark box from either start position (see Table 4.5). Sex did effect time spent in the light area when started in the light ($F_{(1,40)}$=5.074, p=0.030) as well as the dark positions ($F_{(1,40)}$=38.689, p<0.001) with female mice spending more time in the light on both occasions (light start: female 52.16s±6.05, male 31.88s±5.22; dark start: female 64.81s±6.57, male 30.49s±7.25). The number of transitions during these tests was also affected by sex (light start: $F_{(1,40)}$=6.187, p=0.017; dark start: $F_{(1,40)}$=18.321, p<0.001) with female mice (light start 5.78±0.61, dark start 7.07±0.58) making more transitions than male mice (light start 3.65±0.52, dark start 3.12±0.69). Female mice (38.35s±7.27) were also quicker to first leave the dark area than male mice (177.18s±25.58, $F_{(1,40)}$=38.689, p<0.001). Sex did not interact with genotype in any of the measures taken in the light-dark box in either start position (see Table 4.5).

4.3.3.4. Successive alleys

VGluT1 HET mice showed no change in time spent in the different alleys of the successive alleys (genotype*alley interaction $F_{(1,80)}$=0.042, p=0.838, Figure 4.7), frequency of entries to each alley (main effect of genotype $F_{(1,40)}$=0.340, p=0.563; genotype*alley interaction $F_{(2,80)}$=0.542, p=0.584, Figure 4.8) or latency to the differing sections (effects of genotype: alley2 $F_{(1,40)}$=0.074, p=0.787; alley3 $F_{(1,40)}$=0.039, p=0.845, Figure 4.9). Sex had no effect in this test (duration: sex*alley interaction $F_{(1,80)}$=0.624, p=0.432; genotype*sex*alley interaction $F_{(1,80)}$=0.124, p=0.726. Frequency: main effect of sex $F_{(1,40)}$=0.102, p=0.752; genotype*sex interaction $F_{(1,40)}$=0.214, p=0.646; sex*alley interaction $F_{(2,80)}$=0.047, p=0.955; genotype*sex*alley interaction $F_{(2,80)}$=0.083, p=0.921. Latency: alley 2: effect of sex $F_{(1,40)}$=0.358, p=0.553; genotype*sex interaction $F_{(1,40)}$=0.272, p=0.603; alley 3: effect of sex $F_{(1,40)}$=0.566, p=0.456; genotype*sex interaction $F_{(1,40)}$=0.746, p=0.393).

4.3.3.5.Open field

VGluT1 HET mice showed no change in behaviour compared to WT mice in any of the measures of the open field (see Table 4.5). Sex affected a number of measures including time spent in the centre (female 7.76s±0.76, male 4.06s±0.77; $F_{(1,40)}$=10.183, p=0.003), the number of entries into the centre (female 12.15±1.13, male 6.00±0.75; $F_{(1,40)}$=14.857, p<0.001), the latency to first enter this area (female 44.11s±6.68, male 76.41±14.04; $F_{(1,40)}$=5.242, p=0.027) and the total distance covered during this test (female 4054.01cm±193.29, male 3355.57cm±182.73; $F_{(1,40)}$=6.352, p=0.016) with female mice moving more than male mice and subsequently entering the centre earlier, more frequently and for longer than male mice. Sex did not interact with genotype in any of these measures (see Table 4.5).

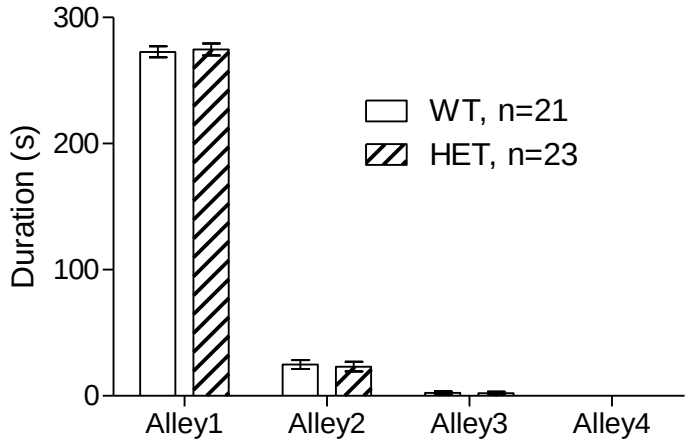


Figure 4.7: The time spent in each alley of the successive alleys by vesicular glutamate transporter 1 heterozygote (HET) and wildtype (WT) mice

Vesicular glutamate transporter 1 heterozygote mice show no change in the successive alleys test. NB: no mice entered alley 4. Presented as mean ± SEM.

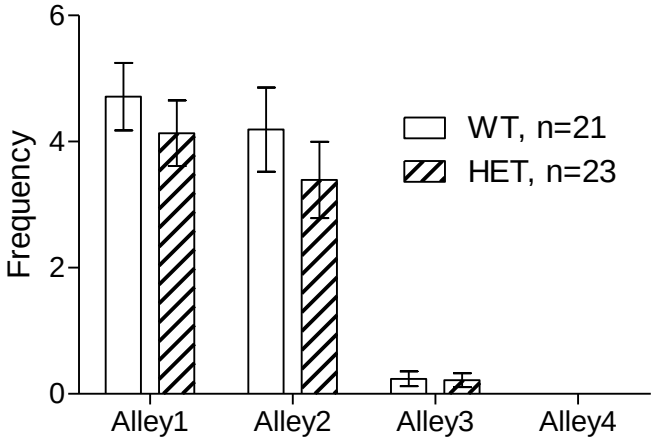


Figure 4.8: The number of entries into each alley of the successive alleys by vesicular glutamate transporter 1 heterozygote (HET) and wildtype (WT) mice

Vesicular glutamate transporter 1 heterozygote mice show no change in the successive alleys test. NB: no mice entered alley 4. Presented as mean ± SEM.

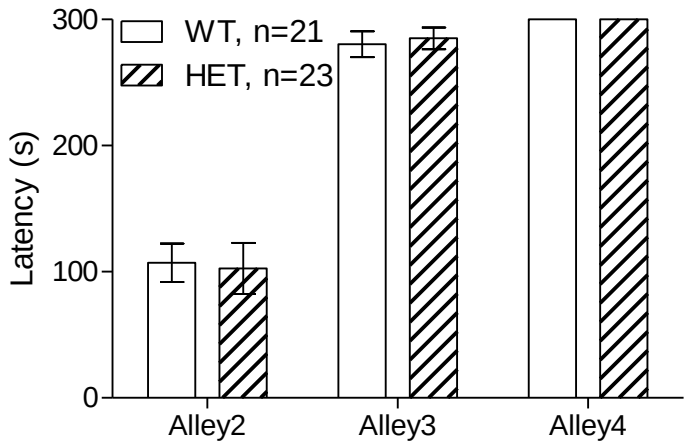


Figure 4.9: The latency to first entry into each alley of the successive alleys by vesicular glutamate transporter 1 heterozygote (HET) and wildtype (WT) mice

Vesicular glutamate transporter 1 heterozygote mice show no change in the successive alleys test. NB: no mice entered alley 4. Presented as mean ± SEM.

4.3.3.6. Hyponeophagia

VGluT1 HET mice showed no alteration in time taken to drink following contact in the hyponeophagia test ($F_{(1,40)}$=0.003, p=0.954). They also showed no change in time to first contact ($F_{(1,40)}$=0.121, p=0.730) or raw latency to drink ($F_{(1,40)}$=0.027, p=0.869, Table 4.5). An effect of sex was seen in latency to drink following contact ($F_{(1,40)}$=5.943, p=0.019) with female mice (17.13s±5.18) being faster to drink than male mice (50.96s±15.04), implying they are less anxious. This is in spite of no effect of sex on latency to first contact ($F_{(1,40)}$=0.754, p=0.390) or raw latency to drink ($F_{(1,40)}$=1.075, p=0.3306). Sex did not interact with genotype in any of these measures (see Table 4.5).

4.3.3.7. Social interaction

In the social interaction test, no effect of genotype was seen in social ($F_{(1,38)}$=3.479, p=0.070) or aggressive encounters (males only $t_{(14)}$=0.031, p=0.976, Table 4.5). Sex did effect social interaction ($F_{(1,38)}$=10.181, p=0.003) with male mice (86.96s±14.61) interacting more than female mice (47.38s±4.10), but this did not interact with genotype ($F_{(1,38)}$=0.944, p=0.337).

4.3.4. Tests of affect

The results for the tests of affect in the VGluT1 HET mice and their WT counterparts are summarised in Tables 4.6-7 and Figures 4.9-12. Univariate ANOVAs using genotype and sex as between-subject factors were used to compare sucrose preference ratio, water intake and sucrose intake in the sucrose preference test and immobility, swimming and climbing in the forced swim test (FST). Repeated

measures ANOVAs were then used to compare the time spent immobile in the FST across the two days of the test using day as the within-subject factor and the same between-subject factors as above. A repeated measures ANOVA was also used to analyse locomotor activity across the diurnal cycle with time as the within-subject factor.

4.3.4.1. Sucrose preference

While genotype did not affect sucrose preference ($F_{(1,40)}$=2.989, p=0.092), VGluT1 HET mice showed a decrease in water intake ($F_{(1,40)}$=6.400, p=0.015; Table 4.6). An effect of sex was seen on sucrose intake ($F_{(1,40)}$=14.385, p<0.001) with female mice (32.37ml±1.10) consuming more sucrose than male mice (25.86ml±1.34), though this was not sufficient to produce an effect of sex on sucrose preference ($F_{(1,40)}$=2.293, p=0.138). No genotype*sex interactions were seen (see Table 4.6).

4.3.4.2. Locomotor activity across the diurnal cycle

Across the diurnal cycle, all mice altered their behaviour (effect of time $F_{(46,1840)}$=35.997, p<0.001; Figure 4.10) but no effects of genotype or interactions with genotype were seen (effect of genotype $F_{(1,40)}$=0.045, p=0.832; genotype*sex interaction $F_{(1,40)}$=0.073, p=0.788; genotype*time interaction $F_{(46,1840)}$=0.927, p=0.612; genotype*sex*time interaction $F_{(46,1840)}$=1.254, p=0.120). Sex showed no main effect ($F_{(1,40)}$=0.055, p=0.815) but did interact with time ($F_{(46,1840)}$=1.900, p<0.001) with male mice showing initial hyperactivity when compared to the female mice, but this

Table 4.6: Results for vesicular glutamate transporter 1 heterozygote (HET) and wildtype (WT) mice in the sucrose preference test

Measure	WT, n=21	HET, n=23	Statistics
Sucrose preference	0.74 ± 0.01	0.77 ± 0.01	Geno $F_{(1,40)}$=2.989, p=0.092
			Sex $F_{(1,40)}$=2.293, p=0.138
			Int $F_{(1,40)}$=1.035, p=0.315
Water Intake (ml)	10.24 ± 0.43	8.68 ± 0.44	**Geno $F_{(1,40)}$=6.400, p=0.015**
			Sex $F_{(1,40)}$=1.694, p=0.200
			Int $F_{(1,40)}$<0.001, p=0.998
Sucrose Intake (ml)	30.34 ± 1.49	29.41 ± 1.29	Geno $F_{(1,40)}$=0.354, p=0.555
			Sex $F_{(1,40)}$=14.385, p<0.001
			Int $F_{(1,40)}$=1.193, p=0.281

Presented as mean ± SEM and analysed with univariate ANOVA.
Geno = effect of genotype, sex = effect of sex, int = genotype*sex interaction.

Vesicular glutamate transporter 1 heterozygote mice show an increase in water intake but not enough to alter their sucrose preference ratio.

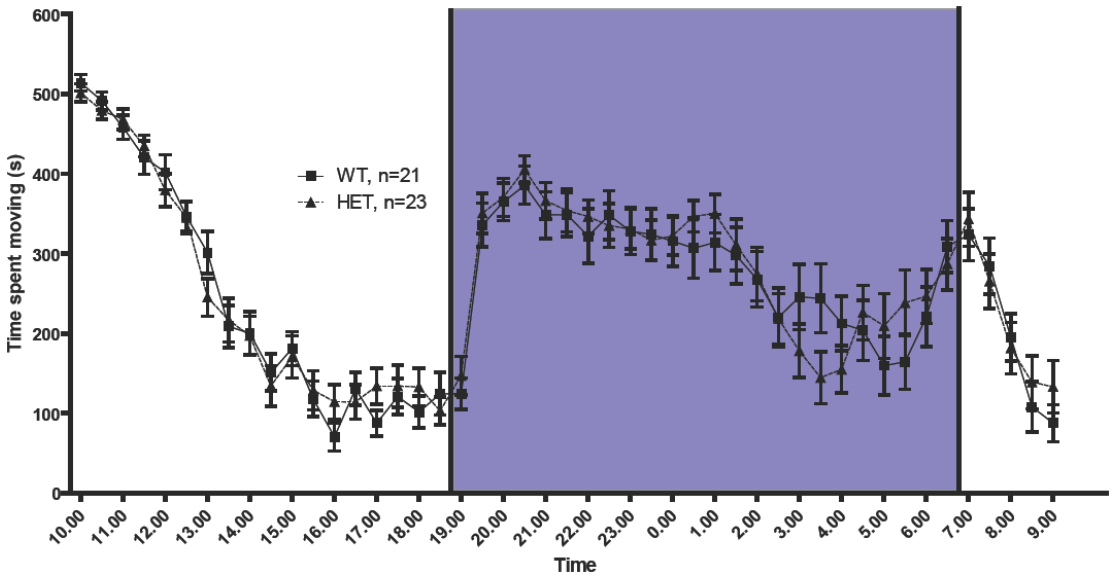


Figure 4.10: Locomotor activity for vesicular glutamate transporter 1 heterozygote (HET) and wildtype (WT) mice over a 23.5 hour period

Vesicular glutamate transporter 1 heterozygote mice show no change in locomotor activity across the diurnal cycle. Shaded area = lights off period, 7pm-7am, presented as mean ± SEM in 47 30min bins.

quickly normalises (Table 4.7). Simple main effects of time were seen in both sexes (female $F_{(46,1194)}$=21.934, p<0.001; male $F_{(46,736)}$=16.786, p<0.001).

4.3.4.3. Forced swim test

On day 1 of the forced swim test, no effects of genotype were seen in swimming ($F_{(1,40)}$=0.413, p=0.524), climbing ($F_{(1,40)}$=0.003, p=0.956) or immobility ($F_{(1,40)}$=0.185, p=0.670; Figure 4.11). The same is true for main effects of sex (swimming $F_{(1,40)}$=0.041, p=0.841; climbing $F_{(1,40)}$=0.280, p=0.600; immobility $F_{(1,40)}$=1.313, p=0.259) and genotype*sex interactions (swimming $F_{(1,40)}$=0.205, p=0.653; climbing $F_{(1,40)}$=0.066, p=0.799; immobility $F_{(1,40)}$=0.740, p=0.395). The same pattern of results was also seen on day 2 (effects of genotype: swimming $F_{(1,40)}$=0.739, p=0.395; climbing $F_{(1,40)}$=0.045, p=0.833; immobility $F_{(1,40)}$=0.745, p=0.393; effects of sex: swimming $F_{(1,40)}$=1.265, p=0.268; climbing $F_{(1,40)}$=0.017, p=0.896; immobility $F_{(1,40)}$=1.258, p=0.269; genotype*sex interactions: swimming $F_{(1,40)}$=0.626, p=0.433; climbing $F_{(1,40)}$=1.320, p=0.257; immobility $F_{(1,40)}$=0.363, p=0.550; Figure 4.12). When looking solely at immobility across the two days, still no main effect of genotype ($F_{(1,40)}$=0.584, p=0.449) or sex were seen ($F_{(1,40)}$<0.001, p=0.994) and there was also no main effect of day ($F_{(1,40)}$=0.310, p=0.581) or interactions involving day and genotype (genotype*day interaction $F_{(1,40)}$=0.186, p=0.669; genotype*sex*day interaction $F_{(1,40)}$=0.049, p=0.825; Figure 4.13). However, an interaction between sex and day was observed ($F_{(1,40)}$=4.631, p=0.037). In agreement with previous analysis, no effect of sex was seen on either day (day 1 $F_{(1,40)}$=1.313, p=0.259; day 2 $F_{(1,40)}$=1.258, p=0.269) but an effect of

Table 4.7: Simple main effect of sex at each time point during the test of locomotor activity over the diurnal cycle in vesicular glutamate transporter 1 heterozygote and wildtype mice

Time	$F_{(1,40)}$	p	Time	$F_{(1,40)}$	p
10am	**17.895**	**<0.001**	10pm	0.004	0.953
10.30am	**9.562**	**0.004**	10.30pm	0.752	0.391
11am	**4.856**	**0.033**	11pm	0.591	0.446
11.30am	0.026	0.872	11.30pm	0.183	0.671
12pm	3.409	0.072	12am	0.602	0.443
12.30pm	0.541	0.466	12.30am	0.023	0.881
1pm	**4.612**	**0.038**	1am	0.015	0.904
1.30pm	0.629	0.432	1.30am	0.000	0.998
2pm	1.627	0.209	2am	0.075	0.786
2.30pm	2.317	0.136	2.30am	0.128	0.722
3pm	1.001	0.323	3am	0.549	0.463
3.30pm	0.505	0.482	3.30am	1.073	0.307
4pm	1.446	0.236	4am	0.545	0.465
4.30pm	0.183	0.671	4.30am	0.578	0.452
5pm	3.002	0.091	5am	**7.154**	**0.011**
5.30pm	3.529	0.068	5.30am	**6.105**	**0.018**
6pm	0.006	0.940	6am	1.975	0.168
6.30pm	2.719	0.107	6.30am	0.060	0.807
7pm	1.114	0.297	7am	2.803	0.102
7.30pm	0.904	0.347	7.30am	1.660	0.205
8pm	**4.978**	**0.031**	8am	**5.118**	**0.029**
8.30pm	0.129	0.721	8.30am	2.283	0.139
9pm	0.088	0.768	9am	0.811	0.373
9.30pm	0.419	0.521			

Male mice are initially more active than female mice but this normalised quickly

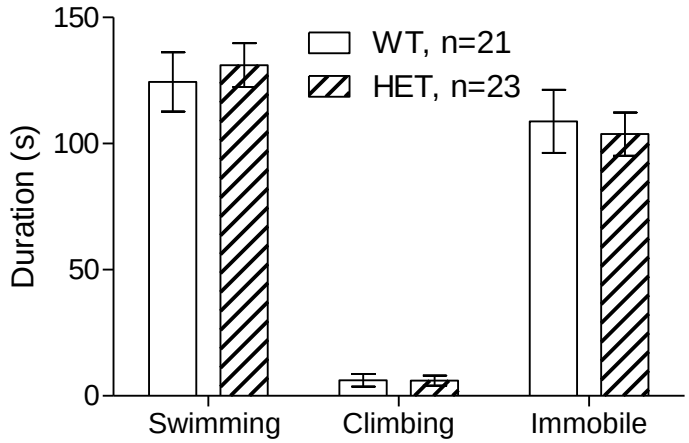


Figure 4.11: Time spent swimming, climbing and immobile in minutes 3-6 on day 1 of the forced swim test by vesicular glutamate transporter 1 heterozygote (HET) and wildtype (WT) mice

Vesicular glutamate transporter 1 heterozygote mice show no change in swimming, climbing or immobility compared to wildtype mice on day 1 of the forced swim test. Presented as mean ± SEM.

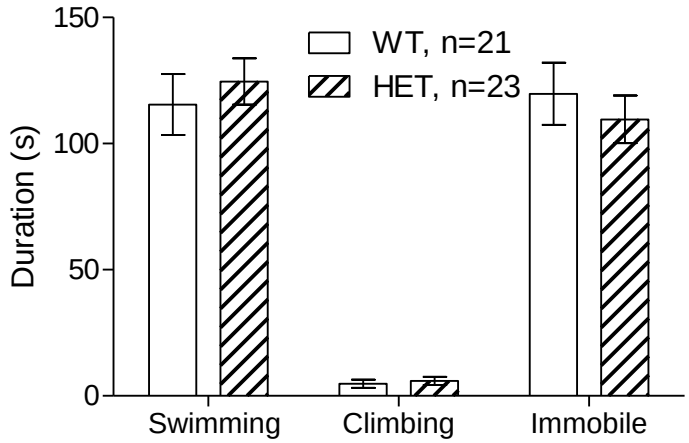


Figure 4.12: Time spent swimming, climbing and immobile in minutes 3-6 on day 2 of the forced swim test by vesicular glutamate transporter 1 heterozygote (HET) and wildtype (WT) mice

Vesicular glutamate transporter 1 heterozygote mice show no change in swimming, climbing or immobility compared to wildtype mice on day 2 of the forced swim test. Presented as mean ± SEM.

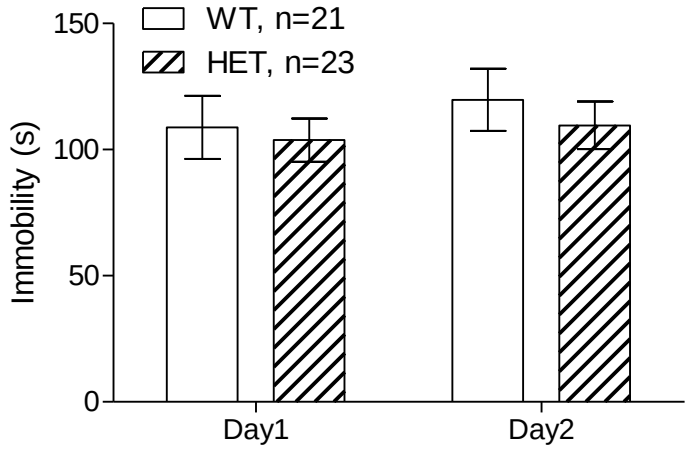


Figure 4.13: Time spent immobile in minutes 3-6 on days 1 and 2 of the forced swim test by vesicular glutamate transporter 1 heterozygote (HET) and wildtype (WT) mice

Vesicular glutamate transporter 1 heterozygote mice show no change in immobility across the two days of the forced swim test compared to wildtype mice and no change across days is seen. Presented as mean ± SEM.

day was seen in female mice (day1 98.92s±9.58, day2 121.20s±9.89; $F_{(1,40)}$=4.723, p=0.036) but not male mice (day1 116.52s±12.02, day2 103.40s±12.41; $F_{(1,40)}$=1.04, p=0.314) with an increase in immobility occurring in the females across the two days of the test.

4.4. Discussion

4.4.1. Summary of results

This chapter aimed to behaviourally phenotype the vesicular glutamate transporter (VGluT) 1 heterozygote (HET) mice with particular focus on tests of emotionality. Male and female VGluT1 HET mice were compared to their wildtype (WT) littermates in several behavioural tests. This revealed the VGluT1 HET mice to be slightly, although not consistently, hypoactive and slightly heavier when compared to WT mice but otherwise showed normal behaviour.

4.4.1.1. Naturalistic behaviour

VGluT1 HET mice showed no alterations in most naturalistic behaviours measured with the only exceptions being body weight and locomotor activity over 2 hours. In these two tests, male VGluT1 HET mice showed a slight increase in body weight as compared to male WT mice and both sexes show hypoactivity in the beam break locomotor test. A decrease in activity may account for the increase in body weight. Otherwise, the VGluT1 HET mice showed normal behaviour in tests of nesting and burrowing, normal food and fluid consumption and no significant alteration in activity in the non-anxious open field in contrast to their hypoactivity in the beam break assessment of locomotor activity. This may be due to the reduced testing period as the data does trend towards a reduction in the VGluT1 HET mice. Overall, the VGluT1 HET mice show broadly normal behaviour in ethological tests, suggesting they do not have globally debilitating deficits.

4.4.1.2. Cognition

VGluT1 HET mice displayed normal cognitive function in the tests performed; spontaneous alternation, the spatial novelty preference Y-maze test and the spatial reference Y-maze, although some genotype*sex interactions were seen. Female VGluT1 HET mice performed better than female WT mice in the spatial novelty preference Y-maze test, while male VGluT1 HET mice performed less well than WT male mice on the appetitively motivated spatial reference memory Y-maze test. Despite the lack of a three way interaction in this task, this deficit appears to only be present at the start of the task and is not evident by the end. The overall high level of performance in these tests suggests these mice, as well as their WT littermates, have intact spatial reference- and working-memory but that subtle, sex-specific alterations in cognition may exist.

4.4.1.3. Anxiety

VGluT1 HET mice show no change in anxiety behaviour in any of the tests performed; the elevated plus maze, the light-dark box, the successive alleys, the open field, hyponeophagia and social interaction but did look less anxious in the black/white alley by having a lower latency to enter the white alley. This is in contrast to previously reported data on light-dark exploration tests of anxiety in these mice (Tordera *et al.*, 2007). That these mice look hypoactive (see Sections 4.3.1 and 4.4.1.1) discounts generalised alterations in locomotion as an alternative explanation for this particular result; their reduced latency is not due to general hyperlocomotion. That no change was seen in the time spent in the white alley, the main measure of this test, as well as no effect of genotype being seen in any other tests of anxiety means this single positive result should be interpreted with caution.

4.4.1.4. Affect

VGluT1 HET mice displayed normal behaviour in the tests performed relating to affect, including the sucrose preference test despite a decrease in water intake. No changes were seen in the forced swim test (FST) or activity across the diurnal cycle.

4.4.1.5. The vesicular glutamate transporter 1 heterozygote mouse phenotype

The VGluT1 HET mice show only a minor reduction in locomotor activity and an increase in body weight as compared to their WT littermates and no other robust alterations in behaviour. This shows the VGluT1 HET mice to be broadly normal and not an appropriate model of depression.

4.4.2. Limitations and future directions

The lack of any behavioural differences in these mice in tests of naturalistic behaviour or cognition remove many possible confounds to the tests of emotionality. The slight decrease in locomotion seen over 2 hours in the beam-break test is not seen in the non-anxious open field, implying that over short periods of being exposed to a novel environment this reduction in movement is not a major issue. This should therefore not act as a confound to other behavioural tests, exemplified by the reduced latency to enter the white alley in the black/white alley. Nonetheless, given that no alterations are seen in the tests of emotionality themselves, these control measures are of less interest. Indeed, given this lack of any alteration in emotionality in these mice, there is little use in continuing to explore alterations relating to depression in them. What would be worth confirming is that there is, indeed, a reduction in

expression in VGluT1 as reported in previous studies investigating the behaviour of these mice (Tordera *et al.*, 2007, Garcia-Garcia *et al.*, 2009). However, this result would not alter the fact that, in my hands, these mice show no phenotype of interest for further investigation.

Several effects of sex were seen and in this regard it is worth noting that the differing housing conditions of the two sexes may, in part, account for these changes rather than being purely effects of sex alone. Despite this, that statistically significant effects of sex could be detected confirms that the behavioural measures being utilised are able to detect differences in performance between groups of animals and so would be suitable for detecting effects of genotype if they were present.

4.4.3. Implications for previous findings

This phenotyping of the VGluT1 HET mice failed to confirm the increase in immobility in the FST and increased anxiety seen in some tests reported by others (Tordera *et al.*, 2007, Garcia-Garcia *et al.*, 2009). In addition to factors such as possible differences in the level of enrichment received by mice and inter-experimenter reliability, a specific issue relating to the genetic background of the mice used may explain these discrepancies. The construct of these mice was made initially using 129/ola oocytes (Wojcik *et al.*, 2004). Prior to behavioural work, they were backcrossed onto a near-congenic C57Bl/6N background (Garcia-Garcia *et al.*, 2009). The mice used here were several generations later than those used in the published papers and were therefore on a relatively more congenic background i.e. were of a subtly different genetic makeup. This in itself will alter the performance of both the VGluT1 HET mice as well as the WT control mice. On a molecular level, it may also

influence the consequences of the genetic alteration made. Both of these issues are compounded further as, to maintain the colony, VGluT1 HET mice will be selected for. This will lead to genes around this loci also being carried through the generations which will both impact on behaviour as well as interact with other genetic code that may differ from that present in that of the loci's original genetic background strain (Crawley, 1996, Gerlai, 1996, Wolfer and Lipp, 2000). This issue of "drifting" genetic backgrounds has also been encountered with other glutamate-related mutant mice, namely the GluN2A knockout mice (Bannerman *et al.*, 2008). This generational effect has also diminished the differences seen in the FST with these mice in the laboratory where this phenotype was first reported (Tordera, personal communication).

In short, in my hands, these mice show normal behaviours in all tests of emotionality and cognition. This does not agree with previous findings and implies these mice have only subtle and selective impairments if any.

4.4.4. Conclusions

The VGluT1 HET mice do not show any alterations in emotionality in my hands. While this does question the robustness of the original findings, there are other possible explanations for this failure to replicate such as changes in other interacting genes within the genetic background of these mice. Nonetheless, these animals do not provide a phenotype to warrant further investigation into emotionality.

CHAPTER 5: Assessing behavioural and neurobiological emotional responses in the 5-HT transporter overexpressor mice using oxygen amperometry

5.1. Introduction

Since studies in previous chapters did not reveal a reliable model of altered emotionality in the glutamate mutants tested, work moved on to the 5-HT transporter (5-HTT) overexpressor (OE) mice. These mice have previously been shown to have reduced anxiety (Jennings *et al.*, 2006, Line *et al.*, 2010). This chapter further investigates emotionality in these mice by combining the 5-HTT OE mice with the oxygen amperometry technique, as well as local field potential measures of neuronal electrical activity. This was done in a fear conditioning paradigm to assess neuronal responses to an emotional stimulus. This chapter introduces fear conditioning as a way of investigating emotional learning, its neurobiology and how amperometry and local field potentials can be used to measure neuronal activity.

5.1.1. Fear conditioning

Fear conditioning is a form of Pavlovian conditioning, involving the association a previously neutral cue (the conditioned stimulus, CS+) with an unconditioned stimulus (US, in this case a footshock) that results in a fear response. The formation of this association allows for the investigation of emotional learning, relevant to mood disorders and those at-risk of developing these problems in which this process may be altered (eg Chan *et al.*, 2007). For instance, a tendency to maintain negative emotional learning may form part of the pathogenesis of depression (Harmer, 2008). By using a second, control stimulus that is never paired with a US, the CS-, the

specific response to the CS+ can be dissociated from general stimulus response. Typically in rodents, this involves the pairing of a light or tone with a footshock that results in the conditioned response (CR) of freezing to the cue. Freezing is the species-specific conditioned response to foot shock and can therefore be used as a behavioural output measure of conditioning to the CS+ in the absence of the US. An association is also made between the testing context and US, and the level of cue and context conditioning can be assessed separately. This is achieved by exposing animals after training to the CS+ in a novel context to assess cue conditioning in isolation from context conditioning, whilst reintroducing the mice to the training context without any CS+ presentations can be used to assess context conditioning.

The tenacity of the associations made can also be assessed through extinction, which occurs in response to repeated presentation of the CS+ with no US. Extinction involves new learning that overrides previously-established associations rather than being an inactive forgetting process. This extinction process is seen behaviourally as a reduction in the conditioned freezing response to cue presentation. Extinction has been shown in rodents to rely on frontal input to the amygdala (Morgan *et al.*, 1993, Quirk *et al.*, 2000, Rosenkranz and Grace, 2001, Milad and Quirk, 2002, Quirk *et al.*, 2003, Milad *et al.*, 2004). The main target for this input is the basal nucleus of the amygdala (Grace and Rosenkranz, 2002, Sah and Lopez De Armentia, 2003), but frontal input also stimulates intercalated neurones to dampen amygdala processing and output (Pare *et al.*, 2004, Berretta *et al.*, 2005, Likhtik *et al.*, 2008).

5.1.2. The amygdala

The neural circuitry involved in fear conditioning has been extensively studied and the amygdala is thought to have a central role (LeDoux, 2000, Davis and Whalen, 2001). The amygdala is an almond-shaped cluster of interconnected, neurochemically heterogeneous nuclei in the medial temporal lobe that has diverse reciprocal connections to cortical and limbic areas (Ottersen, 1982, Cavada *et al.*, 2000, Pitkanen *et al.*, 2000). These nuclei are roughly subdivided into three complexes; i) the basolateral complex (BLA), consisting of the basolateral and lateral nuclei (LA), ii) the cortical-like group including the periamygdaloid cortex and iii) the centromedial group, consisting of the central (CeN) and medial nuclei. Additionally, there are GABAergic intercalated cells between the basolateral and centromedial groups and an amygdalohippocampal area between the amygdala and the hippocampus.

Evidence suggests that lesions to the amygdala results in an inability to learn and/or express fear, both in terms of behavioural and non-behavioural responses (Blanchard and Blanchard, 1972, Kim *et al.*, 1993, Goldstein *et al.*, 1996, Cousens and Otto, 1998). These effects are also found following reversible inactivation of the amygdala using the $GABA_A$ agonist muscimol if given before but not after training (Muller *et al.*, 1997, Wilensky *et al.*, 1999). A freezing deficit has also been seen if muscimol is infused just prior to testing (Muller *et al.*, 1997) which may result from a generalised reduction in fearful responding (Hitchcock *et al.*, 1989). This highlights the differing roles the amygdala plays throughout the acquisition and expression of learned fear.

The most prominent model of amygdala connectivity and its role in fear conditioning is the serial model (LeDoux, 2000). The LA receives sensory input from the thalamus and cortex and is thought to be the site of convergence for the CS and US as it is the point at which the sensory information that comprise these stimuli first comes together (LeDoux *et al.*, 1990, Romanski *et al.*, 1993). This information is then fed to the basolateral nucleus where hippocampal inputs also converge, which are thought to provide contextual information (Phillips and LeDoux, 1992, Maren *et al.*, 1996, Calandreau *et al.*, 2005). Neuroplasticity at this site is key in forming these associations (Fanselow and Kim, 1994). The establishment of potentiated states such as long-term potentiation (LTP) within the amygdala (Schroeder and Shinnick-Gallagher, 2005), specifically within the BLA (Apergis-Schoute *et al.*, 2005), is thought to be important. The basolateral nucleus then innervates the CeN which subsequently activates other areas such as the lateral hypothalamus responsible for the behavioural and physiological responses that make up learned fear (LeDoux *et al.*, 1988).

More recently, an alternative model for amygdala circuitry has been proposed known as the parallel model (Killcross *et al.*, 1997). This views the BLA complex and CeN, not as two processing centres on a single circuit, but rather as parts of two dissociable and parallel processing circuits. This was demonstrated using a conditioning paradigm involving two levers that were initially paired with food reward. Rats were exposed to this set up until good rates of lever pressing were seen. Rats then received a selective lesion of either the BLA, the CeN, a lesion of both of these regions, or underwent a sham operation. Following the surgery, rats were reintroduced to the operant boxes, although the consequences of their lever presses had been altered. Rats pressing one of the levers initiated the presentation of a CS+

tone that was associated with a shock, whereas pressing the other lever led to a control, CS- presentation. Cue presentations, while dependent on responding, were controlled by a variable interval schedule to ensure equal CS+ and CS- presentations between groups regardless of rates of responding.

Sham operated rats robustly shifted their responses in favour of the lever that resulted in the CS-, a learning process known as conditioned punishment. When a CS+ was presented, sham rats showed a general reduction in lever pressing, so called conditioned suppression. Rats with lesions of both the BLA and CeN displayed impairments in both these forms of conditioning. Conditioned punishment was impaired specifically in the BLA lesion group and not the CeN lesion group. Conditioned suppression, conversely, was impaired following CeN lesions but not following BLA lesions. This demonstrates a double dissociation between the BLA and CeN and these two forms of conditioning. As the BLA lesion includes disruption to the lateral nucleus, information required during conditioned suppression must reach the CeN directly and not via what is seen in the serial model as the input to the amygdala as a whole. This alternative parallel model of amygdala connectivity therefore demonstrates that our understanding of this part of the brain and its role in conditioning is not yet complete.

Neuroimaging studies of fear conditioning in humans also show differential amygdala responses to conditioned versus control cues (Buchel *et al.*, 1998, LaBar *et al.*, 1998, Morris *et al.*, 2001, Phelps *et al.*, 2001, Cheng *et al.*, 2003). This amygdala activity is proportional to behavioural measures of fear (LaBar *et al.*, 1998, Phelps *et al.*, 2001), being greatest during acquisition of the association (Buchel *et al.*, 1998, LaBar *et al.*, 1998). There is also evidence for three distinct anatomical regions within

the amygdala; US processing involves the lateral amygdala, CS processing the ventral amygdala and CS response processing the dorsal amygdala (Morris *et al.*, 2001). Evidence for the role of frontal cortical input into the amygdala during extinction has also been collected using neuroimaging, with prefrontal activity seen during extinction (Knight *et al.*, 2004, Phelps *et al.*, 2004, Milad *et al.*, 2005, Delgado *et al.*, 2008) that is inversely proportional to amygdala activity (Ochsner *et al.*, 2002, Kim *et al.*, 2003). Human imaging studies have also shown amygdala activity in response to emotionally salient cues such as fearful, but not neutral, faces (Breiter *et al.*, 1996, Morris *et al.*, 1996). These responses can be investigated without any conditioning period, although whether this response to fearful faces is an unconditioned response or the result of associations made early in life is not clear.

5.1.3. The 5-HT transporter and emotional responses

The amygdala receives direct input from the dorsal raphe 5-HT nucleus in the brain stem (Wang and Aghajanian, 1977) and contains several sub-types of 5-HT receptor (Barnes and Sharp, 1999). The majority of studies addressing the role of 5-HTT in modulating amygdala activity in response to emotional stimuli have made use of selective serotonin reuptake inhibitors (SSRIs) during fear conditioning. More recently, attention has turned towards genetic influences on 5-HTT activity and the consequences this has on amygdala reactivity.

5.1.3.1. SSRI studies

5.1.3.1.1. Rodent studies

The majority of rodent fear conditioning studies using SSRIs have found that acute administration of this class of drugs to rats results in a decrease in the expression of learned fear (Hashimoto *et al.*, 1996, Inoue *et al.*, 1996a, Inoue *et al.*, 1996b, Li *et al.*, 2001b, but see also Van Dijken and colleagues, 1992) when given either during acquisition (Inoue *et al.*, 1996a) or in the test phase (Hashimoto *et al.*, 1996, Inoue *et al.*, 1996b, Li *et al.*, 2001b). These results point towards an important role for the 5-HTT, and 5-HT neurotransmission, in both of these phases of fear conditioning.

However, there is conflicting evidence for this reduction in fear conditioning following SSRI treatment. Citalopram given acutely, either during training or the test period of fear conditioning, has been shown to exacerbate fear responses (Burghardt *et al.*, 2004, Burghardt *et al.*, 2007). This exacerbation of fear has also been found following acute fluoxetine administration prior to testing (Burghardt *et al.*, 2007). This acute increase in fear responses may relate to the apparent anxiogenic effects of acute SSRI treatment found in rodents (Kshama *et al.*, 1990, Griebel *et al.*, 1994) and also the worsening of depressive symptoms seen clinically with acute SSRI treatment (eg Cusin *et al.*, 2007). These studies implicate 5-HTT functionality in the acquisition and expression of fear as well as other emotional behaviours, although the exact relationship between this functionality and its behavioural consequences remains unclear.

5.1.3.1.2. Human studies

In human neuroimaging studies, amygdala activity in response to negative cues such as fearful faces is often used as measure of emotionality. Again, conflicting results have been found relating to the role of the 5-HTT in this activity. One study has shown that infusion of citalopram potentiates amygdala activity in response to angry or fearful faces in a face-matching task (Bigos *et al.*, 2008). This pharmacological blockade of the 5-HTT, resulting in heightened amygdala reactivity, is potentially in agreement with evidence that individuals with low 5-HTT protein expression display greater amygdala responses to fearful faces (Rhodes *et al.*, 2007). Studies showing 5-HT transporter gene linked polymorphic region (5-HTTLPR) *s* allele carrying healthy volunteers, who would be predicted to also have lowered 5-HTT expression, also show increased amygdala responses (Hariri *et al.*, 2002, Hariri *et al.*, 2005). This implies that low 5-HTT functionality results in increased amygdala activity, whether due to genetic or pharmacological reasons.

However, a reduction in amygdala reactivity to fearful faces following SSRI treatment has also been seen. This has been demonstrated both in previously depressed individuals (Anderson *et al.*, 2010) as well as healthy volunteers following acute (Anderson *et al.*, 2010) or 7 days of SSRI treatment (Harmer *et al.*, 2006). Again, this demonstrates the controversy concerning links between 5-HTT function and its consequences in measures relevant to emotionality.

Behaviourally, a single oral dose of citalopram in healthy volunteers was found to increase recognition of fearful faces (Bhagwagar *et al.*, 2004a, Browning *et al.*, 2007), as well as their baseline startle response (Browning *et al.*, 2007). These results imply a heightened response and vigilance towards negative emotional cues, as might be expected given the noted agitation seen clinically following the onset of SSRI treatment. However, a single dose of citalopram in healthy volunteers has also been shown to be sufficient to shift cognitive bias towards the positive, as assessed by recall of more positive than negative words (Browning *et al.*, 2007). Intriguingly, the opposite result to that seen in healthy volunteers is noted in depressed individuals in the fearful faces task; depressed patients usually display enhanced fearful faces recognition, but this is normalised following a single dose of citalopram (Bhagwagar *et al.*, 2004a). This implies some desirable, clinically-relevant rapid-onset actions do occur following a single dose of an SSRI, challenging the dogma that these drugs take time for noticeable effects to occur. This also further demonstrates the contradictory nature of the literature relating to the 5-HTT and mood.

5.1.3.2. 5-HT transporter genetic variation and fear conditioning

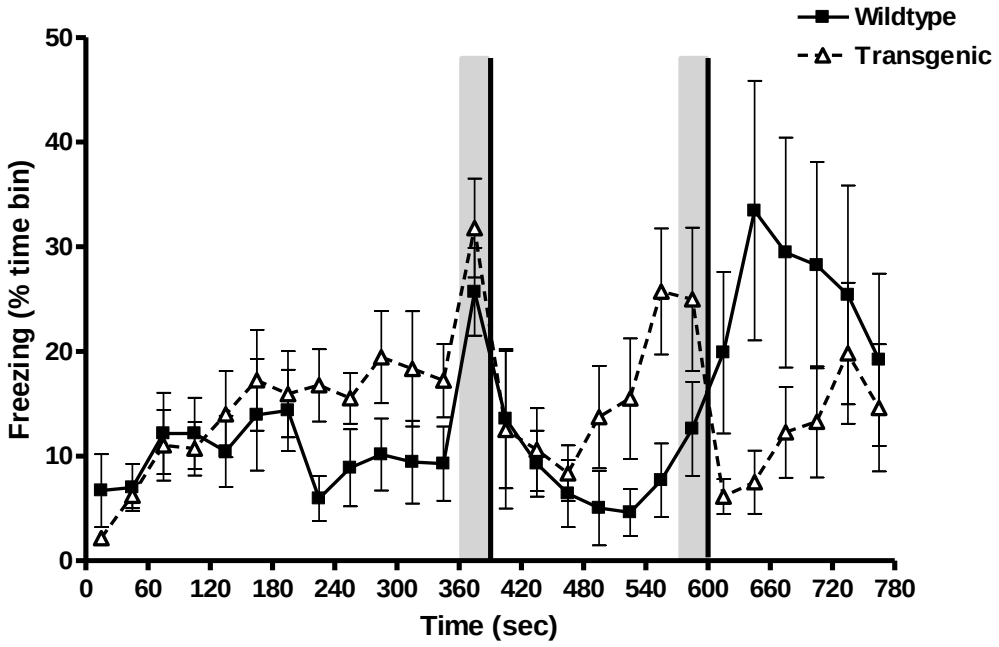
5.1.3.2.1. Transgenic mouse studies

5-HTT knockout (KO) mice have previously been demonstrated to have increased anxiety, as discussed in Section 1.3.1.3. These mice have also been used in a fear conditioning study using a three day paradigm. On day 1, the conditioning day, mice were exposed to three tone-shock pairings using a co-terminating 2s 0.6mA footshock and a 30s 3kHz pure tone. This was then followed by two consecutive extinction days in a novel context with 40 30s tone presentations (in the absence of

any footshocks) with a 5s inter-stimulus interval. 5-HTT KO mice showed normal conditioning during training and normal extinction on the first test day (Wellman *et al.*, 2007). However, on the second day of extinction, both groups showed an increase in freezing at the start of the session as compared to the end of the first extinction session, but this increase was greater in the 5-HTT KO mice as compared to their wildtype (WT) littermates. Across the session, the 5-HTT KO mice extinguished freezing behaviour in a manner similar to their WT counterparts. This suggests that 5-HTT KO mice can acquire, express and extinguish fear learning normally, but that their recall of this extinction learning is impaired. This extinction recall has previously been shown to be dependent on frontal cortical input to the amygdala (Quirk *et al.*, 2000). Wellman and colleagues (2007) link this weakened extinction recall to the increased incidence of post-traumatic stress disorder in *s* allele carriers (Lee *et al.*, 2005), an anxiety disorder characterised by impaired extinction of negative emotional memories (DSM IV-TR, 2000).

The 5-HTT overexpressor (OE) mice show a corresponding decrease in anxiety, again presented in Section 1.3.1.3. However, there is yet to be a published report of their performance in fear conditioning, although this has been performed in this laboratory (Line, unpublished, see Figure 5.1). This study involved a training session of two tone-shock pairings and separate tests of both cue and context conditioning conducted 24hrs later according to a counterbalanced design. Context conditioning was assessed by re-exposing the conditioned mice to the conditioning context for 5min and assessing differences in freezing between WT and 5-HTT OE mice. Assessment of cue conditioning was carried out in a novel context and involved two

A



B

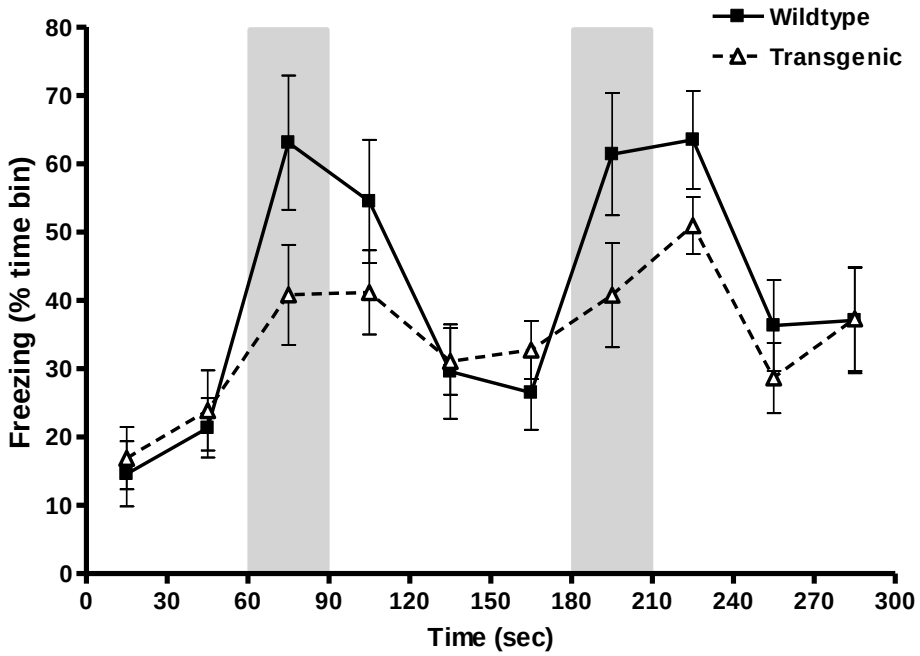


Figure 5.1: Sample data from the D.Phil thesis of Dr Samantha Line displaying the freezing levels of male 5-HT transporter overexpressor (transgenic) mice and wildtype mice during the training (A) and cue conditioning testing (B) sessions in a fear conditioning paradigm

The 5-HT transporter overexpressor mice showed decreased levels of freezing in response to the conditioned cue, as well as a reduction in freezing towards the end of the training session of the fear conditioning paradigm. Taken from S. Line D.Phil thesis (2008).

CS+ presentations with no shock delivery. 5-HTT OE mice demonstrated deficits in both these forms of fear conditioning. This may reflect a deficit in the expression of fear or, alternatively, a deficit in fear conditioning. This reduction in fear acquisition is supported by the reduced freezing of the 5-HTT OE mice relative to WT mice observed at the end of the conditioning session. This provides some evidence for a complementary increase in fear learning in 5-HTT KO mice and reduction of this emotional learning in 5-HTT OE mice, although it is the processes of extinction and acquisition that are altered in these mouse lines, respectively. This implies the consequences of altered 5-HTT protein expression levels on emotional learning may not be a simple relationship. Nonetheless, these results match the predictions made on the basis of human studies.

5.1.3.2.2. Human studies

Findings from animal studies of an association between reduced fear acquisition and increased 5-HTT expression are supported by human fear conditioning studies that have assessed 5-HTTLPR genotype (Garpenstrand *et al.*, 2001, Armbruster *et al.*, 2009). These studies used two distinct visual cues, one of which was paired with an electric shock (to become the conditioned stimulus, the CS+), the other not (the CS-). Participants were initially shown these cues before conditioning, then given eight presentations of each stimulus, the CS+ being reinforced, followed by a further eight presentations in extinction, involving no shocks. Skin conductance was used as the measure of conditioning. *l* allele carriers were found to condition less well in these cue fear conditioning studies. Both groups extinguished at the same rate, despite differing baselines. This adds to the body of literature from human imaging studies suggesting markers of neuronal activity in response to emotional cues are sensitive to

5-HTT manipulation (eg Hariri *et al.*, 2002, Hariri *et al.*, 2005). Together, these studies link the *l* allele, predictive of increased 5-HTT functionality, to both a decrease in fear conditioning as well as reduced amygdala reactivity to negative stimuli. These behavioural and neurobiological findings fit with the central role of amygdala function in emotional processing and show 5-HT neurotransmission can modulate these processes.

The study presented in this chapter attempts to recapitulate the findings of Rhodes and colleagues (2007). This study combined both PET imaging to assess individual's 5-HTT binding levels and fMRI to measure amygdala reactivity to an emotional cue, described further in Section 1.3.1.2.

5.1.4. Amperometry

Using fMRI in rodents would involve the immobilisation of the animal and is therefore not conducive to studying behaviour. However, an analogous signal can be measured in freely-moving rodents using constant potential amperometry (CPA) to record tissue oxygen concentrations (Lowry *et al.*, 2010). Recordings of region tissue oxygen in the brain are thought to provide strong correlates to neuronal activity (Huettel *et al.*, 2004). While the firing of local neuronal populations contributes to this signal (Smith *et al.*, 2002), it is inputs to the area of interest that is thought to be the greatest contributor to the blood-oxygen level dependent (BOLD) signal that underlies fMRI (Mathiesen *et al.*, 2000, Caesar *et al.*, 2003, Offenhauser *et al.*, 2005, Goense and Logothetis, 2008).

CPA involves the implantation of a carbon paste electrode (CPE) into a region of interest and applying the diffusion-limited reduction potential of oxygen, -650mV, to give a current output proportional to the regional oxygen concentration. A reference electrode in a similar media held at a known voltage and an auxiliary electrode to improve signal consistency complete the three electrode system. *In vitro*, this has been shown to be highly selective for the detection of oxygen over other compounds present in the brain (Lowry *et al.*, 1996). The electrical current subsequently detected also correlates strongly with the fMRI BOLD signal *in vivo* (Lowry *et al.*, 2010). The advantage this system has over fMRI, however, is that CPA can be used in freely moving and behaving rodents.

Measurements of oxygen levels in the brain have been investigated using these techniques since the 1950's (Clark *et al.*, 1958) and have been utilised to demonstrate that changes in regional oxygen concentration can be altered by electrical activity (McCreery *et al.*, 1990) and correlate with neurotransmitter release (Kennedy *et al.*, 1992, Zimmerman *et al.*, 1992). The more modern use of CPEs allows for consistent results over (at least) several months (O'Neill and Fillenz, 1985) as compared to bare metal electrodes which are prone to unwanted reactivity *in vivo* leading to "surface poisoning". Much like the BOLD measure used in fMRI, the detection of oxygen using these techniques is dependent on the balance between oxygen delivery and utilisation and so relies on the "overshoot" of the former being much greater than the latter as a consequence of regional brain activity (Leniger-Follert and Lubbers, 1976, Fox *et al.*, 1988, Zimmerman *et al.*, 1992, Lowry and Fillenz, 1997, Bolger and Lowry, 2005).

Given the analogous nature of the signal obtained using CPA to that of fMRI, CPEs can be implanted to allow for *in vivo* measurements of oxygen in awake, freely behaving rodents to give a highly translational tool with which to investigate correlates of neuronal activity. This can be combined with tasks analogous to those used in human imaging studies or, indeed, tasks that would be difficult to perform in humans either due to practical or ethical issues. Furthermore, this oxygen signal can be correlated during these tasks to other measures obtainable in rodent studies but not in humans, such as measures of local neuronal electrical activity. Additionally, these measures can be taken following application of experimental compounds, either in preclinical investigation or to probe the consequences of specific pharmacological manipulation of individual components of the nervous system. Most pertinently, this measure of neuronal activity during behavioural activity can be taken in transgenic mice with specific genetic alterations in isolation from further variation due to genetic (owing to their near-homogeneous genetic background) or environmental (owing to their identical living conditions) factors. This level of control is simply impossible in the human population and would allow for investigations into the consequences of genetic variation on neuronal activity. This amperometric signal, combined with 5-HTT mutant mice and fear conditioning, may therefore be a way in which the contradictory and variable literature on the effect of 5-HTT functionality on neuronal activity during emotionally-driven tasks can be addressed.

5.1.5. Local field potentials

In addition to the BOLD signal correlate of neuronal activity, there have been some studies looking at neuronal population activity during fear conditioning by investigating local field potentials (LFPs). LFPs are a region-specific form of EEG measured by implanting wire electrodes into the area of interest. Two components comprise this signal; action potentials very close to the electrode tip as well as slower, smaller electrical changes in a larger volume around the electrode (Legatt *et al.*, 1980). Measuring LFPs therefore allows for assessment of electrical activity within discrete areas of the brain, a more direct correlate of neuronal firing than those that rely on oxygen. The invasive nature of this method, however, means it is not possible to utilise routinely in human studies. However, LFPs have been measured in other primates as well as in rodents. LFPs have been found to correlate with BOLD signal in studies of primate visual cortex (Logothetis *et al.*, 2001, Goense and Logothetis, 2008). Generally, a shift towards the higher, more active frequency bands is seen with an increase in the BOLD response. In experimental animals, a shift away from the low delta band (generally defined as 0.1-4Hz) to the theta band (4-12Hz) is seen in the BLA following fear conditioning (Pare and Collins, 2000). In context fear conditioning, this is synchronised to hippocampal theta (Seidenbecher *et al.*, 2003, Pape *et al.*, 2005). This synchrony decreases and is replaced by greater synchrony between the amygdala and the frontal cortex during extinction (Pape *et al.*, 2005). This highlights the use of LFPs in measuring synchronising signals across brain regions and their potential use in investigating connections important in neuronal processing.

5.1.6. Aims of this chapter

The 5-HT transporter (5-HTT) overexpressor (OE) mice have previously been used in behavioural tests which show that they have altered emotionality (Jennings *et al.*, 2006, Line *et al.*, 2010, Line, personal communication). This chapter aimed to assess neuronal activity during an emotionally-driven task in the 5-HTT OE mice to probe the underlying neurobiology of this altered behaviour. To this end, basolateral amygdala (BLA) activity during a fear conditioning task in the 5-HTT OE mice and their WT counterparts was recorded via constant potential amperometry and assessments of local field potentials. This gives measures of BLA oxygen and neuronal electrical activity, respectively. Principally, this chapter aimed to establish alterations in these signals in the 5-HTT OE mice, relative to WT mice, in response to a CS previously paired with shock that recapitulates the results of neuroimaging tasks performed in humans. The results from these human imaging studies leads to the hypothesis that increased 5-HTT levels will lead to attenuated measures of neuronal activity in the amygdala during this task. Differences in the behavioural fear responses of these two genotypes were also assessed.

5.2. Methods

5-HT transporter (5-HTT) overexpressor (OE) mice (Jennings *et al.*, 2006) and their wildtype (WT) littermates were implanted with electrodes to record changes in tissue oxygen and local field potentials (LFPs) in response to conditioned cues. This was achieved through electrochemical and electrophysiological recordings within the basolateral amygdala (BLA) of these mice during a differential fear conditioning paradigm.

5.2.1. Subject preparation

5.2.1.1. Animals

Two cohorts of male 5-HTT OE mice (Jennings *et al.*, 2006) and their WT littermates were used for these experiments of which a total of 13 mice per group were used in the final analysis. These numbers were arrived at as only animals that gave good electrical signals throughout the experiment were used. These mice were bred from female CBA WT and male OE mice on a C57Bl/6 background and maintained in The University of Oxford in holding rooms on a 12/12hr light/dark cycle (lights on at 7am and off at 7pm). Prior to surgery, mice were housed in groups of 2-4 with *ad libitum* food and water and were typically of 5 months of age at the time of surgery. Following surgery, mice were individually housed to prevent damage to the headcap and allowed to recover for at least one week before initial electrode conditioning (see Section 5.2.2.2).

5.2.1.2. Surgery

Mice were aseptically, bilaterally implanted with a carbon paste electrode (CPE, gifted by Eli Lilly, UK) in the BLA of one hemisphere to measure tissue oxygen and a platinum wire electrode in the BLA of the contralateral hemisphere to measure LFPs. CPEs were constructed from Teflon-coated 250µm diameter silver wire (277µm coated diameter) with the insulation pulled down the wire to produce a 2mm cavity which was subsequently packed with carbon paste. The co-ordinates used for implantation were targeted towards the BLA and were initially -1.35mm anterior/posterior, ±3.00mm medial/lateral and -4.80mm dorsal/ventral relative to bregma. These were subsequently moved to -1.35mm anterior/posterior, ±3.10mm medial/lateral and -5.00mm dorsal/ventral relative to bregma to place the electrodes more centrally in the BLA. These coordinates were based on the work of others in this laboratory as well as pilot surgeries and differ significantly from those given in published mouse brain atlases (Franklin and Paxinos, 1997). Additionally, wire auxiliary and reference electrodes were implanted into parietal cortex to complete the amperometric circuit. Each of these electrodes was topped with a gold pin (PlasticsOne Inc) to allow for a connection to a recording lead to be made and all of this was secured in a headcap constructed to ensure the electrodes remained in place with minimal discomfort to the animals. Surgeries were performed by myself and Dr Stephen McHugh, each of us assisting the other when not the primary surgeon to maintain sterility.

Briefly, mice were anaesthetised in an induction chamber filled with 3-4% isoflurane at a flow rate of 2-3l/min O_2. Mice were then injected subcutaneously (s. c.) with 0.1ml 5mg/ml meloxicam (Metacam, Boehringer Ingelheim) to provide analgesia throughout and beyond surgery, their scalp shaved and the exposed pate cleaned with Hibiscrub (4% w/v chlorhexidine gluconate, Regent Medical Ltd), before being secured in a stereotaxic frame (Kopf Model 940 (single arm) or 942 (double arm) small animal stereotaxic instrument with digital display console). A homeothermic heat pad (Harvard Apparatus) was placed below the mice and body temperature monitored throughout surgery. A surgical level of anaesthesia was maintained using isoflurane at concentrations ranging from 1-3% at a flow rate of approximately 1.5l/min O_2. Sterile eye drops (Viscotears, Novartis) were used to prevent corneal drying throughout surgery.

An incision was made to expose the skull, and bregma and lambda were measured and used to ensure the head was level. 4% lidocaine hydrochloride (Xylocaine, AstraZeneca UK Ltd) was topically applied to the exposed tissues of the scalp to provide local anaesthetic to the surgical area before the skull was lightly abraded to improve dental cement adhesion, then cleaned and dried with 70% ethanol. The positions of the holes to be drilled were then marked out and drilled using a dental drill (Technobox 810, BienAir, Figure 5.2). Bone dust was intermittently removed using saline which also ensured the skull was kept moist and soft enough to prevent the bone from splintering. Following the drilling of all six holes, three skull screws were secured in place, the auxiliary and reference electrodes, closely apposed, were placed superficially into cortex and the first amygdala electrode positioned

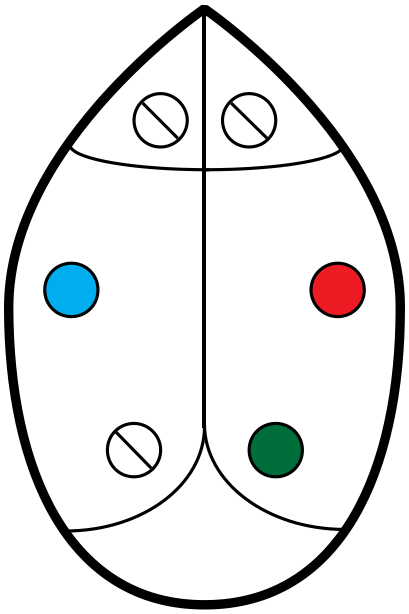


Figure 5.2: A diagrammatic representation of example positions of the electrode and screw placements in the amperometry surgery

The above example shows the relative positions of the two basolateral amygdala electrodes to measure oxygen and local field potentials (red and blue), the auxiliary and reference electrodes (placed together, green) and the three skull screws (crossed circles). The left/right position of electrode placements (oxygen and local field potentials) was counterbalanced across groups.

stereotaxically. These three electrodes were then cemented in place using Kemdent Simplex Rapid dental cement (Associated Dental Products Ltd) before then inserting and cementing the second amygdala electrode. A base layer of cement was then built up to cover the skull and ensure the screws were also surrounded by cement. This ensured they would hold the headcap in place. The gold pin of each electrode was then positioned into a plastic pedestal (PlasticsOne Inc) and cemented securely. This was then positioned atop the base structure on the skull with excess wire coiled below the pedestal and cemented in place with no wire being left exposed. The skull surface was also well covered with the headcap, leaving little-to-no exposed tissue from the initial incision.

Mice were then given 1ml 0.9% saline s. c. as recovery fluids, allowed to come round under pure O_2 at a flow rate of 1.5l/min, before being placed in a heated recovery chamber (Beta Medical Scientific), maintained at 37°C, for several hours. During this time, mice were intermittently observed until normal ambulation was seen. At this point, mice were given a further 0.1ml meloxicam s. c. to provide lasting analgesia throughout the night. Mice received one final injection of 0.1ml meloxicam s. c. the following morning. Mice were then monitored using distress sheets which include assessments of the animal's appearance and weight, normal and provoked behaviours, any clinical signs of infection as well as the state of the headcap and incision. This monitoring continued for at least one week, typically for 3-4 weeks.

5.2.2. Electrochemistry and electrophysiology

5.2.2.1. Potentials

Potentials were applied to the implanted electrodes using a four channel potentiostat (a customised Biostat unit to be compatible with the PlasticsOne system, ACM Instruments, Cumbria, UK). The animals were connected to the potentiostat via a plastic-coated six core cable attached to the pedestal of the mouse and a commutator above the conditioning chamber (all PlasticsOne components). This was set into a counter-weighted arm (MedAssociates) to facilitate free movement of the animal. The commutator was subsequently connected to the potentiostat via a second six core cable (PlasticsOne).

Tissue oxygen was recorded using three-electrode constant potential amperometry with a potential of -650mV, the diffusion-limited potential of oxygen, being applied to the CPE. A potential of 0V was applied to the wire electrode to record LFPs.

The signals resulting from the experiments were recorded via a Powerlab (Model 8/30, ADInstruments) in Chart (version 5, AD Instruments) at a sample rate of 4kHz throughout all sessions. Events were recorded onto this trace via TTL (Transistor-Transistor Logic) pulses being delivered by the Med-PC programme controlling the behavioural paradigm.

5.2.2.2. Signal stability

Several steps were taken to promote tissue oxygen signal stability *in vivo*. Mice were first given one week to recover post-surgery to allow any damaged tissue to heal. Following this, but at least one week prior to the fear conditioning paradigm, the potentials were applied to the electrodes for two hours to allow the electrodes to condition. This was done in a neutral context (see Section 5.2.3.1) with the animal's own bedding present during this time. Previous evidence suggests that the response properties of CPEs are more variable in the first few hours after the potential is first applied but that the signal stabilizes after conditioning (Vanderkooi *et al.*, 1991). One hypothesis for this stabilizing effect is that, during conditioning, oil is removed from the surface of the CPE, which makes the surface more powder-like and increases the rate of charge transfer (Ormonde and O'Neill, 1989, O'Neill, 1993, Kane and O'Neill, 1998).

Finally, whenever a polarizing potential is applied to a CPE, even after electrode conditioning, a large capacitance current is observed that typically reduces rapidly (within seconds) and settles within approximately 10min. To control for this, the potentials were applied to the electrodes for at least 10min before the start of each trial to allow the signal to settle to a stable baseline. This, again, was done in the neutral context to prevent large amounts of habituation to the training and test contexts that may result in generally inactive animals throughout the sessions, thus confounding the freezing measure used, as well as to prevent latent inhibition of the context. Mice were then moved to the training or testing context, as appropriate.

5.2.3. Differential fear conditioning paradigm

A fear conditioning paradigm using a conditioned (CS+) and non-conditioned (CS-) stimulus was used. This was to enable the recording of signals that specifically result from a stimulus previously paired with a foot shock and to dissociate this from signals in response to other, non-emotional cues. Three operant chambers (Mouse Modular Test Chambers, ENV-307A, Med Associates Inc) controlled by MED-PC IV programming were used to isolate cue- from context-conditioning. Freezing behaviour was taken as the behavioural output, assessed automatically using a software program that compares two sample frames taken every second and measures the number of altered pixels. A threshold of <0.5% altered pixels was taken as being indicative of freezing. This approach has been previously validated (Richmond *et al.*, 1998).

The cues used were a pure tone of 2900Hz and white noise, delivered using an audio stimulus generator (ANL-926, Med Associates) controlled by MED-PC. The assignment of each auditory cue as the CS+ and CS- was counterbalanced for each genotype (CS+: white noise, n=7 per group; tone, n=6 per group). The paradigm used lasted five days (Figure 5.3).

5.2.3.1. Pre-exposure

A pre-exposure day was performed to measure unconditioned behavioural, amperometric and LFP responses to the tones used. Mice were exposed to the cues to be used but without shocks in an operant box with opaque sides that was not placed in a noise-attenuating chamber (the neutral context). This chamber had a solid floor created by placing a cardboard insert over the existing grid flooring (ENV-307A-GF).

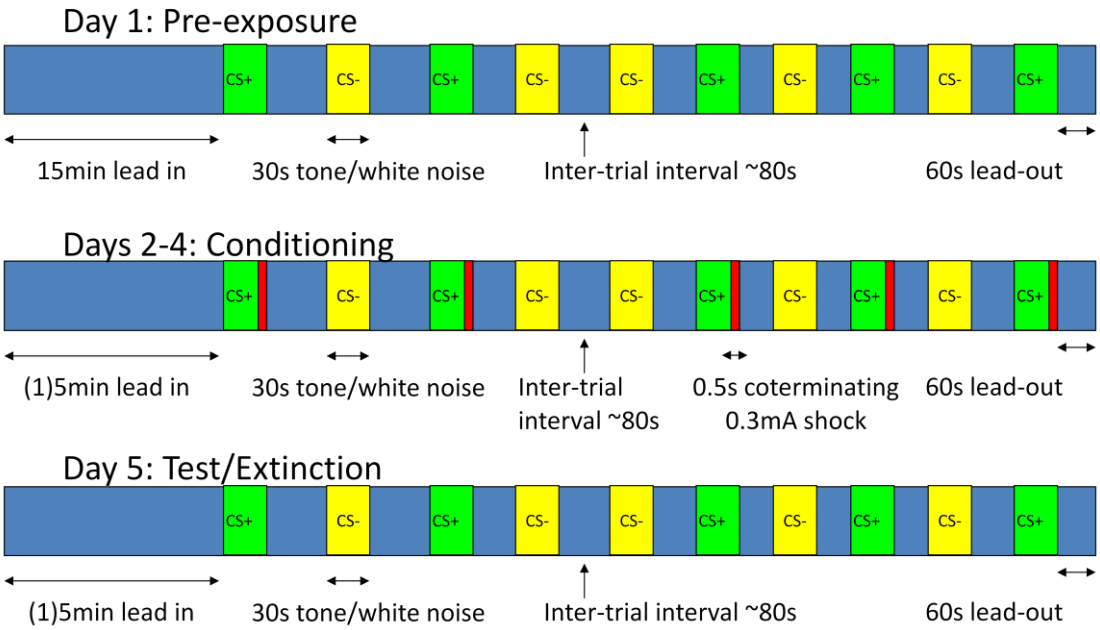


Figure 5.3: Differential fear conditioning paradigm

Mice were trained and tested over a five day protocol. On day 1, mice were pre-exposed to the stimuli to be used in a neutral context. There were then three consecutive days of conditioning during which a shock was paired with one of the cues but not with the others so as to establish a conditioned (CS+) and unconditioned (CS-) stimulus. This was done following a 10min period in the neutral context before being placed into the conditioning context. The mice were tested and extinguished on day 5 in a novel context using the same cues but with no shocks.

The pre-exposure session lasted 33min with a lead-in period in which no cues were presented of 15min. Following this, a 30s presentation of the stimulus that would serve as the CS+ for that animal occurred. An inter-trial interval (ITI) then occurred, followed by a 30s presentation of what would become the CS- for that animal. This pattern of 30s cue and pseudorandom length intervals then continued for the duration of the session which ended in a 60s lead-out. Five presentations of each cue type occurred during this time. The pseudorandom order of CS type and ITI length can be found in Table 5.1 and Table 5.2, respectively. The mean length of the ITI was 80s.

5.2.3.2. Conditioning sessions

Days 2-4 acted as conditioning days. Following the 10min electrode conditioning session in the neutral context, mice were placed in an operant chamber within a noise-attenuating cabinet that would act as the training context. Two distinct contexts were used, the allocation of which was counterbalanced across groups. One context consisted of black and white striped walls on the inside of the cabinet and a sandalwood scent (Body Shop Home Fragrance Oils), whilst the other had solid grey walls and a lavender scent (also Body Shop Home Fragrance Oils). These scents were created by applying one drop of the oil to a paper towel placed below the grid floor of the operant chamber.

Table 5.1: Cue type in the order presented during each session of the differential fear conditioning paradigm

	Conditioned Stimulus (CS) type									
Session	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Pre-exposure	+	-	+	-	-	+	-	+	+	-
Training 1	-	+	-	+	-	+	+	-	-	+
Training 2	+	-	-	+	-	+	-	+	+	-
Training 3	+	-	+	-	-	+	+	-	-	+
Test/extinction, CS- first	-	+	-	+	-	-	+	+	-	+
Test/extinction, CS+ first	+	-	+	-	+	+	-	-	+	-

The order of CS+ and CS- presentation was pseudorandomised across trials but kept consistent between animals to allow for better comparison of the resultant signals. The order of presentation during extinction was counterbalanced to remove order effects from the resultant analysis of the first presentation of each CS type.

Table 5.2: Inter-trial interval length in order during each session of the differential fear conditioning paradigm

	Inter-trial Interval (ITI) length (s)								
Session	**1-2**	**2-3**	**3-4**	**4-5**	**5-6**	**6-7**	**7-8**	**8-9**	**9-10**
Pre-exposure	70	95	60	90	80	100	65	75	85
Training 1	75	90	60	70	95	100	65	85	80
Training 2	65	100	90	75	85	60	80	70	95
Training 3	95	65	90	80	85	70	60	75	100
Test/extinction, CS- first	70	100	60	90	65	85	75	95	80
Test/extinction, CS+ first	70	100	60	90	65	85	75	95	80

The length of the delay between each CS presentation was pseudorandomised around an average of 80s to minimise any temporally-related learning. The order of CS presentation during extinction was counterbalanced but the inter-trial interval was kept constant for both groups.

A 5min lead-in was followed by a 30s cue presentation and the session proceeded as above with a mean ITI duration of 80s and five presentations of each cue type. CS+ presentations were accompanied by a co-terminating 0.5s 0.3mA scrambled footshock, delivered through the grid floor (ENV-307A-GF, driven by the ENV-414SA Aversive Stimulator via the ENV-307A-QD harness), while CS- presentations continued to be unreinforced. Cues were delivered at a measured amplitude of 82dB as compared to a background level of 45dB (assessed with an Environment Meter, Model N09AQ, Precision Gold). Again, the order of cue presentation and ITI sequence for each day can be found in Table 5.1 and Table 5.2, respectively. The total length of these sessions was 23min.

5.2.3.3. Extinction

Day 5 acted as the test/extinction day. Mice were once again given a 10min period in the neutral context to allow for signals to stabilise. Mice were then placed in a novel context. This context was the compartment described above not used for that animal's training; i.e., those trained in the operant chamber with black and white walls and a sandalwood scent were tested in the chamber with grey walls and a lavender scent, and *vice versa*. The session then resembled that of one of the training days, with the exception that no shocks were delivered. The order of cue type presentation was counterbalanced across genotypes, so half of each group were presented with the CS+ first, the other half the CS- first. Details of the order of the remaining CS presentations and the ITI lengths can, once again, be found in Table 5.1 and Table 5.2, respectively.

5.2.4. Histology

Following testing, mice were sacrificed and brain tissue was prepared as detailed in Section 3.2.1.2.2. The BLA is identifiable as lying within a defined area between the white matter tracts resulting from the bifurcation of the external capsule (Franklin and Paxinos, 1997). Placements of amperometric and LFP electrodes are displayed in Figure 5.4. The majority of placements were in the basolateral amygdala with some in the medial complex. Individual oxygen responses were plotted to ensure that signals from placements in the basolateral and central nuclei did not differ (data not shown). One LFP electrode tract was only evident in the lateral globus pallidus, although this may not have been the final point of the electrode. Nonetheless, the data from this placement were excluded from the analysis. The location of three LFP electrodes and one oxygen electrode could not be precisely located owing to tissue damage but had tracts leading to the amygdala and were therefore included in the analysis.

5.2.5. Data analysis

All mice (n=13 per group) were included for behavioural analysis. Data from the LFP electrode placed in the lateral globus pallidus were excluded from all analysis and poor signals were excluded from oxygen and LFP analysis for individual animals on a session by session basis. This resulted in oxygen signals being excluded for one WT mouse on training day 2, two WT mice on training day 3 and one WT and one 5-HTT OE mouse during extinction. In addition to the poor placement in one 5-HTT OE mouse, a further two WT mice and one OE mouse were excluded on training day 2 and one WT and two OE mice on training day 3 with regards to the LFP measures.

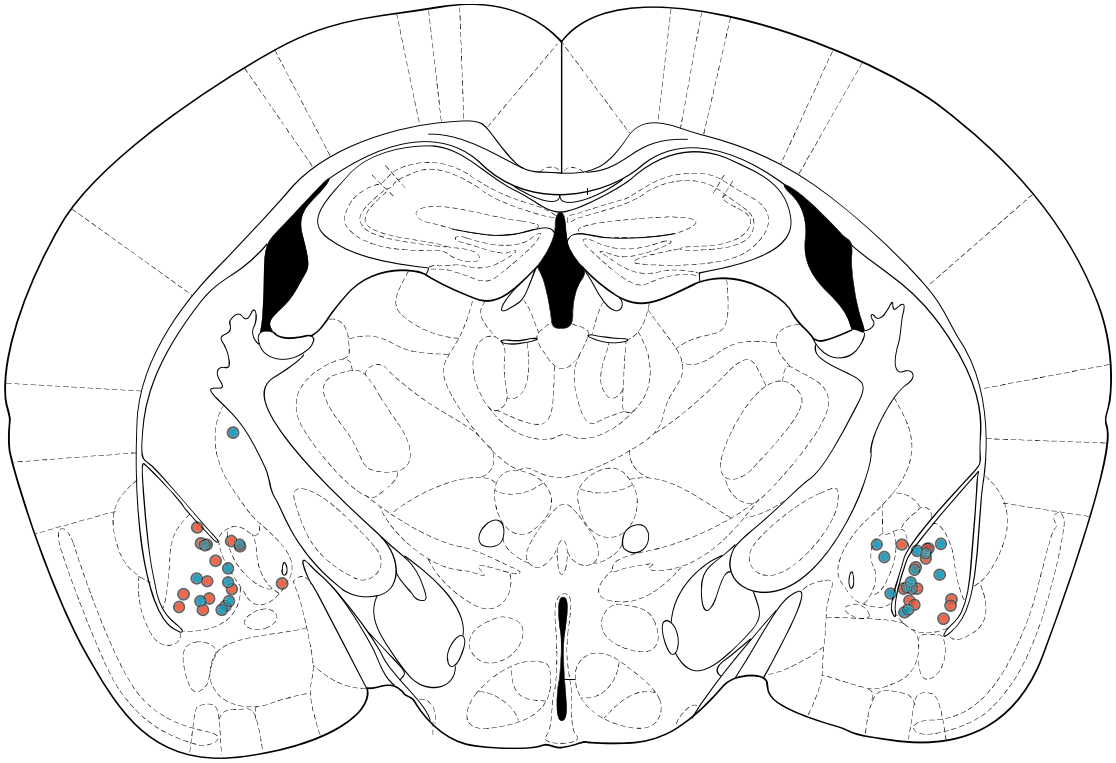


Figure 5.4: Electrode placements for oxygen (red) and local field potential (LFP, blue) electrodes in 5-HT transporter overexpressor and wildtype mice used in fear conditioning

The majority of placements were in the basolateral nucleus of the amygdala with some in the central complex. One LFP placement appeared to be in lateral globus pallidus and so these data were excluded from further analysis. Three LFP electrodes and one oxygen electrode could not be located.

5.2.5.1. Freezing

The main behavioural measure for fear conditioning was the change in freezing between the 30s prior to CS onset and the 30s during CS presentation (freezing during CS presentation – freezing prior to CS presentation). This was then averaged across all five presentations of each CS type (CS+ and CS-) for each genotype on each day. This measure, as well as the raw data of percentage freezing, was split into 10s time bins for the final two days of the paradigm to allow further investigation into the behavioural response across cue presentation.

5.2.5.2. Oxygen signals

Oxygen data obtained during the 30s cue presentations were normalised to the 5s prior to CS onset (see Figure 5.5) and is presented in 2s time bins averaged across all five presentations of each CS type (CS+ and CS-) for each genotype on each day. The first presentation of each CS type was also analysed separately during the extinction day to remove any effect of within-session extinction from this measure. Planned comparisons of the CS+ signal were also made on each day, as well as during the first CS presentations during extinction, as it was this signal in which changes were expected to be observed. Statistical analysis was performed on the 15 time bins (2s each) that occurred during CS presentation.

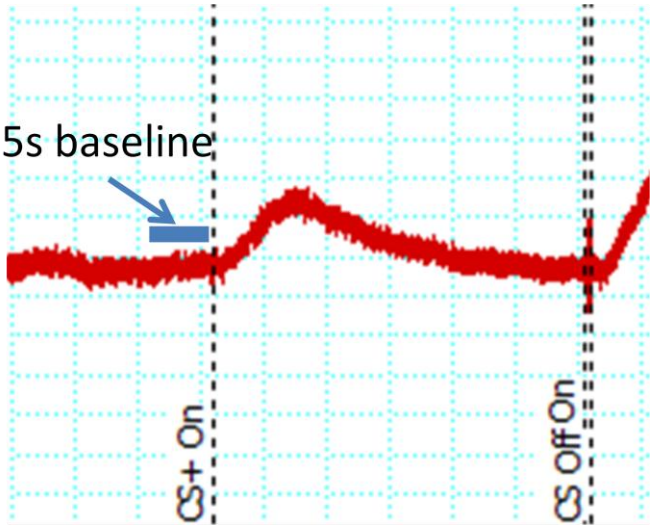


Figure 5.5: A sample trace of the raw oxygen signal obtained during the differential fear conditioning paradigm

The oxygen signal was collected at a high sample rate and the response during the 30s cue presentation normalised to a 5s baseline period just prior to CS onset. This was subsequently processed into 2s time bins. Also visible in this trace is the artefact produced by shock delivery.

5.2.5.3. Local field potentials

Raw LFP recordings were split into frequency bands by taking the modulus of the signal resulting from a bandpass filter performed in Chart (see Figure 5.6). Delta was defined using a bandpass between 1-4Hz and (low) theta as 4-8Hz. The lower value of delta was set at 1Hz, as lower frequency values would have diminished the temporal resolution of the responses measured. The values for the frequency bands chosen were based on work by Pape and colleagues (Seidenbecher *et al.*, 2003, Pape *et al.*, 2005). These data were then normalised by dividing the theta values by the delta values to produce a theta:delta ratio. This ratio was averaged across all five presentations of each CS type (CS+ and CS-) for each genotype on each day and are, again, presented in 2s time bins. The first presentation of each CS type was also analysed separately during the extinction day to remove any effect of within-session extinction from this measure. This normalisation to a theta:delta ratio was required to control for between-subject variability in the power of the LFP signal obtained as well as to reduce the effect electrical noise on this LFP signal. Statistical analysis was performed on the 15 time bins (2s each) that occurred during CS presentation.

Because alterations in the theta:delta ratio appeared to be present outside of CS presentation periods on training day three and during the extinction session, statistical analyses were also performed on the 8s pre-CS presentation period. The means of the theta:delta values during this 8s window were taken and compared using an ANOVA with CS type (to be presented) and genotype as between-subject factors. This was then also done for freezing levels of the mice included in LFP

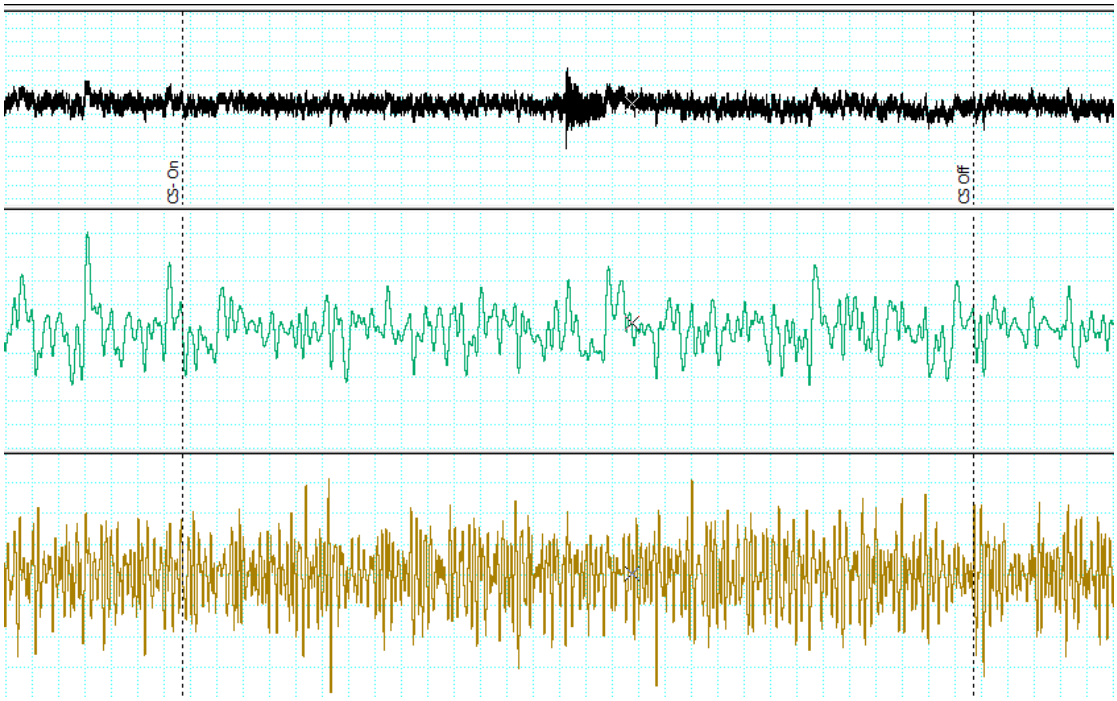


Figure 5.6: Sample trace of raw and processed local field potential signals obtained during the differential fear conditioning paradigm

The raw signal can be seen in black at the top of this trace. This was then bandpass filtered into delta (1-4Hz, light blue in the centre) and theta (4-8Hz, mustard at the bottom) bands. To normalise these signals, a theta:delta ratio was calculated and is presented in 2s time bins in the subsequent figures in this chapter.

analysis to ensure any effects seen were not due to differences in locomotion. T-tests were also used to compare levels prior to the first CS of the day regardless of valence, i.e. before any stimuli had been presented. This was to control for the effect CS presentation may have on subsequent signals obtained. Finally, these pre-CS changes were investigated across the paradigm by performing a repeated measures ANOVA including day as a within-subject factor and the same between-subjects factors given above.

5.2.5.4. Statistical analysis

Repeated measures ANOVAs with genotype as a between-subject factor and CS type with time bin or day as within-subject factors were used where appropriate. When an interaction was found to be significant in an ANOVA, simple main effects analyses were also done with Least Significant Difference (LSD). $p<0.05$ was considered statistically significant and all statistics were done using SPSS version 15.

5.3. Results

5.3.1. Behaviour

The freezing difference score was analysed using a repeated measures ANOVA with day or time bin and CS type as within-subject measures and genotype as a between-subjects factor.

Levels of freezing in response to the CS+ rose over the paradigm, whereas mice were moving more to the onset of the CS- than at baseline by then end of the five days (Figure 5.7). This did not differ between the genotypes. The difference between these behavioural responses to the different CS types was most obvious in the last two days of the paradigm, the final day of training and the extinction session.

Statistically, no differences were seen between genotypes across the differential fear conditioning paradigm (effect of genotype $F_{(1,24)}$=1.221, p=0.280; genotype*day interaction $F_{(4,96)}$=0.546, p=0.702; genotype*CS type interaction $F_{(1,24)}$=0.458, p=0.505; genotype*day*CS type interaction $F_{(4,96)}$=0.047, p=0.996) but an effect of CS type was seen ($F_{(1,24)}$=18.864, p<0.001). The alteration in behaviour across the paradigm was supported by a day*CS type interaction ($F_{(4,96)}$=8.352, p<0.001), with no overall effect of day ($F_{(4,96)}$=0.665, p=0.618). Simple main effects revealed that behavioural responses to both the CS+ (simple main effect of day $F_{(4,21)}$=4.030, p=0.014) and CS- ($F_{(4,21)}$=8.102, p<0.001) altered over days due to mice gradually freezing more to CS+ presentation and being more active during CS- presentation than at baseline. The different responses to the CS types was statistically significant on training day three

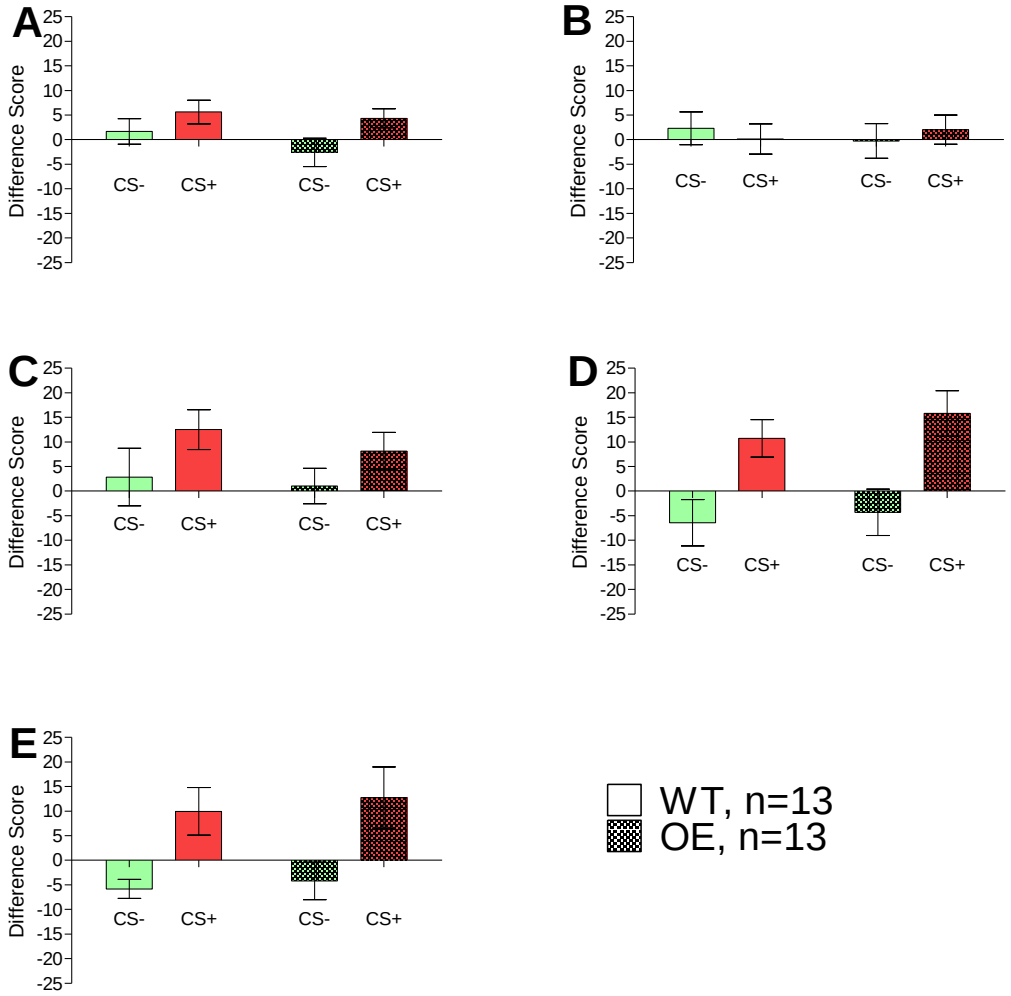


Figure 5.7: Difference score for freezing during 30s CS presentation minus freezing in the 30s prior to CS presentation in the five day differential fear conditioning paradigm for 5-HT transporter overexpressor (OE) mice and their wildtype (WT) littermates

Mice respond relatively little to the tones used during pre-exposure (A), and on the first day of training (B). On the second training day (C), mice can be seen to be freezing more in response to the CS+, although this is not statistically significantly different from their response to the CS- until training day three (D). These differential freezing responses are also seen during extinction (E), with mice freezing in response to the CS+ but moving more than at baseline during CS- presentation.

and the extinction day (simple main effects of CS type: pre-exposure $F_{(1,24)}$=2.760, p=0.110; training day 1 $F_{(1,24)}$=0.001, p=0.982; training day 2 $F_{(1,24)}$=2.078, p=0.162; training day 3 $F_{(1,24)}$=14.397, p=0.001; extinction $F_{(1,24)}$=21.138, p<0.001).

The graph representing freezing during pre-exposure (Figure 5.7A) does appear to show an increase in responding to the CS+ and not to the CS-. As this is before any conditioning has occurred, this could be a source of concern. It is therefore worth emphasising that no statistically significant effects were seen on behaviour during this day of the paradigm (p=0.110). Additionally, the assignment of which cue was used as the CS+ for each given animal was counterbalanced across groups, so this apparent difference does not represent an unconditioned aversion to any specific cue. No difference is seen in behaviour on day one of training, further supporting the suggestion that no differential behavioural response to the cues was seen before conditioning had occurred, although behaviour on day one of training may be confounded by the response to receiving shocks.

To further investigate the behavioural response on the final two days of the paradigm, freezing was split into 10s time bins (Figure 5.8). This was done both using the difference score and the raw percentage freezing measure. In general, a difference between the CS types is still seen but behaviour did not alter significantly across the 30s of cue presentation. This was supported statistically by an effect of CS being detected (difference score: training day three $F_{(1,24)}$=14.398, p=0.001; extinction $F_{(1,24)}$=21.139, p<0.001. Percentage freezing: training day three $F_{(1,24)}$=7.835, p=0.010; extinction day $F_{(1,24)}$=11.149, p=0.003), but no effect of time bin being seen (difference

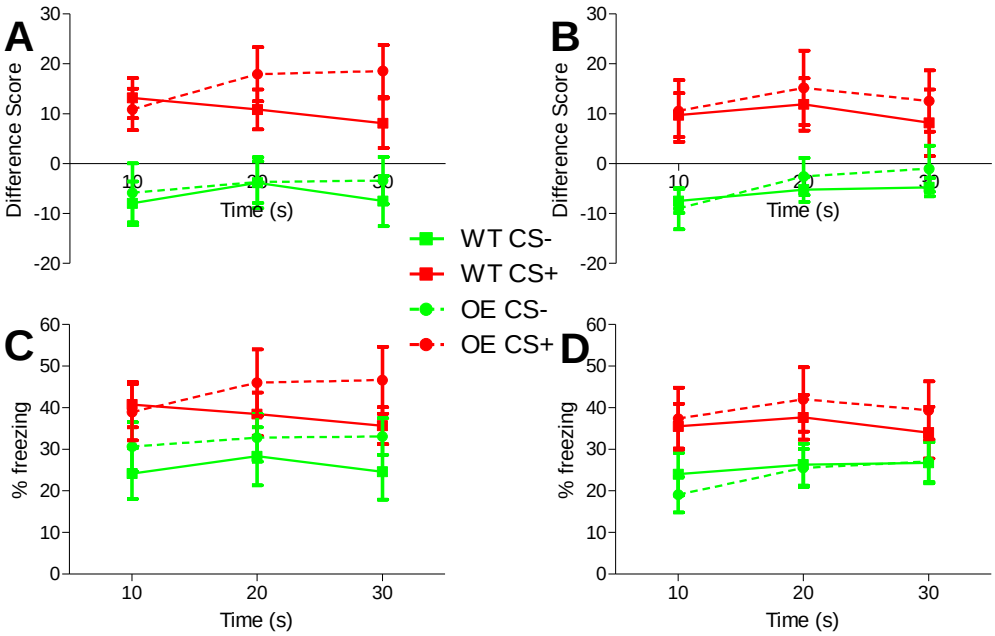


Figure 5.8: Freezing in 10s time bins in response to the CS+ (red) and CS- (green) during day three of training (A and C) and the extinction day (B and D) expressed as a difference score of freezing pre-CS and during CS (A and B) or percentage freezing (C and D)

The wildtype (WT) and 5-HT transporter overexpressor (OE) mice did not differ in the time course of their freezing responses to the two cues during these days.

score: training day three $F_{(2,48)}$=1.935, p=0.156; extinction $F_{(2,48)}$=3.047, p=0.057. Percentage freezing: training day three $F_{(2,48)}$=1.935, p=0.156; extinction day $F_{(2,48)}$=3.047, p=0.057) and no time bin*CS type interaction was seen (difference score: training day 3 $F_{(2,48)}$=0.034, p=0.967; extinction $F_{(2,48)}$=1.039, p=0.362. Percentage freezing: training day 3 $F_{(2,48)}$=0.034, p=0.967; extinction $F_{(2,48)}$=1.039, p=0.362). Genotype also showed no main effect (difference score: training day 3 $F_{(1,24)}$=0.807, p=0.378; extinction $F_{(1,24)}$=0.177, p=0.678. Percentage freezing: training day 3 $F_{(1,24)}$=0.672, p=0.420; extinction $F_{(1,24)}$=0.024, p=0.878) and no interaction with CS type (difference score: training day 3 $F_{(1,24)}$=0.092, p=0.764; extinction $F_{(1,24)}$=0.030, p=0.864. Percentage freezing: training day 3 $F_{(1,24)}$=0.011, p=0.916; extinction $F_{(1,24)}$=0.535, p=0.471).

However, genotype*time bin interactions were seen on training day three (difference score $F_{(2,48)}$=3.440, p=0.040. Percentage freezing $F_{(2,48)}$=3.440, p=0.040) but not during extinction (difference score $F_{(2,48)}$=0.989, p=0.380. Percentage freezing $F_{(2,48)}$=0.989, p=0.380). Simple main effects analysis did not reveal any more detail on this interaction (difference score: simple main effects of genotype: time bin 1 $F_{(1,24)}$=0.001, p=0.981; time bin 2 $F_{(1,24)}$=0.578, p=0.454; time bin 3 $F_{(1,24)}$=3.635, p=0.069. Simple main effects of time bin: WT $F_{(2,23)}$=1.621, p=0.219; SERT OE $F_{(2,23)}$=3.170, p=0.061. Percentage freezing: simple main effects of genotype: time bin 1 $F_{(1,24)}$=0.110, p=0.743; time bin 2 $F_{(1,24)}$=0.532, p=0.473; time bin 3 $F_{(1,24)}$=1.795, p=0.193. Simple main effects of time bin: WT $F_{(2,23)}$=1.621, p=0.219; SERT OE $F_{(2,23)}$=3.170, p=0.061). No three way interaction was seen (difference score: training day 3 $F_{(2,48)}$=2.097, p=0.134; extinction $F_{(2,48)}$=0.033, p=0.967. Percentage freezing: training day 3 $F_{(2,48)}$=2.097, p=0.134; extinction $F_{(2,48)}$=0.033, p=0.967).

5.3.2. Amperometry

The oxygen signal normalised to the 5s pre-CS period was analysed using repeated measures ANOVAs with time bin and CS type as within-subject measures and genotype as a between-subjects factor on each day. The results from these analyses are summarised in Table 5.3. Planned comparisons of CS+ responses were also performed with time bin as a within-subject factor and genotype as a between-subjects factor.

5.3.2.1. Pre-exposure

During pre-exposure (n=13 per group, Figure 5.9), oxygen levels in the amygdala were seen to respond to CS onset with an increase in signal (as seen by an effect of time bin) but this was not modulated by genotype or CS type (Table 5.3). All oxygen responses therefore appeared similar; an initial increase in oxygen signal that slowly decreased throughout CS presentation. This was also reflected in the planned comparison of CS+ responses between genotypes, which showed an effect of time bin ($F_{(14,336)}$=3.367, p<0.001) but no modulatory effect of genotype (effect of genotype $F_{(1,24)}$=0.015, p=0.904; genotype*time bin interaction $F_{(14,336)}$=0.862, p=0.601).

Table 5.3: Initial statistical analysis of the oxygen signal obtained during the differential fear conditioning paradigm

Session	Time bin	Effect of... Genotype	CS type
Pre Exposure	**$F_{(14,336)}$=6.084, p<0.001**	$F_{(1,24)}$<0.001, p=0.998	$F_{(1,24)}$=0.678, p=0.419
Training 1	**$F_{(14,336)}$=8.344, p<0.001**	$F_{(1,24)}$=0.006, p=0.938	$F_{(1,24)}$=1.350, p=0.257
Training 2	**$F_{(14,322)}$=19.418, p<0.001**	$F_{(1,23)}$=0.837, p=0.370	$F_{(1,23)}$=0.050, p=0.825
Training 3	**$F_{(14,308)}$=22.254, p<0.001**	$F_{(1,22)}$=1.072, p=0.312	$F_{(1,22)}$=0.035, p=0.853
Extinction, overall analysis	**$F_{(14,308)}$=24.574, p<0.001**	$F_{(1,22)}$=2.660, p=0.117	$F_{(1,22)}$=0.868, p=0.362
Extinction, first CS presentation	**$F_{(14,308)}$=17.365, p<0.001**	**$F_{(1,22)}$=5.988, p=0.023**	$F_{(1,22)}$=0.007, p=0.935

Session	…interaction Time bin* genotype	Time bin* CS type	Genotype* CS type	Time bin* genotype* CS type
Pre Exposure	$F_{(14,336)}$=0.361, p=0.984	$F_{(14,336)}$=0.309, p=0.993	$F_{(1,24)}$=0.052, p=0.821	$F_{(14,336)}$=0.666, p=0.807
Training 1	$F_{(14,336)}$=0.452, p=0.956	**$F_{(14,336)}$=1.771, p=0.042**	$F_{(1,24)}$=0.404, p=0.531	$F_{(14,336)}$=0.525, p=0.918
Training 2	$F_{(14,322)}$=1.074, p=0.380	**$F_{(14,322)}$=4.759, p<0.001**	$F_{(1,23)}$=1.283, p=0.269	$F_{(14,322)}$=0.586, p=0.876
Training 3	$F_{(14,308)}$=1.126, p=0.334	**$F_{(14,308)}$=4.999, p<0.001**	**$F_{(1,22)}$=7.717, p=0.011**	**$F_{(14,308)}$=2.203, p=0.008**
Extinction, overall analysis	$F_{(14,308)}$=0.506, p=0.929	$F_{(14,308)}$=0.706, p=0.768	$F_{(1,22)}$=0.637, p=0.433	$F_{(14,308)}$=0.387, p=0.978
Extinction, first CS presentation	**$F_{(14,308)}$=2.094, p=0.012**	**$F_{(14,308)}$=3.335, p<0.001**	$F_{(1,22)}$=1.199, p=0.285	$F_{(14,308)}$=0.776, p=0.695

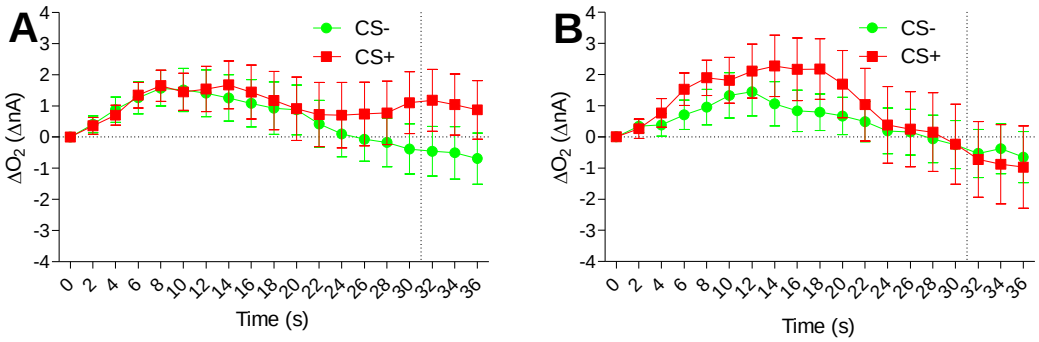


Figure 5.9: Oxygen signals in the amygdala of wildtype (A) and 5-HT transporter overexpressor mice (B) in response to the CS+ (red) and CS- (green) during pre-exposure in the differential fear conditioning paradigm

The amygdala responded to both cues equally during pre-exposure in both wildtype and 5-HT transporter overexpressor mice. Dashed line at 30s indicates CS termination. Note the position of 0 (no change) on the Y-axis.

5.3.2.2.Training

5.3.2.2.1. Day one

On day one of training (n=13 per group, Figure 5.10), oxygen levels in the amygdala rose in response to CS presentation in both genotypes and this response differed subtly between the two CS types (time bin*CS type interaction $F_{(14,336)}$=1.771, p=0.042. See also Table 5.3). The shape of the oxygen response to the CS+ consisted of a positive peak and a clear return to baseline, whereas the CS- response involved an initial increase in the oxygen signal that plateaued and did not return to baseline. Statistically, this is represented by effects of CS type being most evident at the end of CS presentation (Table 5.4).

The planned comparison of CS+ responses showed an effect of time bin ($F_{(14,336)}$=8.248, p<0.001) but no effects involving genotype (effect of genotype $F_{(1,24)}$=0.153, p=0.699; genotype*time bin interaction $F_{(14,336)}$=0.491, p=0.938), implying that the oxygen response to CS+ presentation is similar in both genotypes during training day one.

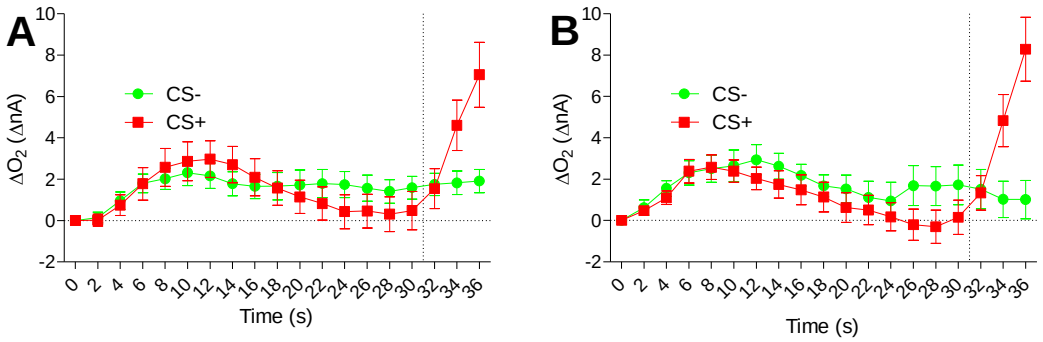


Figure 5.10: Oxygen signals in the amygdala of wildtype (A) and 5-HT transporter overexpressor mice (B) in response to the CS+ (red) and CS- (green) during day one of training in the differential fear conditioning paradigm

The oxygen levels in the amygdala respond differently to the CS+ and CS-, mainly due to the more pronounced return to baseline in the CS+ response, but this does not differ between genotypes. Dashed line at 30s indicates CS termination. Note the position of 0 (no change) on the Y-axis.

Table 5.4: Simple main effect of CS type for each time bin of the oxygen signal in the amygdala for wildtype and 5-HT transporter overexpressor mice during day one of training in the differential fear conditioning paradigm

Time post CS onset (s)	$F_{(1,24)}$	p	Direction of effect
2	0.298	0.590	
4	0.787	0.384	
6	0.002	0.964	
8	0.164	0.689	
10	0.034	0.855	
12	0.004	0.951	
14	0.001	0.976	
16	0.039	0.846	
18	0.253	0.619	
20	1.534	0.228	
22	1.844	0.187	
24	3.168	0.088	
26	**6.615**	**0.017**	**CS+<CS-**
28	**7.239**	**0.013**	**CS+<CS-**
30	**4.932**	**0.036**	**CS+<CS-**

The oxygen levels in the amygdala differ between the CS+ and CS- in the final three 2s time bins of CS presentation.

5.3.2.2.2. Day two

On day two of training (WT n=12, 5-HTT OE n=13; Figure 5.11), different CS oxygen responses continued to be seen with a large positive peak in response to the CS+ and a smaller response to the CS-. In WT mice, the CS+ response returned to baseline, whereas in the 5-HTT OE mice the oxygen signal in response to the CS+ fell below baseline. Statistically, the differential response to the CS types is supported by the presence of a time bin*CS type interaction ($F_{(14,322)}$=4.759, p<0.001), although simple main effects analysis did not find an effect of CS type at any specific time point (all p>0.05). No effects of genotype were seen (Table 5.3). This lack of a genotype difference was also seen in the planned comparison of CS+ responses (effect of time bin ($F_{(14,322)}$=15.355, p<0.001; effect of genotype $F_{(1,23)}$=1.579, p=0.222; genotype*time bin $F_{(14,322)}$=1.061, p=0.392).

5.3.2.2.3. Day three

On day three of training (WT n=13, 5-HTT OE n=11; Figure 5.12), the oxtgen responses appeared to differ greatly between genotypes as well as to the different CS types within each genotype. In the WT mice, the CS+ response showed a large positive response which then returned to baseline, whereas the CS- showed only a small initial response that also fell back to baseline. In the 5-HTT OE mice, the CS+ response showed a positive peak that quickly fell substantially below baseline. The CS- response showed an initial peak similar in magnitude to the CS+ response which then slowly declined to baseline.

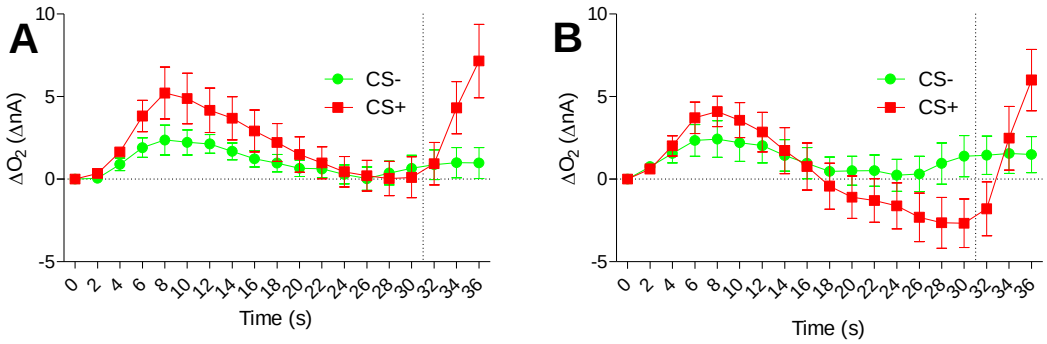


Figure 5.11: Oxygen signals in the amygdala of wildtype (A) and 5-HT transporter overexpressor mice (B) in response to the CS+ (red) and CS- (green) during day two of training in the differential fear conditioning paradigm

Differential oxygen responses were seen to the CS+ and CS- on day two of training although this did not differ between genotypes. Dashed line at 30s indicates CS termination. Note the position of 0 (no change) on the Y-axis.

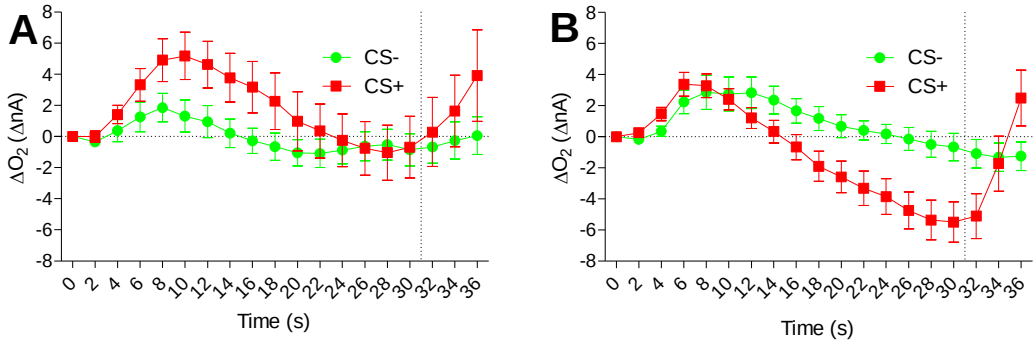


Figure 5.12: Oxygen signals in the amygdala of wildtype (A) and 5-HT transporter overexpressor mice (B) in response to the CS+ (red) and CS- (green) during day three of training in the differential fear conditioning paradigm

The amygdala responded more so to the CS+ than the CS-. In the wildtype mice, this is due to a larger positive peak whereas the 5-HT transporter overexpressor mice show an exaggerated negative tail in their CS+ response. Dashed line at 30s indicates CS termination. Note the position of 0 (no change) on the Y-axis.

Statistically, this difference in amygdala oxygen responses between genotypes and to different CS types was seen in the appropriate interaction terms (genotype*CS type interaction $F_{(1,22)}$=7.717, p=0.011; time bin*CS type interaction $F_{(14,308)}$=4.999, p<0.001) as well as in a time bin*genotype*CS type interaction ($F_{(14,308)}$=2.203, p=0.008. See also Table 5.3). This was due to the CS responses in the 5-HTT OE mice still differing from one another, but this was due to the CS+ signal being lower than the CS- response, whereas in the WT mice, a more positive response was seen to the CS+ in comparison to the CS-.

Simple main effects analysis supports a difference between the genotypes in response to the CS+ (simple main effect of genotype $F_{(1,22)}$=4.764, p=0.040) and suggests that the CS- response does not differ between the genotypes ($F_{(1,22)}$=1.575, p=0.223). The larger positive peak of the WT mice and the more exaggerated tail in the 5-HTT OE mice in response to the CS+ are also supported by simple main effects of CS type split by genotype and time bin (Table 5.5). Importantly, this showed both genotypes to show different signals in response to the CS+ in comparison to the CS-; in the case of the WT mice this was in the form of a larger positive peak, and in the case of the 5-HTT OE mice it was the negative tail that sets the CS+ response apart from the response to the CS-.

In line with these findings, the planned comparison between CS+ responses shows an effect of genotype ($F_{(1,22)}$=4.764, p=0.040), time bin ($F_{(14,308)}$=18.408, p<0.001) and an interaction between these two factors ($F_{(14,308)}$=2.047, p=0.015). Simple main effects revealed this is due to the difference between the genotypes being most apparent as the CS+ oxygen response began to fall (Table 5.6).

Table 5.5: Simple main effect of CS type for each genotype and time bin of the oxygen signal in the amygdala for wildtype (WT) and 5-HT transporter (5-HTT) overexpressor (OE) mice during day three of training in the differential fear conditioning paradigm

Genotype	Time post CS onset (s)	$F_{(1,22)}$	p	Direction of effect
	2	1.288	0.269	
	4	3.407	0.078	
	6	3.372	0.080	
	8	**5.540**	**0.028**	**CS+>CS-**
	10	**8.086**	**0.009**	**CS+>CS-**
	12	**8.223**	**0.009**	**CS+>CS-**
	14	**8.456**	**0.008**	**CS+>CS-**
WT	**16**	**7.294**	**0.013**	**CS+>CS-**
	18	3.680	0.068	
	20	1.512	0.232	
	22	0.782	0.386	
	24	0.145	0.707	
	26	0.005	0.944	
	28	0.073	0.790	
	30	0.007	0.933	
	2	2.537	0.126	
	4	**4.612**	**0.043**	**CS+>CS-**
	6	1.205	0.284	
	8	0.127	0.725	
	10	0.075	0.786	
	12	1.872	0.185	
	14	3.227	0.086	
OE	16	3.958	0.059	
	18	**4.822**	**0.039**	**CS+<CS-**
	20	**4.662**	**0.042**	**CS+<CS-**
	22	**6.155**	**0.021**	**CS+<CS-**
	24	**6.988**	**0.015**	**CS+<CS-**
	26	**8.067**	**0.010**	**CS+<CS-**
	28	**7.519**	**0.012**	**CS+<CS-**
	30	**6.664**	**0.017**	**CS+<CS-**

Differences between the CS responses was most different during the initial peak of the CS+ response for the wildtype mice but in the negative tail of the CS+ for the 5-HT transporter overexpressor mice.

Table 5.6: Simple main effect of genotype for each time bin of the oxygen response in the amygdala for wildtype (WT) and 5-HT transporter (5-HTT) overexpressor (OE) mice to CS+ presentation on day three of training in the differential fear conditioning paradigm

Time post stimulus onset (s)	$F_{(1,22)}$	p	Direction of effect
2	0.356	0.557	
4	0.004	0.952	
6	0.002	0.968	
8	1.169	0.291	
10	3.049	0.095	
12	**4.769**	**0.040**	**WT>OE**
14	**4.406**	**0.048**	**WT>OE**
16	**4.816**	**0.039**	**WT>OE**
18	**4.500**	**0.045**	**WT>OE**
20	2.971	0.099	
22	3.395	0.079	
24	3.277	0.084	
26	3.812	0.064	
28	4.052	0.057	
30	**4.376**	**0.048**	**WT>OE**

The return to baseline (and below) is more pronounced in the 5-HTT OE mice than WT mice.

Overall, this suggests a diminished oxygen response in the 5-HTT OE mice in response to the CS+. However, this response still differs from the 5-HTT OE mice's response to the CS-, but this is a more negative response as compared to the positive peak seen in the WT response to the CS+ in comparison to the CS-.

5.3.2.3.Extinction

5.3.2.3.1. Overall analysis

During extinction (n=12 per group, Figure 5.13), the genotypes once again differed in their responses to CS presentation. The WT mice showed an initial positive peak in response to the CS+ which fell slightly below baseline and showed a smaller peak in response to the CS- which also fell slightly below baseline. The 5-HTT OE mice showed a similar response to both the CS+ and CS-; a small initial positive peak that rapidly falls below baseline. The initial statistical analysis revealed no statistically significant effects except for that of time bin (Table 5.3). When the CS- was excluded and the planned comparison of CS+ responses performed, an effect of genotype was seen, reflecting a larger positive peak to the CS+ in WT mice as compared to 5-HTT OE mice (effect of time bin $F_{(14,308)}=16.757$, $p<0.001$; effect of genotype $F_{(1,22)}=4.695$, $p=0.041$; genotype*time bin interaction $F_{(14,308)}=0.626$, $p=0.843$). Again, this implies an attenuated response in the 5-HTT OE mice to CS+ presentation as compared to the WT mice.

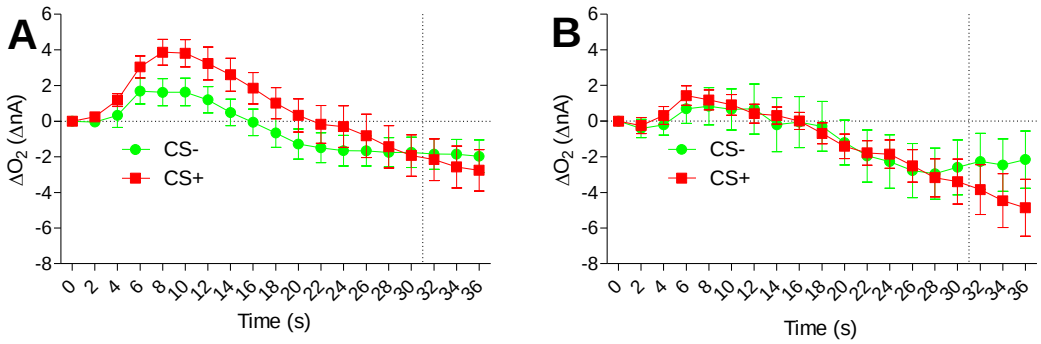


Figure 5.13: Oxygen signals in the amygdala of wildtype (A) and 5-HT transporter overexpressor mice (B) in response to the CS+ (red) and CS- (green) during the extinction session of the differential fear conditioning paradigm collapsed across all CS presentations

Overall the responses during extinction did not differ between CS type or genotype although the CS+ response was much greater in the wildtype mice as compared to the 5-HT transporter overexpressor mice. Dashed line at 30s indicates CS termination. Note the position of 0 (no change) on the Y-axis.

5.3.2.3.2. First CS presentation

The oxygen response to the first CS presentations of each type (which were fully counterbalanced) during the extinction session was also assessed (Figure 5.14). This was done as no overall differences were seen in the responses averaged across all five presentations which may be due to within-session extinction which could mask any group differences. The amygdala oxygen responses to these first presentations were seen to differ between CS+ and CS- as well as between genotypes in a similar manner to training day three. In the WT mice, an initial positive peak in response to the CS+ was seen which fell slightly below baseline. A smaller peak in response to the CS- was seen which gradually returned to baseline. The 5-HTT OE mice displayed a similar initial peak to both the CS+ and CS- but a more dramatic negative tail, falling substantially below baseline, following this peak during CS+ presentation as compared to CS- presentation.

A difference between the genotypes in their responses to the different CS types is supported statistically (time bin*CS type interaction $F_{(14,308)}=3.335$, $p<0.001$; effect of genotype $F_{(1,22)}=5.988$, $p=0.023$; time bin*genotype interaction $F_{(14,308)}=2.094$, $p=0.012$. See also Table 5.3). The exaggerated negative tail of the 5-HTT OE mice also resulted in overall differences between the genotypes towards the end of CS presentation (simple main effects of genotype, Table 5.7). The planned comparison of CS+ responses also showed this effect of genotype (effect of time bin $F_{(14,308)}=17.847$, $p<0.001$; effect of genotype $F_{(1,22)}=4.833$, $p=0.039$; genotype*time bin interaction $F_{(14,308)}=2.642$, $p=0.001$). Simple main effects revealed this is due to differences between the genotypes being most apparent at the end of CS+ presentation (Table 5.8).

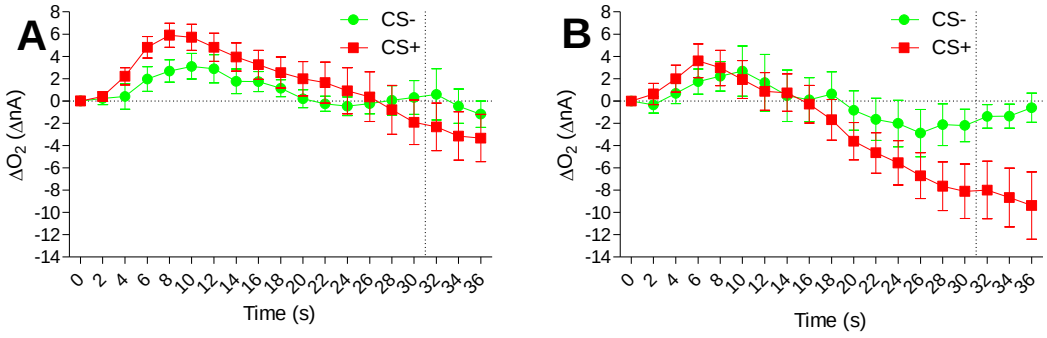


Figure 5.14: Oxygen signals in the amygdala of wildtype (A) and 5-HT transporter overexpressor mice (B) in response to the first CS+ (red) and CS- (green) during the extinction session of the differential fear conditioning paradigm

Amygdala oxygen responses differed between CS types and genotypes, with wildtype responses generally being greater than those of the 5-HT transporter overexpressor mice particularly towards the end of CS presentation. This was due to the more exaggerated drop in the oxygen signal of the 5-HT transporter overexpressor mice as compared to the wildtype mice. Dashed line at 30s indicates CS termination. Note the position of 0 (no change) on the Y-axis.

Table 5.7: Simple main effect of genotype for each time bin of the oxygen signal in the amygdala for wildtype (WT) and 5-HT transporter (5-HTT) overexpressor (OE) mice to the first CS presentation during the extinction session of the differential fear conditioning paradigm

Time post CS onset (s)	$F_{(1,22)}$	p	Direction of effect
2	0.044	0.836	
4	0.001	0.979	
6	0.329	0.572	
8	2.349	0.140	
10	2.374	0.138	
12	3.496	0.075	
14	3.236	0.086	
16	**5.537**	**0.028**	**WT>OE**
18	3.177	0.088	
20	**6.100**	**0.022**	**WT>OE**
22	**5.747**	**0.025**	**WT>OE**
24	**4.482**	**0.046**	**WT>OE**
26	**6.037**	**0.022**	**WT>OE**
28	**4.715**	**0.041**	**WT>OE**
30	**4.784**	**0.040**	**WT>OE**

The amygdala oxygen responses differed between genotypes towards the end of CS presentation.

Table 5.8: Simple main effect of genotype for each time bin of the oxygen response in the amygdala for wildtype (WT) and 5-HT transporter (5-HTT) overexpressor (OE) mice to the first CS+ presentation during the extinction session of the differential fear conditioning paradigm

Time post CS onset (s)	$F_{(1,22)}$	p	Direction of effect
2	0.046	0.832	
4	0.023	0.881	
6	0.464	0.503	
8	2.340	0.140	
10	3.383	0.079	
12	3.560	0.072	
14	2.317	0.142	
16	2.786	0.109	
18	3.414	0.078	
20	**5.979**	**0.023**	**WT>OE**
22	**6.022**	**0.023**	**WT>OE**
24	**5.112**	**0.034**	**WT>OE**
26	**5.494**	**0.029**	**WT>OE**
28	**4.929**	**0.037**	**WT>OE**
30	3.858	0.062	

The amygdala oxygen response to the CS+ differed between genotypes towards the end of CS presentation.

5.3.3. Local field potentials

The theta:delta ratio was analysed using repeated measures ANOVAs with time bin and CS type as within-subject measures and genotype as a between-subjects factor on each day. A summary of this analysis is presented in Table 5.9.

5.3.3.1. Pre-exposure

During pre-exposure (WT n=13, 5-HTT OE n=12; Figure 5.15), the theta:delta ratio did not respond in either genotype to CS presentations of any type. No statistically significant effects were seen (see Table 5.9). The planned comparison of CS+ responses also showed no effects, although there was a trend towards an effect of time bin (effect of genotype $F_{(1,23)}$=0.271, p=0.607; effect of time bin $F_{(14,322)}$=1.658, p=0.063; genotype*time bin interaction $F_{(14,322)}$=1.037, p=0.416). This is in contrast to the oxygen response which showed a small positive peak in response to CS presentation prior to conditioning regardless of CS type or genotype (see Section 5.3.2.1).

Table 5.9: Initial statistical analysis of the theta:delta ratio signal obtained during the differential fear conditioning paradigm

Session	Time bin	Effect of... Genotype	CS type
Pre Exposure	$F_{(14,322)}$=1.108, p=0.349	$F_{(1,23)}$<0.001, p=0.584	$F_{(1,23)}$=0.021, p=0.885
Training 1	**$F_{(14,322)}$=4.083, p<0.001**	$F_{(1,23)}$=1.397, p=0.249	**$F_{(1,23)}$=7.714, p=0.011**
Training 2	**$F_{(14,280)}$=5.294, p<0.001**	$F_{(1,20)}$=0.557, p=0.464	**$F_{(1,20)}$=6.919, p=0.016**
Training 3	**$F_{(14,280)}$=5.912, p<0.001**	**$F_{(1,20)}$=6.170, p=0.022**	**$F_{(1,20)}$=7.333, p=0.014**
Extinction, overall analysis	$F_{(14,308)}$=1.396, p=0.153	**$F_{(1,22)}$=6.967, p=0.015**	**$F_{(1,22)}$=14.925, p=0.001**
Extinction, first CS presentation	**$F_{(14,308)}$=1.824, p=0.034**	$F_{(1,22)}$=0.389, p=0.539	**$F_{(1,22)}$=7.198, p=0.014**

Session	…interaction Time bin* genotype	Time bin* CS type	Genotype* CS type	Time bin* genotype* CS type
Pre Exposure	$F_{(14,322)}$=0.810, p=0.658	$F_{(14,322)}$=1.139, p=0.322	$F_{(1,23)}$=0.006, p=0.938	$F_{(14,322)}$=1.159, p=0.306
Training 1	**$F_{(14,322)}$=4.083, p<0.001**	**$F_{(14,322)}$=3.710, p<0.001**	$F_{(1,23)}$=2.354, p=0.139	$F_{(14,322)}$=1.467, p=0.122
Training 2	$F_{(14,280)}$=0.417, p=0.969	**$F_{(14,280)}$=5.301, p<0.001**	$F_{(1,20)}$<0.001, p=1.000	$F_{(14,280)}$=1.582, p=0.084
Training 3	$F_{(14,280)}$=0.837, p=0.629	**$F_{(14,280)}$=5.302, p<0.001**	$F_{(1,20)}$=1.800, p=0.195	**$F_{(14,280)}$=1.891, p=0.027**
Extinction, overall analysis	$F_{(14,308)}$=0.516, p=0.923	$F_{(14,308)}$=0.944, p=0.512	$F_{(1,22)}$=0.011, p=0.918	$F_{(14,308)}$=0.858, p=0.605
Extinction, first CS presentation	$F_{(14,308)}$=0.533, p=0.913	$F_{(14,308)}$=0.987, p=0.466	$F_{(1,22)}$=0.147, p=0.705	**$F_{(14,308)}$=1.761, p=0.044**

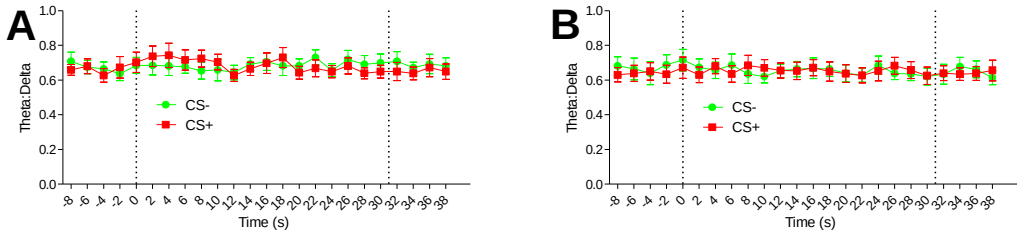


Figure 5.15: Theta:delta ratio in the amygdala of wildtype (A) and 5-HT transporter overexpressor mice (B) in response to the CS+ (red) and CS- (green) during pre-exposure in the differential fear conditioning paradigm

During pre-exposure, amygdala local field potentials did not differ in response to different CS types or between genotypes. Dashed lines indicate CS onset at 0s and termination at 30s.

5.3.3.2.Training

5.3.3.2.1. Day one

On the first day of training (WT n=13, 5-HTT OE n=12; Figure 5.16), the theta:delta ratio was seen to respond to CS+ presentation in both genotypes. The nature of this response differed subtly between the genotypes, with the CS+ response in the WT mice being due to a negative tail at the end of CS+ presentation whereas in the 5-HTT OE mice a more pronounced positive peak is seen at the beginning of CS presentation. This is represented statistically by an overall effect of CS type ($F_{(1,23)}$=7.714, p=0.011), a time bin*CS type interaction ($F_{(14,322)}$=3.710, p<0.001), as well as a genotype*time bin interaction ($F_{(14,322)}$=4.083, p<0.001. See also Table 5.9). A robust difference between the genotypes is only seen in one specific time bin (simple main effects of genotype, see Table 5.10).

This genotype*time bin interaction is collapsed across CS type, although this genotype*time bin interaction was also seen in the planned comparison of CS+ responses (effect of time bin $F_{(14,322)}$=5.039, p<0.001; effect of genotype $F_{(1,23)}$=0.430, p=0.518; genotype*time bin interaction $F_{(14,322)}$=1.976, p=0.019). Again, simple main effects of genotype were only seen in a single time bin (Table 5.11). Nonetheless, this implies a subtly different response to CS+ presentation between the genotypes.

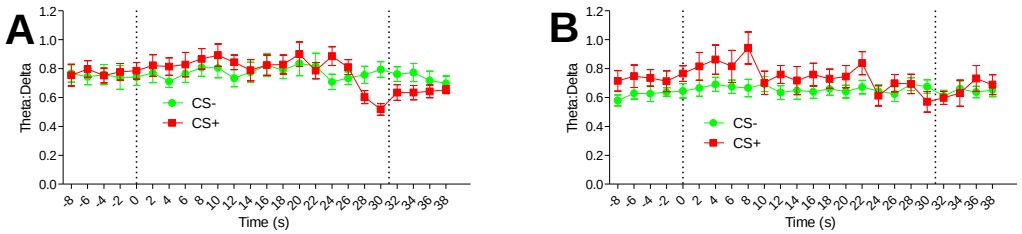


Figure 5.16: Theta:delta ratio in the amygdala of wildtype (A) and 5-HT transporter overexpressor mice (B) in response to the CS+ (red) and CS- (green) during day one of training in the differential fear conditioning paradigm

Amygdala local field potentials respond to CS+ but not CS- presentations although the form of this response differs subtly between genotypes. Wildtype mice show a reduction in the their theta:delta signal at the end of CS+ presentation, whereas the 5-HT transporter overexpressor mice show an initial increase in their theta:delta ratio in response to CS+ presentation. Dashed lines indicate CS onset at 0s and termination at 30s.

Table 5.10: Simple main effect of genotype for each time bin of the theta:delta ratio for wildtype (WT) and 5-HT transporter (5-HTT) overexpressor (OE) mice during day one of training in the differential fear conditioning paradigm

Time post CS onset (s)	$F_{(1,23)}$	p	Direction of effect
2	0.421	0.523	
4	0.033	0.856	
6	0.291	0.595	
8	0.121	0.731	
10	3.330	0.081	
12	2.136	0.157	
14	1.096	0.306	
16	1.862	0.186	
18	2.284	0.144	
20	3.523	0.073	
22	0.306	0.585	
24	**4.957**	**0.036**	**WT>OE**
26	3.577	0.071	
28	0.044	0.835	
30	0.241	0.628	

Effects of genotype in specific time bins are very few during day one of training.

Table 5.11: Simple main effect of genotype for each time bin of the theta:delta ratio for wildtype (WT) and 5-HT transporter (5-HTT) overexpressor (OE) mice for the planned comparison of the CS+ response during day one of training in the differential fear conditioning paradigm

Time post CS onset (s)	$F_{(1,23)}$	p	Direction of effect
2	0.003	0.956	
4	0.179	0.676	
6	0.007	0.933	
8	0.350	0.560	
10	2.947	0.099	
12	1.110	0.303	
14	0.351	0.559	
16	0.397	0.535	
18	1.103	0.304	
20	1.797	0.193	
22	0.277	0.603	
24	**8.175**	**0.009**	**WT>OE**
26	1.976	0.173	
28	1.309	0.264	
30	0.425	0.521	

Differences between genotypes in local field potential responses to CS+ during day one of training in specific time bins are very few.

5.3.3.2.2. Day two

On day two of training (n=11 per group, Figure 5.17), both genotypes showed an initial increase in the theta:delta ratio in response to CS+ presentation and a drop in this signal towards the end of CS presentation relative to the CS- response. This was seen statistically in effects driven by CS type (effect of CS type $F_{(1,20)}$=6.919, p=0.016; CS type*time bin interaction $F_{(14,280)}$=5.301, p<0.001). While the signal during CS- presentation did not change over time (simple main effects time bin $F_{(14,294)}$=0.457, p=0.954), the CS+ response did change over time ($F_{(14,294)}$=7.242, p<0.001) due to the initially greater signal to the CS+ decreasing towards the end of cue presentation to below the CS- signal. Simple main effects of CS type were present at specific time points and representing different directions of effect at each end of CS presentation (see Table 5.12. See also Table 5.9).

The planned comparison of CS+ responses detected an effect of time bin ($F_{(14,280)}$=7.243, p<0.001) due to the initial increase and slight decrease in the theta:delta ratio during CS+ presentation, but this was not influenced by genotype (effect of genotype $F_{(1,20)}$=0.407, p=0.531; genotype*time bin interaction $F_{(14,280)}$=1.004, p=0.449). This further supports a response to the CS+ in the LFP signal of the amygdala that did not differ between the genotypes.

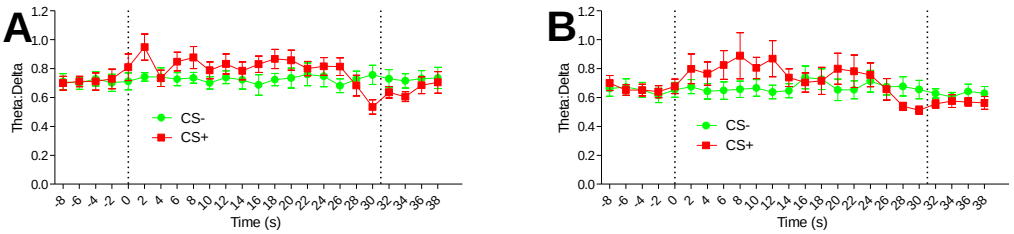


Figure 5.17: Theta:delta ratio in the amygdala of wildtype (A) and 5-HT transporter overexpressor mice (B) in response to the CS+ (red) and CS- (green) during day two of training in the differential fear conditioning paradigm

On day two of training, both genotypes were seen to show responsive local field potential signals to the CS+ but not to the CS-. Dashed lines indicate CS onset at 0s and termination at 30s.

Table 5.12: Simple main effect of CS type for each time bin of the theta:delta ratio for wildtype and 5-HT transporter overexpressor mice during day two of training in the differential fear conditioning paradigm

Time post CS onset (s)	$F_{(1,20)}$	p	Direction of effect
2	**9.042**	**0.007**	**CS+>CS-**
4	1.408	0.249	
6	**12.251**	**0.002**	**CS+>CS-**
8	**4.671**	**0.043**	**CS+>CS-**
10	**10.595**	**0.004**	**CS+>CS-**
12	**6.921**	**0.016**	**CS+>CS-**
14	**4.701**	**0.042**	**CS+>CS-**
16	2.491	0.130	
18	2.474	0.131	
20	**6.380**	**0.020**	**CS+>CS-**
22	1.612	0.219	
24	1.426	0.246	
26	3.878	0.063	
28	**7.395**	**0.013**	**CS+<CS-**
30	**21.728**	**<0.001**	**CS+<CS-**

Local field potential signals differed in their response to the differing CS types over a large proportion of CS presentation. Initially, this was due to the CS+ resulting in a greater theta:delta ratio that dropped towards the end of CS presentation to below the CS- response.

5.3.3.2.3. Day three

On the final day of training (WT n=12, 5-HTT OE n=10; Figure 5.18), both genotypes again showed an increase in the theta:delta ratio to CS+ presentation that decreased towards the end of CS presentation. However, the overall levels of theta:delta appeared to be lower in the 5-HTT OE mice. This was supported statistically by an overall effect of genotype ($F_{(1,20)}$=6.170, p=0.022). An effect of CS type was still present ($F_{(1,20)}$=7.333, p=0.014), as was a CS type*time bin interaction ($F_{(14,280)}$=5.302, p<0.001. See also Table 5.9). A genotype*CS type* time bin interaction was seen ($F_{(14,280)}$=1.891, p=0.027) to account for the early rise in signal in the WT mice in response to the CS+ that then returned to CS- levels as compared to the more stable CS+ response of the 5-HTT OE mice. This is also seen in simple main effects analysis (simple main effects of CS type, Table 5.13).

Effects of genotype are seen throughout CS- presentation but only the first time bin in response to the CS+ (Table 5.14). This suggests a baseline reduction in theta:delta in the 5-HTT OE mice but that this is less apparent during their response to the CS+. This is further supported by the planned comparison of CS+ responses which showed no influence of genotype (effect of time bin $F_{(14,280)}$=7.013, p<0.001; effect of genotype $F_{(1,20)}$=1.979, p=0.175; genotype*time bin interaction $F_{(14,280)}$=1.532, p=0.099).

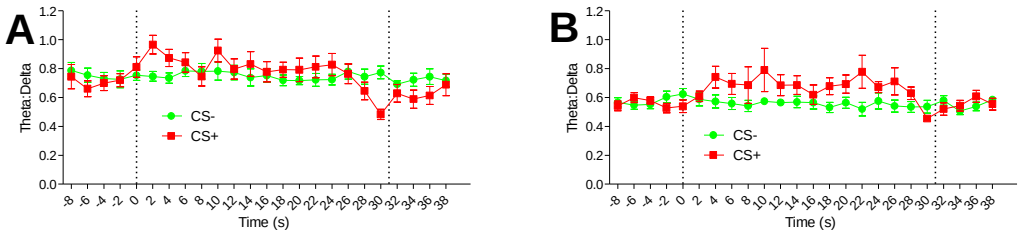


Figure 5.18: Theta:delta ratio in the amygdala of wildtype (A) and 5-HT transporter overexpressor mice (B) in response to the CS+ (red) and CS- (green) during day three of training in the differential fear conditioning paradigm

By day three of training, 5-HT transporter overexpressor mice were seen to show lower levels of theta:delta than wildtype mice but both genotypes responded to CS+ presentation. Dashed lines indicate CS onset at 0s and termination at 30s.

Table 5.13: Simple main effect of CS type for each time bin by genotype of the theta:delta ratio for wildtype (WT) and 5-HT transporter (5-HTT) overexpressor (OE) mice during day three of training in the differential fear conditioning paradigm

Genotype	Time post CS onset (s)	$F_{(1,20)}$	p	Direction of effect
	2	**28.096**	**<0.001**	**CS+>CS-**
	4	**6.546**	**0.019**	**CS+>CS-**
	6	1.373	0.255	
	8	0.200	0.660	
	10	1.786	0.196	
	12	0.195	0.663	
	14	1.518	0.232	
WT	16	0.329	0.573	
	18	3.419	0.079	
	20	1.144	0.297	
	22	1.365	0.256	
	24	2.278	0.147	
	26	0.053	0.820	
	28	**4.754**	**0.041**	**CS+<CS-**
	30	**58.359**	**<0.001**	**CS+<CS-**
	2	0.179	0.677	
	4	**8.102**	**0.010**	**CS+>CS-**
	6	**6.358**	**0.020**	**CS+>CS-**
	8	2.651	0.119	
	10	3.463	0.078	
	12	4.192	0.054	
	14	1.957	0.177	
OE	16	1.191	0.288	
	18	**11.360**	**0.003**	**CS+>CS-**
	20	2.803	0.110	
	22	**9.376**	**0.006**	**CS+>CS-**
	24	1.599	0.221	
	26	**5.257**	**0.033**	**CS+>CS-**
	28	3.666	0.070	
	30	3.903	0.062	

Differences in the local field potential responses to the different CS types were most apparent during the positive CS+ response at CS onset which fell below the CS- signal towards CS termination in the wildtype (WT) mice and at onset and sporadically throughout CS presentation in the 5-HT transporter overexpressor (OE) mice.

Table 5.14: Simple main effect of genotype for each time bin by CS type of the theta:delta ratio for wildtype (WT) and 5-HT transporter (5-HTT) overexpressor (OE) mice during day three of training in the differential fear conditioning paradigm

Time post CS onset (s)	CS Type	$F_{(1,20)}$	p	Direction of effect
2	**CS-**	**7.778**	**0.011**	**WT>OE**
	CS+	**21.108**	**<0.001**	**WT>OE**
4	**CS-**	**8.318**	**0.009**	**WT>OE**
	CS+	1.930	0.180	
6	**CS-**	**15.060**	**0.001**	**WT>OE**
	CS+	2.251	0.149	
8	**CS-**	**11.918**	**0.003**	**WT>OE**
	CS+	0.190	0.668	
10	**CS-**	**8.182**	**0.010**	**WT>OE**
	CS+	0.697	0.414	
12	**CS-**	**12.385**	**0.002**	**WT>OE**
	CS+	1.234	0.280	
14	**CS-**	**5.135**	**0.035**	**WT>OE**
	CS+	1.723	0.204	
16	**CS-**	**8.284**	**0.009**	**WT>OE**
	CS+	2.612	0.122	
18	**CS-**	**13.082**	**0.002**	**WT>OE**
	CS+	2.147	0.158	
20	**CS-**	**10.526**	**0.004**	**WT>OE**
	CS+	0.858	0.365	
22	**CS-**	**9.873**	**0.005**	**WT>OE**
	CS+	0.067	0.799	
24	**CS-**	**5.123**	**0.035**	**WT>OE**
	CS+	2.869	0.106	
26	**CS-**	**12.000**	**0.002**	**WT>OE**
	CS+	0.218	0.646	
28	**CS-**	**9.797**	**0.005**	**WT>OE**
	CS+	0.042	0.840	
30	**CS-**	**14.068**	**0.001**	**WT>OE**
	CS+	0.441	0.514	

Differences in the local field potentials of the two genotypes are seen in response to the CS- across the whole stimulus presentation but only in the first time bin in response to the CS+.

Because of this overall main effect of genotype, and inspection of Figure 5.18, the pre-CS period was also investigated. The mean theta:delta ratio of the 8s window prior to CS onset presented in Figure 5.18 was taken and compared between genotypes. No effects of CS type (to be presented) were seen (effect of CS type $F_{(1,20)}$=2.746, p=0.113, genotype*CS type interaction $F_{(1,20)}$=1.608, p=0.219) but a significant effect of genotype was present ($F_{(1,20)}$=7.610, p=0.012). This demonstrates that this reduction in theta:delta signal in the 5-HTT OE mice is present during this session irrespective of CS presentation. That there is no difference between CS type is reassuring as during this period no CS is actually present. The difference in the theta:delta ratio between groups at this time was not due to a difference in freezing levels in the animals included in LFP analysis (effect of genotype $F_{(1,20)}$=0.012, p=0.913; effect of CS type $F_{(1,20)}$=3.131, p=0.092; genotype*CS type interaction $F_{(1,20)}$=0.325, p=0.575, data not shown). Furthermore, this difference was apparent before the first CS presentation of the day ($t_{(20)}$=2.212, p=0.039) so was not a consequence of within-session CS presentations or shock delivery.

5.3.3.3. Extinction

5.3.3.3.1. Overall analysis

During extinction (WT n=13, 5-HTT OE n=11; Figure 5.19), 5-HTT OE mice were once again seen to have overall lower levels of theta:delta signal than WT mice (effect of genotype $F_{(1,22)}$=6.967, p=0.015), but both genotypes still showed an increase in theta:delta during CS+ presentation (effect of CS type $F_{(1,22)}$=14.925, p=0.001). No other effects were seen (see Table 5.9).

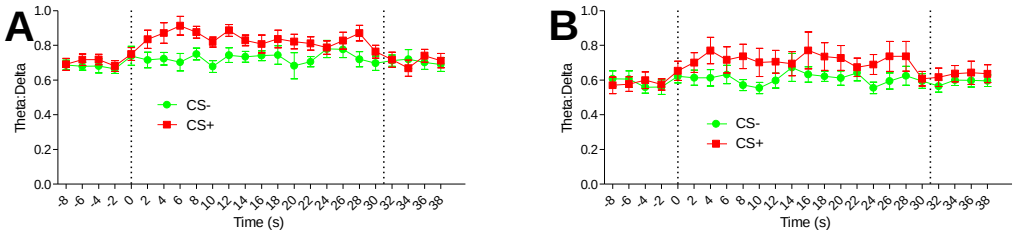


Figure 5.19: Theta:delta ratio in the amygdala of wildtype (A) and 5-HT transporter overexpressor mice (B) in response to the CS+ (red) and CS- (green) during the extinction session of the differential fear conditioning paradigm collapsed across all CS presentations

Theta:delta signals are greater to the CS+ than CS- in both genotypes although there is an overall reduction in the theta:delta ratio in the 5-HT transporter overexpressor mice before and during CS presentation. Dashed lines indicate CS onset at 0s and termination at 30s.

The planned comparison between CS+ responses showed no significant effects, although there was a trend for an effect of genotype (effect of genotype $F_{(1,22)}$=3.883, p=0.062; effect of time bin $F_{(14,308)}$=1.574, p=0.085; genotype*time bin interaction $F_{(14,308)}$=0.495, p=0.935). Due to the overall differences between the genotypes, the pre-CS presentation period was once again subjected to statistical analysis as detailed above. Again, no effects of CS type (to be presented) were seen, as would be expected (effect of CS type $F_{(1,20)}$=0.434, p=0.517, genotype*CS type interaction $F_{(1,20)}$=0.685, p=0.417), but a significant effect of genotype was present ($F_{(1,20)}$=7.425, p=0.012). This again implies that this reduction in theta:delta signal in the 5-HTT OE mice is present during this session at times other than CS presentation. This was not accounted for by differences in freezing levels (effect of genotype $F_{(1,22)}$=0.003, p=0.956; effect of CS type $F_{(1,22)}$=2.136, p=0.158; genotype*CS type interaction $F_{(1,20)}$=0.048, p=0.828, data not shown).

5.3.3.3.2. First CS presentation

When assessing the responses to the first CS presentations alone (Figure 5.20), an increase in the theta:delta ratio was again seen during CS+ presentation relative to the CS- response (effect of CS type $F_{(1,22)}$=7.198, p=0.014. See also Table 5.9). While no main effect of genotype was seen ($F_{(1,22)}$=0.389, p=0.539), a genotype*time bin*CS type interaction was present ($F_{(14,308)}$=1.761, p=0.044). Simple main effects analysis revealed differences between the genotypes only in select time bins towards the end of CS+ and CS- presentation (simple main effects of genotype, Table 5.15). Both genotypes showed an increased theta:delta signal to the CS+ at some specific time points throughout CS presentation (simple main effects of CS type, Table 5.16).

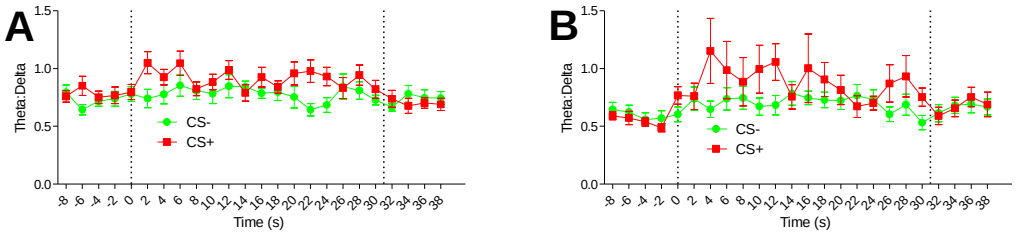


Figure 5.20: Theta:delta ratio in the amygdala of wildtype (A) and 5-HT transporter overexpressor mice (B) in response to the first CS+ (red) and CS- (green) during the extinction session of the differential fear conditioning paradigm

The first CS presentation of extinction shows an increased CS+ theta:delta ratio in both genotypes. Dashed lines indicate CS onset at 0s and termination at 30s.

Table 5.15: Simple main effect of genotype for each time bin by CS type of the theta:delta ratio for wildtype (WT) and 5-HT transporter (5-HTT) overexpressor (OE) mice during first CS presentation during the extinction session of the differential fear conditioning paradigm

Time post CS onset (s)	CS Type	$F_{(1,22)}$	p	Direction of effect
2	CS-	<0.001	0.993	
	CS+	3.831	0.063	
4	CS-	1.354	0.257	
	CS+	0.719	0.406	
6	CS-	0.745	0.398	
	CS+	0.055	0.817	
8	CS-	0.260	0.616	
	CS+	0.089	0.769	
10	CS-	0.915	0.349	
	CS+	0.304	0.587	
12	CS-	1.523	0.230	
	CS+	0.166	0.688	
14	CS-	0.274	0.606	
	CS+	0.074	0.788	
16	CS-	0.315	0.580	
	CS+	0.073	0.790	
18	CS-	0.432	0.518	
	CS+	0.198	0.661	
20	CS-	0.058	0.812	
	CS+	0.814	0.377	
22	CS-	1.352	0.257	
	CS+	**5.001**	**0.036**	**WT>OE**
24	CS-	0.189	0.668	
	CS+	**5.098**	**0.034**	**WT>OE**
26	CS-	3.312	0.082	
	CS+	0.051	0.824	
28	CS-	1.082	0.310	
	CS+	0.003	0.956	
30	**CS-**	**7.114**	**0.014**	**WT>OE**
	CS+	0.375	0.547	

Differences between the genotypes were most apparent towards the end of CS+ presentation owing to a drop in the theta:delta signal in 5-HTT OE mice that is not seen in WT mice. The WT mice also display a slight increase in the theta:delta ratio at the end of CS- presentation that is not seen in the 5-HTT OE mice, resulting in an effect of genotype in the final time bin of CS- presentation.

Table 5.16: Simple main effect of CS type for each time bin by genotype for the theta:delta ratio for wildtype (WT) and 5-HT transporter (5-HTT) overexpressor (OE) mice during first CS presentation on day one of extinction in the differential fear conditioning paradigm

Genotype	Time post CS onset (s)	$F_{(1,22)}$	p	Direction of effect
	1	**5.358**	**0.030**	**CS+>CS-**
	2	0.587	0.452	
	3	1.383	0.252	
	4	0.019	0.892	
	5	0.340	0.566	
	6	0.864	0.363	
	7	0.466	0.502	
WT	8	0.569	0.459	
	9	0.301	0.589	
	10	2.453	0.132	
	11	**9.455**	**0.006**	**CS+>CS-**
	12	**8.925**	**0.007**	**CS+>CS-**
	13	0.009	0.924	
	14	0.778	0.387	
	15	1.073	0.311	
	1	0.021	0.885	
	2	**5.786**	**0.025**	**CS+>CS-**
	3	1.975	0.174	
	4	1.058	0.315	
	5	2.995	0.098	
	6	**5.185**	**0.033**	**CS+>CS-**
	7	0.119	0.733	
OE	8	1.656	0.212	
	9	3.880	0.062	
	10	0.411	0.528	
	11	0.628	0.437	
	12	0.107	0.746	
	13	3.096	0.092	
	14	2.219	0.150	
	15	**5.066**	**0.035**	**CS+>CS-**

Differences in theta:delta response is seen only in select time bins within each genotype.

These subtle effects were not seen in the planned comparison of CS+ responses, which showed no statistically significant effects but a trend towards an effect of time bin (effect of time bin $F_{(14,308)}$=1.612, p=0.075; effect of genotype $F_{(1,22)}$=0.060, p=0.810; genotype*time bin interaction $F_{(14,308)}$=1.304, p=0.203).

The pre-CS period to this first presentation also showed an effect of genotype ($F_{(1,22)}$=19.987, p<0.001) with no effects of CS type, as would be expected (effect of CS type $F_{(1,22)}$=0.005, p=0.947; genotype*CS type interaction $F_{(1,22)}$=2.450, p=0.132). As this first presentation was fully counterbalanced, this rules out order effects as well as shocks as possible confounds to this difference between the genotypes. Again, differing levels of freezing were also ruled out (effect of genotype $F_{(1,22)}$=0.210, p=0.651; effect of CS type $F_{(1,22)}$=1.192, p=0.287; genotype*CS type $F_{(1,22)}$=1.192, p=0.287). The pre-CS period prior to the first CS presented regardless of valence also showed a significant effect of genotype ($t_{(22)}$=3.921, p=0.001).

5.3.3.4. Pre-CS presentation alterations

To further explore the differences noted above in pre-CS signals, further statistical analysis was conducted on this period of time across days. Genotype had no influence on the 8s pre-CS window selected on the days prior to the final day of training (effects of genotype: pre-exposure $F_{(1,23)}$=0.046, p=0.833; training day one $F_{(1,23)}$=0.213, p=0.213; training day two $F_{(1,20)}$=0.774, p=0.390. Genotype*CS type interactions: pre-exposure $F_{(1,23)}$=0.374, p=0.536; training day one $F_{(1,23)}$=3.582, p=0.071; training day two $F_{(1,20)}$=0.085, p=0.774). However, an effect of the CS type to pre presented was unexpectedly seen during the first day of training but not on the other days (pre-exposure $F_{(1,23)}$=1.723, p=0.202; training day one $F_{(1,23)}$=6.599, p=0.017;

training day two $F_{(1,20)}$=0.191, p=0.667). Nonetheless, the baseline difference seen between genotypes was only seen in the final day of training and the extinction session.

A repeated measures ANOVA was also performed on the baseline signals of all mice that gave reliable signals on every day of the fear conditioning paradigm (WT n=11, 5-HTT OE n=10). There was a strong trend for an overall effect of genotype across all days ($F_{(1,19)}$=4.343, p=0.051), but this did not interact with day ($F_{(4,76)}$=1.295, p=0.280). This implies the 5-HTT OE mice always show a lowered theta:delta ratio as compared to WT mice. Simple main effects analysis was conducted regardless of the lack of an interaction owing to the *a priori* prediction that WT signals were potentiated across days whereas the 5-HTT OE signals were not. This proved not to be the case; WT mice showed no simple main effect of day ($F_{(4,40)}$=0.766, p=0.547) whereas the 5-HTT mice did ($F_{(4,36)}$=2.998, p=0.031). This is due to the signals in the 5-HTT OE mice dropping over the days, although a subtle increase in signal is also seen in the WT signals across days, resulting in effects of genotype only in the final two days of the paradigm (Table 5.17).

Aside from a CS type*day interaction ($F_{(4,76)}$=3.761, p=0.008) accounted for by the unexpected effect of CS type during training day one only (simple main effects of CS type: pre-exposure $F_{(1,19)}$=1.793, p=0.196; training day one $F_{(1,19)}$=6.814, p=0.017; training day two $F_{(1,19)}$=0.240, p=0.630; training day three $F_{(1,19)}$=1.701, p=0.208; extinction $F_{(1,19)}$=0.517, p=0.481), no other effects were seen in this longitudinal analysis (effect of day $F_{(4,76)}$=1.762, p=0.145; effect of CS type $F_{(1,19)}$=1.773, p=0.199; genotype*CS type interaction $F_{(1,19)}$=1.773, p=0.199; genotype*day*CS type interaction $F_{(4,76)}$=1.749, p=0.148).

Table 5.17: The pre-CS period theta:delta ratio for wildtype (WT) and 5-HTT transporter (5-HTT) overexpressor (OE) mice on each day of the differential fear conditioning paradigm with the simple main effects of genotype detected on each day

Day	**WT, n=11**	**OE, n=10**	**$F_{(1,19)}$**	**p**
Pre Exposure	0.68 ± 0.05	0.61 ± 0.04	1.175	0.292
Training 1	0.76 ± 0.07	0.65 ± 0.05	1.795	0.196
Training 2	0.71 ± 0.05	0.67 ± 0.04	0.428	0.521
Training 3	**0.74 ± 0.06**	**0.56 ± 0.02**	**7.742**	**0.012**
Extinction	**0.71 ± 0.03**	**0.58 ± 0.04**	**9.119**	**0.007**

The theta:delta ratio prior to CS onset in the 5-HTT OE mice fell over the days of the fear conditioning paradigm while the WT signals increased slightly, resulting in differences between the genotypes on the final two days of the paradigm.

5.4. Discussion

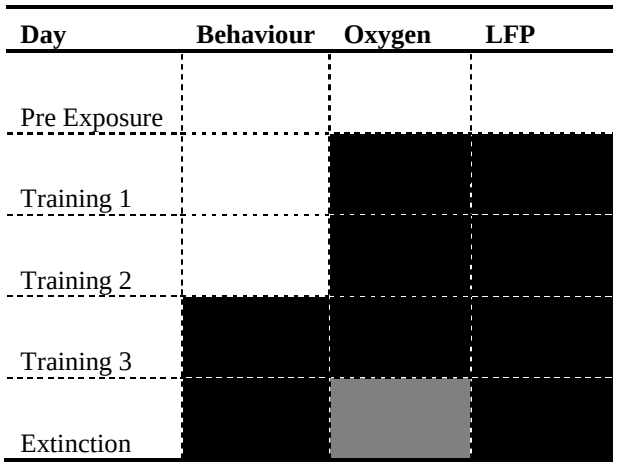
This chapter presented measures of neuronal activity in the basolateral amygdala (BLA) in the 5-HT transporter (5-HTT) overexpressor (OE) and wildtype (WT) mice in response to an emotional cue. This was achieved through use of constant potential amperometry (CPA) to measure regional tissue oxygen levels. This was paired with measures of local field potentials (LFPs) to give a direct measure of neuronal activity. Both of these signals, in addition to a behavioural readout, were taken throughout a differential fear conditioning paradigm. Alterations in the neuronal signals taken were detected across time as well as between CS types and between genotypes. A reduced positive oxygen peak was seen in the 5-HTT OE mice compared to WT mice following conditioning. 5-HTT OE mice also showed a more exaggerated negative tail in the oxygen signal following the initial peak. This resulted in the oxygen signals of the 5-HTT mice still distinguishing between CS type, but because the CS+ response is more negative than that to the CS-. An overall reduction in the LFP measure taken was also seen in the 5-HTT OE mice on the final two days of the paradigm. However, both genotypes showed equivalent differential behavioural responses to the CS+ and CS-, freezing more to the CS+ after the tone-shock association was made and, eventually, moving more than at baseline in response to the CS-.

5.4.1. Summary of results

Behaviourally, mice of both genotypes increased their freezing to the CS+ across sessions whilst moving more in response to the onset of the CS-. In line with this, WT mice were seen to show greater neuronal responding to the CS+ than the CS- in both measures of BLA activity. The oxygen response to the CS+ consisted of an initial rise followed by a decline back to baseline by the end of CS+ presentation. The CS- also led to an initial rise in oxygen that was smaller than that to the CS+ following conditioning. In the LFP signal, an increase in the theta:delta ratio was seen in response to the CS+ that remained relatively constant throughout CS presentation. CS- presentation did not affect the theta:delta ratio. These changes in the measures of neuronal activity preceded the altered behavioural responses (Table 5.18).

5-HTT OE mice also showed good behavioural discrimination between the CS types but showed altered neuronal signals as compared to the WT mice (see Table 5.19). The peak oxygen response in the BLA of the 5-HTT OE mice was diminished compared to WT mice and the oxygen signal dropped substantially below baseline after this peak more dramatically than was seen in the WT mice. While this demonstrates a difference between the genotypes in their responses to the CS+, it is worth noting that the 5-HTT OE mice still show different oxygen responses to the CS+ and the CS-. However, the form of this difference is a more negative signal in response to the CS+ than CS-, whereas the WT mice show a more positive response to the CS+.

Table 5.18: Days of the differential fear conditioning paradigm in which differences in the various measures taken were seen between the different CS types



Black squares indicate differences being present. Grey squares indicate differences were only detected in the CS+ planned comparison. Alterations in the measures of neuronal activity were seen before changes in behaviour were noted. These differences always showed an increase to the CS+ as compared to the CS- in freezing, oxygen and theta:delta, respectively.

Table 5.19: Days of the differential fear conditioning paradigm in which differences in the various measures taken were seen between the genotypes

Day	Behaviour	Oxygen	LFP
Pre Exposure			
Training 1			■
Training 2		■	
Training 3		■	■
Extinction		■	■

Black squares indicate differences being present. While no differences in behaviour were seen between genotypes throughout the differential fear conditioning paradigm, differences were seen in the measures of neuronal activity. This was first seen as an increased initial theta:delta spike in response to the CS+ in 5-HT transporter overexpressor mice. Later, an overall reduction in this measure was seen in these mice as compared to wildtype mice. 5-HT transporter overexpressor mice also showed a lower early positive peak and more pronounced late tail in their CS+ oxygen response on several days of this paradigm.

The 5-HTT OE mice also showed changes in the theta:delta ratio measure of LFPs in the BLA. This was initially seen as an increased positive peak in response to CS+ presentation as compared to WT mice on training day one. During training day three and the extinction day, however, the 5-HTT OE mice displayed an overall reduction in this LFP measure compared to WT mice. This was true both during CS presentation as well as in periods outside of cue delivery. This pre-CS difference could not be explained by differences in the levels of freezing between the groups during the pre-CS periods. Analysis of this pre-CS difference across time suggested that the difference apparent on the final day of training and during extinction was due to a reduction in the theta:delta ratio of the 5-HTT OE mice over the days of the fear conditioning paradigm.

The greater oxygen response seen in the WT mice to the CS+ as compared to the CS- is in line with the greater increase in amygdala BOLD signals seen to emotional as compared to control cues in humans (Buchel *et al.*, 1998, LaBar *et al.*, 1998, Morris *et al.*, 2001, Phelps *et al.*, 2001, Cheng *et al.*, 2003). That the CS+ response is somewhat reduced during the extinction day relative to the final day of training also supports the fMRI findings that the amygdala is most active during fear acquisition (Buchel *et al.*, 1998, LaBar *et al.*, 1998). Analysis of the oxygen response to the first CS presentations during extinction also implies within-session extinction of the signals may have occurred. The reduction in the initial positive oxygen response seen in the 5-HTT OE mice as compared to WT mice would also fit with the claim that increased 5-HTT activity diminishes amygdala reactivity to emotional cues. This is based on the observed negative correlation in humans between amygdala reactivity during an emotional face-matching task and 5-HTT binding in the amygdala (Rhodes *et al.*, 2007) as well as amygdala activity and genetic markers of 5-HTT expression levels

(Hariri *et al.*, 2002, Hariri *et al.*, 2005). These latter studies demonstrate a decrease in amygdala reactivity in individuals with the *l* 5-HTT gene allele, thought to be predictive of increased 5-HTT protein expression.

The LFP signal seen during CS+ presentation also corresponds to previous literature demonstrating increased theta oscillations in the lateral amygdala of cats following fear conditioning (Pare and Collins, 2000). Pare and Collins showed a selective increase in theta during CS+ following conditioning that was not seen in response to a control cue. That a comparable CS+ response is seen in both genotypes, but a difference is seen in the oxygen response to CS+ presentation between genotypes, may be the result of these two measures of neuronal activity not being truly comparable. This is because, as discussed in Sections 5.1.4 and 5.1.5, regional tissue oxygen in the brain correlates best to the sum of the inputs to the region of interest, whereas the LFP signal is mostly comprised of local neuronal firing. This rate of firing will be modulated by afferent inputs, but will also comprise signals initiated within the region. The oxygen and LFP signals will therefore correlate, but measure subtly different components of the brain's activity.

The baseline reduction in the theta:delta ratio seen in the 5-HTT OE mice following conditioning (and possibly before) may be indicative of a general inhibition of activity of this area. This may correlate with the reduced oxygen signals in response to a CS+ seen in both these rodents as well as humans with increased 5-HTT expression (Rhodes *et al.*, 2007). However, the reduction in the theta:delta ratio is seen regardless of CS presentation. Another possible explanation would be a potentiation of neuronal activity in this area as conditioning occurred, thought to be necessary for consolidation of the cue-shock association (Apergis-Schoute *et al.*, 2005,

Schroeder and Shinnick-Gallagher, 2005), is occurring in the WT mice but not the 5-HTT OE mice. However, analysis of this baseline difference across the paradigm suggests it is a reduction in the 5-HTT OE signal over time that accounts for the difference between the genotypes seen, not an increase in the WT signal. This implies there may be active inhibition of amygdala activity occurring in the 5-HTT OE mice. This may be due directly to lowered 5-HT tone from the brain stem 5-HT nuclei. Alternatively, this may be due to the known frontal cortical-mediated inhibition of amygdala activity (eg Grace and Rosenkranz, 2002). Support for modulation of this coupling being influenced by 5-HTT has been shown in a relative decrease in the coupling of the amygdala and cingulate in individuals with the *s* allele of the 5-HTT gene (Pezawas *et al.*, 2005). The possibility of altered inputs to the amygdala would need to be further explored by monitoring frontal or brain stem activity during this fear conditioning paradigm in the 5-HTT OE mice.

The initial increase in the theta:delta ratio seen in the 5-HTT OE mice relative to the WT CS+ response on day one of training is difficult to explain. This genotype difference was present only on a single day and simple main effects found only one specific time bin in which a genotype effect was present (see Table 5.10). This time bin, surprisingly, was towards the end of CS presentation, not during the initial response to the CS+. This apparent increase in LFP signal in the 5-HTT OE mice in response to the CS+ may therefore require further investigation before being considered a robust alteration.

Overall, behavioural discrimination between the CS+ and CS- was seen in both genotypes. Different oxygen signals were also seen to each CS type in both genotypes. However, the form of this discrimination in the oxygen signals differed between genotypes; the WT mice showed a larger positive peak response to the CS+ than the CS-, whereas the 5-HTT OE mice showed a more pronounced negative tail in their CS+ oxygen response, resulting in a signal that is generally lower than that seen in response to the CS-. Both genotypes also displayed an increase in the theta:delta ratio LFP signal in response to the CS+ as compared to the CS-. However, the 5-HTT OE mice showed a general reduction in this signal regardless of CS presentation. This implies a dampening of the electrical activity in this area in the 5-HTT OE mice.

5.4.2. Limitations

5.4.2.1. Differences between these results and previous studies

Previous work in this laboratory suggests that in a more traditional fear conditioning paradigm, 5-HTT OE mice show reduced freezing to a tone previously paired with a shock during extinction and towards the end of the conditioning session (see Section 5.1.3.2.1). This may either be due to a deficit in conditioning or expression of this learned fear. This deficit in fear conditioning in the 5-HTT OE mice was not seen in the paradigm used in this chapter. There are procedural differences between these two experiments that may account for some variability, such as the use of a CS- as well as pre-exposure to the cues used in this study. However, the greatest contributor to this difference is likely to be that the mice used here may have been over-trained,

having received 15 tone-shock pairings as compared to two in the previous study. This may obscure any behavioural differences that may have been seen otherwise.

The lack of a behavioural difference between the groups is also at odds with human studies that find a good correlation between amygdala signals and behavioural readouts of fear (LaBar *et al.*, 1998, Phelps *et al.*, 2001). These studies do not assess the involvement of the 5-HTT, however. Correlations between the 5-HT transporter gene linked polymorphic region (5-HTTLPR) and measures of fear have been demonstrated (Garpenstrand *et al.*, 2001, Armbruster *et al.*, 2009). However, these studies typically use physiological measures of fear such as skin conductance rather than truly behavioural responses such as freezing. The nature of human and animal fear conditioning experiments are also quite different; human studies have to be conducted under a degree of informed consent, lessening the degree to which this is a fear-inducing procedure. Ethical considerations aside, this may alter the signals measured. Again, these differences may account for some of the variance between this study and the human literature. Over-training of the mice may further explain the discrepancies seen and the differences between human and rodent fear conditioning may make comparisons across species difficult.

The lack of a behavioural difference in this amperometric study does have some advantages, however. First, it ensures that the signals obtained in response to the CS+ are made to a cue the mice have successfully paired with an aversive outcome. Had the tone-shock association not been made by the 5-HTT OE mice, the nature of the CS+ oxygen response in these mice would have been unclear; it could not have been said to be a response to a conditioned cue. That no difference is seen in the levels of freezing produced in response to CS+ presentation also rules out the

possibility that the measures of neuronal activity obtained are simply correlates of locomotion. This also better recapitulates the results obtained in human fMRI studies using fearful faces. In such studies, volunteers are asked to match an emotional face with one from a selection of other faces exhibiting the same emotion. In these tasks, no behavioural differences have been reported due to altered 5-HTT expression. However, differences are seen in amygdala reactivity that correlates with 5-HTT functionality (eg Rhodes *et al.*, 2007). The measures of neuronal activity obtained are therefore not predictive of behavioural output, but do indicate that 5-HT in the amygdala is influencing the way in which the brain responds to emotional cues.

On the question of comparing human and rodent literature, it is worth noting that procedures such as the face matching task use their cues, primarily, to produce amygdala activity and not to evoke conscious emotional responses; fearful faces are not used with the intention of frightening human subjects. The aim of rodent fear conditioning is the production of a learned emotional response to a conditioned cue. However, in this study, this conditioned cue is also being relied upon to produce amygdala activity in the mice. The nature of the behavioural responses seen in human face matching tasks and rodent fear conditioning may therefore be of a different nature, but the respective cues of both paradigms induce amygdala activity. This signal may therefore be more comparable between species than the behavioural measures obtained.

5.4.2.2. Electrode placement

One possible concern over the validity of the results obtained would be the placements of the electrodes. This is largely well controlled in this experiment with most of the electrodes being placed in the BLA (see Figure 5.4). However, some of the electrode placements included in analysis were in the central amygdala. These nuclei are thought to have a distinct role from the BLA. Nonetheless, the recording field of similar electrodes to those used here is thought to be equal to twice the diameter of the wire (eg Offenhauser *et al.*, 2005). In this case, the 250µm carbon paste electrode used would encapsulate an area of 500µm, large enough that these more medially positioned electrodes would also record some BLA activity. Importantly, the individual traces obtained from these more medial placements also did not differ from those obtained from placements located within the BLA. Overall, the signals obtained can definitely be said to be from the amygdala, and the largest contributing nucleus is likely to be the BLA. The differing contributions made by the lateral and central nuclei would be a fascinating undertaking using CPA. However, this investigation would require a dedicated experiment and cannot fully be commented on based on the results obtained in this study. A more appropriate behavioural paradigm that tests the parallel model of amygdala connectivity could be used in such an investigation to increase the potential interest of the findings.

5.4.3. Implications for previous findings

The differing results obtained here compared to the previous fear conditioning experiment conducted in these mice does not directly question the freezing deficit demonstrated in that study in 5-HTT OE mice (see Section 5.4.2.1). Instead, procedural differences are likely to account for the lack of a genotype effect in behaviour seen in this study. However, the altered amygdala signals seen here may underlie the previously demonstrated behavioural differences seen under other conditions.

Importantly, the study presented here does offer some evidence for a correlation between 5-HTT function and amygdala reactivity to emotional cues regardless of behavioural output. This is in agreement with the Rhodes and colleagues study (2007) that demonstrated a negative correlation between 5-HTT binding and amygdala activity in response to fearful and angry faces. The advantage this combined PET and fMRI study has over many human studies investigating 5-HTT influence on emotional responses is that it estimates 5-HTT binding availability within specific brain regions. A larger but more conflicting data set exists attempting to correlate polymorphisms in the 5-HTT gene to emotionality (see Section 1.3.1.2). However, such genetic polymorphisms are several steps removed from the final functional 5-HTT found on the membrane that acts to clear 5-HT from the synapse.

Investigations of single genetic polymorphisms in humans may be confounded by the heterogeneous nature of other genetic factors and the environmental influences that may impact upon protein expression levels. This may lead to discrepancies between the effects of these polymorphisms predicted by *in vitro* studies and the

actual protein expression that results from an individual's genetic code. In short, *l* allele carriers may not express more 5-HTT protein than those who are *s* carriers owing to the numerous other influences that may affect expression levels. The relative homogeneity of the genetic and environmental influences on rodents allow for better investigation of the consequences of individual genetic alterations. This present study therefore supports a role for the 5-HTT in amygdala reactivity to emotional cues in a system in which 5-HTT protein expression has been demonstrated to be elevated (Jennings *et al.*, 2006). Discrepancies between protein expression and genetic markers thought to predict 5-HTT levels may account for the conflicting data obtained in humans.

By recapitulating the findings of Rhodes and colleagues (2007), this study also has wider implications. This is, to our knowledge, the first use of CPA to measure tissue oxygen in transgenic mice. That the signals obtained appear analogous to the findings of a human fMRI study (Rhodes *et al.*, 2007) goes some way to validating this technique as a translational tool. This therefore has implications, not only for past findings, but also on the validity of future studies that make use of this measure of tissue oxygen.

It has been shown that acute fluoxetine delivery can suppress theta activity in the hippocampus of rabbits (Kudina *et al.*, 2004). This demonstrates that manipulation of the 5-HTT can affect theta oscillations in other systems. However, the direction of the effect is the same seen in the 5-HTT OE mice in the current study, implying that both an increase and decrease of 5-HTT activity results in a reduction in theta activity. This discrepancy may be due to the differences seen between acute and chronic manipulations of a neurotransmitter system. Furthermore, neurodevelopmental

changes in transgenic lines may lead to further discrepancies even in comparison to chronic pharmacological manipulations. This possibility could be further investigated through use of pharmacological interventions that block or enhance 5-HTT function, such as through use of SSRIs and the atypical antidepressant tianeptine, which increases 5-HTT functionality (Mennini *et al.*, 1987), respectively. This could be done using both acute and chronic regimes to investigate the consequences of long-term modulation of 5-HTT functionality in isolation from possible neurodevelopmental confounds. Using the 5-HTT KO mice in the fear conditioning and amperometric paradigm detailed in this chapter would also further the work of replicating human fMRI studies in mice by covering the whole range of 5-HTT expression levels available.

This difference in the theta:delta ratio between the genotypes is most apparent in the final two days of the paradigm used. This implies it may not baseline levels of theta oscillations in the amygdala that have been altered by manipulating 5-HTT levels, but the way in which they alter in response to conditioning. One possible explanation based on previous findings would be a failure for the circuitry within the amygdala to enter a potentiated state, i.e., a reduction in the level of long term potentiation (LTP) established following conditioning. The establishment of such a state within the amygdala has been shown to be integral to fear conditioning (Kim *et al.*, 1991, Apergis-Schoute *et al.*, 2005, Schroeder and Shinnick-Gallagher, 2005). To fully address this possibility, synaptic plasticity within these animals would need to be investigated. However, the data collected here more strongly implicate a suppression of activity in the 5-HTT OE mice, not a potentiation of WT signals. The input into the amygdala responsible for this would also need further investigation. The frontal cortex is a likely candidate for such a modulatory area as it is known to

suppress amygdala activity during extinction (eg Grace and Rosenkranz, 2002) and can be modulated by 5-HTT expression levels (Pezawas *et al.*, 2005). Alternatively, this diminished activity may be a direct consequence of lowered 5-HT tone from the brain stem 5-HT nuclei.

5.4.4. Future directions

Several future experiments present themselves to more fully understand the effect of 5-HTT levels on neuronal markers of emotional processing. As only the 5-HTT OE mice have been used so far, repeating this experiment using the 5-HTT KO mice, both homozygotes and heterozygotes, would complete the gradient of expression levels available to us. By manipulating 5-HTT functionality using SSRIs, both in WT and transgenic mice, would also further our understanding of the role of 5-HTT in neuronal responses to emotional cues. This would also help disentangle the influence of acute, chronic and life-long alterations to 5-HTT functionality in these emotional processes and address whether the differences seen here are due to 5-HTT expression levels during development or solely due to differences in 5-HTT expression levels in the adult animals.

Areas of the brain other than the amygdala may also be involved in the behavioural paradigm used here. The work presented in this chapter, mirroring human imaging studies, shows no difference in behavioural performance in an emotionally-driven task but altered signals in the amygdala of the 5-HTT OE mice as compared to WT mice. It is therefore tempting to speculate that another area of the brain is compensating for the relative inactivity of the amygdala in both humans and rodents with increased expression levels of 5-HTT. Alternatively, it may be that activity in

other regions is causing the altered amygdala signals seen here. Such areas, if they exist, are yet to be identified in human imaging studies. Measures of immediate early genes (IEGs) such as fos would allow for brain-wide measures of activity following CS+ presentation in rodents. This approach lacks the temporal resolution of CPA and cannot take advantage of within-subject comparisons of CS+ and CS- responses, however. An IEG study could therefore be performed to assess what areas of the brain respond to a CS+ in 5-HTT OE and WT mice. Differences between the genotypes would provide candidate areas for further investigation using the techniques detailed in this chapter.

The Deakin and Graeff hierarchical defence model (1991) suggests areas of the brain involved in panic and vigilance responses exist in a balance influenced by 5-HT activity. This model is an attempt to give a brain-wide account for the role of 5-HT in emotionality. Principally, this model takes into account the regional variation in 5-HT receptor type expression that leads to 5-HT release being (generally) excitatory or (generally) inhibitory in different areas of the brain. This, in turn, leads to 5-HT promoting neuronal activity in certain areas whilst inhibiting that of others. The Deakin and Graeff model puts 5-HT as key in inhibiting panic responses whilst facilitating vigilant anxiety behaviour. Both of these behaviours form part of a complete defensive system; it is sometimes appropriate to run and sometimes more appropriate to continue cautiously. The regulation of these two conflicting behaviours acts as a gating mechanism to ensure one is favoured over the other in response to threats in an appropriate and rapid manner.

Given the reduction in 5-HT functionality in the 5-HTT OE mice (Jennings *et al.*, 2006, Jennings *et al.*, 2008), this model would predict that these mice would, in relation to WT mice, show an increase in panic responses and a decrease in avoidance behaviours. The decrease in unconditioned anxiety in these mice (Jennings *et al.*, 2006, Line *et al.*, 2010) suggests that there is a reduction in the 5-HTT OE mice's vigilant responses. Although yet to be quantified, these mice have also been found to be hyper-responsive to handling, both during this study and those conducted previously by other experimenters (Line, Jennings, personal communications). This may be indicative of the increase in panic responses predicted by the Deakin and Graeff model. The "panic" areas of their brains may therefore be predicted to be more active. Such areas, for instance the periaqueductal grey, may therefore show increased activity in 5-HTT OE mice in response to negative emotional cues.

Support may have been found for this shift towards panic responses in the 5-HTT OE mice by investigating the unconditioned response to shock in the fear conditioning paradigm used here. However, the response to the first delivered shock did not differ between genotypes (data not shown). This lack of a difference in shock response is in agreement with the previous fear conditioning study using the 5-HTT OE mice (Line, 2008). A heightened response to shock may have been expected if a shift has occurred towards panic defensive responses. However, this measure may not be sensitive enough to detect such a difference.

Different overall levels in the local field potential measures taken in the 5-HTT OE mice following, and possibly before, conditioning suggest a reduction in electrical activity within the amygdala that may be due to modulation of amygdala activity by other brain regions or may be a direct consequence of lowered 5-HT synaptic activity in the amygdala due to the overexpression of the 5-HTT protein. Although not supported by the data presented here, an alternative explanation would be a reduction in plasticity within the amygdala. LTP has not yet been investigated in these animals and may provide a further stratum of understanding to the altered levels of activity within the amygdala in animals with increased levels of 5-HTT protein. Similarly, no electrophysiological measures of plasticity have been made in the 5-HTT KO mice, although some morphological work has been done. The 5-HTT KO mice have been shown to have a greater spine density within the BLA compared to WT mice which may underlie, in part, the increased tenacity of fear conditioning in these animals (Wellman *et al.*, 2007). Further study is therefore required in both the 5-HTT OE and 5-HTT KO mice regarding plasticity. However, the limited work done in the 5-HTT KO mice may indicate greater connectivity within the amygdala in those with low levels of 5-HTT, leading to greater regional activity. The reduction in the theta:delta ratio in 5-HTT OE mice may predict a complementary decrease in amygdala connectivity. Both histological and electrophysiological investigation in 5-HTT OE mice may therefore further explain the shift in LFP signal seen in this study.

Finally, the use of CPA to measure tissue oxygen is in its infancy. This technique offers a signal seemingly homologous to the BOLD signal that underlies fMRI. CPA measurements can, however, be obtained in freely behaving animals in a way that is not possible with other imaging techniques. This therefore provides a translational tool that lends itself to behavioural neuroscience. The experiments that could be

performed using this technique to further probe neuronal functioning as a whole are therefore not limited to the subject of this thesis, but are much more wide-ranging.

5.4.5. Conclusions

The 5-HTT OE mice show altered activity in the basolateral amygdala in response to an emotionally salient cue, as measured by regional oxygen levels as well as LFPs. This is in spite of their behavioural responses being similar to those of WT mice. These alterations in measures of neuronal activity are most obvious following conditioning to the CS+. CS- oxygen signals are identical in both genotypes. The exaggerated fall seen in the oxygen response to the CS+ in the 5-HTT OE mice still differs from their response to the CS-, but in a way that is different from the more-positive CS+ response seen in WT mice. The lower peak oxygen response to the CS+ in 5-HTT OE mice as compared to WT mice mirrors findings in humans that increased levels of 5-HTT expression result in reduced amygdala reactivity to fearful or angry faces (Rhodes *et al.*, 2007). This provides evidence of a link between 5-HTT functionality and amygdala reactivity to emotionally salient cues. Furthermore, this chapter and the paradigm used in it provide a platform on which to base several future experiments relevant to emotionality and translational research in general.

CHAPTER 6: General discussion

This thesis has presented data on the behavioural characterisation of three mutant mouse lines with targeted manipulations to the glutamate system, focussing on tests of emotionality, and a fear conditioning paradigm combining 5-HT transporter (5-HTT) overexpressor (OE) mice with recordings of neuronal activity. In this discussion, these data are summarised before the validity of the animals and techniques used are considered. The overall findings and implications of the data to research into depression and its treatment are then presented.

6.1. Summary of findings

6.1.1. Glutamate mutants

The results from the GluA1 knockout (KO) mice (Zamanillo *et al.*, 1999) suggest some alterations in emotional behaviours. These include changes in social behaviours and a reduction in immobility in the forced swim test (FST). However, these changes seem to be dependent on the novelty of the testing environment. This implies that these changes may be further manifestations of the altered habituation noted previously in these animals (Sanderson *et al.*, 2009, Sanderson *et al.*, 2010).

The GluN1 hypomorph (HYPO) mice (Mohn *et al.*, 1999) showed many altered behaviours in naturalistic, cognitive and emotional domains. This finding makes interpretation of their overall phenotype difficult as many of the deficits seen may act as confounds to other tests. In short, the data suggests these mice are so severely and globally impaired, they are of limited use in any field of behavioural research. The

vesicular glutamate transporter (VGluT) 1 heterozygote (HET) mice (Tordera *et al.*, 2007), conversely, showed no changes in emotional behaviour.

Collectively, results from these experiments have some implications for the behavioural testing of genetically modified mice in general. The importance of using a test battery is exemplified by the GluN1 HYPO mice. These mice appear to show a robust antidepressant phenotype in both the forced swim test (FST) and sucrose preference test. This is in line with the antidepressant properties of NMDA receptor antagonists (see Section 3.1.1.3). However, these mice also show pronounced hyperactivity that may confound both of these tests, as well as profound cognitive deficits. The impact that these factors have on other tests is difficult to assess but must be taken into account when considering more complex behaviours.

The finding of a lack of any alterations to emotionality in the VGluT1 HET mice is in contrast to published data (Tordera *et al.*, 2007, Garcia-Garcia *et al.*, 2009). The mice used here were obtained from the same laboratory that performed these previous studies. The discrepancies between the present and previous findings may be due to several reasons. Inter-experimenter differences, as well as differences between institutions, such as in the housing conditions, have all been shown to effect behavioural measures (e.g. Wurbel, 2001, Nithianantharajah and Hannan, 2009). Furthermore, the mice used here are the progeny of those used in the previous studies, but several generations removed. As these mice were maintained on a mixed background that was then backcrossed to be made congenic, different generations may be of a subtly different background strain. This genetic drift may also contribute to the differing results obtained.

6.1.2. 5-HT transporter overexpressor mice during fear conditioning

The differential fear conditioning experiment showed good discrimination between CS+ and CS- in both wildtype (WT) and 5-HTT OE mice in terms of both behavioural measures and measures of neuronal activity. Differences between the genotypes were seen in the neuronal responses to the CS+. In the case of the oxygen response, the 5-HTT OE mice showed a reduction in the initial peak response and a more pronounced negative tail compared to WT mice. This may be analogous to the reduced BOLD response seen in the amygdala in response to fearful faces in individuals with high 5-HTT expression (Rhodes *et al.*, 2007). The 5-HTT OE mice do, however, show different oxygen responses to the CS+ than the CS-, but this in the form of a more negative CS+ response compared to the CS- response, whereas the WT mice show an increased positive peak in response to the CS+ as compared to the CS-.

The oxygen measurements were accompanied by simultaneous monitoring of local field potentials (LFPs) in the BLA and theta and delta rhythms extracted. The theta:delta ratio was found to increase in response to the CS+ in both genotypes, whereas the CS- did not affect the theta:delta ratio measure. This is in keeping with previous studies demonstrating an increase in theta activity in response to a CS+ following fear conditioning (Pare and Collins, 2000). An overall reduction in the theta:delta ratio is seen in the 5-HTT OE mice as compared to WT mice that is most apparent following conditioning. This was true during CS presentation as well as during non-cue presentation periods. Surprisingly, CS+ presentation made this general reduction less apparent. This alteration in LFP signal in the 5-HTT OE mice may be indicative of a modulatory input to the amygdala dampening the electrical

activity in this brain region or be a direct consequence of lowered 5-HT mediated neurotransmission.

The lack of altered behaviour in the 5-HTT OE mice, despite the altered signalling, could be thought of as surprising. However, the human imaging tasks most akin to this fear conditioning paradigm that have been performed to investigate the consequences of 5-HTT expression variability, such as the emotional faces matching task, do not report any behavioural differences between their test subjects either. Despite this, a correlation is still seen between the BOLD signal and 5-HTT expression. That levels of freezing did not differ between WT and the 5-HTT OE mice also rules out the possibility that these alterations in signal are solely due to a difference in locomotor activity. These alteration in oxygen-dependent measures of neuronal activity in both humans and rodents therefore are not predictive of behavioural outcome, but are indicative of a role for 5-HT modulation to amygdala reactivity to emotional cues.

6.2. Validity of these models

To be confident of the conclusions drawn from these data, we must also consider the validity of the techniques employed. This includes the mutant mice used, the tests performed and the methods used to measure neuronal activity.

6.2.1. Animal tests of emotionality

6.2.1.1. The forced swim test

The main test used in Chapters 2-4 to investigate emotionality was the FST. This is near-universal in its use in preclinical investigations into depression. The face validity of this test is often brought into question as it in no way recapitulates any clinically-observed behaviour; psychiatrists do not ask possibly-depressed patients to tread water. However, its sensitivity and selectivity for antidepressant drugs has been demonstrated in a great number of papers using a variety of compounds (see, for instance, the summary table in Cryan *et al.*, 2005). This test and the immobility measure are therefore seen to have great predictive validity as a measure of behavioural despair and its amelioration by clinically-relevant drugs. There remain, however, some questions over this test's usefulness in preclinical research due to its use in normal, non-"depressed" rodents and its sensitivity to acute effects of antidepressant drugs that are not apparent clinically.

Given the lack of a mood-elevating effect of antidepressants in healthy volunteers, the validity of pre-clinical evidence for antidepressant effects in otherwise-normal animals may also be questioned. However, the basis of tests such as the FST is that they are inducing behavioural despair, a depressive-like state. This is supported by the importance of pre-exposure to the FST to improve sensitivity to acute antidepressant treatments (Borsini *et al.*, 1989); prior exposure to a cylinder of water establishes a depressive-like state in the animal that can then be investigated in subsequent exposure to this environment. Without this prior exposure, the FST is less sensitive to antidepressant drug effects (ibid.). The reduction of immobility in this

test by pharmacological intervention is therefore more equivalent to the elevation of mood in a clinical population than simply giving such drugs to volunteers.

That effects are seen in the FST acutely, however, does differ from the clinical picture in which at least one week of treatment is required before effects on mood are seen (Taylor *et al.*, 2006). This is not to deny the acute effects these drugs can have on other emotionally-driven measures such as amygdala reactivity (Anderson *et al.*, 2010) and fearful faces recognition (Bhagwagar *et al.*, 2004a), and an overall effect of cognitive bias (Harmer *et al.*, 2009). Indeed, the acute effects seen in the FST may in some way relate to these measured effects. Nonetheless, the discrepancy between FST and clinical effects is difficult to account for, although it remains true that the acute effects in the FST are seen for most, if not all, clinically active antidepressants. It may be, therefore, that the face validity of the FST is further diminished by this discrepancy but its predictive validity is not. That so few new antidepressants have been developed, however, may be enough to deny its usefulness. Before dismissing this test on the basis of this discrepancy, however, there are clearly many other factors that must be considered in this failure to produce new drugs, including our poor understanding of the neuropathology involved in depression.

Manipulations more sensitive to chronic treatment regimes over acute drug administration, such as chronic mild stress and the accompanying measure of sucrose consumption, may be seen to have greater validity. Chronic mild stress also has its detractors, however (eg Forbes *et al.*, 1996), and high-throughput, acute tests will always be more desirable in an industrial setting to paradigms that take time to perform if they have no greater predictive validity. The validity of the FST therefore hinges on whether or not this discrepancy between the acute effects of putative

antidepressants in this test and the requirement of chronic treatment for clinical efficacy significantly impairs its predictive value. Until this is categorically shown, one way or the other, the FST will remain the questionable gold-standard of preclinical depression research.

6.2.1.2. Other tests relevant to emotionality

The work presented in this thesis does not rely solely on one test, however. This was both an attempt to give a more complete picture of emotionality in the mutant mice used, as well as to provide control measures. The importance of this latter point is best demonstrated by the global alterations in behaviour seen in the GluN1 HYPO mice. Using other emotion-related tests to fully model depression also seemed necessary given the complex nature of clinical depression. That anhedonia is as canonical a symptom as depressed mood in the clinical definition of depression makes assessments of this as important as use of tests such as the FST. Despite this, measures such as sucrose preference are given much less prominence in the literature than assessments of behavioural despair (searches on PubMed for "forced swim test depression" results in several hundred more citations than "sucrose preference depression"). Other possible symptoms associated with depression, such as alterations in weight, sleep and appetite, are so easily measured in a highly-translatable manner in rodents it seems bizarre that these, too, are not routinely investigated when attempting to model depression and antidepressant activity. Sadly, the glutamate mutant mice tested here do not provide good examples of the potential for these additional measures given their overall phenotypes. Nonetheless, if our understanding of this disease is to be advanced, this more complete approach should become more prominent; alterations in a single test such as the FST should

not be considered sufficient to demonstrate a model of depression or a possible antidepressant drug.

6.2.1.3. Fear conditioning

Fear conditioning has been used in a variety of different fields of research including those concerned with learning, emotion and the intersection of these two processes. In this thesis, fear conditioning is used to provide an emotionally salient cue that will lead to amygdala activation. This was done to recapitulate the amygdala activity induced by cues such as fearful and angry faces in human fMRI studies. This is not to equate rodent fear conditioning with the fearful faces paradigms used in humans. However, a cue that produced a robust amygdala response had to be established to enable the investigation of alterations in this response in mice overexpressing the 5-HTT protein. This activity is known to occur in response to a conditioned cue following training, as well as to innate cues such as predatory odours (Li *et al.*, 2004). However, a punctate conditioned cue provides a discrete window in which to investigate neuronal responses that can be compared to a baseline during which either no cue or a control, non-conditioned cue is presented. More diffuse signals would not allow for such time locked, event-related signals to be identified, making fear conditioning perfectly suited for investigation into neuronal responses to emotionally salient cues.

The relationship between fear conditioning and depression is not a simple one, however. Fear conditioning was used here as a way of eliciting amygdala responses to a negative emotional cue. It is the response to such negative stimuli that is of interest in depression. This stems from the idea that part of the psychopathology of depression may be due to cognitive alterations resulting in a shift towards an increased consideration of negative cues and an attenuated consideration of positive cues, so called "cognitive bias" (Beck, 1976, Beck, 2008). More recently, this concept of cognitive bias has been used to explain the delayed measureable effect of antidepressants on mood, despite their acute effects on tasks assessing emotional processing (see, for instance, Harmer, 2010, as well as the published studies of the Harmer lab). This puts fear conditioning and other paradigms assessing emotional learning at the heart of an interesting and active area of research into the underlying processes that cause depression.

6.2.2. Glutamate mutants in depression (and beyond)

The results obtained with the glutamate-related mutants were disappointing in terms of investigating emotionality. The GluA1 KO mice do show some interesting phenotypes, but the transient nature of these behaviours and their likely relation to habituation processes alters our interpretation of these changes. The GluN1 HYPO mice further demonstrate the importance of the glutamate system in a wide range of processes, and suggest that more subtle manipulations of this system are necessary to prevent global impairments. This subtlety may be achieved either through manipulation of other proteins or through spatially, cell type, and/or temporally specific transgenic lines. The VGluT1 HET mice did not display any alterations in emotional behaviours. Overall, the three mouse lines with direct manipulations of

the glutamate system tested do not give any categorical answers with regard the role of glutamate in emotionality.

The wide-ranging alterations in behaviour seen in the GluN1 HYPO mice do, however, impinge on other areas of research. The large amount of literature on these mice as a model of schizophrenia may need to be reconsidered in light of the global changes seen in their behaviour. When viewed against this background of far-reaching cognitive and behavioural impairment, the specificity of the deficits in pre-pulse inhibition and social interaction reported previously are called into question. That is not to dispute a possible role for NMDA receptor-mediated neurotransmission in schizophrenia, but to suggest that the GluN1 HYPO mouse line does not provide reliable evidence for such a role. Furthermore, this offers further support to the more wide-ranging approach to behavioural phenotyping elaborated on in Section 6.2.1 and implicit across this thesis.

Future work on depression taking advantage of glutamate-related transgenic mouse lines may still shed light on the role of this system in emotionality. The possibility of developmental problems or compensation in the case of the GluN1 HYPO mice and the VGluT1 HET mice, respectively, suggest inducible lines would be advantageous. As our knowledge of glutamate-related alterations in the clinical condition improve, spatially- or cell type-specific inducible lines may better recapitulate the behaviours involved in depression without resulting in other, non-specific impairments. Again, this specificity is paramount in the legitimacy of a model of any psychiatric disease.

The use of the 5-HTT OE mouse in this thesis, with the resulting possibility of neurodevelopmental alterations, is justified by the noted variation in expression level of the 5-HTT across the human population. This may, in part, be driven by genetic polymorphisms such as the 5-HT transporter gene linked polymorphic region (5-HTTLPR) polymorphism in the 5-HTT gene. If such variation were shown across the human population in elements of the glutamate system, use of more traditional mutant mice with modified glutamate systems may be justified. Such polymorphisms are yet to be found, however, and are likely to result in more subtle behavioural effects than were observed in, for instance, the GluN1 HYPO mice.

6.2.3. 5-HT transporter overexpressor mice and emotionality

The 5-HTT OE mice have been shown to have brain-wide increases in 5-HTT expression that correlate with the range of expression levels seen in humans (Jennings *et al.*, 2006). Their use in investigating changes in emotionality is therefore relevant to the individual variance seen across the general population. That direct measures of 5-HTT binding have been made in these mice makes the current study more akin to human studies that have done the same (i.e. Rhodes *et al.*, 2007), as opposed to those that rely on genetic polymorphisms that may or may not alter 5-HTT protein expression levels. The discrepancies that can arise between gene allele and protein levels due to the influences of environment and other genes or polymorphisms may account for the conflicting nature of these studies attempting to correlate emotionality and single polymorphisms. Experimental rodents on a fixed genetic background and in controlled living environments reduce these influences. This makes the use of transgenic mice a valuable tool in investigating the effects of life-long alterations to one gene in isolation from other factors.

The use of constant potential amperometry (CPA) as a means to measure local tissue oxygen levels is in its infancy, however. This makes its use more controversial and its validation an ongoing process. However, CPA has been shown to be capable of giving a signal that is selective for oxygen and correlates well with fMRI (see Section 5.1.4). The success of the study presented in this thesis in recapitulating changes seen in a human fMRI study (Rhodes *et al.*, 2007) may prove to be an early example of its validation in the neurosciences.

6.3. The influence of glutamate and 5-HT on emotionality

The results obtained using the mutant mice with direct manipulations of the glutamate system do not give a clear message in terms of the influence glutamate has on affect. Previous data and some of the results obtained here do suggest a role for glutamate in emotionality. However, these constitutive mutant mouse models do not appear to be an appropriate tool with which to investigate this relationship.

The 5-HTT OE mice have previously been shown to have alterations in emotional behaviour. In this thesis, these mice with increased expression of the 5-HTT protein have been shown to have altered amygdala responses to an emotionally salient cue. This data supports a relationship between 5-HTT expression levels and amygdala reactivity, and so a possible link between this protein and emotionality. By using transgenic mice, this has been done in a way that is not confounded by factors that may contribute to the conflicting nature of many of the studies performed in humans. This strengthens this finding and implies 5-HTT expression levels may prove an interesting measure in assessing an individual's risk in developing depression, but that this needs to be assessed in a highly controlled manner.

However, in this study a dissociation was seen between the behavioural measure of this association and the resulting amygdala signals to some extent; no differences between the genotypes was seen in the freezing responses, but was seen in the oxygen signal taken. It is therefore worth emphasising that the 5-HTT OE mice did show different oxygen responses to the CS+ and CS-, in line with differing behavioural responses to these two cues, but the form this difference in the oxygen signals took differed from that seen in the WT mice. The 5-HTT OE mice showed a more negative response to the CS+ as compared to the CS-, whereas the WT mice show a greater positive peak oxygen response to the CS+ as compared to the CS-. The role of the 5-HTT protein in modulating amygdala reactivity to negative emotional cues is therefore supported by this study. However, the relationship between this neuronal response and associative learning is not as clear. Again, this is also the case of human imaging studies investigating the role of the 5-HTT in responses to fearful and angry faces (Hariri *et al.*, 2002, Hariri *et al.*, 2005, Rhodes *et al.*, 2007).

6.4. Implications

The most clear implications from the work on the glutamate mutants is that the GluN1 HYPO mice are simply too globally impaired to be of use in this or other areas of behavioural research such as into schizophrenia. Further to this, it is clear that NMDA receptor-mediated neurotransmission is integral to a number of, if not all, behavioural processes. Overall, it seems mutants of the glutamate system from throughout development may not be the most appropriate tool with which to investigate the role of glutamate in emotionality.

The greatest implications of this work, therefore, come from the 5-HTT OE mice. The use of CPA as a method for measuring tissue oxygen managed to recapitulate findings from human fMRI studies. This is an important step in the validation of this translational tool and the 5-HTT OE mouse line, and may prove to be an important study in supporting the role of the 5-HTT protein in mood modulation. Furthermore, it may help to launch this exciting technology as a mainstay in neuroscience research.

6.5. Future directions

It is clear that the GluN1 HYPO mice and the VGluT1 HET mice are of limited future use in this area. The cognitive account for the alterations seen in the GluA1 KO mice means they are of some interest, but may always offer non-emotionality-related explanations for the results obtained. However, the work done with the 5-HTT OE mice does warrant further investigation.

The interest in the consequences of alterations in the expression levels of 5-HTT in the general population make 5-HTT mutant mice desirable transgenic lines for investigations into emotionality. While some have questioned the relationship between 5-HTT polymorphisms and measures of emotionality, measures of actual 5-HTT binding have been shown to correlate well with amygdala reactivity (Rhodes *et al.*, 2007). By using transgenic mice, the consequences of alterations to the gene encoding the 5-HTT protein can be investigated in isolation from other factors. To complete the recapitulation of the human study, 5-HTT KO mice, both homozygote and heterozygote, could be put through the same protocol used. This would then complete the gradient of 5-HTT expression levels available for investigation.

The use of CPA to measure oxygen *in vivo* is in its infancy and there are innumerable studies that could be done less connected to this project that would nonetheless be fascinating owing to its translational potential. Translational work such as is possible with this technique is surely the aim of preclinical work and may contribute greatly to our understanding of sadly misunderstood psychiatric conditions such as depression.

6.6. Summary

This thesis has presented extensive emotional phenotyping of three lines of genetically altered mice with direct manipulations of the glutamate system. These proved to present with behaviours not suited to further investigation or failed to provide definitive answers on the role of this system in depression. Work therefore moved on to mice previously demonstrated to have behavioural phenotypes of interest in this domain, the 5-HT transporter overexpressor mice.

Measurements of basolateral amygdala activity in WT and 5-HTT OE mice in response to an emotionally salient cue were performed using constant potential amperometry to measure tissue oxygen in addition to measures of local field potentials during a differential fear conditioning paradigm. The results obtained demonstrated that increased expression of the 5-HT transporter alters amygdala reactivity. This was shown, not only through a reduction in the peak oxygen response and exaggerated negative tail to a conditioned cue, but also in a reduction in the theta:delta ratio in the basolateral amygdala of these animals compared to wildtype mice following conditioning. These results for the oxygen signal mimicking results obtained in human neuroimaging studies (Rhodes *et al.*, 2007). The reduced

electrical activity may be due to a modulatory input to the amygdala dampening activity in this region.

The data obtained give no definitive information on the role of glutamate in affect. It does, however, highlight the difficulties in studying this ubiquitous neurotransmitter in specific disorders and the methods that may prove useful in future endeavours to investigate it. The final experiment does provide evidence for a link between 5-HTT expression levels and emotionally-driven neuronal responses. The use of constant potential amperometry in this study goes some way to validating this highly translatable tool for future experiments into emotionality. It is this validation that is probably the greatest finding of this project.

CHAPTER 7: Bibliography

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