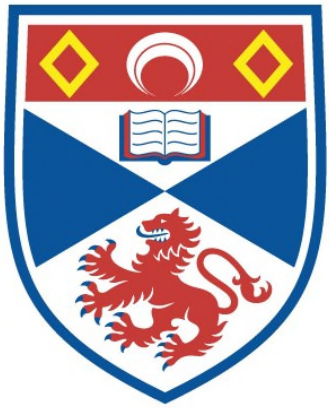
# BEHAVIOURAL PHENOTYPING OF MICE WITH GENETIC ALTERATIONS OF THE $GABA_A$ RECEPTOR

**Nicola Stefanie Lisa Foister**

**A Thesis Submitted for the Degree of PhD**
**at the**
**University of St Andrews**



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# Behavioural phenotyping of mice with genetic alterations of the $GABA_A$ receptor

**A thesis submitted for the degree of PhD**

**Submitted August 2009**

**By Nicola Stefanie Lisa Foister**

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# Declaration

I, Nicola Stefanie Lisa Foister, hereby certify that this thesis, which is approximately 33000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.
I was admitted as a research student in September, 2005 and as a candidate for the degree of PhD in September, 2006; the higher study for which this is a record was carried out in the University of St Andrews between 2005 and 2009.

Date Signature of candidate

I, Professor Verity Brown, hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate for the degree of PhD in the University of St Andrews and that the candidate is qualified to submit this thesis in application for that degree.

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# Acknowledgements

Well I have arranged to take this thesis for printing at 2pm this afternoon. It has taken a while and there have been a few hiccups along the way but I've made it at last, but not without the help of some incredible people!

Firstly I would like to thank my supervisors – yes all four of them! Professor Verity Brown without whom my confidence crises would probably have prevented this thesis from ever being finished, Dr Delia Belelli and Professor Jeremy Lambert for the unending supply of mice and enthusiasm for this project and unrelenting support and finally but far from least, Professor David Balfour, who's day to day supervision has been hugely appreciated – thank you for making me feel so welcome in your laboratory! Learning from these four unique individuals for the time of my PhD has set me in firm standing for the future – thank you, all of you!

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I would also like to thank my family – my mum, Claire, and her husband Donald who have supported me in so many ways. My brothers Ben and James who are a constant source of entertainment and make sure I maintain my sense of humour – thank you!

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# Contents

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# Abbreviations

| | |
|---|---|
| BDZ | Benzodiazepine |
| CD | Compound Discrimination |
| CDRev | Compound Discrimination Reversal |
| CER | Conditional Emotional Response |
| CNS | Central Nervous System |
| ED | Extra Dimensional |
| EDRev | Extra Dimensional Reversal |
| EEG | Electroencephalography |
| EPM | Elevated Plus Maze |
| GABA | γ – aminobutyric acid |
| $GABA_AR$ | γ – aminobutyric acid type A receptor |
| $GABA_BR$ | γ – aminobutyric acid type B receptor |
| $GABA_CR$ | γ – aminobutyric acid type C receptor |
| GABA-T | γ – aminobutyric acid type transaminase |
| GAD | Glutamate Decarboxylase |
| GAT | γ – aminobutyric acid transporter |
| HBC | hydroxypropyl - β - cyclodextrin |
| ID | Intra Dimensional |
| IDRev | Intra Dimensional Reversal |
| IPSC | Inhibitory Post Synaptic Current |
| IPSP | Inhibitory Post Synaptic Potential |
| LORR | Loss Of Righting Reflex |
| nREM | non Rapid Eye Movement |
| OF | Open Field |

| | |
|---|---|
| PTZ | Pentyleletetrazol |
| REM | Rapid Eye Movement |
| rpm | rotations per minute |
| SD | Simple Discrimination |
| SWS | Slow Wave Sleep |
| THIP | 7 – tetra hydroisoxazolo [5,4-c]pyridin-3-ol |
| VGAT | Vesicular Neurotransmitter Transposters |

# Abstract

GABA is the main inhibitory neurotransmitter of the central nervous system. $GABA_ARs$ are multimeric transmembrane receptors, which are composed of 5 subunits. It is known that there are 19 subunits that can make up the $GABA_ARs$, allowing for a vast array of receptor subtypes. In addition to the GABA binding site $GABA_ARs$ have distinct allosteric binding sites for benzodiazepines, barbiturates, ethanol, certain general anaesthetics and neuroactive steroids. The molecular heterogeneity of the $GABA_AR$ is accompanied by distinct pharmacological profiles of the different receptor subtypes. The advance of transgenic mouse models has allowed the functional significance of this heterogeneity to be studied *in vivo*. Therefore, this thesis utilises a variety of transgenic mouse models carrying either mutations or deletions of certain subunits to study the functional significance of the receptor heterogeneity.

Mice lacking the α1 subunit ($\alpha1^{-/-}$), carrying a point mutation of the α1 subunit (α1H101R), and mice lacking the δ subunit ($\delta^{-/-}$) have been utilised to investigate the role of these subunits in the sedative actions of benzodiazepines and the $GABA_AR$ agonist THIP. Although there are limitations to the interpretation of these results due genetic background of the $\alpha1^{-/-}$ and α1H101R, experiments suggest that the α1H101R mutation is not behaviourally silent as previously suggested and provide further evidence that the α1 subunit mediates the sedative properties of benzodiazepines. These experiments also reveal that the extrasynaptic δ containing receptors are responsible for mediating the sedative effects of

THIP, and these findings combined with evidence from collaborators, implicates the thalamus as an anatomical mediator of these effects.

An investigation of the putative cognitive enhancing effects of THIP using an attentional set-shifting task for mice suggested that pre-treatment with THIP reduces the number of errors to reach criterion. $\delta^{-/-}$ mice could not be trained to perform the task, therefore further behavioural investigation of these mice was performed, which suggested a heightened level of anxiety and reduced motivation for a food reward.

This thesis has furthered our understanding of the functional role of $GABA_AR$ subtypes. With the advance in genetic manipulations that allow for regionally selective mutations of the receptor the anatomical structures involved in these functions can be identified.

# 1 General Introduction

*"The genetic approaches of antisense technology and transgenics might also be useful to manipulate the expression of individual subunits [of the $GABA_A$ receptor], the effects of which can be determined using a variety of biochemical, electrophysiological or even behavioral techniques. Clearly the next leap forward will be from determination of the composition of receptor subtypes to the understanding of their roles in brain function"*

McKernan and Whiting, 1996

## *1.1 Thesis Objective*

More than a decade after McKernan and Whiting's review (1996) of the various $GABA_A$ receptor subtypes in the brain, the use of transgenic mice lacking certain receptor subunits or carrying point mutations of the receptor are used extensively to investigate the physiological and pharmacological significance of the large heterogeneity of this receptor in the mammalian central nervous system (CNS).

This thesis forms part of a larger on going study to pharmacologically, electrophysiologically and behaviourally characterise mice carrying mutations of $GABA_A$ receptors to further understand the functional significance of $GABA_A$ receptor heterogeneity and the role of GABAergic inhibition in behaviour.

In order to understand the functional significance of the receptor heterogeneity, it is necessary to appreciate the properties and function of GABAergic neurones and GABA receptors. Therefore, the remainder of this Chapter focuses on the characteristics of GABAergic neurones and what is currently known about the physiological and functional significance of certain receptor subtypes.

## *1.2 GABA and GABA Receptors*

### 1.2.1 GABA and GABA Receptors – A 50 year history

The identification of the amino acid γ-aminobutyric acid (GABA) was first published in 1950 (Roberts and Frankel, 1950). It was later shown that GABA produced inhibitory effects on neurones (Bazemore*, et al.*, 1956) and induced membrane hyperpolarisation (Kuffler and Edwards, 1958).

In the mid 60s, GABA was shown to mimic the changes in membrane potential and the decrease of input resistance of cells normally shown during electrically-evoked inhibitory post synaptic potentials (IPSPs) in cat sensorimotor cortex neurones (Krnjevic and Schwartz, 1966; 1967). It was not until the 1970s that the first pharmacological evidence of GABA as an inhibitory neurotransmitter arose. Synaptic inhibition and GABA-evoked inhibition in the cerebellum and hippocampus were both blocked by bicuculline (plant alkaloid and competitive antagonist of the GABA receptor; Curtis*, et al.*, 1970a; Curtis*, et al.*, 1970b). In addition, GABA concentrations were found to be higher in inhibitory neurones than in excitatory neurones (Roberts*, et al.*, 1970).

In the 1980s, the patch clamp technique (developed by Neher and Sakmann, 1976) was used to demonstrate that spinal cord and hippocampal neurones maintained in cell culture expressed single, GABA-activated, bicuculline sensitive $Cl^-$ channels (Hammill*, et al.*, 1983).

The receptor now known to be responsible for the mediation of the GABA-evoked response became known as the $GABA_A$ receptor ($GABA_AR$) the first two subunits (α1 and β1) of which were identified by Schofield*, et al.*, 1987. The receptor was shown to be a member of the cys-loop ligand gated ion channel family. This family of receptors also includes the nicotinic acetylcholine, $5HT_3$ receptors and glycine receptors. It is now known that the $GABA_AR$ is responsible for the mediation of the majority of fast inhibitory neurotransmission within the mammalian CNS. Although this thesis concentrates on $GABA_ARs$, there are also two other forms of GABARs which are considered briefly.

The $GABA_B$ receptor ($GABA_BR$) is a bicuculline insensitive GABAR. In contrast to the $GABA_AR$, the $GABA_BR$ modulates the activation of $K^+$ and inhibition of $Ca2^+$ channels, through G-protein coupling (Hill & Bowery, 1981). $GABA_BRs$ are insensitive to bicuculline but are selectively activated by L-baclofen (an agonist specific to mammalian $GABA_BRs$). There are two isoforms (1a and 1b) of $GABA_BRs$ which can form heteromeric receptor complexes (for review see Marshall*, et al.*, 1999) and share structural homology with metabotropic glutamate receptors (Kaupmann*, et al.*, 1997).

The $GABA_C$ receptor ($GABA_CR$) was first identified in 1984 and was found to be insensitive to bicuculline and baclofen (Drew*, et al.*, 1984). Activation of the $GABA_CR$ (by the binding of GABA), in common with $GABA_ARs$, results in rapid activation of the $Cl^-$ conducting channels but

the pharmacological profile of these two receptors is somewhat different (for review see Bormann, 2000 or Zhang, D*, et al.*, 2001). The $GABA_CR$ is expressed largely in the vertebrate retina but is also found in the spinal cord and cerebellum. Traditionally, the $GABA_CR$ was thought to comprise of ρ subunits (1-3) (Bormann, 2000; Koulen*, et al.*, 1998; Zhang, D*, et al.*, 1995); however, more recent evidence suggests that certain $GABA_C$ subunits can also co-assemble with $GABA_AR$ subunits (such as γ2) and these can form functional heteromeric receptors in *Xenopus laevis* oocytes (Pan and Qian, 2005) and central synapses (Harvey*, et al.*, 2006; Milligan*, et al.*, 2004).

### 1.2.2 Synthesis, metabolism, release and uptake of GABA

GABA is synthesised (Figure 1.1) from the primary excitatory neurotransmitter glutamate by the enzyme glutamate decarboxylase (GAD) (Basile*, et al.*, 2004). Two GAD isoforms have been identified. The first, GAD65, is found in axons and terminal boutons whereas the second, isoform GAD67, has a wide-ranging subcellular distribution (for review: Martin and Rimvall, 1993; Waagerpeterson*, et al.*, 1999). GABA is transported into vesicles by vesicular neurotransmitter transporters (VGAT). Reuptake of GABA, following activation of pre and postsynaptic receptors, from the synaptic cleft is performed by high affinity GABA transporters (GAT), which are expressed on the presynaptic terminals and glial cells. GAT1 and GAT3 transporters are common in the CNS; GAT2 transporters, are less common in the CNS, but are present on astrocytes. GABA transporters are important in regulation of GABA

overspill onto extrasynaptic receptors while maintaining the supply of presynaptic GABA (for review: Borden, 1996; Conti*, et al.*, 2004). Importantly, it has recently been demonstrated that GATs can reverse direction and function to promote non-vesicular release of GABA (Wu*, et al.*, 2007), suggesting that these transporters play a role in mediating ambient GABA concentrations and thus regulating the amount of GABA available to activate extrasynaptic $GABA_AR$s. GABA is then either recycled to synaptic vesicles or metabolised by the mitochondrial enzyme GABA transaminase (GABA-T) to form succinic semialdehayde, which is later converted to succinate and fed into the Kreb's cycle (Figure 1.1).

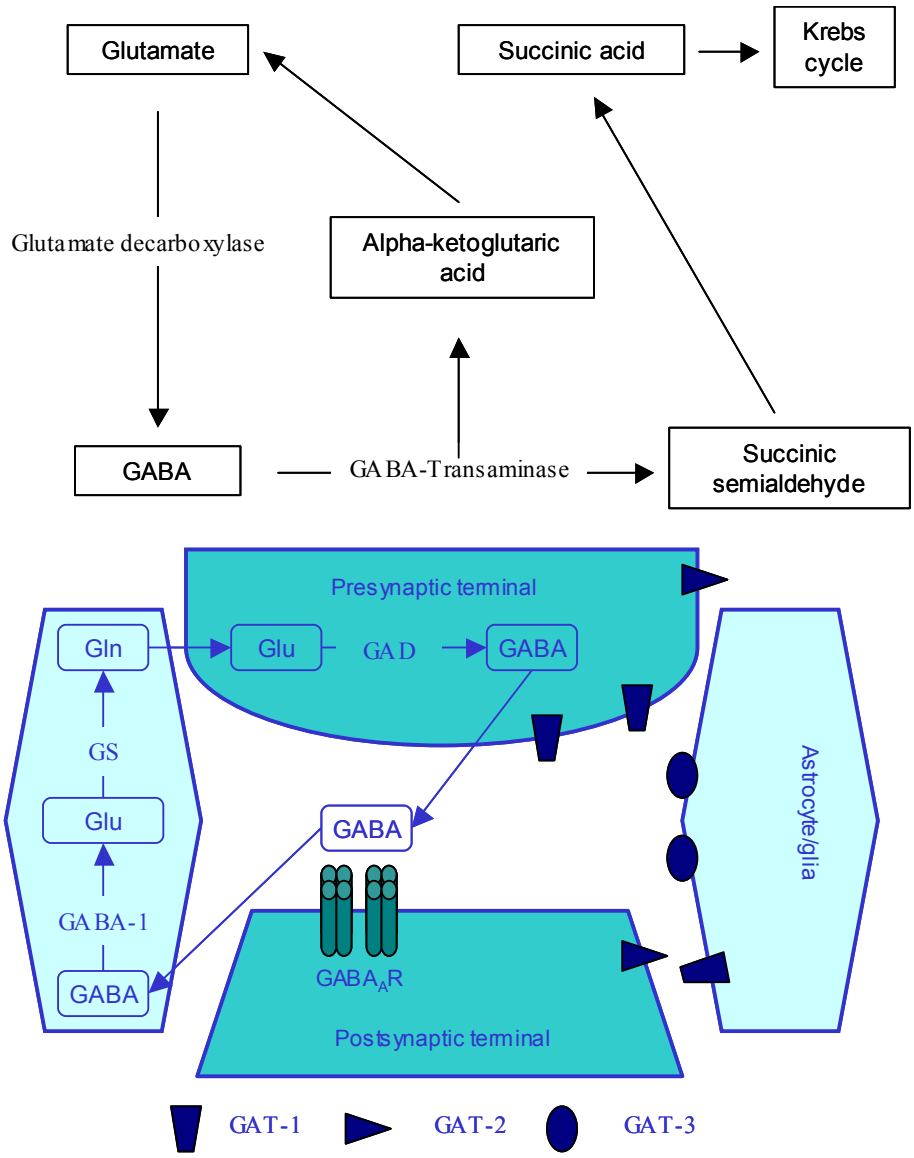


***Figure 1.1: Schematic representation of GABA synthesis, release and reuptake by GABA transporters (GAT).***

*GABA is synthesised by glutamate decarboxylase (GAD) from glutamate (Glu) (TOP) using GABA-α-oxoglutarate transaminase (GABA-1) and glutamte synthase (GS). GABA is then transported to the vesicles and released from the synapse acts upon the receptors on the postsynaptic neurone. GABA transporters (GATs) quickly remove GABA from the synaptic cleft to astrocytes or glia. GABA is then metabolised to glutamate or rapidly recycled into vesicles (BOTTOM; Schematic adapted from Conti, et al., 2004).*

### 1.2.3 $GABA_AR$ structure and heterogeneity

$GABA_ARs$ are multimeric transmembrane receptors which are pentameric assemblies and the 5 subunits are arranged around a central pore (Baumann*, et al.*, 2001; Nayeem*, et al.*, 1994). The $GABA_AR$ consists of a large extracellular N-terminal domain containing cysteine residues common to the cys loop family of receptors. There are also 4 α-helical transmembrane domains with a large intracellular loop between the third and fourth transmembrane domains and a short extracellular C-terminal domain (Figure 1.2).

The $GABA_AR$ was first identified in the early 1980s (Sigel*, et al.*, 1983) and the first 2 subunits (α and β) were cloned soon after (Schofield*, et al.*, 1987). However, receptors formed from these 2 subunits alone, which were activated by GABA and antagonised by bicuculline, were not sensitive (using electrophysiological recordings) to the allosteric effects of benzodiazepines (BDZs). Only with successful cloning, in the late 80s, of the γ2 subunit were fully functional BDZ sensitive receptors identified and this subunit was found to be crucial to the binding of benzodiazepines (Pritchett*, et al.*, 1989). It is now known that there are 19 subunits that can make up the receptor (6α, 3β, 3γ, δ, ε, π, and 3ρ) (Davies*, et al.*, 1997; Hedblom and Kirkness, 1997; Macdonald and Olsen, 1994).

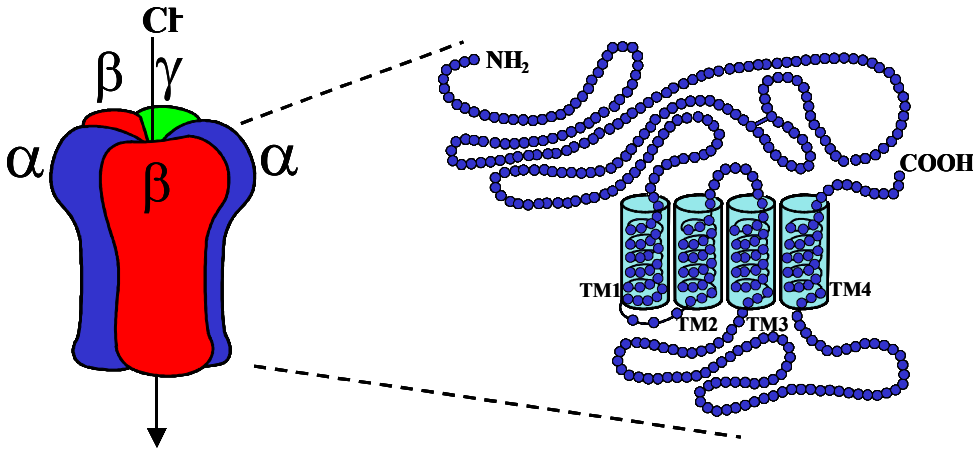


***Figure 1.2: Schematic representation of the pentameric assembly of the $GABA_AR$ and the structure of an individual receptor.***

***Left:*** *Typical pentameric structure of the $GABA_AR$ which surrounds a central aqueous pore.* ***Right:*** *The typical structure of a $GABA_AR$ subunit consists of a large extracellular N terminal domain (ECD) and 4 large transmembrane domains (TM1-4). Figure provided by J Lambert.*

The number of subunit combinations is theoretically very large and complex; however, *in vivo* the subunit composition of $GABA_A$Rs is governed by a number of factors including developmental and regional. Indeed, it has been shown that despite the large number of possibilities of receptor subtypes only approximately 30 exist *in vivo* due to the rules governing temporal and anatomical location (Sieghart and Sperk, 2002). Using *in situ* hybridisation and immunocytochemistry studies, detailed knowledge of the expression of certain subunits is now known. Studies have shown that some subunits such as α1, β2 and γ2 are widely distributed throughout the CNS whereas some exhibit a more distinct regional expression (Figure 1.3). For example, α4 is predominantly expressed in the thalamus and to a lesser extent the dentate gyrus, cortex and basal ganglia whereas the α6 subunit is predominantly expressed in the granule cells of the cerebellum. In both cases these receptor subunits preferentially, although not exclusively, co-assemble with the regionally restricted δ subunit. The α5 subunit is largely restricted to the hippocampus. The most common subunit composition α1β2γ2 (Figure 1.4) accounts for ~50% of all $GABA_A$Rs (McKernan and Whiting, 1996; Mohler, 2007).

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***Figure 1.3: Digital images showing regional distribution of $GABA_AR$ subunits.***

*Immunocytochemistry analysis of the regional distribution of various $GABA_AR$ subunits. α1, β2 and γ2 are widely distributed throughout the CNS. δ and α5 are more regionally specific (Image from Fritschy and Mohler, 1995).*

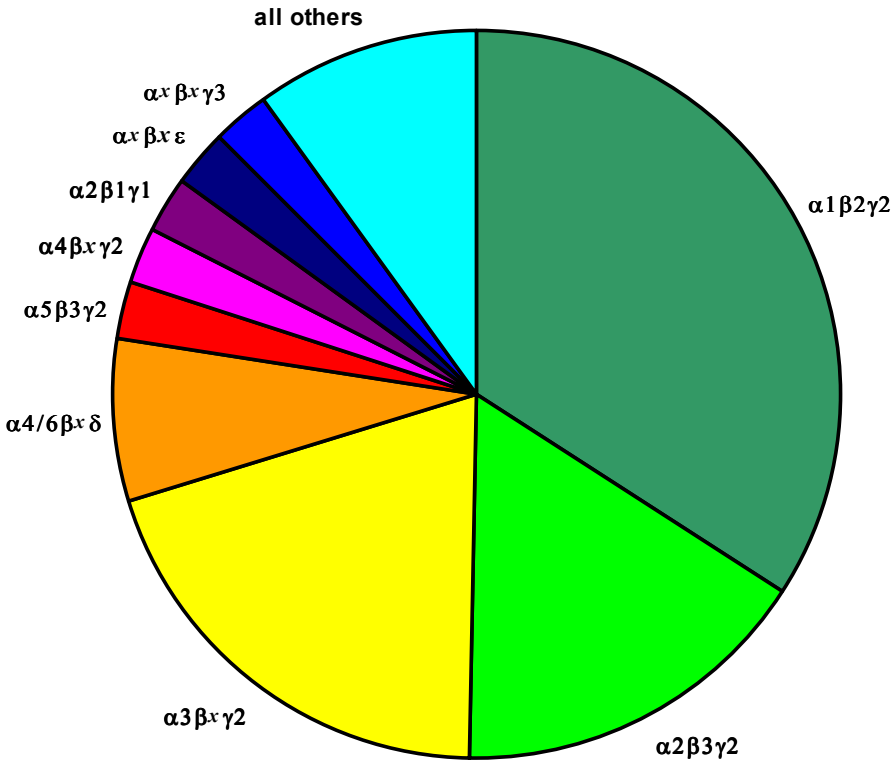


***Figure 1.4: Pie chart demonstrating the relative contributions of major certain $GABA_A$ receptor isoforms.***

*The overwhelming majority of receptors consist of the synaptically located α1β2γ2. Receptor subtypes containing the δ subunit are less common. These estimations on the relative contributions of certain $GABA_AR$ isoforms were derived from immunoprecipitation studies using subunit specific antibodies. Adapted from Whiting, 2003.*

Subunit composition also varies within a neurone (synaptic or extrasynaptic), and it is now widely known that receptor subtypes expressed at different cellular locations mediate 2 forms of GABAergic inhibition. These are phasic inhibition, which is mediated by transient activation of synaptically located receptors, and tonic inhibition, which is mediated by the sustained exposure of low levels of GABA on receptor subtypes expressed at peri- or extrasynapic locations (Figure 1.5). The γ2 subunit has a distinctive role in receptor clustering at synaptic locations (Essrich*, et al.*, 1998) and is therefore found synaptically (albeit not exclusively). However, there are examples of the γ2 (for example while paired with α5β2/3) in extrasynaptic locations in the dentate gyrus granule cells and hippocampal pyramidal cells, as well as some cortical layers. In this position, the γ2 subunit, is responsible for the mediation of tonic inhibition (Ade*, et al.*, 2008; Caraiscos*, et al.*, 2004; Zhang, N*, et al.*, 2007a). Other receptor isoforms containing the γ2 subunit (α2β3γ2 and α3β2γ2) which are predominantly located in the hippocampus, striatum and cortex (McKernan and Whiting, 1996), are synaptically located. The more regionally specific δ containing $GABA_A$Rs (highly expressed in the thalamus, dentate gyrus granule cells, cortex and cerebellar granule cells (Sieghart and Sperk, 2002)) are exclusively expressed extrasynaptically and perisynaptically (Nusser*, et al.*, 1998; Peng*, et al.*, 2004b; Wei*, et al.*, 2003a; Zhang, N*, et al.*, 2007a). δ containing $GABA_A$Rs have a relatively high affinity for GABA and have limited desensitisation making these receptors ideal for mediating tonic inhibition (Farrant and Nusser, 2005). In addition in the granule cells of the dentate gyrus and cerebellum,

perisynaptic δ containing $GABA_AR$s can become activated by “spillover” GABA, thus contributing to a slow, long lasting decay component of inhibitory postsynaptic currents (Rossi and Hamann, 1998; Wei*, et al.*, 2003a).

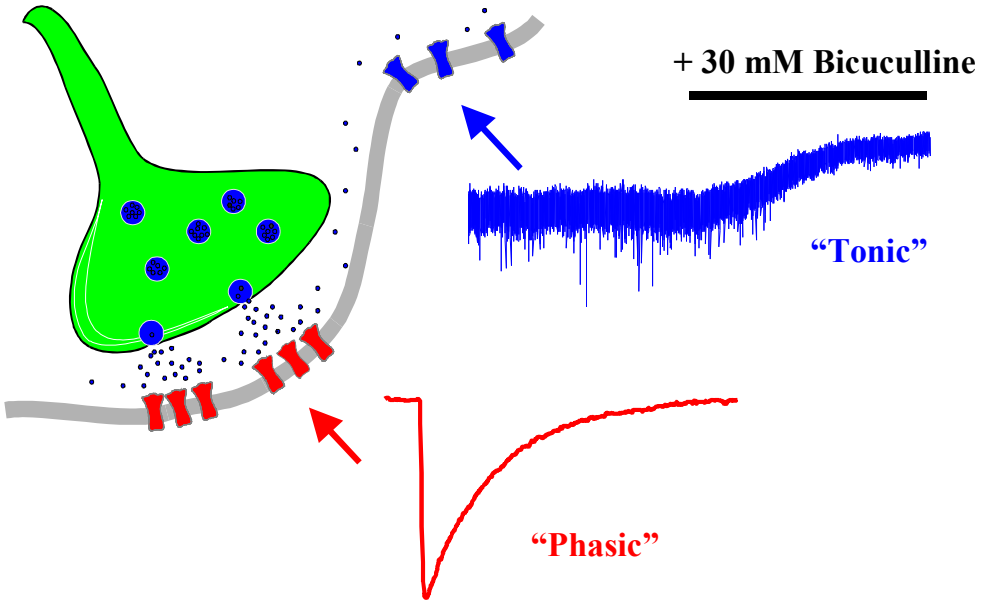


***Figure 1.5: Schematic representation of phasic and tonic inhibition mediated by $GABA_A$ receptors located at synaptic and extra synaptic locations.***

*Vesicular release of GABA into the synaptic cleft activates synaptic $GABA_A$Rs resulting in an inhibitory postsynaptic current, which exhibits fast activation and deactivation kinetics: this form of inhibition is known as phasic inhibition. Spill over GABA can activate peri and extrasynaptic receptors; these receptors exhibit limited desensitisation to GABA. In the majority of cases, phasic inhibition is mediated by γ2 containing synaptic receptors whereas in some neurons tonic inhibition is mediated by extrasynaptic δ containing $GABA_A$Rs. Image provided by J Lambert.*

## *1.3 Pharmacology of $GABA_AR$s*

In addition to the GABA binding site, the $GABA_AR$ has distinct allosteric binding sites for benzodiazepines (BDZs), barbiturates, inhaled anaesthetics and neuroactive steroids. These drugs typically enhance receptor function and in agreement have anxiolytic, relaxing, sedative and anti-convulsive effects and are used to treat various clinical conditions (Table 1.1). Ligands other than GABA that can interact with $GABA_AR$s fall into 3 categories; those that activate the receptor (agonists), those that inhibit the receptor (antagonists) and those that can increase or decrease a response to GABA (positive/negative allosteric modulators). The relative affinity of these drugs to the $GABA_AR$s is often crucially dependent on subunit composition (Table 1.1).

This section will review the pharmacology of distinct $GABA_AR$ subtypes with respect to BDZs, etomidate, THIP and the neuroactive steroid alphaxalone.

| Binding Site | Drug | Function | Receptor Action | Reference |
|---|---|---|---|---|
| Benzodiazepine | Zolpidem | Hypnotic | Preferential affinity for α1 subtypes | Dämgen and Lüddens, 1999 |
| | Zaleplone | Hypnotic | Preferential affinity for α1 subtypes | Dämgen and Lüddens, 1999 |
| | Indiplon | Hypnotic | Preferential affinity for α1 subtypes | Foster, et al., 2004 |
| | L - 838 417 | Anxiolytic | Affinity for α1, α2, α3, α5 and partial agonist at α2, α3, α5 subtypes. Lacks efficacy at α1 | McKernan, et al., 2000 |
| | TPA 023 | Anxiolytic | Partial agonist α2, α3 and antagonist at α1 and α5 subtypes | Atack, et al., 2006 |
| | TPA 003 | Anxiolytic | Partial agonist at α3 subtypes | Dias, et al., 2005 |
| | A3 IA | Anxiogenic | Inverse agonist at α3 subtypes | Atack, et al., 2005 |
| | L - 655 708 | Memory Enhancer | Partial inverse agonist at α5 subtypes | Chambers*, et al.*, 2004; Dawson*, et al.*, 2006; Navarro*, et al.*, 2002; Sternfeld*, et al.*, 2004 |
| GABA | THIP | Hypnotic | Partial agonist α1 & α3, agonist at α5 and super agonist at α4β3δ subtypes | Storustovu and Ebert, 2003 |
| Others | Neurosteroids | Anxiolytic Sedative Anaesthetic | High sensitivity at δ and α1β1, α3β1 subtypes | Belelli*, et al.*, 2002; Brown, N*, et al.*, 2002 |
| | Propofol | Sedative Anaesthetic | Acts on β2 and β3 subtypes | Rudolph and Antkowiak, 2004b |
| | Etomidate | Sedative Anaesthetic | Preferentially acts on β2 and β3 subtypes vs β1 | Hill-Venning*, et al.*, 1997 |

***Table 1.1: Table showing a selection of $GABA_A$ receptor subtype ligands.***

*Binding site, ligand name, ligand action and the interaction with recombinant $GABA_A$Rs is given. Adapted from Rudolph and Mohler, 2006.*

### 1.3.1 Benzodiazepines

The first benzodiazepine (BDZ), chlordiazepoxide, was identified in 1954 and was later marketed under the brand name Librium. This development was followed almost a decade later by diazepam (Valium) in 1963. Since the discovery of these classic BDZs, their anticonvulsant, anxiolytic, muscle relaxant, sedative and hypnotic properties have been utilised for the treatment of anxiety, muscle spasms, insomnia, seizures and alcohol withdrawal. These drugs were used clinically many years before any knowledge of the molecular mechanisms of their effects was identified. In the late 1970s, and early 1980s, however, BDZs were found to be positive allosteric modulators of $GABA_AR$s and to enhance the effects of GABA by acting on distinct sites of the $GABA_AR$. BDZs have no direct effect on channel conductance or opening time. They do, however, increase the frequency of opening, prolonging the decay time of mIPSCs, which results in increased $Cl^-$ influx causing hyperpolarisation and increased inhibition (Eghbali*, et al.*, 1997; Study and Barker, 1981; Vicini*, et al.*, 1987). BDZ binding to the $GABA_AR$ is dependent on the presence of an αβγ combination of subunits (Pritchett*, et al.*, 1989). The type of α subunit affects BDZ pharmacology. α1, 2, 3 and 5 containing receptors are essential together with a γ2 or γ3 subunit for BDZ binding; indeed, the BDZ binding pocket is believed to be at the α-γ subunit interface (see below). In contrast α4 and α6 containing receptors are insensitive to BDZs (Wisden*, et al.*, 1991).

Specific BDZ site agonists have provided the opportunity to characterise the properties of given receptors. Zolpidem, although not a BDZ in itself, is a BDZ site agonist, which is selective for α1 containing $GABA_AR$s at concentrations up to 100nM, but becomes active at α2 and α3 containing receptors at 1μM. In addition, α5 containing $GABA_AR$s show very low affinity for zolpidem (Dämgen and Lüddens, 1999; Pritchett*, et al.*, 1989). Drugs such as zolpidem can be used to characterise the functional roles of different receptor subtypes. For example, since zolpidem is highly selective for the α1 subtype and has a strong sedative profile, one could speculate that α1 subtypes contribute to the sedative effects of BDZs.

BDZ binding to $GABA_AR$s is dependent on a single amino acid residue. This histidine, at the 101 position (mouse numbering), is found in α1, α2, α3, and α5 subunits. α4 and α6 subunits have an arginine at the 101 position (Wieland*, et al.*, 1992). Indeed, a point mutation of histidine to arginine in recombinant receptors leads to a loss of affinity to diazepam (Benson*, et al.*, 1998a). Molecular evidence from mutagenesis studies has now shown that the binding pocket for BDZs is at the cleft between the γ and α subunits and that the γ subunit is essential for BDZ binding (Sigel and Buhr, 1997).

### 1.3.2 General Anaesthetics - Etomidate

William Morton performed the first public demonstration of general anaesthesia in 1846; however the molecular mechanisms of general anaesthesia have only recently started to be understood.

The $GABA_AR$ is known to play an important role in the mediation of the effects of the anaesthetics etomidate, propofol and the barbiturate pentobarbitone. These drugs are positive allosteric modulators of the $GABA_AR$. Additionally at higher concentrations they have GABA-mimetic properties i.e. they can directly activate the receptor itself in the absence of GABA (Belelli*, et al.*, 1996; Evans and Hill, 1978; Peters*, et al.*, 1988). For the purposes of this thesis, the mechanisms of etomidate will be reviewed closer.

Etomidate has no effect on the single channel conductance, but increases the probability of the channel being open for longer (Yang and Uchida, 1996) and, in common with other modulators of the receptor, etomidate prolongs the decay of mIPSCs resulting in increased inhibitory control (Belelli*, et al.*, 2003).

Like BDZs, the subunit composition of the receptor has been demonstrated to be vitally important in the actions of etomidate. However, in contrast with BDZs, it is the β subunit which primarily dictates etomidate sensitivity, not the α subunit. It has also been established that the γ subunit is not essential for etomidate binding and that the identity of the β subunit and subsequently the α subunit are likely to be more important (Ernst*, et al.*, 2005; Hemmings*, et al.*, 2005). Etomidate has greater potency on β2/β3 containing receptors than on β1 (Figure 1.6) containing receptors (Hill-Venning*, et al.*, 1997). By changing the asparagine at the 289 position of the β2 subunit to a serine of the β1

subunit the, GABA modulatory and GABA mimetic effects of etomidate were reduced (Belelli et al, 1997). Furthermore, changing this to a methionine (the amino acid present at the homologous position on the etomidate and propofol insensitive Drosophila receptor) completely abolishes the electrophysiological effects of etomidate (Belelli*, et al.*, 1997; Krasowski*, et al.*, 1998).

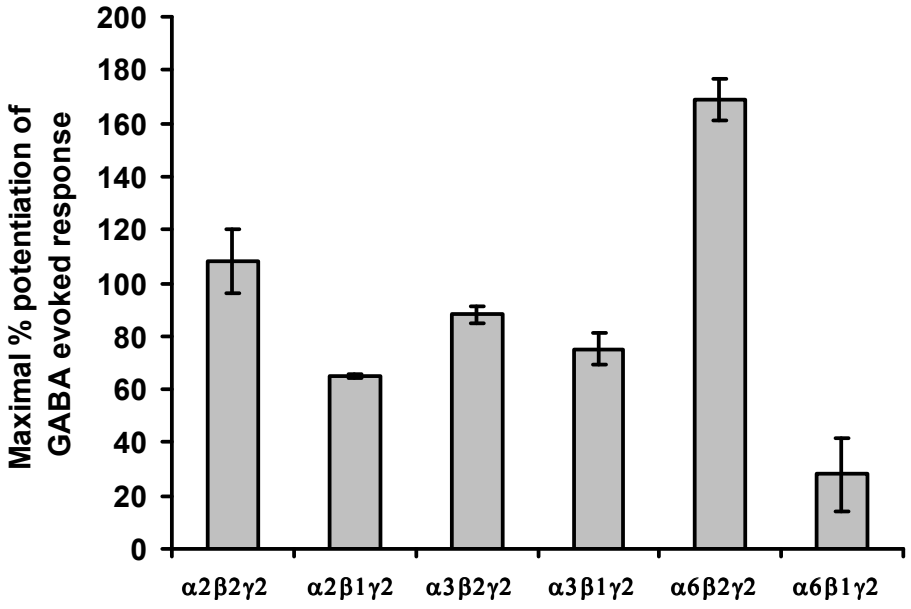


***Figure 1.6: Graph showing the percentage of the maximum response to GABA of etomidate at different recombinant $GABA_A$ receptors in Xenopus laevis oocytes.***

*Etomidate is more potent on β2 containing receptors than those containing β1. Data derived from Hill-Venning, et al., 1997.*

### 1.3.3 THIP

7-Tetra hydroisoxazolo[5, 4-c]pyridin-3-ol (THIP), also know with the trade name gaboxadol, has recently been developed (and undergone clinical trials) as a treatment for insomnia (Wafford and Ebert, 2006; Wafford and Ebert, 2008).

THIP has been shown to act preferentially upon δ containing $GABA_A$Rs with higher efficacy and potency than GABA itself (Adkins*, et al.*, 2001; Belelli*, et al.*, 2005; Brown, N*, et al.*, 2002; Drasbek*, et al.*, 2007). δ containing receptors are located predominately in extra and peri synaptic locations (Nusser*, et al.*, 1998; Peng*, et al.*, 2004a; Wei*, et al.*, 2003b; Zhang, N*, et al.*, 2007b). Furthermore, it has been demonstrated that extrasynaptic receptors in the relay neurones of the ventral thalamus are activated by relatively low concentrations (~30nM) of THIP and that THIP has no effect on the synaptic currents at least up to the concentration of 3μM (Belelli*, et al.*, 2005; Cope*, et al.*, 2005a; Jia*, et al.*, 2005). This specificity for δ containing $GABA_A$Rs makes THIP a useful pharmacological tool when investigating the functional roles of δ containing $GABA_A$Rs. The δ subunit pairs preferentially with either α4 subunits in the thalamus, dentate gyrus and cortex and α6 subunits in the cerebellum. Indeed, the large inward current induced by THIP administration in thalamic relay neurones and dentate gyrus granule cells is absent in $\alpha4^{-/-}$ mice (Chandra*, et al.*, 2006). THIP has different potencies at these α4 and α6 containing receptor subtypes (Storustovu and Ebert, 2006) being significantly more potent amongst the δ

containing isoforms (Figure 1.7). THIP is more efficacious and potent at α6δ receptors than at α4δ receptors (Storustovu and Ebert, 2006). Indeed, the level of activation of α6β3δ containing receptors is 15% of the relative GABA activated maximum. In contrast, activation of α4β3δ receptors is 50% of the relative GABA activated maximum (1h post 10mg/kg subcutaneous injection; Figure 1.7).

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***Figure 1.7: Figure showing the concentration response curves for THIP obtained from Xenopus laevis oocytes***.

*THIP interacts with greater potency and efficacy at δ containing receptors than γ receptors. Furthermore, THIP acts with greater potency and efficacy at α6δ than at α4δ receptors. Figure from Storustovu and Ebert, 2006.*

### 1.3.4 Neurosteroids

Neuroactive steroids and neurosteroids are well known for their hypnotic, sedative and anaesthesia inducing properties but at lower doses they exhibit anxiolytic, analgesic and anticonvulsant properties (for review: Belelli and Lambert, 2005; Lambert*, et al.*, 1995). In the early 1980s, it was found that the synthetic neurosteroids and intravenous anaesthetic 5α-pregnan-3α-ol-11,20-dione (alphaxalone) potently enhanced the function of $GABA_AR$s (Harrison, NL and Simmonds, 1984). In addition, endogenously occurring neurosteroids, including 5α-pregnan-3α-ol-20-one (5α3α), 5β-pregnan-3α-ol-20-one (5β3α) and 5α-pregnan-3α,21-diol-20-one (THDOC), were recorded to be potent positive allosteric modulators of the $GABA_AR$ (Belelli and Lambert, 2005; Callachan*, et al.*, 1987; Harrison, NL*, et al.*, 1987; Herd*, et al.*, 2007; Peters*, et al.*, 1988).

Single channel conductance is not altered by neurosteroids but they do increase the probability of the GABA-gated channel being in the open state (Barker*, et al.*, 1987; Callachan*, et al.*, 1987; Lambert*, et al.*, 1987; Twyman and Macdonald, 1992; Zhu and Vicini, 1997). Neurosteroids also have GABA-mimetic properties i.e. they can activate the $GABA_AR$ receptors in the absence of GABA (Callachan*, et al.*, 1987; Lambert*, et al.*, 1987). Neurosteroids bind at a different site from BDZs (Callachan*, et al.*, 1987; Gee*, et al.*, 1988; Peters*, et al.*, 1988).

In contrast to BDZs, etomidate and THIP, the receptor subunit composition has less of an impact on the action of neurosteroids in

recombinant expression studies (Belelli*, et al.*, 2002; Lambert*, et al.*, 2003). The effects of neurosteroids upon native receptors exhibit heterogeneity across different neuronal populations. The effects of neurosteroids on synaptic receptors results in a prolongation of the mIPSC decay time whereas on extrasynaptic receptors neurosteroids promote longer openings, which manifests as an increase of the bicuculline sensitive tonic conductance (Stell*, et al.*, 2003; Zhu and Vicini, 1997).

Recently, the role of extrasynaptic receptors in the mediation of the effects of neurosteroids has received a lot of interest. Recombinantly expressed δ containing $GABA_A$Rs are highly sensitive to neurosteroids (Belelli*, et al.*, 2002; Brown, N*, et al.*, 2002; Wohlfarth*, et al.*, 2002) when paired with the α4 or the α6 subunits (Figure 1.8). However, there is clear heterogeneity in sensitivity of native peri- or extrasynaptic δ containing $GABA_A$Rs and some are insensitive to high concentrations of endogenous neurosteroids (Porcello*, et al.*, 2003).

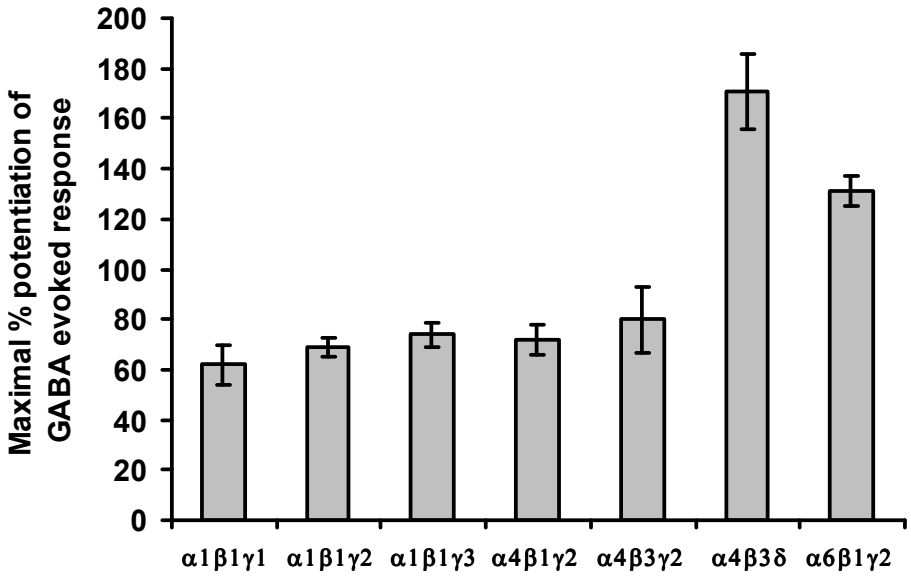


***Figure 1.8: Histogram showing the percentage of the maximum response to GABA of 5α3α at different recombinant $GABA_A$ receptors in* Xenopus laevis *oocytes.***

*5α3α is more efficacious on δ containing receptors than those containing γ. Data derived from Belelli, et al., 2002*

## *1.4 Behavioural tests*

There are a number of common behavioural tests, which are used to study the functional significance of $GABA_AR$ heterogeneity. Before examining some of the evidence for a functional role of $GABA_AR$ heterogeneity a selection of relevant behavioural tests will be presented.

### 1.4.1 Elevated Plus Maze

The elevated plus maze (EPM) is traditionally considered a test of anxiety like behaviours in rats and mice (Pellow 1985 & Lister 1987). The maze consists of four arms, arranged as a cross (hence the name 'plus (+) maze'), so that there is a 90-degree angle between each arm. The arms are either enclosed by walls (closed arms) or left open (open arms). This model is based upon a rodent aversion to open spaces, which presents as greater movement in the closed arm entries in comparison with open arm entries. A reduction in anxiety presents itself as an increase in time spent in the open arms, and a higher number and/or proportion of open arm entries in comparison with closed arm entries. Closed arm entries and total arm entries are commonly used to compare locomotor activity. Following treatment with an anxiolytic drug (such as a BDZ) rodents make an increased amount of time spent in the open arms, suggesting a decrease in anxiety. The opposite pattern is observed following anxiogenic drugs. It is important to note however that there is evidence that an anxiolytic effect of a drug in rodents as measured by the EPM does not always translate to the human, furthermore drugs known to be anxiolytic in humans do necessarily have an anxiolytic profile in the EPM.

Another limitation of the EPM is introduced by repeat testing. It is known that the effect of BDZs on the number of open arm entries may not be observed upon second exposure to the EPM (Rodgers & Shepherd, 1992) and that a second exposure to the EPM may depend on different factors of anxiety than the first (File, 1993; Holmes *et al*, 1998). It is therefore important to take these issue into account when using the EPM.

### 1.4.2 Open Field

The open field test in laboratory animals is often used as a fundamental index of behaviour. Open field studies are used to study an animal's response to novel environment and exploratory motivation. It assesses locomotor activity, exploratory and anxiety like behaviours. The open field generally consists of an open area (of any given size, but usually square or round) that is surrounded by walls to prevent the rodent from escaping. The open field examines the conflict between the desire to explore a novel environment and the fear of novel/open spaces. Like the EPM the open field can be used to assess anxiolytic/anxiogenic properties of drugs by measuring the degree the rodent avoids the centre of the open field and stays close to the walls (thigmotaxis). The open field can also be used to measure locomotor activity responses to drugs (such as known sedatives) and behavioural responses to novelty (habituation).

### 1.4.3 Rotarod

The rotarod is used to assess motor-coordination and balance. In this test the rodent is required to walk upon a rotating drum, the speed of which

can be set to vary depending on the purpose of the experiment. The speed of the drum can also be fixed or set to accelerate over time. Rodents with deficits in motor-coordination and balance will fall off the rotating drum after shorter times or at slower speeds than rodents carrying no deficits. The nature of the rotarod also allows the testing of sedative properties of drugs – sedated rodents will also fall from the rotating drum at slower speeds than those treated with vehicle. The accelerating rotarod allows for improvements in motor coordination as well as deficits, however the fixed speed rotarod is primarily designed to detect depletions in motor performance (Jones & Roberts, 1968).

## *1.5 Functional significance of $GABA_AR$ heterogeneity*

As discussed above, $GABA_AR$ heterogeneity has a strong influence on the pharmacological properties of the receptor. The advance of genetically altered mouse models has allowed the functional significance of this heterogeneity to be expressed *in vivo*. There are two forms of genetically altered mouse model commonly used in electrophysiological and behavioural studies, the first of which is the knock-out mouse. Various knock out models have been developed whereby a specific receptor subunit gene has been deleted or inactivated. However, due to compensatory mechanisms, interpretation of results obtained from knock out mice can be difficult. The second model used is the knock in mouse. Here a point mutation is inserted into the gene encoding for the particular receptor subunit rendering the subunit insensitive to a subunit-selective pharmacological agent without influencing the receptor's function and

ideally the sensitivity to the neurotransmitter (Benson*, et al.*, 1998b; Wieland*, et al.*, 1992). It is perhaps worth noting here that the term knock-in can also be used in reference to a genetic modification in which a known gene is inserted into the genome in its entirety. However, for the purpose of this thesis knock-in refers to the insertion of a point mutation of the receptor. Electrophysiological and behavioural studies using both kinds of genetic manipulation have been used to elucidate the functional significance of the different receptor subtypes and will be discussed further in relation to BDZs, etomidate, THIP and neurosteroids.

### 1.5.1 Benzodiazepines

Using knock-in mice, the subunits responsible for the mediation of many of the behavioural effects of BDZs have been uncovered (Figure 1.9). Knock-in mice carrying the H101R point mutation in the α1 subunit were used to show that the sedative, anterograde amnesic and partially the anticonvulsant actions of diazepam are mediated primarily by a component of α1 containing receptors (McKernan*, et al.*, 2000; Rudolph*, et al.*, 1999). Using a similar approach, α2 and α3 containing receptors have been implicated in the mediation of the anxiolytic effects of BDZ (Dias*, et al.*, 2005; Low*, et al.*, 2000; McKernan*, et al.*, 2000; Yee*, et al.*, 2005). α2 containing receptors have also been associated with the mediation of the myorelaxant and anticonvulsant actions of diazepam (Crestani*, et al.*, 2001; Low*, et al.*, 2000). Recently, both α2 and α3 subunits have been linked with the analgesic effects of BDZs (Knabl*, et al.*, 2008). α5 containing receptors have been shown to underpin

impairments in spatial and temporal memory functions associated with BDZ treatment (Collinson*, et al.*, 2002) and partially the myorelaxant properties of diazepam (Crestani*, et al.*, 2001). However, these specialised *in vivo* studies have highlighted some inconsistencies in the literature, in particular the role of α1 in sedation and α2/α3 in anxiety, and these will be reviewed more closely.

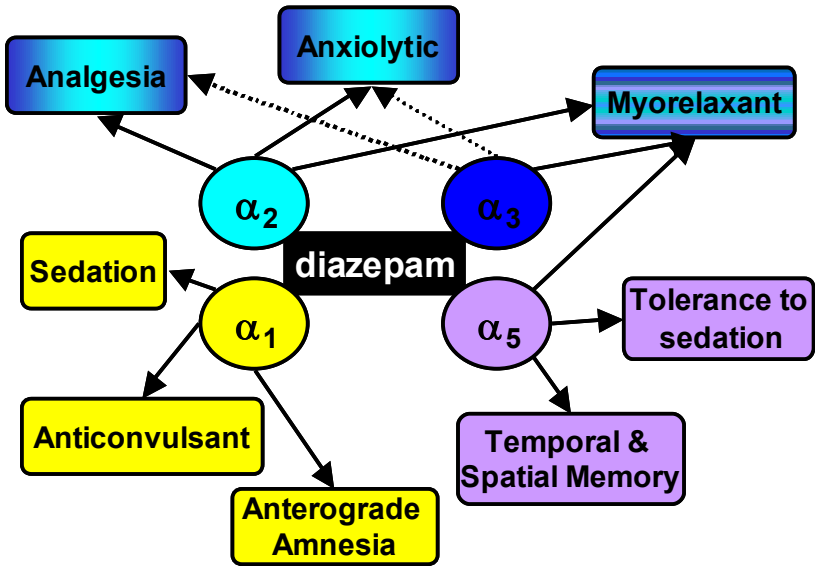


***Figure 1.9: Schematic highlighting the subunits responsible for the mediation of some of the behavioural effects of the benzodiazepine diazepam.***

*The sedative, anticonvulsant and amnesic properties of diazepam have been linked with α1 containing $GABA_AR$. The analgesic, anxiolytic and myorelaxant properties of diazepam have been associated with α2 and α3. Adapted from a diagram provided by J Lambert.*

#### *1.5.1.1 The α1 subunit and benzodiazepines*

Recent *in vivo* studies using transgenic mouse models have produced conflicting evidence for the role of the α1 subunit in the role of the sedative effects of BDZs.

The simplest of these transgenic models is the α1 knockout mouse ($\alpha1^{-/-}$). In the $\alpha1^{-/-}$, the α1 subunit is deleted. The generation of $\alpha1^{-/-}$ mice showed that the genetic removal of this subunit is not lethal to mice (Sur*, et al.*, 2001). In this $\alpha1^{-/-}$ mouse, more than 50% of the $GABA_A$Rs (as measured by $H^3$ muscimol binding) are lost, demonstrating that the $\alpha_1$ subunit is the most abundant in the mammalian brain (Sur*, et al.*, 2001).

Electrophysiological (whole-cell patch clamp) recordings from the Purkinje neurones derived from $\alpha1^{-/-}$ mice revealed the absence of spontaneous GABAergic inhibitory post-synaptic currents. Equally whole cell evoked currents and GABA gated currents are absent (Sur*, et al.*, 2001). Less than 50% of cerebellar Purkinje neurones responded to GABA and those that did showed significantly reduced amplitude and potency when compared to neurones derived from wild type mice (Sur*, et al.*, 2001). Deletion of the α1 subunit leads to a complete reduction of synaptic activity in the ventral basal neurones of the thalamus from postnatal day 18 onwards (Peden*, et al.*, 2008).

The behavioural phenotype of $\alpha1^{-/-}$ mice superficially is similar to that of wild types, however some substantial phenotypic alterations are caused

by the removal of the α1 subunit. $\alpha1^{-/-}$ mice are under represented in litters suggesting embryos/early postnatal pups are less viable than wild type littermates (Sur*, et al.*, 2001). $\alpha1^{-/-}$ pups are less well groomed, although this trait was found to improve post weaning (Sur*, et al.*, 2001). Furthermore, $\alpha1^{-/-}$ mice have lower body weights than their wild type counter parts and this trait persists into early adulthood (Sur*, et al.*, 2001).

One notable phenotypic trait is the presence of an involuntary tremor in the hind legs of $\alpha1^{-/-}$ mice, which is absent in wild types (Kralic*, et al.*, 2005). This tremor is not a resting tremor, but rather appears more like intentional tremor, as it becomes more evident upon tail suspension or movement. Upon muscle relaxation the tremor is no longer detectable. Non-sedative doses of ethanol were found to eliminate this tremor but diazepam and allopregnanolone had no effect in this regard (Kralic*, et al.*, 2005). Possibly, as a result of this tremor, the performance of the $\alpha1^{-/-}$ mouse on the rotarod was reduced to approximately 40% of that of wild types (Kralic*, et al.*, 2005). However, baseline locomotor activity and exploration/habituation to a novel environment were found to not be different from wild types (Reynolds*, et al.*, 2003a; Sur*, et al.*, 2001).

As mentioned above, BDZ site ligands with high affinity for α1-containing receptors, such as zolpidem, are more sedative than those selective for the other α subunits, suggesting that the α1 subtype is important in mediating the sedative effects of BDZs. Somewhat surprisingly then, $\alpha1^{-/-}$ mice are found to be more susceptible to the motor impairing effects of

diazepam, as low doses of diazepam that were found to increase the activity of wild type mice significantly reduced the activity of $\alpha1^{-/-}$ mice (Kralic*, et al.*, 2002a; Reynolds*, et al.*, 2003a). Furthermore, the sedative-hypnotic effect of diazepam, as measured by loss of righting-reflex, was dramatically increased whereas hypnotic effect of zolpidem was reduced (Kralic*, et al.*, 2002a), an observation that may be anticipated by the selectivity of this ligand for the α1 containing subtype.

The anxiolytic effect of diazepam was evident in both wild type and $\alpha1^{-/-}$ mice. Interestingly, the $\alpha1^{-/-}$ mouse showed a greater increase of percentage open arm entries and time in open arms of the EPM following 0.6mg/kg diazepam than wild type mice. This change in behavioural effects of diazepam may be caused by an up regulation of α2 and α3 containing receptors, both of which have been implicated in the mediation of the anxiolytic effects of diazepam (Atack*, et al.*, 2005; Atack*, et al.*, 2006; Dias*, et al.*, 2005; Dixon*, et al.*, 2007; Dixon*, et al.*, 2008; Low*, et al.*, 2000; McKernan*, et al.*, 2000; Morris*, et al.*, 2006). A significant increase in the time spent in open arms and open arm entries was detected in wild types following 10mg/kg diazepam but not in $\alpha1^{-/-}$ mice (Kralic*, et al.*, 2002a). It is plausible that the sedative properties of diazepam may have interfered with the determination of anxiolytic effects of this drug in the $\alpha1^{-/-}$ mouse at this dose.

Knock-out mice provide the simplest way to investigate the functional role of receptor composition *in vivo*. However, this model exhibits clear

disadvantages; for example, genetic compensatory adaptations can occur to correct a subunit deletion, thus complicating interpretation of results from such studies. An alternative, more elegant and subtle way of studying the functional significance of the receptor composition is provided by way of knock-in mice. Knock-in mice carry a specific point mutation of the desired receptor subunit rendering the subunit insensitive to the corresponding ligand. In the case of the α1 subunit, mutation of histidine at the 101 position to an arginine (the α1H101R mutation) renders the receptor insensitive to BDZs.

The α1H101R mouse is generated by a point mutation at the 101 position from histidine (H) to an arginine (R) (McKernan*, et al.*, 2000; Rudolph*, et al.*, 1999). In α1H101R mice, the α1 subunit and all the other major subunits (α2, α3, α5, β2/3 and γ2) of $GABA_ARs$ are expressed at normal levels, and immunohistochemical distribution of the subtypes is also comparable to wild types (Rudolph*, et al.*, 1999). The point mutation results in a decrease in the number of BDZ binding sites (McKernan*, et al.*, 2000; Rudolph*, et al.*, 1999). Gating properties of the H101R receptor were assessed using whole-cell patch clamp recordings from cerebellar Purkinje neurones. The response to GABA has been demonstrated to be the same in α1H101R derived neurones as wild types, but the potentiation of this response by diazepam is reduced in cerebellar Purkinje neurones obtained from α1H101R mice (Rudolph*, et al.*, 1999).

The α1H101R mouse has no differences in weight, balance, feeding, core temperature and baseline locomotor activity/response to a novel environment when compared to wild type mice (McKernan*, et al.*, 2000; Rudolph*, et al.*, 1999). The functional consequences of the α1H101R knock-in are likely to be apparent only in the context of the actions of a BDZ.

The myorelaxant effects of diazepam were tested using the grip strength paradigm. A decrease in grip strength following injection with diazepam was observed in both wild type and α1H101R mice, thus suggesting the myorelaxant effects of diazepam are not mediated by the α1 containing $GABA_A$Rs (McKernan*, et al.*, 2000).

Two independent laboratories have investigated the motor impairing effects of diazepam, as measured using the rotarod. At a speed of 16RPM, the α1H101R mice remained unaffected by diazepam (at doses up to 10mg/kg) and performed better than wild types on the rotarod at all doses tested. The α1H101R mice only became significantly affected by diazepam at 30mg/kg (McKernan*, et al.*, 2000). In contrast, at a lower speed (2RPM) α1H101R mice were found to be susceptible to the motor impairing effects of diazepam to the same extent of wild types (Rudolph*, et al.*, 1999).

Diazepam significantly reduced the locomotor activity of wild type mice; however, no reduction in locomotor activity was observed in α1H101R

mice at doses below 30mg/kg (McKernan*, et al.*, 2000). In fact, in a novel environment, a dramatic *increase* in locomotor activity was observed in α1H101R mice following treatment with 3mg/kg diazepam (Crestani*, et al.*, 2000b; McKernan*, et al.*, 2000). This increase in activity may reflect reduced anxiety in the novel environment unmasked by the loss of sedation apparent in diazepam treated α1H101R mice. Alternatively, it is conceivable that the alerting/arousing effect of the novel environment again reduced the impact of the sedating and motor impairing effects of diazepam in much the same manner as it appeared to in the rotarod test. Consistent with this suggestion is the fact there was no increased activity in α1H101R mice when tested in a familiar environment. This increase in activity was not observed in α1H101R mice tested in a familiar environment (Rudolph*, et al.*, 1999).

In the light/dark choice test of anxiety, both wild type and α1H101R mice have been shown to exhibit a similar increase in the amount of time spent in the light compartment following treatment with diazepam (Rudolph*, et al.*, 1999). Both genotypes also show an increase in the percent of open arm entries and time in open arms following treatment with diazepam in the EPM (Rudolph*, et al.*, 1999).

The anticonvulsant effect of diazepam (as measured by pentylenetetrazole-induced tonic convulsions) was vastly reduced in the α1H101R mouse when compared with wild types (Rudolph*, et al.*, 1999).

Diazepam is known to potentiate the sedative effects of ethanol and this effect was observed in both α1H101R mice and wild type mice (Rudolph*, et al.*, 1999)

Diazepam is also known to be amnesic, but using a step-through passive avoidance test, this effect of diazepam was absent in α1H101R mice, with the mice failing to show the expected reduction in time spent in the punished environment (Rudolph*, et al.*, 1999).

Evidence from the $\alpha1^{-/-}$ and the α1H101R mice to the sedative actions of BDZs is somewhat contradictory. A direct comparison of these mice would be helpful in unravelling the origins of the differences between these mice.

#### *1.5.1.2 The α2 and α3 subunits and the anxiolytic effects of benzodiazepines*

α2 and α3 point mutated mice (α2H101R mice and α3H126R mice) show the same sedative and motor impairing effects of diazepam as observed in wild type mice (Low*, et al.*, 2000). However, by using the light/dark choice test and the EPM, it was shown that in α2H101R mice there was no anxiolytic effect of diazepam (Low*, et al.*, 2000). In contrast, when treated with diazepam, α3H126R mice were still similarly susceptible to the anxiolytic effects of diazepam as wild types (Low*, et al.*, 2000). This evidence suggested that α2 containing $GABA_A$Rs are responsible for the anxiolytic properties of BDZs. Contrasting pharmacological evidence has

shown that α3 containing $GABA_A$Rs are additionally responsible for mediating the anxiolytic properties of BDZs. An inverse agonist ($\alpha_3$IA) that is specific for α3 containing $GABA_A$Rs was shown to have anxiogenic (increased anxiety) effects on wild type rats in the EPM (Atack*, et al.*, 2005). An α3 selective agonist (TP-003), which has no affinity for α1 containing receptors (therefore does not result in sedation), has been shown to have anxiolytic effects in rodents and non-human primates (Dias*, et al.*, 2005). It has been suggested that the receptor subunit responsible for the mediation of anxiolytic properties is largely dependent on receptor occupancy. TP-003 requires a receptor occupancy of at least 75% therefore it is thought that at lower occupancy α2 may be mediating the anxiolytic effects or potentiate the effects mediated by α3 containing $GABA_A$Rs. Using a conditional emotional response (CER) paradigm, it has been shown again that both α2 and an unidentified subunit are partly responsible for the anxiolytic properties of BDZs (Morris*, et al.*, 2006). Diazepam no longer had an anxiolytic effect on α2H101R mice in the CER task again suggesting that α2 is responsible for the anxiolytic effect of these drugs (Morris*, et al.*, 2006). However, a compound (L-838417), which has agonist properties at α2, α3 and α5, maintains its anxiolytic nature in α2H101R, mice again suggesting that α3 is important in the anxiolytic properties of BDZs.

### 1.5.2 Etomidate

Using knock-in mice the subunits responsible for the mediation of many of the behavioural effects of the anaesthetic etomidate have been

uncovered (Figure 1.10). Knock-in mice carrying the N265S point mutation in the β2 or β3 subunit have shown that the sedative and hypnotic effects of etomidate are mediated by the β2 containing receptors whereas the β3 containing receptor mediates the immobility-inducing effects and to a lesser extent the hypnotic effects of this anaesthetic (Jurd*, et al.*, 2002; Reynolds*, et al.*, 2003b; Rudolph and Antkowiak, 2004a). In addition, hypothermic action and respiratory depressant actions of etomidate have been linked to β2 and β3 containing receptors (Cirone*, et al.*, 2004; Zeller*, et al.*, 2005).

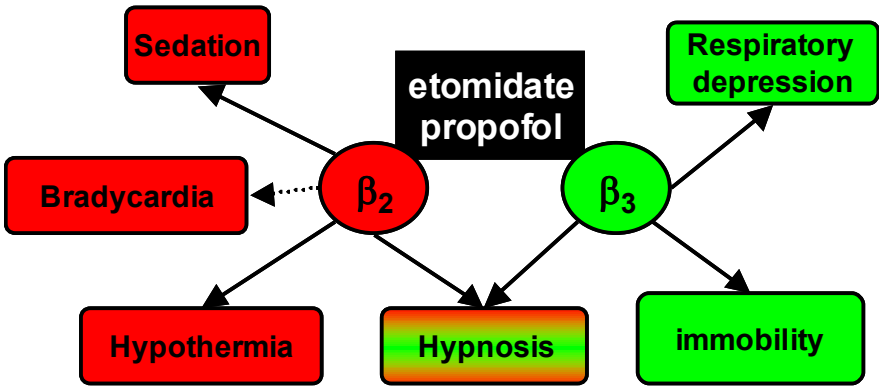


***Figure 1.10: Schematic demonstrating the subunits implicated in some of the behavioural effects of the anaesthetics etomidate and propofol.***

*The identity of β subunit isoform dictates the properties of etomidate and propofol. Adapted from a diagram provided by J Lambert.*

### 1.5.3 THIP

As discussed above, THIP is selective for δ containing $GABA_A$Rs. Interestingly, this is in contrast to BDZs, as their sedative actions are mediated through the α1 and γ2 subunits containing receptor isoforms, which have typically a synaptic localisation.

In wild type mice, THIP induces dramatic alterations in the electroencephalography (EEG) profile during the wakeful period and non-rapid eye movement (nREM) periods of the sleep-wake cycle. These changes are absent in the $\delta^{-/-}$ mouse suggesting a role for these extra synaptic $GABA_A$Rs in mediating the sleep enhancing properties of THIP (Winsky-Sommerer*, et al.*, 2007). THIP (30mg/kg s.c.) produces a loss of righting reflex (LORR) in mice. In $\delta^{-/-}$ mice the latency to the onset of LORR is increased and the duration of LORR decreased suggesting the δ containing $GABA_A$Rs are, at the very least, involved in mediating the anaesthetic actions of THIP (Boehm II*, et al.*, 2006). Although little work has been carried out investigating the other behavioural effects of THIP in $\delta^{-/-}$ mice, a comprehensive study has reported on the behavioural effects of THIP in the $\alpha4^{-/-}$ mouse, this subunit is known to pair with δ in the thalamic nuclei and dentate gyrus granule cells. The ataxic effects of THIP, as measured by fixed-speed rotarod, were abolished (10mg/kg) and significantly reduced (15mg/kg) in $\alpha4^{-/-}$ mice. Furthermore, the sedative effects, as measured by a decrease in locomotor activity, of THIP (10mg/kg) were also abolished. Furthermore, $\alpha4^{-/-}$ mice were largely insensitive to the analgesic effects of this drug as measured by the

radiant tail-flick assay (Chandra*, et al.*, 2006). Behavioural data from the $\delta^{-/-}$ mouse are lacking in this respect.

### 1.5.4 Neurosteroids

As discussed above, recent evidence suggests that neurosteroids, although not as subtype selective as the other pharmacological agents can potentiate the function of α4δ and α1δ. In agreement with this observation, $\delta^{-/-}$ mice are less susceptible to the sleep inducing effects of alphaxalone and pregnanolone and the anxiolytic effect of ganaxalone (Mihalek*, et al.*, 1999).

Using a sleep time assay, it has been shown that $\delta^{-/-}$ mice are resistant to the sleep inducing effects of alphaxalone (8 and 16mg/kg, i.v.) and pregnanalone (8mg/kg, i.v.) and the anxiolytic effect of ganaxalone was found to be absent in $\delta^{-/-}$ mice (Mihalek*, et al.*, 1999). Low dose ganaxalone, known to prolong pentylenetetrazol (PTZ) seizures, was less effective in $\delta^{-/-}$ mice (Mihalek*, et al.*, 1999).

## *1.6 Genetically modified mice*

Genetically modified mice are commonly being used to establish links between specific genes and their control on brain functions and behaviour. The mouse makes an important model organism for these behavioural studies because it allows for the investigation of relationships between specific behaviours and the electrophysiological, biochemical or cellular mechanisms which underpin them. However, studies (and their subsequent interpretation) using genetically modified mice can be hampered by a variety of factors including genetic background (including breeding strategies and the "flanking gene" problem), laboratory environment, experimental design (including the sex and age of the animals) and pleiotropy. There are a number of ideals which can be aspired in regards to these factors and these factors and potential solutions have been reviewed extensively in the literature (Bailey*, et al.*, 2006; Brown, MJ and Murray, 2006; Crabbe*, et al.*, 1999; Kafkafi*, et al.*, 2005; van Meer and Raber, 2005; Wolfer*, et al.*, 2002; Yoshiki and Moriwaki, 2006). These ideals, however, are not always practical for a number of reasons such as availability of space for colonies, staff availability and financial.

### 1.6.1 Genetic background

In 1996 a Banbury workshop produced a series of recommendations, which were subsequently published in 1997, on the genetic background in genetically modified mice (Silva*, et al.*, 1997). Some of these recommendations are of particular interest to this thesis.

Controlling for the genetic background of modified mice is essential. It is known that the behavioural profile of mice is dependent on strain (see for example: Balogh*, et al.*, 1999; Colacicco*, et al.*, 2002; Crawley*, et al.*, 1997; Homanics*, et al.*, 1999; Rodgers*, et al.*, 2002; Wehner and Silva, 1996). Indeed, some mutations result in different phenotypes when the genetic background is different (some examples are given in: Doetschman, 2009 and Silva*, et al.*, 1997). For this reason the genetic background of mice should be known and easily reproduced. In the ideal scientific community mice would be maintained on a common genetic background to improve inter – laboratory reliability. The mutated line should not be maintained by continuous inbreeding of homozygous individuals from an F2 generation as this could result in genetic drift and likely phenotypic differences, which may not be as a direct result of the mutation of interest. For this reason, mice should be maintained in congenic lines maintained by backcrossing on both C57/BL6 and/or 129 backgrounds (both strains are commonly used in the generation of genetically modified mice). It is worth noting however that it has since been suggested that limiting to the C57/BL6 or 129 backgrounds may in itself limit the detection of effects of given mutations, and a background strain should be selected on solid and informed genetic basis (Crawley*, et al.*, 1997; Frankel, 1998). Ideally mice for phenotyping would be the result of the crossing of two congenic lines with a defined hybrid F1 background. However, the characterisation of F2 mice produced from the crossing of the original chimera and C57/BL6, followed by inter-crossing of their heterozygous offspring can be a good conciliation between the

important control of genetic background and pressures on space, time and finances. Indeed, this is the technique used throughout this thesis.

## *1.7 Thesis Aims*

This thesis forms part of a larger on going study to pharmacologically and behaviourally characterise mice carrying mutations of $GABA_AR$s to further understand the functional significance of $GABA_AR$ heterogeneity and the role of GABAergic inhibition in behaviour. The main aims of this thesis are:

1. To investigate the differences between $\alpha1^{-/-}$ and α1H101R mice in regards to the sedative properties of BDZs.
2. To investigate the role of the extrasynaptic $GABA_AR$ in the behavioural effects of THIP.
3. To develop mouse behavioural models in the laboratory and use these to further understand the functional role of $GABA_AR$ heterogeneity.

To achieve these aims, behavioural studies of mice lacking or carrying point mutations of the synaptic, widely distributed α1 subunit ($\alpha1^{-/-}$ and α1H101R) and mice lacking the extrasynaptic, regionally restricted δ subunit ($\delta^{-/-}$) have been carried out.

In Chapter 3, a direct comparison of the effects of BDZs in $\alpha1^{-/-}$ and α1H101R mice is performed. In Chapter 4, the behavioural effects of THIP in $\alpha1^{-/-}$ and $\delta^{-/-}$ mice are investigated.

In Chapter 5, the potential cognitive enhancing properties of THIP are investigated using an attentional set shifting task for mice. This task is optimised for mice in Chapter 5 and also used to assess attentional set shifting in $\delta^{-/-}$ mice. Further behavioural analysis of the $\delta^{-/-}$ mouse is carried out in Chapter 6.

# 2 General Methods

*“The multidisciplinary nature of behavioural neuroscience has attracted contributions from diverse sources, but this had led to problems. Almost every laboratory has its own preferred methods of studying behavioural processes, and the newcomer is faced with a bewildering choice of techniques to measure ‘motor activity’, ‘motivation’, and the like”*

Sahgal, 1992

In Behavioural Neuroscience II

## *2.1 Animals*

The experimental subjects used in this thesis were wild type (WT) and genetically altered (GA) mice. Mice were aged between 3-6 months at the time of behavioural testing and weighed approximately 20-40 grams. They were group housed (litter mates), with environmental enrichment in the form of cardboard tubes, houses and nesting material. At all times in the home cage they had free access to standard rodent chow and water. The colony was maintained on a 12 hr. alternating light/dark schedule, with lights on at 6:30 AM (380+/-20 Lux). All animal procedures were carried out under UK Home Office license (Animal (Scientific Procedures) Act 1986).

In studies using only WT mice, C57 black subline 6 (C57/BL6J) mice were used (Charles River, UK). In studies using GA mice, they were either lacking the α1 or δ subunits of the $GABA_AR$ ($\alpha1^{-/-}$ and $\delta^{-/-}$ respectively) or carrying a point mutation from histidine to arginine at the 101 position (α1H101R knock in mice). The $\alpha1^{-/-}$ mice, α1H101R and WT controls were generated on a mixed C57BL/6J-129SvEv background at the Merck Sharp and Dohme Research Laboratories at the Neuroscience Research Centre in Harlow (Sur *et al.*, 2001; McKernan *et al*, 2000). In the experiments reported here the same WT controls were used for both $\alpha1^{-/-}$ and α1H101R mice. These WT controls were bred by backcrossing onto the C57BL/6J genetic background for more than 10 generations. Mice used for experiments were obtained from homozygous WT breeding

pairs bred for two generations from the corresponding heterozygous breeding pairs maintained at the University of Dundee, Medical School Resource Unit. $\delta^{-/-}$ mice and WT controls ($\delta^{+/+}$) were generated on a mixed C57BL/6J-129Sv/SvJ background at the University of Pittsburgh (Mihalek *et al.*, 1999). Experiments were conducted on the first two generations of homozygous-bred WT, $\alpha1^{-/-}$, α1H101R and $\delta^{-/-}$. Breeding pairs were derived from the corresponding heterozygous mice bred at the University of Dundee, Medical School Resource Unit.

### 2.1.1 Genotyping

The mice were supplied to this project with a known genotype[1] however a brief description of the procedures used is provided. Genotyping was carried out using a genotyping kit from Sigma (Extract-N-Amp Tissue PCR kit, Sigma-Aldrick, UK). A biopsy was taken from the ear of each mouse using an ear punch (Vet Tech, UK) and sample collected into sterile epindorf tube. To extract DNA 100μl of extraction solution and 25μl of tissue preparation solution was added to each ear sample. Samples were mixed using a vortex and then briefly centrifuged (10 seconds) to ensure ear tissue was in the extraction solutions. Samples were then incubated at 21ºC for 10 minutes and then at 95ºC for 3 minutes using a heat block. 100μl of neutralisation solution was then asses to each sample and samples were stored on ice. For each sample 15μl of PCR solution (10μl Reaction Mix, 0.3μl of forward primer, 0.3μl of reverse

[1] Although NF carried out this procedure to learn the technique for practical purposes the genotyping of mice was largely carried out by members of Professor J Lambert and Dr D Belleli's laboratories.

primer, 0.1μl of neomycin cassette reverse primer and 4.3μl of double distilled $H_2O$) was prepared and 5μl of extracted DNA was added to the PCR solution in sterile PCR tubes. Samples were then briefly mixed using a vortex before being placed in PCR machine. The PCR programme used consisted of 1 cycle at 94ºC for 5 minutes, 35 cycles of 94ºC for 30 seconds, 60ºC for 30 seconds and 72ºC for 7 minutes, 1 cycle at 72ºC for 7 minutes and a final hold at 4ºC. To each PCR sample 4μl of 6X loading buffer was added. The PCR product and a 1Kb ladder were run on a 1% agarose XTBE agarose gel, 1.5% ethidium bromide was used for visualisation under ultra violet light. The position of the bands on the gel were used to identify knock out versus wild type animals.

## *2.2 Behavioural Testing*

The following behavioural tests are common to Chapters 3, 4 and 6. Details for each individual experiment are described in the appropriate Chapters.

### 2.2.1 Locomotor Activity

The locomotor activity of mice was measured using an Activity Monitor (Benwick Electronics, Norfolk, UK) using infrared light beams to detect movement (Figure 2.1). The chamber was opaque plastic with dimensions of 32 x 20 x 19cm. Arranged to form a grid covering the length and width of the cage were two sets of beams. The lower beams were 2cm above the grid floor and so could detect the animals' horizontal locomotion. The upper set of beams were 7cm above the lower beams, and were positioned for optimal detection of rearing. The beams were arranged in a 1inch grid. Activity was measured by beam breaks and recorded as counts of activity. A mouse was considered mobile if the central position of the animal changed by more than 2inches, i.e. if there were two consecutive beam breaks, but not if the same beam was broken twice; this reduced the effect of static movements (such as head bobbing and grooming) on the measure of locomotor activity. To habituate the animals to the testing box each animal was placed in the activity monitor for 20mins over a series of days and the locomotor activity was logged in 5 bins. Two forms of habituation are observed in rodents, the first is when mice habituation within session (intrasession) where the rodent becomes familiar to the environment and a reduction in locomotor activity is

observed over time within the single session. The second is when rodents habituate between sessions (intersession) where the rodent will show a reduction in locomotor activity between sessions if they have memory of the previous exposure to the testing environment. Locomotor activity can therefore be used to evaluate the extent and rate of habituation (for a comprehensive review of habituation in rodents, see Leussis & Bolivar, 2006). Activity was also measured after drug administration and used as an indicator of sedation induced by drugs presumed to be sedative.

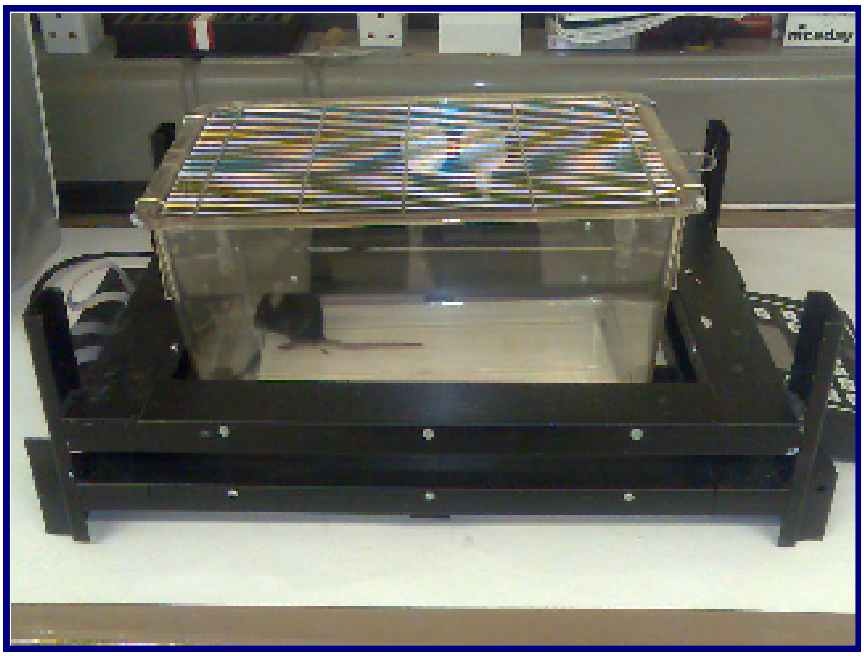

***Figure 2.1: Photograph showing activity monitor and cage used to measure locomotor activity.***

### 2.2.2 Elevated Plus Maze

The maze used here was made of black Perspex (Figure 2.2). There are four arms, each arm is 5cm wide and extends 50cm from a central, 5 x 5cm, 'start location' where the four arms meet. The entire maze is elevated to a height of 90cm.

Mice were placed on the central platform facing a closed arm and their movements over 5mins were recorded using a digital video camera mounted on a static tripod. This allowed the experimenter to observe the behaviour without being close to the experimental area. The videos were watched and scored by two observers, the first (NF) was aware of the drug treatment/mouse genotype and the second (SV) was independent and blind to condition[2]. The number of open and closed arm entries and time in open and closed arms were recorded from the videotapes. All double scoring was analysed for inter-rater reliability and found to have a high correspondence ($p > 0.05$).

Given the value of transgenically altered mice and to reduce the number of animals used for experimental purposes, repeated measure designs were utilised at times in this thesis. To see if there was any effect of test session, the first and second exposures were included as a factor ('session') in the statistical model for analysis. In experiments where a repeated measures design was utilised, mice were exposed to the EPM

2With thanks to Svetlana Vronskaya for second scoring of EPM videos.

only twice and second exposure took place at least 7 days after the first to reduce the effect of the first exposure upon the second.

*Figure 2.2: Photograph showing elevated plus maze.*

### 2.2.3 Open Field

In the present experiments, the open field that was used was made from clear Plexiglas measuring 90cm x 90cm with 50cm walls (Figure 2.3). The open field was designed such that it was possible to change the visual environment within the maze. For example, the clear walls and base allowed the addition of a high contrast pattern. Open field behaviour was measured using a digital video recorder mounted on a static tripod. As a measure of anxiety, the number of visits to the centre of the open field and the amount of time spent by the external walls (thigmotaxis) was measured. Again videos were double scored, once by an observer who was blind to mouse condition/genotype and once by NF. All double scoring was analysed for inter-rater reliability and found to have a high correspondence ($p > 0.05$).

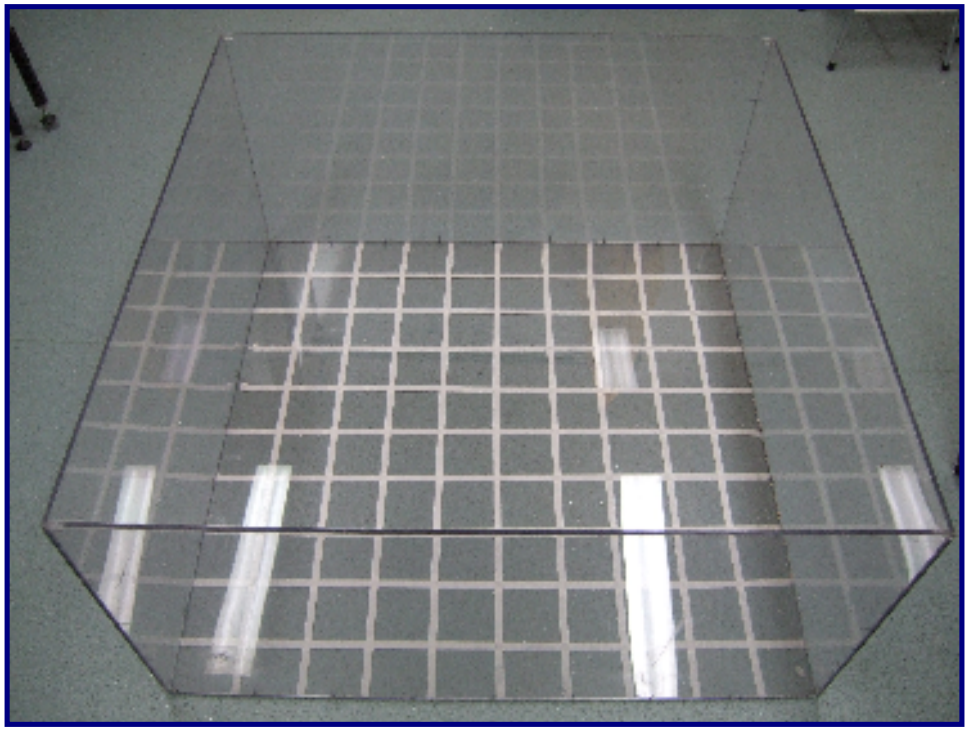

*Figure 2.3: Photograph showing open field.*

### 2.2.4 Rotarod

The rotarod is used to assess motor coordination and therefore can detect ataxic effects of drugs or other treatments, including genetic alterations (Dunham & Miya, 1957). The rotarod consists of a horizontal beam with dividers to keep multiple mice apart from (and out of sight of) each other. The rotarod can be used in two different ways. When used with a fixed speed, mice are repeatedly, over 2 60min sessions (2 separate days), placed onto the bar, which is rotating at 6rpm until they are able to remain on the rod for at least 180secs. On the day, of test the mice are placed back on the rotarod at the same speed and tested at various points (up to 150mins post injection) in 15min intervals. The time on the rotarod at each time point is recorded (maximum 180secs).

Alternatively, the rod can be made to accelerate: the mouse is placed on the rotarod rotating at 6rpm until they remained on the rod for 180secs. On the day of test, mice are placed on the rotarod and the speed is accelerated by 4.4rpm/min to a maximum speed of 50rpm and maximum time of 10mins. The accelerating rotarod allows for improvements in motor coordination as well as deficits; however the fixed speed rotarod is primarily designed to detect depletions in motor performance (Jones & Roberts, 1968). The rotarod (Ugo Basile, Italy) used in these experiments consisted of a 3cm diameter rod split into 5 sections, allowing the testing of up to 5 mice at once (Figure 2.4). Below the rods were pressure plates so that when the mouse fell from the rod the plate would be flipped and the inbuilt timer stopped.

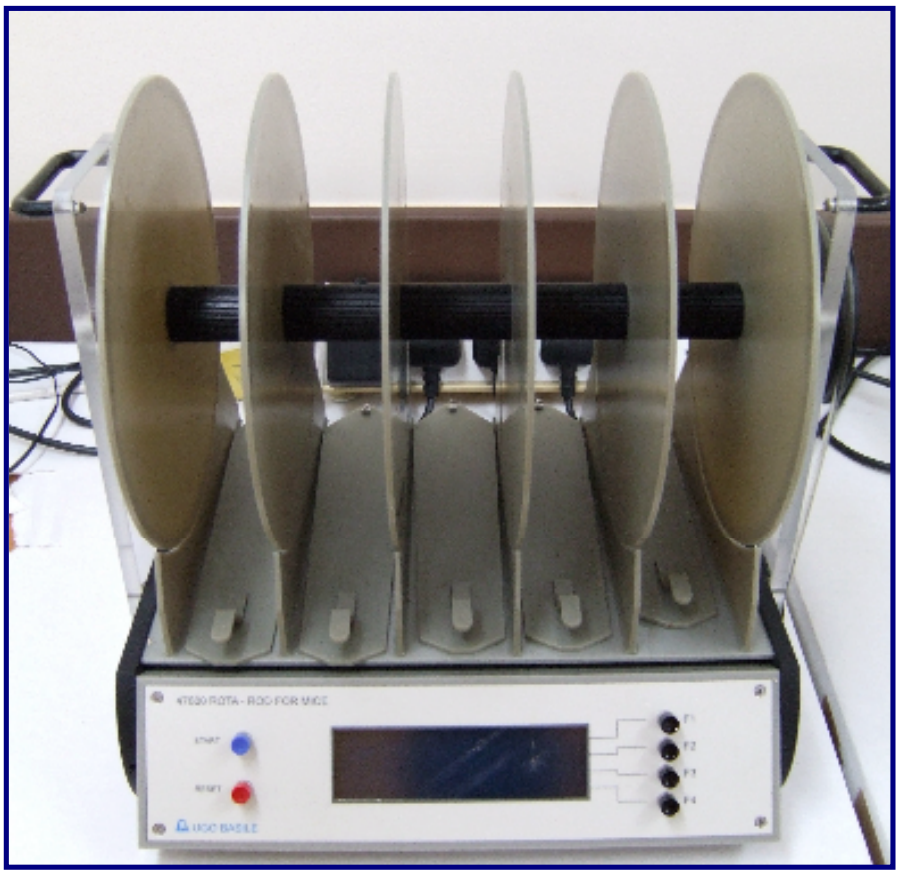

*Figure 2.4: Photograph showing rotarod.*

## 2.3 Drug Preparation

All drugs (see Table 2.1) were prepared daily and administered by intraperitoneal injection (i.p.) at a maximum volume of 100µl/10g of mouse.

| Drug Name | Generic Name | Source | Presumed Action | Dose Range | Vehicle Concentration |
|---|---|---|---|---|---|
| Chlordiazepoxide | Librium | Sigma UK | Anxiolytic Sedative | 5 - 30mg/kg | 0.9% NaCl |
| Etomidate | Hypnomidate | Janssen-Cilag Ltd, UK | Sedative Anaesthetic | 5mg/kg | 17.5% propylene glycol |
| Alphaxalone | Alfaxan | Vetoquinol UK Ltd | Sedative Anaesthetic Anxiolytic | 2.5 - 20mg/kg | 6.4% 2α-hydroxypropyl-β-cyclodextrin |
| THIP | Gaboxadol | Bjarke Ebert (Lundbeck) | Hypnotic Anxiolytic | 2 - 30mg/kg | 0.9% NaCl |

***Table 2.1: Table showing drugs used in this thesis.***

*Generic name, source, presumed action, dose range used in this thesis and vehicle information is provided.*

# 3 The sedative and anxiolytic properties of chlordiazepoxide in $\alpha1^{-/-}$ and α1H101R mice

*"Although BZ-site agonists are proven safe and efficacious drugs, one key issue limiting their utility is their multiple actions. Here we used gene-targeting approaches to demonstrate that the α1 subtype of the GABAA receptor was mainly responsible for the sedative/motor effects of non-selective BDZ such as diazepam, whereas other subtypes were primarily responsible for the anxiolytic activity."*

McKernan, et al., 2000

*"Deletion of the α1 subunit increased the sensitivity to the motor-impairing effects of diazepam. This result may indicate an alteration in benzodiazepine-induced sedation, muscle relaxation or both."*

Kralic*, et al.*, 2002a

## *3.1 Objective*

To investigate the role of the α1 subunit in the sedative actions of BDZs using $\alpha1^{-/-}$ and α1H101R mice.

## *3.2 Introduction*

As discussed in Chapter 1 (see Section 1.3.1.1), there is contradictory evidence from $\alpha1^{-/-}$ and α1H101R mice as to the role of the α1 receptor subtype in the mediation of the sedative actions of BDZs. Although evidence from studies of α1H101R mice suggests that the α1 subunit mediates the sedative effects of BDZs (Crestani*, et al.*, 2000a; McKernan*, et al.*, 2000; Rudolph*, et al.*, 1999), the $\alpha1^{-/-}$ mouse appears to be sedated to a greater extent by BDZs (Kralic*, et al.*, 2002a; Reynolds*, et al.*, 2003a). Laboratory differences may account for the conflicting results and these two transgenic models have not been directly compared under identical experimental conditions.

Therefore, to investigate the differences in the literature, a direct comparison of the baseline locomotor activity, exploratory behaviour and anxiety (as measured by the EPM) of these two transgenic models has been carried out. Further to this, an investigation of the functional role of the α1 subunit in the sedative properties of the BDZ chlordiazepoxide is made using locomotor activity as a measure of sedation. A comparison of anxiolytic properties of chlordiazepoxide in these mice has also been carried out to help elucidate possible causes of differences.

## *3.3 Methods*

### 3.3.1 Animals

α1H101R, $\alpha1^{-/-}$ and wild type mice from the same colony were used for all experiments. The wild types for α1H101R and $\alpha1^{-/-}$ mice were the same (for further information on mice see Section 2.1). Mice were all adult, aged between 3-5 months, and weighing approximately 20-30 grams. Both male and female mice were used. All animals were maintained in similar environmental conditions, with nesting material and cardboard tubes as environmental enrichment.

### 3.3.2 Locomotor Activity and Sedation

The locomotor activity of 35 (18♂/17♀) mice (12 {6♂/6♀} α1H101R, 11 {5♂/6♀} $\alpha1^{-/-}$ and 12 {6♂/6♀} wild types) was measured using the methods described in Section 2.2.1. To habituate the animals to the testing chamber, each animal was placed in the chamber for 20mins per day for 12 consecutive days logged in 4 bins of 5mins each, so the data could be analysed with session quartile as a factor. Following 12 days of habituation, each mouse was then injected with 1 of 3 doses of chlordiazepoxide (5mg/kg, 15mg/kg, or 30mg/kg) or its saline vehicle (0.9% NaCl) by the intraperitoneal route (i.p.). Locomotor activity was measured for 20mins starting 30mins after the injection, the stage at which blood levels of chlordiazepoxide could be expected to be at plateau. Each mouse received all 4 treatments using a counter-balanced design with at least 48h intervals between each injection.

Both counts of beam breaks of the lowest horizontal beams (indicating locomotion) and counts of vertical beam breaks (indicating rearing) were recorded. By looking at rearing, one can infer differences in exploration and potentially account for any differences in changes in locomotion, for example invoking changes in patterns of exploration and/or habituation.

### 3.3.3 EPM

Following a 7-day rest period, each mouse was then treated with either 10mg/kg chlordiazepoxide or its saline vehicle (0.9% NaCl, i.p.). 30mins after injection each mouse was exposed to the EPM for 5mins (for more details on EPM protocol, see Section 2.2.2). Following another period of rest (again 7 days), the mice were then injected with either 10mg/kg chlordiazepoxide or its saline vehicle so that those that received chlordiazepoxide 1 week earlier received saline vehicle and vice versa. This procedure was carried out using a counter balanced design. The amount of time spent in open arms and the number of open arm entries were used as indications of anxiety (Section 2.2.2).

### 3.3.4 Analysis

Data are presented as the mean with standard error of the mean (±SEM). Mixed ANOVAs were used to look for interactions and significant differences between groups. The Mauchly's test for sphericity was used and when appropriate the Huynh-Feldt Correction for heterogeneity of variance was applied (adjusted p values will be reported). Post-hoc

analyses were carried out using planned comparisons where appropriate. Statistical analysis was carried out using SPSS for windows version 12.

## *3.4 Results*

### 3.4.1 Baseline Locomotor Activity and Habituation

There were no significant differences between the baseline locomotor activity of male and female mice (Figure 3.1) in either genotype confirmed by a lack of a genotype x sex interaction (interaction of genotype and sex: $F(1,29) = 3.3$, n.s.) and no main effect of sex ($F(2,29) = 0.23$, n.s.). Therefore the data from male and female mice were pooled for analysis.

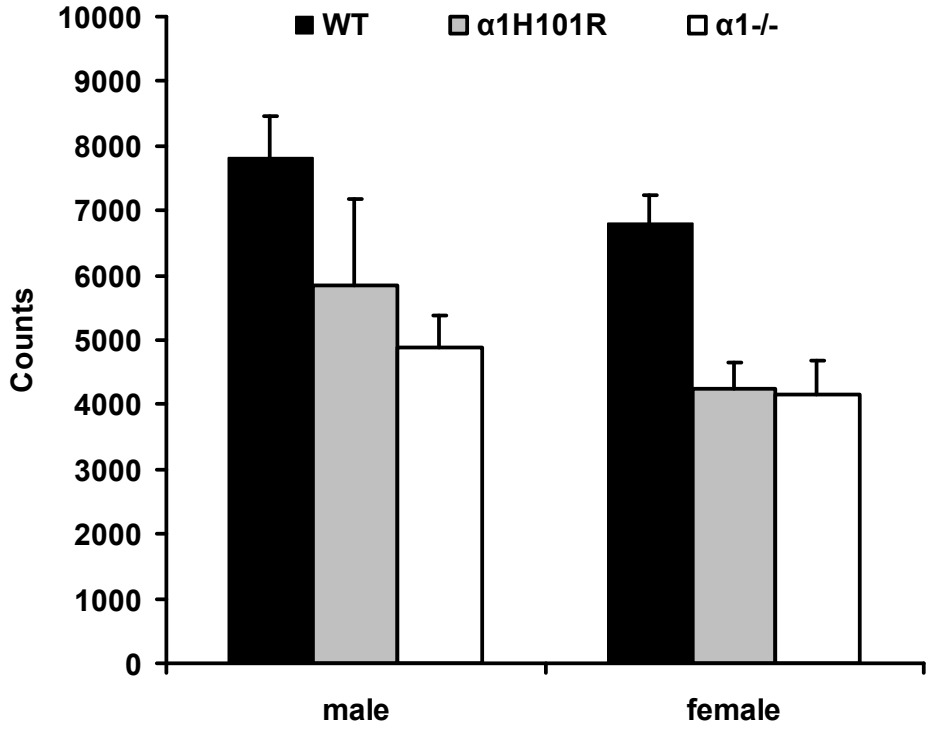


***Figure 3.1: Locomotor activity is not affected by sex.***

*Histogram showing total number (±SEM) of locomotor activity counts as measured by beam breaks recorded over 12 day habituation period in male and female wild type (WT, n = 12), α1H101R (n = 12) and $\alpha 1^{-/-}$ (n = 11) mice. No differences in activity levels were found between males and females in any genotype ($p > 0.05$).*

Overall, there was both inter- and intra-session habituation over the 12 days of measurement, with a decrease in activity during each session, which diminished as locomotion declined generally over days. However, this pattern was genotype dependent as reflected in a 3-way interaction of genotype, day and time (days x time x genotype, $F(66,1056) = 1.68$, $p < 0.01$). As can be seen in Figure 3.2, on day 1, α1H101R mice showed an over all lower level of activity than either the $\alpha1^{-/-}$ or wild type mice (main effect of genotype, $F(2,32) = 5.3$, $p < 0.01$). This effect of genotype accounted for 44% of the total variance. Also, on day 1, all genotypes showed a reduction in locomotor activity throughout the session confirmed by a significant main effect of time ($F(3,96) = 46.8$, $p < 0.01$) which accounted for 42% of the total variance. An interaction of genotype and time ($F(6,96) = 2.3$, $p = 0.04$) accounts for only 2% of the variance on day 1 and is likely due to the greater decline in locomotor counts over the 4 times in the $\alpha1^{-/-}$ mice when compared to α1H101R and wild type mice. The $\alpha1^{-/-}$ mice showed a greater decline in locomotor activity over the 12 days compared to the other groups, such that by day 12 (Figure 3.3) they had the lowest levels of activity (confirmed by a main effect of genotype ($F(2,32) = 7.5$, $p < 0.01$, which accounted for 66% of the variance). By day 12, there was still a decline in locomotor counts evident throughout the session (main effect of time, $F(3,96) = 12.9$, $p < 0.1$, accounting for 20% of the variance), which was not affected by genotype (interaction of genotype and time: $F(6,96) = 1.4$, n.s.).

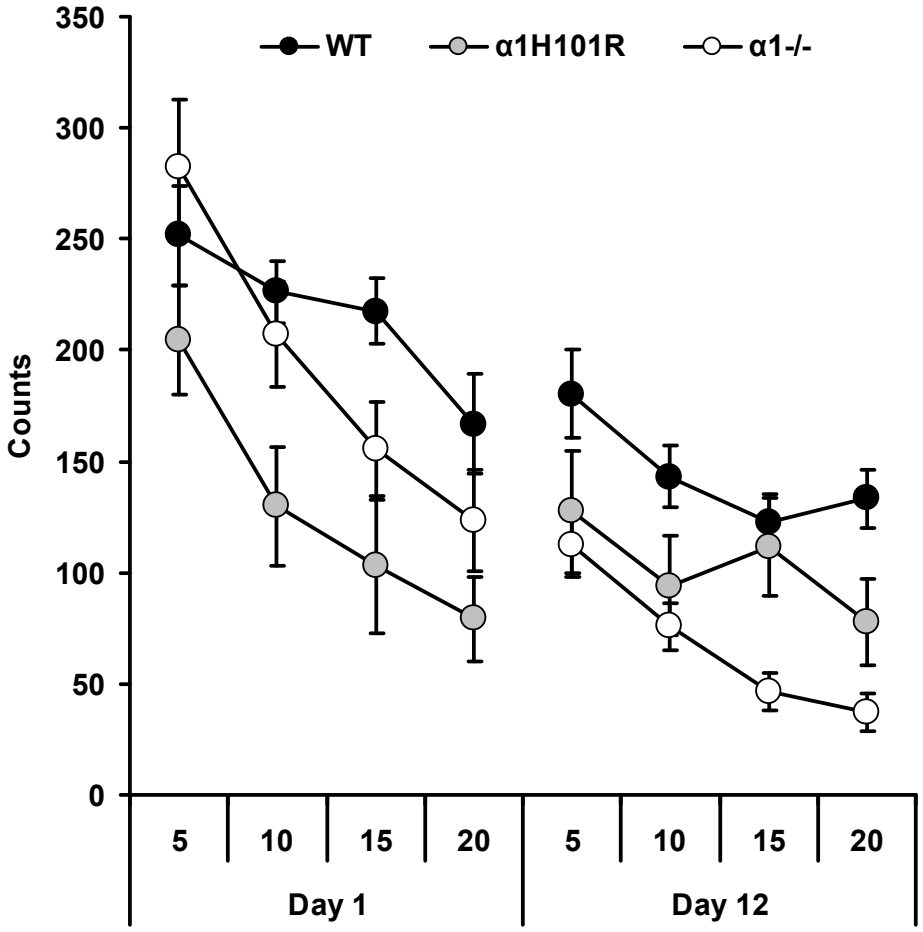


***Figure 3.2: Locomotor activity reduced within session in wild type, α1$^{-/-}$ and α1H101R mice.***

*Line graph showing the number (±SEM) of locomotor activity counts recorded as recorded by beam breaks on day 1 and day 12 of habituation in wild type (WT, n = 12), α1$^{-/-}$ (n = 11) and α1H101R (n = 12) mice. The locomotor activity of all mice was lower on day 12 when compared with day 1. Locomotor activity was dependent on genotype and time ($p < 0.01$).*

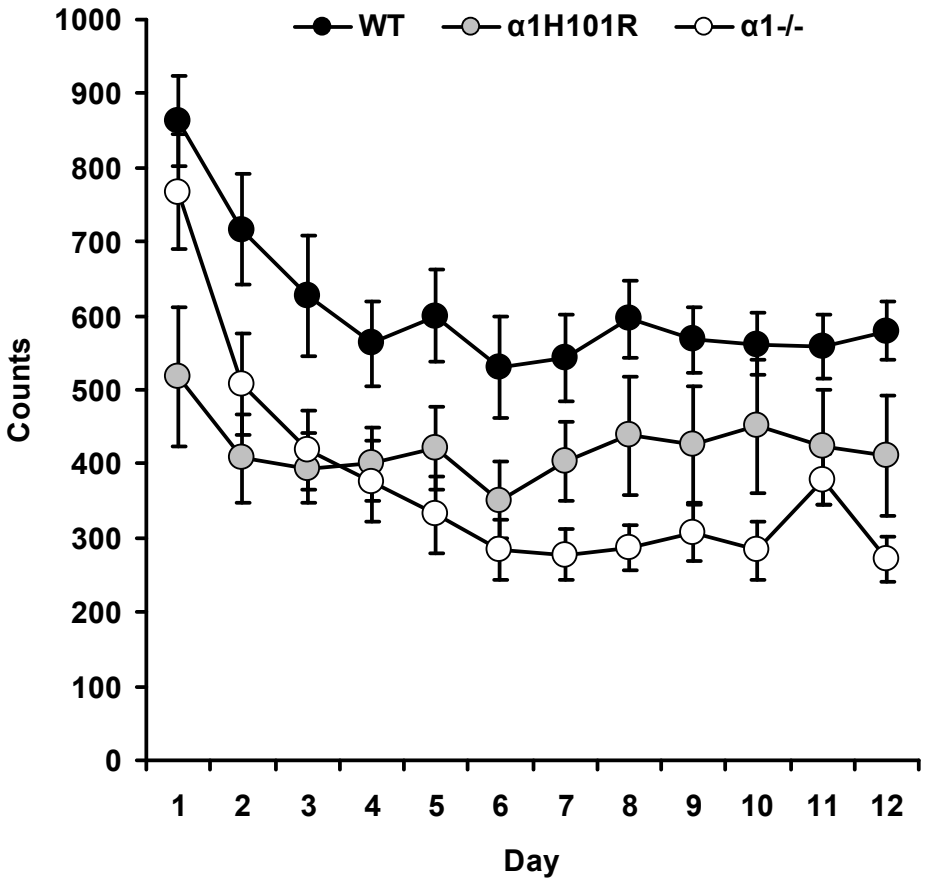


***Figure 3.3: Locomotor activity reduced over day in wild type and $\alpha 1^{-/-}$ mice but not α1H101R mice.***

*Line graph showing the mean (±SEM) number of locomotor activity counts as measured by beam breaks recorded over 12 day habituation period in wild type (WT, n = 12, $\alpha 1^{-/-}$ (n = 11) and α1H101R (n = 12). The locomotor activity of mice depended on genotype and day ($p < 0.01$). $\alpha 1^{-/-}$ mice showed a greater decline in locomotor counts over days compared to wild type ($p < 0.01$). From an initially lower level of counts, α1H101R mice did not decline further ($p > 0.05$).*

### 3.4.2 Exploratory Rearing

The experimental design also allowed study of exploratory rearing presented by the 3 genotypes. Males and females did not rear to different extents (sex, $F(1,29) = 2.955$, n.s.) therefore data were collapsed over sex for further analysis. As can be seen in Figure 3.4, both $\alpha1^{-/-}$, α1H101R mice reared significantly less than wild type mice, (main effect of genotype, $F(2,29) = 18.681$, $p < 0.01$; confirmed by planned pairwise comparisons; WT > α1H101R and $\alpha1^{-/-}$, $p < 0.05$). Rearing did decrease within the session (main effect of time $F(3,87) = 3.307$, $p < 0.05$) but the overall level of rearing did not diminish over the 12 test sessions (main effect of day: $F(11, 319) = 1.743$; all interactions not significant).

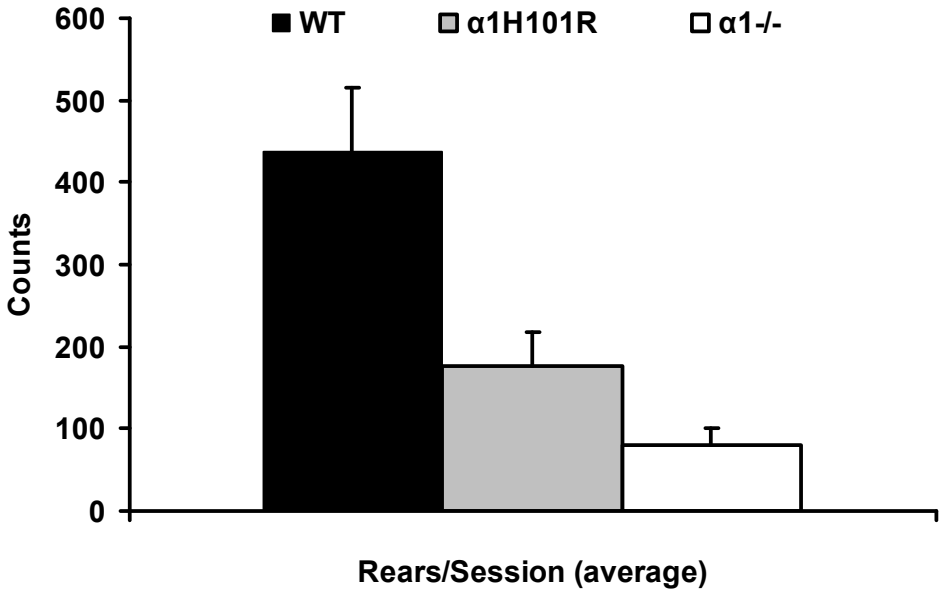


***Figure 3.4: Wild type mice reared more than $\alpha1^{-/-}$ and α1H101R mice.***

*Histograms showing number (±SEM) of rearing counts as measured by beam breaks recorded in wild type (WT, n = 12), $\alpha1^{-/-}$ (n = 11) and α1H101R (n = 12) mice over 12 days of habituation. Both $\alpha1^{-/-}$ and α1H101R mice demonstrated less rearing than wild types ($p < 0.05$).*

### 3.4.3 The effect of chlordiazepoxide on locomotor activity

1 wild type mouse died before chlordiazepoxide treatment could be completed. There was no effect of sex (sex, $F(1,32) = 0.778$, $p > 0.05$), therefore data were collapsed across sex for further analysis.

Injections of chlordiazepoxide (5, 15, 30mg/kg) dose-dependently decreased locomotion (Figure 3.5A) in wild types and $\alpha1^{-/-}$ mice (albeit with these mice starting from a lower baseline, replicating the effect observed during habituation as described in Section 3.4.1). Chlordiazepoxide did not have the same dose-dependent effect in the α1H101R mice (confirmed by an interaction of dose x time x genotype, $F(18,279) = 3.160$, $p < 0.001$). Simple main effects (dose) analysis confirmed an effect of dose in both wild type (dose, $F(3,10) = 14.282$, $p < 0.001$) and $\alpha1^{-/-}$ mice (dose, $F(3,10) = 6.576$, $p < 0.01$). There was a trend in the data to suggest that chlordiazepoxide was not without effect in the α1H101R mice, with a slight rise in locomotor activity following injection with 5mg/kg chlordiazepoxide, in the first 5mins (Figure 3.5B) of the testing session (confirmed by a main effect of dose: $F(3,33) = 4.22$, $p < 0.05$). This effect was not detected when comparing the whole 20min testing period ($F(3,33) = 2.5$, $p = 0.07$).

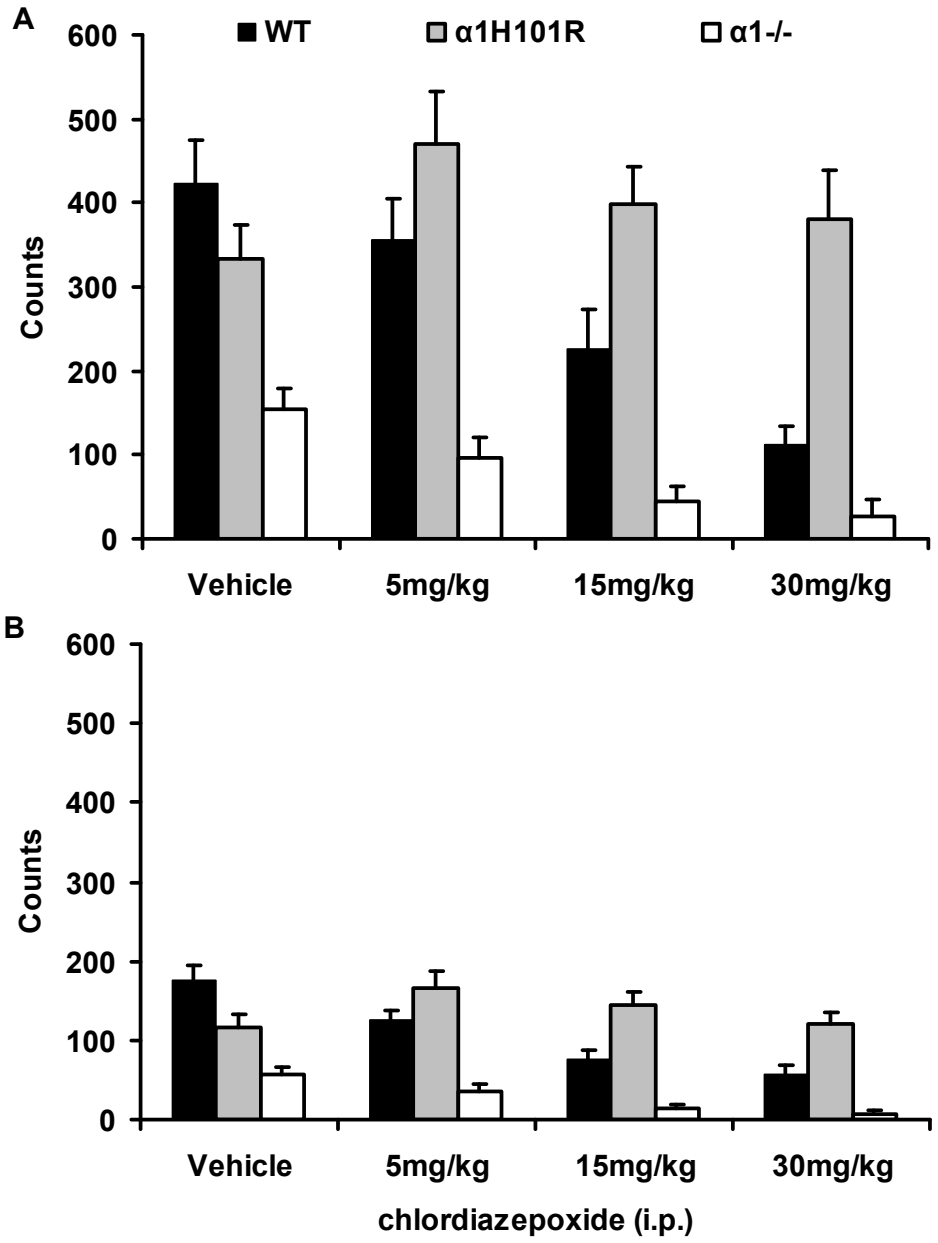


***Figure 3.5: Chlordiazepoxide reduced locomotor activity in wild type and α1$^{-/-}$ mice but not α1H101R mice.***

*Histograms showing the number (±SEM) of locomotor activity counts as measured by beam breaks recorded in wild type (WT, n = 11), α1$^{-/-}$ (n = 11) and α1H101R (n = 12) during the entire 20 minute session (A) and the first 5 minutes (B) following injection with chlordiazepoxide (5, 15, 10mg/kg) or its saline vehicle. Chlordiazepoxide reduced locomotor activity in wild type and α1$^{-/-}$ mice ($p < 0.01$) but was without effect in α1H101R mice ($p > 0.05$).*

### 3.4.4 The effect of chlordiazepoxide on EPM behaviour

It has been reported that some of the behavioural effects of BDZ administration may be associated with state dependent learning and that the effects measured on second exposure to the EPM are different to those measured on the first exposure (for review of the role of learning and anxiety in the EPM, see File, 1993). Therefore an initial analysis was used to determine any effect of order. Initial analysis showed no effect of sex (sex, $F(1,22) = 0.077$, $p > 0.05$) or order of testing (order, $F(1,22) = 1.830$, $p > 0.05$). This finding allows us to analyse both sexes and mice first tested with either saline or chlordiazepoxide together. Due to the sedative nature of chlordiazepoxide, “closed arm” entries, as well as open arm entries, were considered to correct for the effect of baseline differences in locomotor activity induced by chlordiazepoxide treatment.

From Figure 3.6A, it can be seen that following chlordiazepoxide the total number of arm entries made by $\alpha1^{-/-}$ mice is lower than that made by wild types and α1H101R mice under baseline conditions (main effect of genotype: $F(2,31) = 4.618$, $p < 0.05$; confirmed by planned pairwise comparisons, WT = α1H101R > $\alpha1^{-/-}$, $p < 0.05$) thus replicating the lower level of locomotor activity described in Sections 3.4.2 and 3.4.3.

The total number of arm entries made by $\alpha1^{-/-}$ mice and the time spent in the open arms following treatment with chlordiazepoxide is substantially decreased (from an average of 6 entries to only 1) when compared to vehicle treated mice resulting in very little variance in the chlordiazepoxide

treated $\alpha1^{-/-}$ group. Therefore the effect of chlordiazepoxide in the $\alpha1^{-/-}$ mice was not analysed further (see discussion).

The number of open arm entries following chlordiazepoxide increased in both wild type and α1H101R mice (confirmed by main effect of drug: $F(1,21) = 10.288$, $p < 0.01$) and this effect did not differ significantly between genotypes (no drug x genotype interaction: $F(1,21) = 0.001$, n.s.).

Initial examination of Figure 3.6B reveals that under baseline conditions α1H101R and wild type mice spent comparable amounts of time in open arms (no effect of genotype: open; $F(2,21) = 3.16$, closed; $F(2,21) = 0.819$, both n.s.). The amount of time spent in open arms following chlordiazepoxide administration in wild type and α1H101R mice was increased (confirmed by main effect of drug: $F(1,21) = 7.45$, $p < 0.05$) but the effect of chlordiazepoxide did not differ between genotypes (no genotype x drug interaction: $F(1,21) = 1.29$, n.s.).

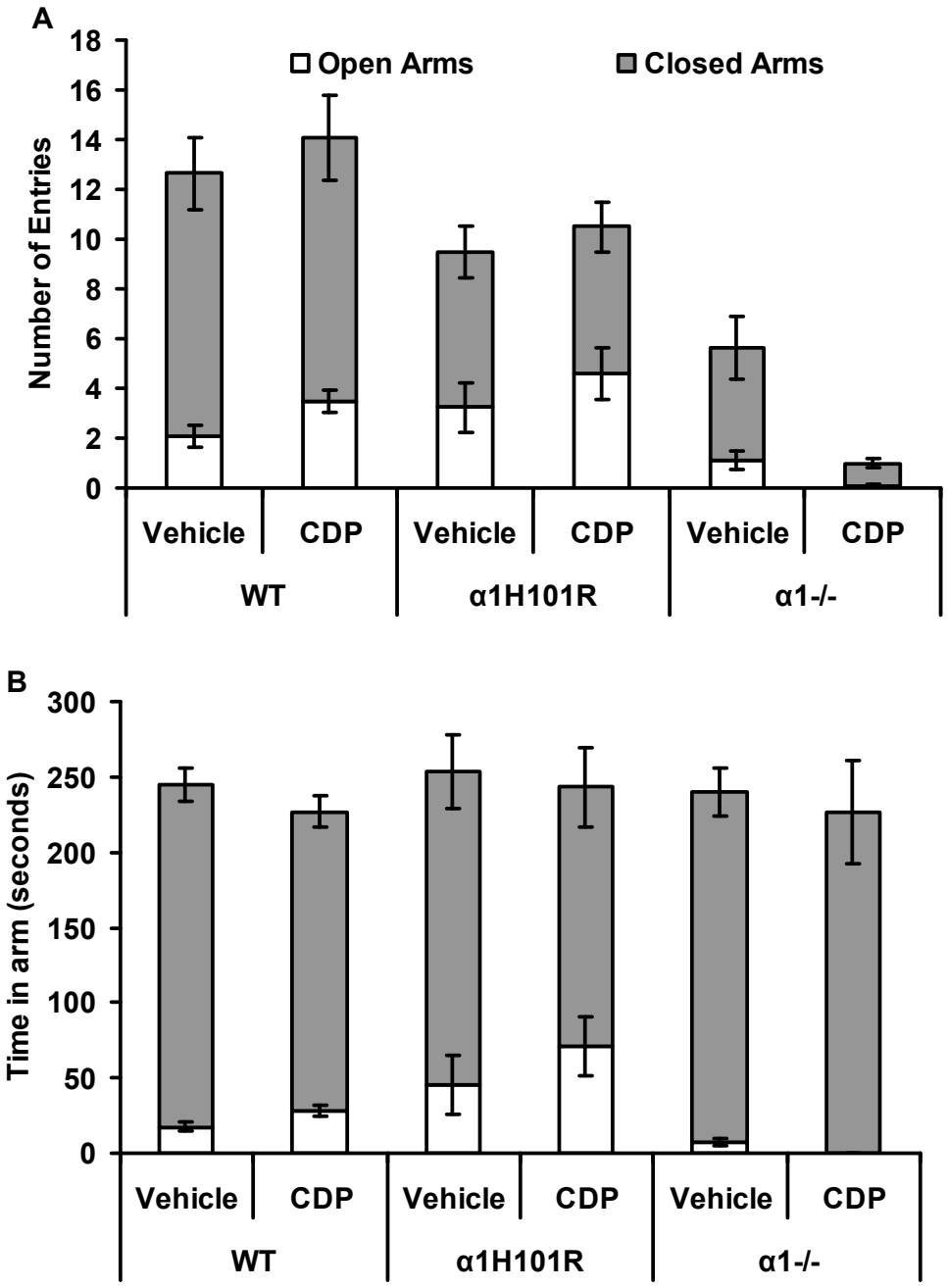


***Figure 3.6: Chlordiazepoxide increased open arm entries and time in open arms of wild type and α1H101R mice.***

*Stacked histogram showing number (±SEM) of open and closed arm entries (A) and time in each arm (B) of wild type (WT, n = 11), α1H101R (n = 12) and $\alpha 1^{-/-}$ (n = 11) mice in the EPM following chlordiazepoxide (10mg/kg). Chlordiazepoxide increased the number of open arm entries ($p < 0.01$) and time spent in open arms ($p < 0.05$) in wild type and α1H101R mice.*

## *3.5 Discussion*

### 3.5.1 Baseline Locomotor Activity and Habituation

Baseline locomotor activity and rearing in α1H101R and $\alpha1^{-/-}$ mice was measured. Previous evidence has suggested no baseline differences in α1H101R (McKernan*, et al.*, 2000; Rudolph*, et al.*, 1999) or $\alpha1^{-/-}$ (Reynolds*, et al.*, 2003a; Sur*, et al.*, 2001) when compared to wild type counterparts. In contrast, we found substantial differences. α1H101R showed lower locomotor activity than wild types in a novel environment and little reduction in locomotor activity over the 12 days. This suggests a lack of intersession habituation. However, α1H101R did show a reduction in locomotor activity within session suggesting intrasession habituation. α1H101R showed a lower baseline activity than wild types on initial exposure to the novel testing environment. A lack of a burst of spontaneous activity on initial exposure to a novel environment could indicate a high level of anxiety accompanied by a low motivation to explore. A high level of anxiety in these mice can be ruled out by the presence of bursts of spontaneous activity in the first 5 minutes of a session culminating in intrasession habituation and no significant difference in baseline anxiety was uncovered using the EPM. Furthermore, no baseline differences in anxiety have been reported previously using the EPM and the light dark box test of anxiety (Rudolph*, et al.*, 1999). Intrasession habituation does not rely on a memory component, as there is no extended retention period (for review of habituation in rodents, see Leussis and Bolivar, 2006). Intersession habituation however does have a memory component: if an animal does not retain information about an

environment, each subsequent exposure would not differ from the initial exposure. Reduced intersession habituation could, therefore, be due to impaired memory for the environment in the α1H101R mice. Supporting this suggestion is the lower levels of rearing observed in these mice: as rearing is an exploratory behaviour, reduced rearing would reduce the opportunity to sample, and thus remember, the environment. Therefore, the most parsimonious account would be that reduced exploration – rather than impaired memory *per se* – is responsible for the reduction in intersession habituation.

It is somewhat surprising to detect a behavioural phenotype in α1H101R mice. GABAergic inhibition should not be altered and, due to the specificity of the knock-in mutation, any functional phenotype should only be detectable under the presence of a BDZ. The presence of endogenous ligands for the BDZ site on the $GABA_AR$ could explain a detectable phenotype in the α1H101R mouse. A fashionable idea in the 1980s and early 90s that has recently fallen out of favour in the literature, is the presence of endogenous ligands for the BDZ site (endozepines). BDZs such as diazepam have been found in animal tissues and foods (Avallone*, et al.*, 2001; Avallone*, et al.*, 1996; Medina*, et al.*, 1988; Sangameswaran and de Blas, 1985; Unseld*, et al.*, 1989). Although the origin of these is unclear, environmental contamination by synthetic BDZs has been ruled out (Sangameswaran and de Blas, 1985; Sangameswaran*, et al.*, 1986; Unseld and Klotz, 1989). Indeed endozepines have been found in extracts from *Matricaria chamomilla* (camomile tea), which bind to BDZ sites and

produce sedation in rats (Avallone*, et al.*, 1996). Several molecules have been identified which act upon the BDZ binding site of the $GABA_AR$ (for example inosine, hypoxanthine, nicotinamide and β-carboline); however these have low affinity to the receptor, exist in low concentrations and/or possess inverse agonist properties at the $GABA_AR$ and therefore are unlikely to result in the observations seen here.

Baseline locomotor activity in a novel environment of $\alpha1^{-/-}$ mice appears similar to wild types; however, $\alpha1^{-/-}$ showed a greater level of inter session habituation which resulted in a much lower baseline activity level by day 12. Although previous evidence has not shown differences in baseline locomotor activity (Sur*, et al.*, 2001), a significant deficit in rotarod performance has been previously demonstrated (Kralic*, et al.*, 2005). It is possible that a degree of ataxia could account for lower levels of activity once the initial urge to explore a novel environment has passed. One notable phenotypic trait of the $\alpha1^{-/-}$ mouse is the presence of an involuntary tremor in the hind legs (Kralic*, et al.*, 2005). This tremor is not a resting tremor but rather appears more like intentional tremor, as it becomes more evident during movement or upon tail suspension (Kralic*, et al.*, 2005; personal observations). This tremor could account for the decreased performance on the rotarod and the reduced levels of activity observed here. This essential tremor could also account for the reduced amounts of exploratory rearing exhibited in these mice.

### 3.5.2 Sedative Responses to Chlordiazepoxide

We tested the sedative effects of chlordiazepoxide in α1H101R and $\alpha1^{-/-}$ mice; this is the first investigation of the role of the α1 subunit in BDZ induced sedation in α1H101R and $\alpha1^{-/-}$ mice under the same conditions. Chlordiazepoxide reduced activity in wild type but did not reduce activity in α1H101R mice. This is in agreement of previously published work (Crestani*, et al.*, 2000b; McKernan*, et al.*, 2000; Rudolph*, et al.*, 1999), which showed that the sedative effects of diazepam are absent in α1H101R mice. Combined, this evidence confirms that the sedative actions of BDZs are mediated by α1 containing $GABA_A$Rs. There was a slight increase in activity of α1H101R mice following injection with low dose chlordiazepoxide. This has been demonstrated previously using diazepam in a novel environment (Crestani*, et al.*, 2000b; McKernan*, et al.*, 2000) but has not been shown when the mice were tested in a familiar environment (Rudolph*, et al.*, 1999) suggesting an uncovering of the anxiolytic effects of diazepam in the novel environment in the absence of the sedative properties of this drug. In the current experiment, although α1H101R mice were familiar to the testing environment, this increase was still observed. The lack of intersession habituation in the α1H101R mice may account for this.

In contrast, the locomotor activity of the $\alpha1^{-/-}$ mouse was reduced following chlordiazepoxide injection. This again is in agreement with previously published work (Kralic*, et al.*, 2002a; Reynolds*, et al.*, 2003a), which showed that the sedative effects of diazepam are present in the $\alpha1^{-/-}$

mouse. Low doses of diazepam that were found to increase the activity of wild type mice significantly reduced the activity of $\alpha1^{-/-}$ mice (Kralic*, et al.*, 2002a; Reynolds*, et al.*, 2003a), suggesting that the $\alpha1^{-/-}$ mouse was more susceptible to the sedative effects of diazepam. However, the results shown here suggest $\alpha1^{-/-}$ mice are not more susceptible to the effects of chlordiazepoxide but this lower activity is simply a function of lower baseline activity caused by a dramatic reduction in activity upon repeated exposure to the testing environment. The α1 subunit is the single most abundant in the mammalian CNS (Sur*, et al.*, 2001). Deletion of the α1 subunit in the $\alpha1^{-/-}$ mouse leaves opportunity for subunit compensation and indeed there is an up regulation of α2 and α3 peptide expression in these mice (Kralic*, et al.*, 2002b).

### 3.5.3 Anxiolytic Responses to Chlordiazepoxide

We tested the anxiolytic effects of chlordiazepoxide in α1H101R and $\alpha1^{-/-}$ mice in the EPM.

The very low levels of activity in the EPM precluded any meaningful detection of an anxiolytic action of chlordiazepoxide in the $\alpha1^{-/-}$ mice. The dose of chlordiazepoxide chosen was the lowest one expected to induce anxiolytic effects in the wild type mice; however this dose induced a level of sedation rendered the $\alpha1^{-/-}$ mice untestable. It cannot be ruled out that an anxiolytic effect would be uncovered in the $\alpha1^{-/-}$ mice at a lower dose but this would not necessarily be anxiolytic in the wild type. A full dose response to chlordiazepoxide in the EPM would be required to elucidate

the anxiolytic actions of chlordiazepoxide in the $\alpha1^{-/-}$ mouse. The anxiolytic effect of diazepam has been previously demonstrated in $\alpha1^{-/-}$ mice (Kralic*, et al.*, 2002a). Interestingly the anxiolytic effect of diazepam (0.6mg/kg) is greater in $\alpha1^{-/-}$ mice than in wild types (Kralic*, et al.*, 2002a). This is likely due to the up regulation of α2 and α3 containing receptors, both of which have been implicated in the mediation of the anxiolytic effects of diazepam (Atack*, et al.*, 2005; Atack*, et al.*, 2006; Dias*, et al.*, 2005; Dixon*, et al.*, 2007; Dixon*, et al.*, 2008; Low*, et al.*, 2000; McKernan*, et al.*, 2000; Morris*, et al.*, 2006; Morris*, et al.*, 2007). Previous work concluded that a higher dose of diazepam (10mg/kg) had no anxiolytic effect in $\alpha1^{-/-}$ mice. However, given the evidence presented here, it would be parsimonious to conclude that the mice in that study were also sedated at that dose and the sedation prevented the detection of an anxiolytic action (Kralic*, et al.*, 2002a).

Chlordiazepoxide increased open arm entries and the time spent in open arms in both wild type and α1H101R mice. This is consistent with previous experiments investigating the anxiolytic effect of diazepam in α1H1010R mice in the light/dark choice test of anxiety as well as the EPM (Rudolph*, et al.*, 1999).

### 3.5.4 Issues of genetic background

There are some important issues to consider during the interpretation of these results in regards to the genetic background of the wild type comparators and the genetic mutations themselves. As mentioned above

the WT controls for $\alpha1^{-/-}$ and α1H101R mice were the same and generated by a series of backcrossing onto the C57BL/6 genetic background for more than 20 generations when these mice arrived in Dundee. Mice used for experiments were conducted on the first two generations, with breeding pairs derived from the corresponding heterozygous breeding pairs. The two lines are refreshed regularly to avoid genetic drift in the colonies maintaining the two genetic mutations on the same genetic background. However, this does mean that the genetic background of the two strains is likely to be different from the original studies using the $\alpha1^{-/-}$ and α1H101R mice (Crestani*, et al.*, 2000b; Kralic*, et al.*, 2002a; Kralic*, et al.*, 2002b; McKernan*, et al.*, 2000; Reynolds*, et al.*, 2003b; Rudolph*, et al.*, 1999). Perhaps the results shown here provide further evidence that the genetic background of the mice in which a mutation is investigated is extremely important and the potential differences between this study and those previously are a result from differences in genetic background. One way to test this theory is to generate a line of these mutations in a 129 background in addition to the current C57/BL6 to establish if the effects of the α1 mutations shown here are dependent on the genetic background.

## *3.6 Summary*

This chapter has compared the baseline behaviours of $\alpha1^{-/-}$ and α1H101R mice and studied the role of the synaptic α1 subunit in the mediation of the sedative and anxiolytic actions of chlordiazepoxide. Although the data are not conclusive, it has provided further evidence for the role of the a1 subunit in the sedative properties of BDZs and highlighted potential baseline differences between $\alpha1^{-/-}$ and wild type mice. This Chapter has

also provided evidence that the generally considered “silent” knock in mutation may indeed produce a detectable behavioural phenotype. Although further investigation is required, in addition to the role of genetic background in the differences found in the current studies.

In the following chapter, the role of this synaptic α1 subunit and the extrasynaptic δ subunit in the sedative actions THIP are investigated.

# 4 The role of the α1 and δ subunits in the mediation of the behavioural effects of THIP

*"Current data from in vitro and in vivo studies demonstrate that gaboxadol achieves its hypnotic effects via a fundamentally different mechanism from that of the benzodiazepine receptor agonists. Functional selectivity for the tonic current generated by extrasynaptic $GABA_A$ receptors containing the δ-subunit, with little effect on synaptic currents mediated by benzodiazepine receptor agonist-sensitive receptors, uniquely characterizes gaboxadol."*

Wafford and Ebert, 2006

## *4.1 Objective*

To investigate the role of the synaptic α1 and extrasynaptic δ subunits in the mediation of the behavioural effects of THIP.

## *4.2 Introduction*

As discussed in Chapter 1, the behavioural effects of THIP are thought to be mediated by α4δ containing $GABA_A$Rs. The ataxic sedative and analgesic effects of THIP are absent in $\alpha4^{-/-}$ mice (Chandra*, et al.*, 2006). Behavioural data from the $\delta^{-/-}$ mouse is lacking, although it is known that the anaesthetic effects of THIP are reduced in $\delta^{-/-}$ mice (Boehm II*, et al.*, 2006).

In this Chapter, mice lacking the synaptic α1 and extrasynaptic δ subunits are utilised to investigate the role of these subunits in the behavioural effects (ataxia and sedation) of THIP.

The data in this chapter form part of that published in Herd*, et al.*, 2009 (see Appendix 1)

## *4.3 Methods*

### 4.3.1 Animals

Mice lacking the extrasynaptic δ subunit ($\delta^{-/-}$) or the synaptic α1 subunit ($\alpha1^{-/-}$) together with the appropriate wild type ($\delta^{+/+}$ and $\alpha1^{+/+}$ respectively) mice from the same colony, were used for all experiments. For more details on genotypes used in these experiments, please refer to Chapter 2. In studies using only externally bred wild type mice, C57BL/6 mice were used (Charles River). All animals were maintained in similar environmental conditions, with nesting material and cardboard tubes as environmental enrichment.

### 4.3.2 Behaviour

The fixed speed and accelerating rotarod are used to measure ataxia. Locomotor activity is used to measure sedation. For details of these methods, refer to Chapter 2.

## *4.4 Results*

### 4.4.1 The effect of THIP on C57BL/6 mice in the fixed speed rotarod

To establish at what dose THIP induced ataxia in mice, C57BL/6 mice (n = 20) were trained up to 3 times daily on the rotarod at a fixed speed of 6rpm. Training was considered complete when mice were able to stay on the fixed speed rotarod for 180secs. For more details on the fixed speed rotarod, see Chapter 2. Mice were then split into 4 groups (n = 5) and administered 1 of 3 doses of THIP (2, 5 or 10mg/kg) or saline vehicle in a between subjects design.

All mice learned to stay on the fixed speed rotarod for 180secs at 6rpm within 4±1 practice trials. Figure 4.1 shows that 10mg/kg THIP reduced the ability of C57BL/6 mice to stay on the fixed speed rotarod (confirmed by main effect of drug $F(3,16) = 39.6$, $p < 0.01$) in a time dependent manner (confirmed by drug x time interaction: $F(18,96) = 9.630$, $p < 0.01$). The effect of 10mg/kg THIP becomes apparent from 15min post injection (paired t-test, $t = 5.232$, $p < 0.01$) and recovery becoming essentially complete by 90min (paired t-test, $t = 1$, n.s.). Lower doses of THIP had no effect on rotarod performance.

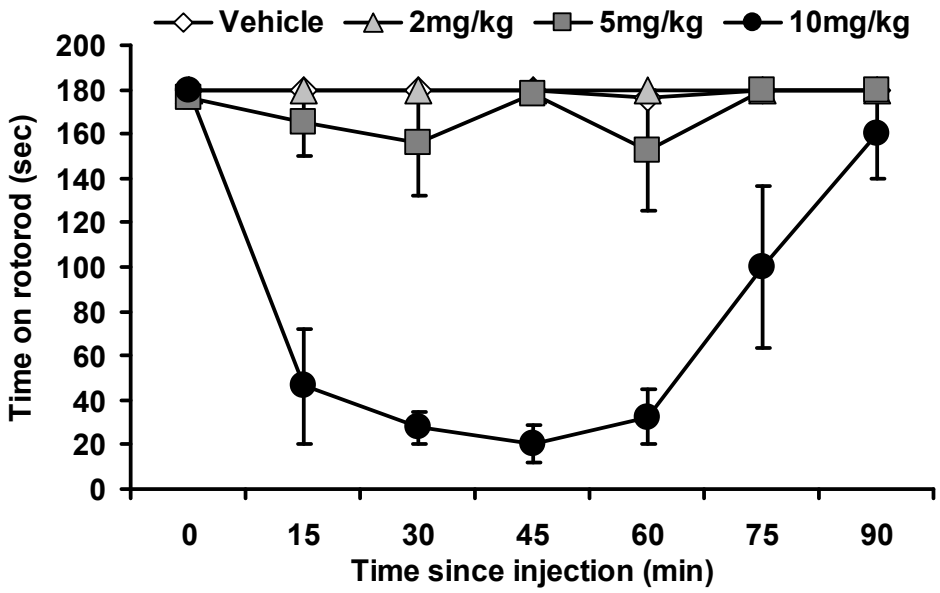


***Figure 4.1: THIP reduces rotarod performance in C57BL/6 mice.***

*Line graph showing mean (±SEM) number of secs mice (n = 5) stayed on rotarod (6rpm) following injection of THIP (2, 5, 10mg/kg) or its saline vehicle over a 90 min time course since injection. 10mg/kg THIP significantly reduced the amount of time C57BL/6 mice could spend on the rotarod from 15mins post injection onwards ($p < 0.01$). Performance recovered by 90mins post injection.*

### 4.4.2 The effect of THIP on $\alpha1^{+/+}$ and $\alpha1^{-/-}$ mice in the fixed speed rotarod

$\alpha1^{+/+}$ and $\alpha1^{-/-}$ mice (n = 8) were trained up to 3 times daily on the rotarod at a fixed speed of 6rpm. Training was considered complete when mice were able to stay on fixed speed rotarod for 180secs. Mice were then injected (i.p.) with 3 doses of THIP (10, 15 and 30mg/kg) or its saline vehicle on 4 separate occasions with at least 48hs between doses. These doses were chosen based on the results in Section 4.4.1 and personal communication with Greg Homanics, University of Pittsburgh, USA. Time on the rotarod was measured up to 150mins post-injection in 15min intervals.

All mice learned to stay on the rotarod for 180secs at 6rpm within 4±1 practice trials. Figure 4.2 shows that THIP reduced the amount of time mice are able to stay on the rotarod and that different doses of THIP have different recovery profiles (confirmed by main effect of dose: $F(2,28) = 9.1$, $p < 0.01$; dose x time interaction: $F(10,280) = 3.5$, $p < 0.01$). There was no difference between $\alpha1^{+/+}$ and $\alpha1^{-/-}$ mice.

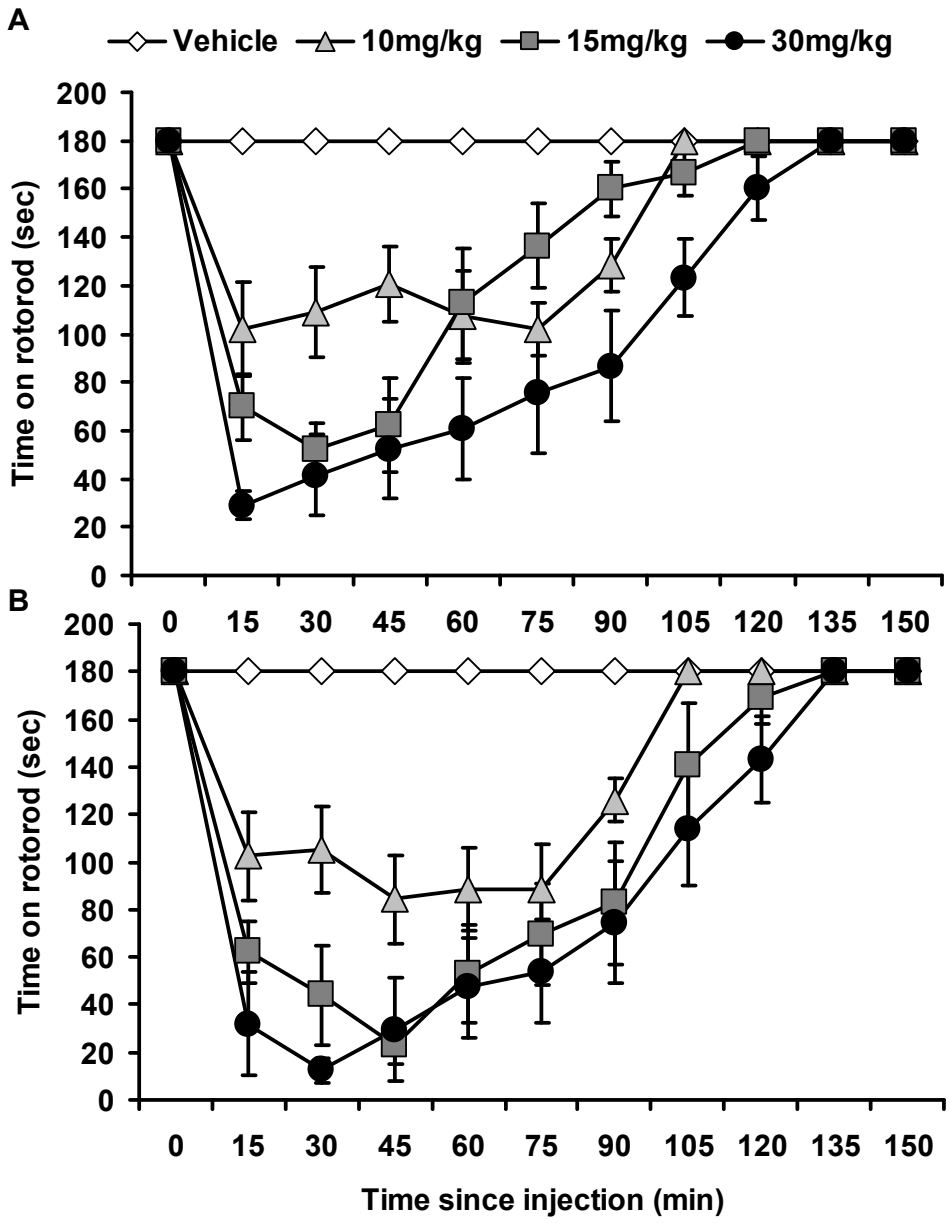


***Figure 4.2: The effect of THIP on rotarod performance in $\alpha1^{+/+}$ and $\alpha1^{-/-}$ mice.***

*Line graphs showing fixed speed rotarod performance in $\alpha1^{+/+}$ (A) and $\alpha1^{-/-}$ (B) mice (n = 8) following injection of THIP (10, 15, and 30mg/kg) or its saline vehicle (i.p). Rotarod performance declined in both $\alpha1^{+/+}$ and $\alpha1^{-/-}$ mice ($p < 0.01$). Different doses of THIP were shown to have different recovery profiles ($p < 0.01$).*

### 4.4.3 Baseline Locomotor Activity of $\delta^{-/-}$ and $\delta^{+/+}$ mice

The locomotor activity of 9 $\delta^{+/+}$, 9 $\delta^{-/-}$ mice (all male) was measured using the methods described in Section 2.1. To habituate the animals to the testing chamber, each animal was placed in the chamber for 20mins for 6 days using a balanced design over genotype (so that a $\delta^{+/+}$ and a $\delta^{-/-}$ were tested simultaneously). On each day, the locomotor activity was logged in 5min bins.

As can be seen from Figure 4.3, baseline locomotor activity did not differ between $\delta^{-/-}$ and $\delta^{+/+}$ mice over the 6 days of habituation (no effect of genotype: $F(1,16) = 0.6$, n.s.). It can also be seen (Figure 4.4) that during day 1 of habituation locomotor activity reduced over time but not on day 6 of habituation (interaction of day and time: $F(15,240) = 5.1$, $p < 0.001$; main effect of time on day 1: $F(3,48) = 17.8$, $p < 0.001$; no effect of time on day 6: $F(3,48) = 0.02$, n.s.). This is largely accounted for by the burst of initial activity recorded in the first 5mins of day 1.

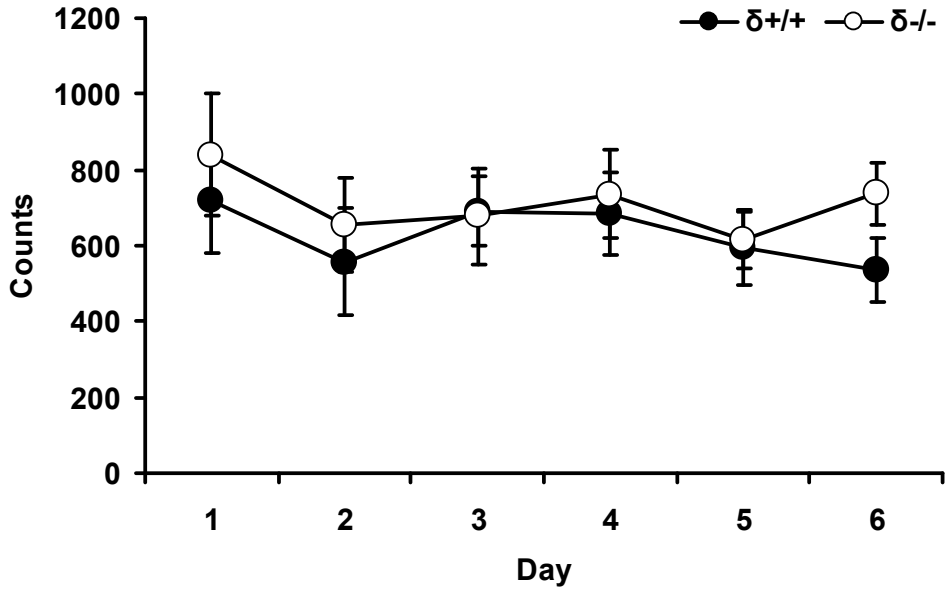


***Figure 4.3: There was no difference in locomotor activity between genotypes.***

*Line graph showing the number (±SEM) of locomotor activity counts recorded over the 6 day habituation period in $\delta^{+/+}$ (n = 9) and $\delta^{-/-}$ (n = 9) mice. There were no differences in locomotor activity between the $\delta^{+/+}$ and $\delta^{-/-}$ mice ($p > 0.05$).*

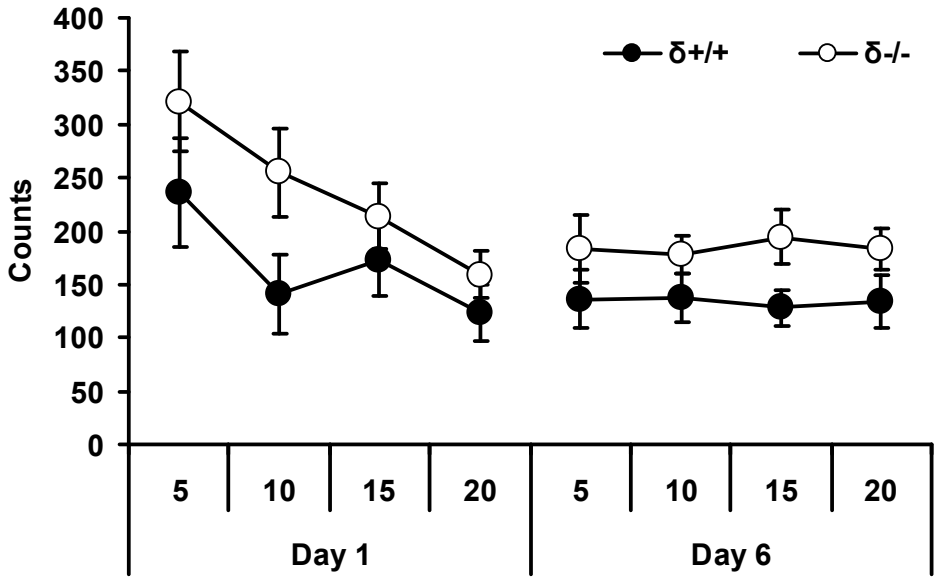


***Figure 4.4: Locomotor activity was less on day 6 when compared with day 1.***

*Line graph showing the number (±SEM) of locomotor activity counts recorded on day 1 and day 6 of habituation in $\delta^{+/+}$ (n = 9) and $\delta^{-/-}$ (n = 9) mice. Locomotor activity of both genotypes reduced over time on day 1 ($p < 0.01$) but not on day 6 ($p > 0.05$). Furthermore, initial activity was less on day 6 than on day 1 ($p < 0.001$) in both genotypes.*

### 4.4.4 Locomotor Activity of $\delta^{+/+}$ and $\delta^{-/-}$ mice following injection with THIP

Following habituation, the behavioural response to sedative drugs known to be sedative in mice was measured using the same techniques described in section 2.1. For information on drug preparation, please refer to chapter 2.

9 $\delta^{+/+}$ and 9 $\delta^{-/-}$ mice were injected with 2, 5 and 10mg/kg THIP or its saline vehicle and locomotor activity recorded 30mins after injection for 20mins. Each mouse received each dose and drugs were given in a repeated measure counter balanced design with at least 48hrs between doses.

As can be seen from Figure 4.5, THIP dose dependently decreased locomotor activity in $\delta^{+/+}$ mice. THIP did not have the same dose dependent effect in the $\delta^{-/-}$ mice (confirmed by a dose x genotype interaction: $F(3,48) = 5.4$, $p < 0.01$). Simple main effects analysis confirmed an effect of THIP in $\delta^{+/+}$ mice ($F(3,24) = 17.4$, $p < 0.01$) but not in $\delta^{-/-}$ mice ($F(3,24) = 0.7$, n.s.). There was no effect of time bin ($F(3,48) = 1$, n.s.; all interactions not significant).

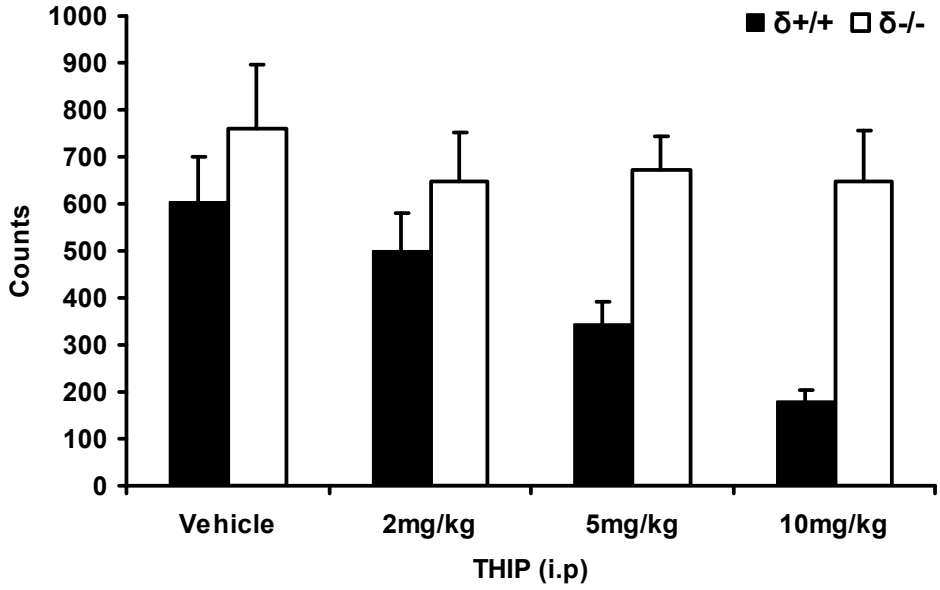


***Figure 4.5: Locomotor activity of $\delta^{+/+}$ but not $\delta^{-/-}$ mice was reduced following injection with THIP.***

*Histogram showing number (±SEM) of locomotor activity counts before and after THIP injection (i.p) in $\delta^{+/+}$ (n = 9) and $\delta^{-/-}$ (n = 9). Locomotor activity was reduced in $\delta^{+/+}$ mice ($p < 0.01$) but not in $\delta^{-/-}$ mice ($p > 0.05$).*

### 4.4.5 Locomotor Activity of $\delta^{+/+}$ and $\delta^{-/-}$ mice following injection with chlordiazepoxide, etomidate and alphaxalone

Half (5 $\delta^{+/+}$ and 5 $\delta^{-/-}$) of the mice previously tested with THIP were then tested to examine the effects of other known sedative drugs in $\delta^{-/-}$ mice. Locomotor activity of these mice was recorded following injection (i.p.) of 5mg/kg etomidate, 10 and 20mg/kg alphaxalone, 30mg/kg chlordiazepoxide and the respective vehicles. There was a gap of 48hrs between different doses of the same drug and at least 5 days between the administrations of different drugs.

#### *4.4.5.1 Chlordiazepoxide*

As can be seen from Figure 4.6, 10mg/kg chlordiazepoxide reduced the locomotor activity of both $\delta^{+/+}$ and $\delta^{-/-}$ mice (confirmed by main effect of drug: $F(1,8) = 14.805$, $p < 0.01$; no effect of genotype: $F(1,8) = 0.915$, n.s.; all interactions not significant).

#### *4.4.5.2 Etomidate*

As can be seen from Figure 4.7, 5mg/kg etomidate reduced the activity of both $\delta^{+/+}$ and $\delta^{-/-}$ mice (confirmed by main effect of drug $F(1,7) = 11.945$, $p < 0.05$; no main effect of genotype: $F(1,7) = 0.939$, n.s.; all interactions not significant).

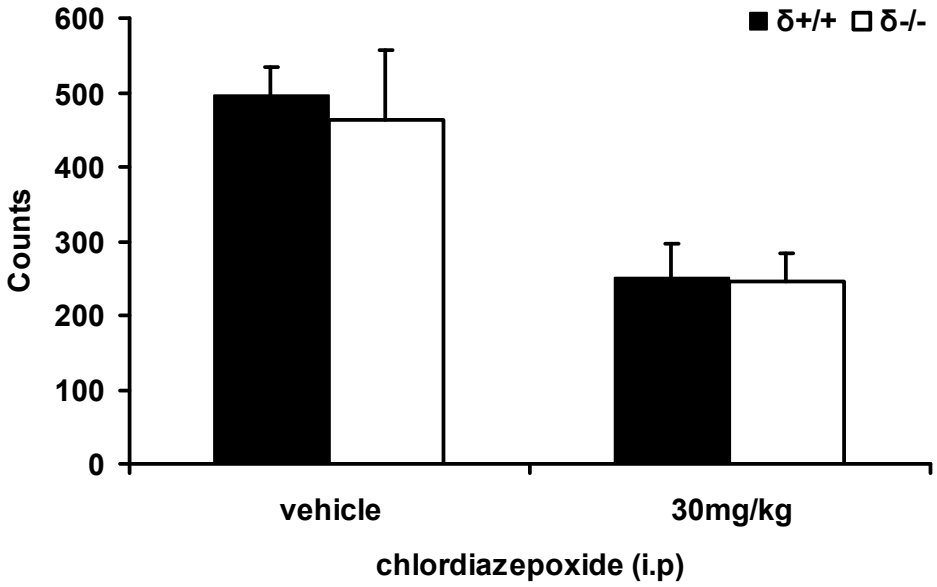


***Figure 4.6: Locomotor activity of $\delta^{+/+}$ and $\delta^{-/-}$ mice was reduced following injection with chlordiazepoxide.***

*Histogram showing number (±SEM) of locomotor activity counts after chlordiazepoxide injection (30mg/kg) or its saline vehicle (i.p) in $\delta^{+/+}$ and $\delta^{-/-}$ mice (n = 5). Chlordiazepoxide reduced locomotor activity ($p < 0.01$) in both genotypes ($p > 0.05$).*

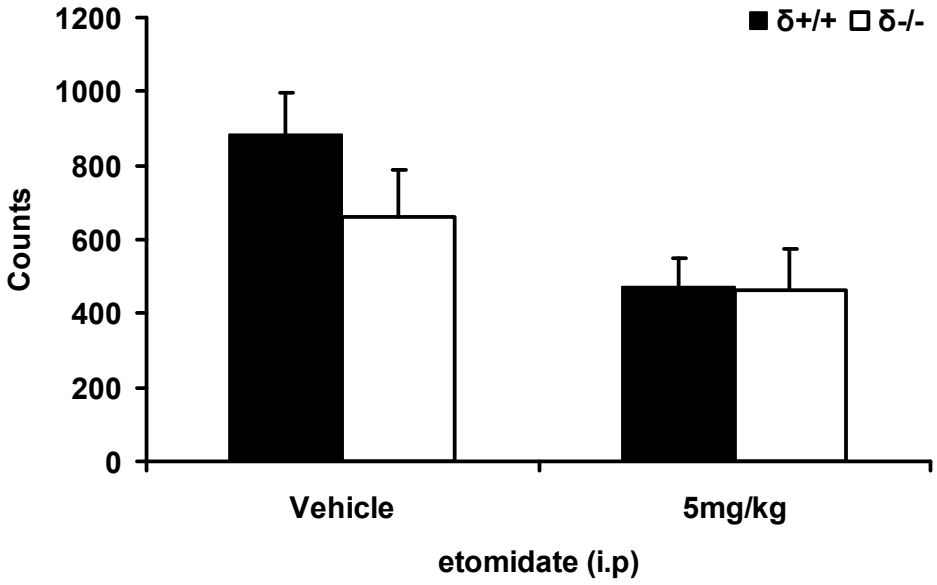


***Figure 4.7: Locomotor activity of $\delta^{+/+}$ and $\delta^{-/-}$ mice was reduced following injection with etomidate.***

*Histogram showing number (±SEM) of locomotor activity counts after etomidate injection (5mg/kg) or its saline vehicle (i.p) in $\delta^{+/+}$ and $\delta^{-/-}$mice (n = 5). Locomotor activity was reduced in both genotypes ($p < 0.05$).*

#### *4.4.5.3 Alphaxalone*

Figure 4.8 shows both 10 and 20mg/kg alphaxalone reduces the locomotor activity of $\delta^{+/+}$ mice. However, it appears that only 20mg/kg reduces the activity of $\delta^{-/-}$ mice (confirmed by a genotype x dose interaction: $F(2,14) = 6.3$, $p < 0.05$) and in fact 10mg/kg alphaxalone may modestly increase the locomotor activity of $\delta^{-/-}$ mice, although the variance of this group is particularly large. Simple main effects analysis confirmed effect of alphaxalone in both $\delta^{+/+}$ ($F(2,8) = 8.166$, $p < 0.05$) and $\delta^{-/-}$ ($F(2,6) = 11.037$, $p < 0.01$) mice. If the data are plotted over the 20min test period (Figure 4.9) it appears that the time since injection is important (confirmed by a main effect of time: $F(3,21) = 16.520$, $p < 0.001$) and that in the first 5mins $\delta^{-/-}$ mice treated with alphaxalone show a modest increase in locomotor activity following injection with alphaxalone. However a time x genotype interaction only approached significance ($F(3,21) = 2.9$, $p = 0.06$) and there was no time x genotype x dose 3 way interaction ($F(6,42) = 0.867$, n.s.).

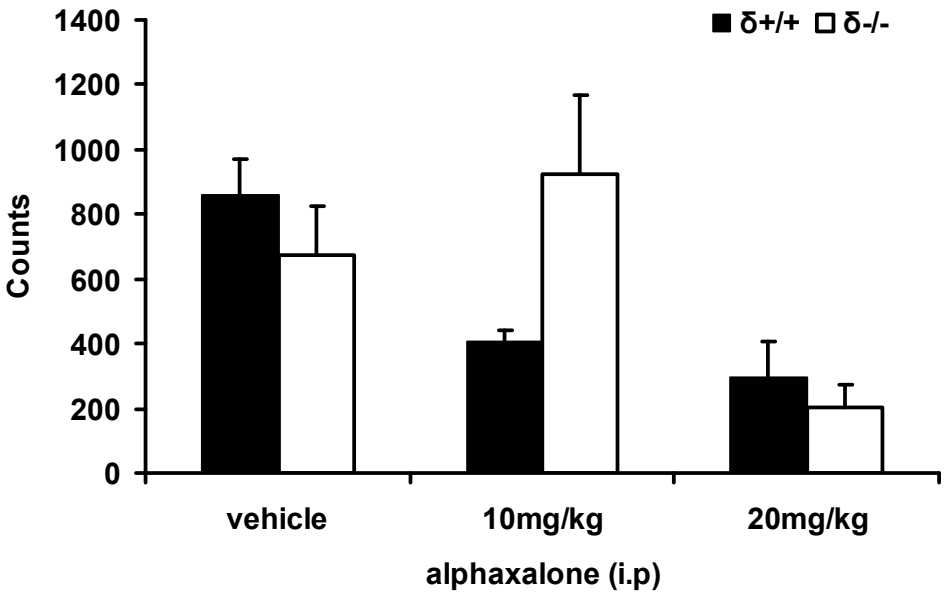


***Figure 4.8: Locomotor activity of $\delta^{+/+}$ and $\delta^{-/-}$ mice following injection with alphaxalone.***

*Histogram showing number (±SEM) of locomotor activity counts recorded after injection with alphaxalone (10, 20mg/kg) and its saline vehicle (i.p) in $\delta^{-/-}$ and $\delta^{+/+}$ mice (n = 5). Locomotor activity following 20mg/kg alphaxalone was reduced in both $\delta^{+/+}$ ($p < 0.05$) and $\delta^{-/-}$ ($p < 0.01$) mice. Locomotor activity following 10mg/kg was genotype dependent ($p < 0.05$) and a reduction in locomotor activity was recorded only in $\delta^{+/+}$ mice.*

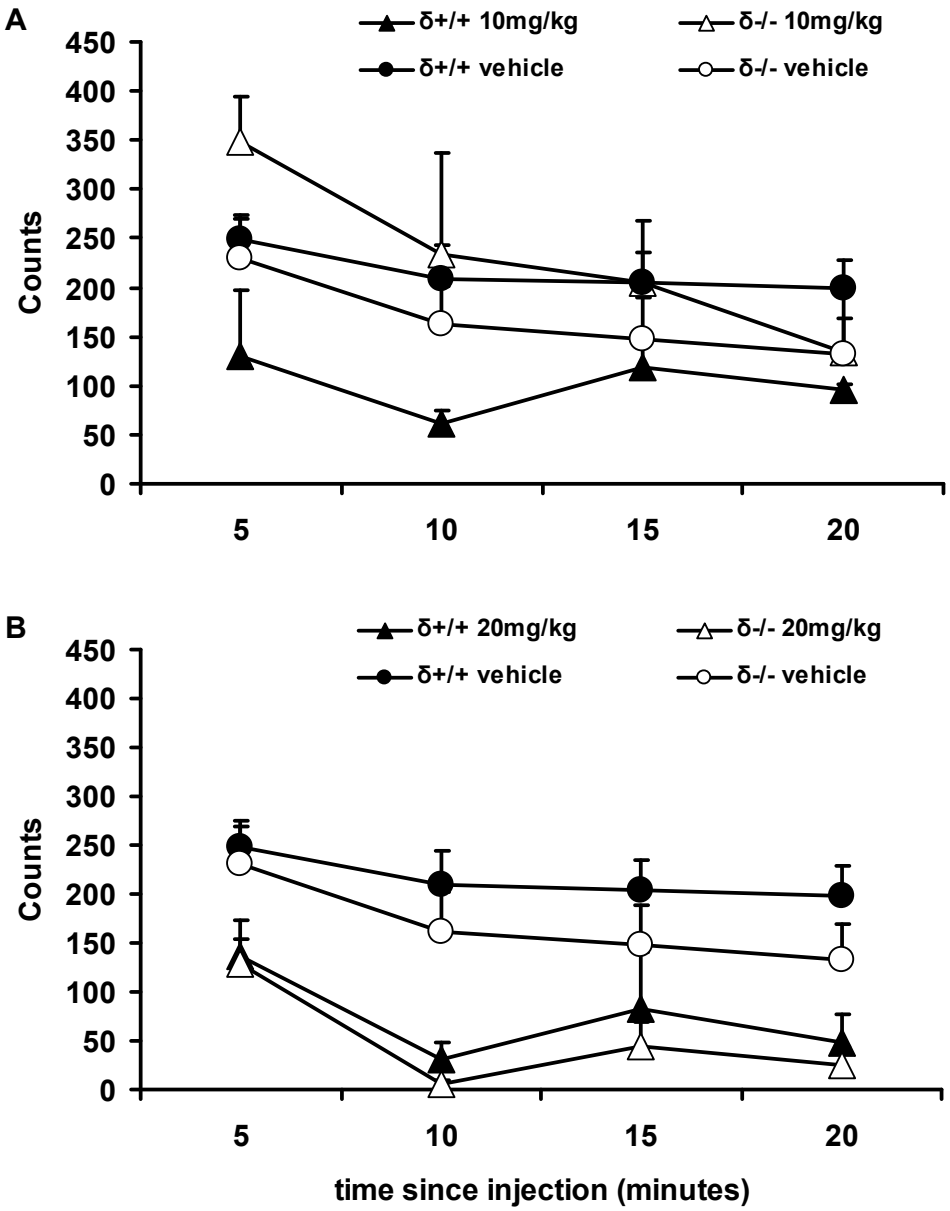


***Figure 4.9: Locomotor activity of $\delta^{+/+}$ and $\delta^{-/-}$ mice following injection with alphaxalone.***

*Line graphs showing number (±SEM) of locomotor activity counts recorded after injection with alphaxalone 10mg/kg (A), 20mg/kg (B) or its saline vehicle in $\delta^{+/+}$ and $\delta^{-/-}$ mice (n = 5). The effect on locomotor activity of alphaxalone was affected by time since injection ($p < 0.001$) and, although it appears 10mg/kg alphaxalone increased locomotor activity in $\delta^{-/-}$ mice in the first 5 mins following injection, there was no three way interaction between time, dose and genotype ($p > 0.05$).*

#### *4.4.5.4 The effect of alphaxalone on C57BL/6 mice in the accelerating rotarod*

To assess if alphaxalone can improve motor performance, C57BL/6 mice (n = 6) were trained to stay on the rotarod for 180secs at 6rpm. Mice were then injected with 3 doses of alphaxalone or its HBC vehicle. Mice were then tested on an accelerating rotarod at set intervals (10, 30 and 60mins after injection) with a maximum speed of 50rpm and an acceleration of 4.4rpm per min for a maximum time of 10mins. Drugs were administered in a repeated measures counter balanced design with at least 48hrs between injections.

The data presented in Figure 4.10 suggests that performance (as measured by time until falling off) on the accelerating rotarod is increased following injection with low dose alphaxalone, supported by a main effect of dose ($F(3,15) = 8.9$, $p < 0.01$). An increase in performance at all 3 doses was confirmed by planned pair wise comparisons ($p < 0.05$). The improvement was not dependent on the time since injection (no main effect of time: $F(2,10) = 0.163$, n.s.).

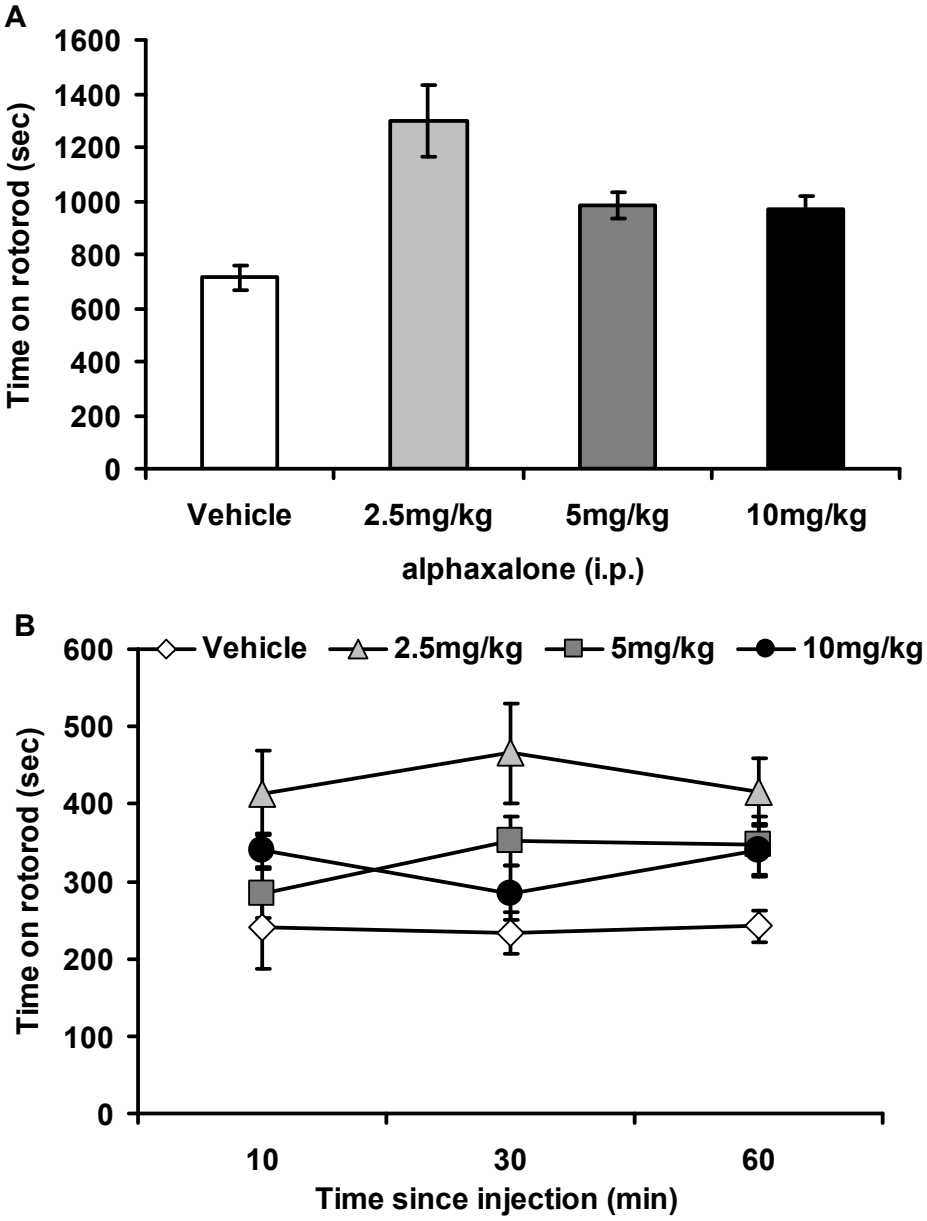


***Figure 4.10: Rotarod performance in C57BL/6 mice following injection with alphaxalone.***

*Histogram (A) showing total (±SEM) time spent on accelerating rotarod post injection with alphaxalone (2.5, 5 and 10mg/kg) or its HBC vehicle (n = 6). Line graph (B) showing time spent on accelerating rotarod at 10, 30 and 60 mins post injection with alphaxalone or its HBC vehicle (i.p). Alphaxalone increased the amount of time spent on the accelerating rotarod in C57BL/6 mice ($p < 0.01$).*

## *4.5 Discussion*

### 4.5.1 The ataxic effects of THIP

THIP was found to be ataxic at doses above 10mg/kg (i.p.) and this ataxic effect was not changed in mice lacking the α1 subunit suggesting that the α1 subunit is not involved in the mediation of the effects of THIP. Furthermore, a role for the α1 subunit in the mediation of THIP-induced current or THIPs action on thalamic excitability has been ruled out (Herd*, et al.*, 2009). THIP has no effect on synaptic currents of the ventral thalamus at relatively high concentrations (Cope*, et al.*, 2005b; Jia*, et al.*, 2005). This is the first demonstration to suggest that deletion of the widely occurring synaptic α1 containing receptors does not alter the motor impairing effects of THIP and further excludes a contribution of the α1δ subtype in the sedative actions of THIP (Glykys*, et al.*, 2007).

### 4.5.2 Baseline locomotor activity of $\delta^{-/-}$ mice

As expected, there were no differences in baseline locomotor activity or habituation patterns between $\delta^{+/+}$ and $\delta^{-/-}$ mice. This is consistent with the deletion of the α4 extrasynaptic subunit (the α4 subunit is co-expressed with the δ subunit in the thalamus). $\alpha4^{-/-}$ mice do not differ in baseline activity from their $\alpha4^{+/+}$ counter parts (Chandra*, et al.*, 2006).

In some ways, it is surprising these mice do not show some sort of motor impairment due to the abundant expression of δ containing $GABA_A$Rs in the cerebellum. Reductions in GABAergic inhibition in the cerebellum have been

previously associated with deficits in the control of movement (Cheron*, et al.*, 2008; Korpi*, et al.*, 1999).

### 4.5.3 Sedative effects of THIP are mediated by δ containing $GABA_ARs$

Previous studies have shown that THIP (30mg/kg) leads to a LORR in $\delta^{+/+}$ and $\delta^{-/-}$ mice, although the duration of the LORR is vastly reduced in $\delta^{-/-}$ mice (Boehm II*, et al.*, 2006). In this study, lower doses of THIP have been investigated for the effects on locomotor activity i.e. sedative rather anaesthetic. The reduction in locomotor activity observed in $\delta^{+/+}$ mice post treatment with THIP is lost in $\delta^{-/-}$ suggesting that the sedative actions of this drug are mediated by the δ containing $GABA_ARs$. Data produced by colleagues showed that THIP induced ataxia in $\delta^{+/+}$ mice at 10 and 30mg/kg but only induced ataxia in $\delta^{-/-}$ mice at 30mg/kg and at this dose the recovery time in $\delta^{-/-}$ was markedly reduced (Figure 4.11). The ataxic response as measured by rotarod to 15mg/kg THIP is also abolished in $\alpha4^{-/-}$ mice (Chandra*, et al.*, 2006). The locomotor activity of $\alpha4^{+/+}$ mice as measured by open-field is also reduced after 10mg/kg THIP injection and this sedative effect is also abolished in $\alpha4^{-/-}$ mice (Chandra*, et al.*, 2006). Recent results from our laboratory (Herd*, et al.*, 2009) have shown that the THIP induced inward current in ventral basal neurones (whole cell patch-clamp recordings) is absent in $\delta^{-/-}$ mice. Moreover the magnitude of tonic and THIP induced currents was significantly reduced. Furthermore, THIP-induced extrasynaptic conductance was dramatically reduced in $\alpha4^{-/-}$ mice (Chandra*, et al.*, 2006), suggesting that these effects are mediated by α4δ containing receptors of the

thalamus. Although both α1 and α4 subunits are abundantly expressed in the thalamic relay neurons, the α4 subunit is partner to the δ subunit (Belelli, *et al*., 2005; Jia, *et al.,* 2005; Chandra, *et al.,* 2006).

Combined these data conclusively reveal the α4δ subtype as the selective molecular target of the actions of THIP.

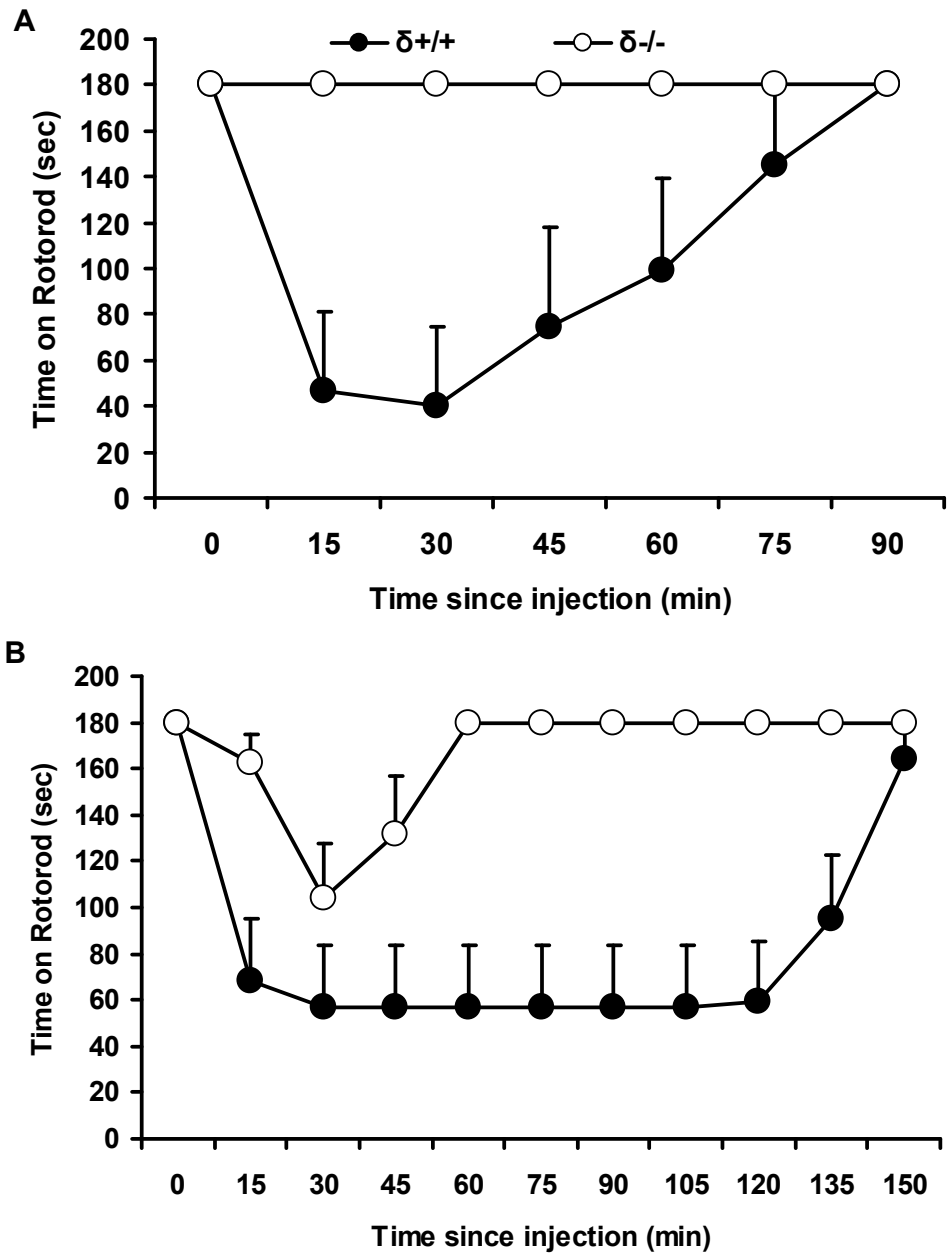


***Figure 4.11: The effect of THIP on rotarod performance in $\delta^{-/-}$ and $\delta^{+/+}$ mice.***

*THIP (10mg/kg) reduced locomotor activity in $\delta^{+/+}$ but not $\delta^{-/-}$ mice (A). THIP (30mg/kg) reduced rotarod performance in $\delta^{+/+}$ and $\delta^{-/-}$ mice (B), but recovery was much faster in $\delta^{-/-}$ mice. Data derived from Herd, et al., 2009 and produced by Dev Chandra and Greg Homanics, University of Pittsburgh.*

Locomotor activity was impaired in $\delta^{+/+}$ mice at doses of THIP as low as 2.5mg/kg; however, impairment on the rotarod was not observed until 10mg/kg. This could simply be explained by the intrinsic differences between the 2 tests; one a measure of spontaneous activity, the other a measure of forced activity and one could hypothesise that a greater inhibitory conductance maybe required to suppress forced in comparison with spontaneous activity. Alternatively, the preferential partnership of δ with α4 (spontaneous activity) in the thalamus and α6 (forced activity) in the cerebellum may contribute to differences between the tests. As mentioned above, the cerebellum is rich in α6δ and altered GABAergic transmission in the cerebellum has been associated with deficits in motor coordination analogous to those reported here and by our collegues (Herd*, et al.*, 2009). Furthermore, it has recently been speculated (Cremers and Ebert, 2007; Ebert*, et al.*, 2008) that the peak brain concentration of THIP following injection with doses similar to those used here would result in a differential activation of a6d subtype (50% of the GABA-evoked maximum) and the α4δ subtype (15-20% of the GABA-evoked maximum). Therefore, one could speculate that a greater dose and level of receptor occupancy would be required to induce rotarod impairment. In support, both thalamic relay neurones and the dorsal striatum (both rich in α4δ subtypes) have been implicated in the control of motor function (for review see Groenewegen, 2003).

It is worth noting, however, that potential compensatory changes in the $\delta^{-/-}$ mouse can lead to difficulty in the interpretation of the results shown here

and those produced by our colleagues (Herd*, et al.*, 2009). For example decreased expression and coupling of the γ2 and α4 subunits have been documented in the $\delta^{-/-}$ mouse (Korpi et al, 2002). Similarly an upregulation of the γ2 subunit has been identified in cerebellar granule cells. The development of neurone specific knock-out mice will help elucidate the relative contributions of α4δ and α6δ subtypes in the behavioural effects of THIP.

### 4.5.4 Removal of the δ subunit does not change behavioural effects of chlordiazepoxide or etomidate.

The reduction in locomotor activity shown in $\delta^{+/+}$ following injection with chlordiazepoxide or etomidate are not changed in the $\delta^{-/-}$ mice, suggesting that the sedative properties of these drugs are not mediated by the extrasynaptic receptors. $\delta^{-/-}$ mice are also not susceptible to the anaesthetic properties of etomidate (Mihalek *et al,* 1999). Electrophysiological studies of the $\delta^{-/-}$ mouse have suggested that the inward current induced by etomidate in wild type DG neurones is mediated by the δ subunit (Herd *et al,* 2008), suggesting that the behavioural data from this study is somewhat at odds with the electrophysiological data. However, it appears that the heterogeneity of the β subunits extrasynaptically and synaptically may go some way to explain these discrepancies, as it is known that the effects of etomidate are strongly determined by the type of β subunit (see Sections 1.3.2 and 1.4.2).

### 4.5.5 Removal of the δ subunit reduces the sedative properties of alphaxalone

$\delta^{-/-}$ mice showed no reduction in locomotor activity following treatment with 10mg/kg alphaxalone. Furthermore, in the first 5mins after alphaxalone injection the drug increased locomotor activity in $\delta^{-/-}$ mice in comparison with $\delta^{+/+}$ mice. $\delta^{-/-}$ mice treated with 20mg/kg showed a significant decrease in locomotor activity throughout the 20min session. These results are in contrast with those previously published. Previous experiments have shown that deletion of the δ subunit dramatically reduced sleep time following 8 and 16mg/kg (i.v) alphaxalone and 8mg/kg (i.v.) pregnanolone (Mihalek*, et al.*, 1999). There are differences in experimental design between the current study and those previously published. The locomotor activity assay used in this study did not measure the same parameters as the sleep time assay. Furthermore, the route of administration is also different; it may be that the amount of neurosteroid available to the receptors using these different administration methods has led to the different behavioural profiles. C57BL/6mice treated with low dose alphaxalone showed improved performance on the rotarod further supporting the idea that alphaxalone might have stimulant properties at low doses. The role of extrasynaptic receptors in the effects of neurosteroids remains unclear but warrants further investigation.

## *4.6 Summary*

In summary, the results presented here show that the δ containing receptor mediates the behavioural effects of THIP and at the very least is involved in mediation of the behavioural effects of neurosteroids.

The regions responsible for the mediation of distinct behavioural effects could be further investigated with the generation of region-selective knock out mice. However, this is beyond the scope of this thesis.

# 5 Optimisation of Attentional Set Shifting task for mice

*"Every one knows what attention is. It is the taking possession by the mind, in clear and vivid form, of one out of what seem several simultaneously possible objects or trains of thought. Focalization, concentration, of consciousness are of its essence. It implies withdrawal from some things in order to deal effectively with others, and is a condition which has a real opposite in the confused, dazed, scatterbrained state which in French is called distraction, and Zerstreutheit in German."*

William James in the Principles of Psychology, 1890

In Principles of Psychology Volume 1

## *5.1 Objective*

To develop an attentional set shifting task for mice, with the view to using this task to investigate the putative cognitive enhancing effects of THIP and the role of GABAergic inhibition in the control of attention.

## *5.2 Introduction*

The previous chapters of this thesis have focused on the role of GABAergic inhibition in the mediation of the sedative properties of BDZs and other GABA selective ligands. However, in this chapter we turn to the functional questions beyond sedation. The role of $GABA_AR$s in the sedative actions of a variety of drugs demonstrated in Chapters 3 and 4 is clear. Indeed, evidence suggests that $GABA_AR$ agonists produce sedation and anaesthesia by engaging the endogenous sleep circuitry (Nelson*, et al.*, 2002; and for review see Franks, 2008).

### 5.2.1 Sleep and Cognition

It is also well understood that lack of sleep can lead to deficits in alertness, vigilance and concentration (Belenky*, et al.*, 2003; Kendall*, et al.*, 2006) as well as higher cognitive processes such as complex decision making (Harrison, Y and Horne, 1999; Harrison, Y*, et al.*, 2007; Killgore*, et al.*, 2006; Killgore and McBride, 2006; Linde*, et al.*, 1999), inhibitory control (Drummond*, et al.*, 2006; Harrison, Y*, et al.*, 2007) and attention (Nilsson*, et al.*, 2005). Indeed, it has recently been suggested and discussed in a recent New Scientist article (Young, 21st February, 2009)

that lack of sleep may be a cause of many psychiatric conditions, including attention deficit disorder rather than a symptom. It could therefore be hypothesised that improved sleep would improve cognitive performance.

THIP has been shown to improve sleep quality by increasing the amount of SWS in patients. Importantly, THIP does not affect the amount or profile of REM sleep (Ebert*, et al.*, 2008; Faulhaber*, et al.*, 1997; Lancel and Faulhaber, 1996; Lancel*, et al.*, 1997). Anecdotal evidence from clinical trials has suggested a potential improvement in next day cognitive performance following treatment with THIP (personal communication, Bjarke Ebert) however there is no direct pre clinical evidence to suggest this is the case.

To investigate the potential role of THIP as a cognitive enhancer (directly or indirectly due to improved sleep), an attentional set shifting task has been used.

### 5.2.2 Behavioural flexibility and the mental set

Behavioural flexibility is the ability to adapt behavioural strategies to face changes in one's environment. Behavioural flexibility will result in the change of behaviour to suit the change in environment. Behavioural flexibility can reflect changes in cognitive state but behavioural and cognitive flexibility are distinct in that cognitive actions are covert and that

behavioural actions are overt. Despite this one can make inferences to cognitive states by observation of behavioural actions.

Mental Set is the frame of mind which allows one to solve a particular problem but may not be the most effective way to do so. For example, if one approaches a door, which has always opened by a push mechanism, but on one new occasion the door does not open upon the first push - does one push harder or try to pull the door? A strong mental set would result in pushing harder, where as a weak mental set would allow for behavioural flexibility and one would try to pull the door. In a predictable environment, an established and strong mental set is beneficial; however, in unpredictable environments, cognitive flexibility is required and mental sets must be easily changed. The mental sets of most relevance here are those of attentional set and learning set.

### 5.2.3 Attentional Sets and Reversal Learning

Attentional sets are formed from previous experience. Following the formation of attention set, the participant become more likely to attend to selective relevant features (dimensions) than to other dimensions of a given stimulus. So, for example, the sorting of objects according to a given feature, or aspect, of the stimulus (colour/shape/texture) leads to the formation of an attentional set.

The Wisconsin card sort task (WCST) and Intradimensional-Extradimensional (ID-ED) task are commonly used to assess attentional processing in patients. In the WCST, the patient is presented with a collection of cards, which differ in various stimuli such as number of objects and shape. The participant must use these stimuli to match the cards correctly without knowing which of the stimuli are relevant to creating the correct match. The participant learns which is the relevant stimulus based on positive reinforcement and learns which is the relevant stimulus by trial and error. Changing the relevant stimulus, thus requiring a shift of attentional set can test the level of attentional set formation. A strong attentional set, will be marked by a large number of errors and slow acquisition of a new attentional set.

Reversal learning is also a form of flexibility. Reversal learning is used here to refer to the unlearning of an association to allow for a new association to be learned. A previously learnt conditioned response to

positive stimuli is extinguished and a negative stimulus becomes a positive stimulus and therefore produces the conditioned response.

### 5.2.4 Attention and human disease

Deficits in attention are common in many illnesses, such as traumatic and non-traumatic brain injuries (stroke, brain tumours), neurodegenerative disorders (Alzheimer's and Parkinson's disease) and mental illness (schizophrenia and attention deficit hyperactivity disorder (ADHD)). Indeed, deficits in attention and executive functions rather than memory are the first observed in neurodegenerative disorders such as Alzheimer's (for review, see Perry and Hodges, 1999). Deficits in attentional functions tend to be related to problems with frontal lobe function and in particular the prefrontal cortex (for review see Dalley*, et al.*, 2004).

### 5.2.5 Attentional Set Shifting in non-human primates and rats

Attentional set shifting until 2000 was largely investigated using primates. This was because it was largely accepted that primates had the forebrain structures (prefrontal cortex) that allowed for such complex behaviours. In primates, the prefrontal cortex mediates attentional set shifting between different perceptual dimensions (Owen et al, 1991; Dias et al, 1996a,b, 1997, Passingham, 1972). Following lesions of the prefrontal cortex, a distinct impairment in extra dimensional (ED) shifting is observed in primates (Dias et al, 1996a,b).

In 2000, using a novel task similar to the human and monkey versions of the WCST, it was shown that rats do indeed have the ability to perform complex shifts of attention and that after bilateral lesions of the medial frontal cortex the same impairments are found in rats as in non-human primates, indicating that the medial frontal cortex in the rat has similar function to the prefrontal cortex in the primate (Birrell and Brown, 2000a; b). Rodents will readily dig to obtain a food reward. This new attentional set shifting task required the rats to dig in bowls to obtain a food reward. The bowls could be changed to differ in several dimensions, the digging medium texture, the digging medium odour and/or the texture of the bowl itself. Rats could perform discriminations and performed inter dimensional (ID) shifts with greater ease than ED shifts. This difference in ID/ED performance indicates the rats were forming attentional sets, and is assumed to be fundamental to the task at large (personal communication, Verity Brown). Since the development of this task, rats have been used commonly to investigate attentional set shifting. Indeed, the original paper has been cited in 160 research articles since 2000 (as of February 2009, ISI Web of Knowledge).

### 5.2.6 Attentional Set Shifting in Mice

The development of genetically altered mice has provided new and unique opportunities to study executive function. However, using mice in this field is exceptionally new. This is highlighted by a PubMed search for “attentional set shifting & mice” which returns 10 main articles (as of

February 2009). This collection of papers suggests that mice do not make an ideal species for behavioural research of this type.

The first of these articles was published in 2002 and was the first attempt at optimisation of this task for mice. Colacicco and colleagues Colacicco*, et al.*, 2002 modified the rat paradigm using two different strains of mice (129Sv/Ev and C57BL/6J). These strains are both commonly used for the generation of transgenic mouse models. Both these strains of mice readily learned to discriminate bowls based on odour and texture of digging medium; however, strain variation was detected at the simple discrimination (SD) and ED shift. C57BL/6J mice took significantly more trials to reach criteria at both of these stages compared with 129Sv/Ev mice. Importantly, there was no difference between the number of trials needed to reach criteria between the ID shift and ED shift in either strain suggesting under the conditions used the mice did not form an attentional set.

This lack of an ID/ED difference in mice was also evident in research investigating the effects of phencyclidine (PCP) on attentional set shifting in mice (Laurent and Podhorna, 2004), although deficits in the task at large were shown to be induced by PCP, an effect also shown in rats.

Furthermore, when mice were tested in a computerised touch-screen version of the task with visual stimuli (Bussey*, et al.*, 2001) there was again no ID/ED difference indicating a lack of formation of attentional set.

The role of dopamine receptors (D2 & D3) in attentional set shifting was investigated using mice lacking D2 or D3 receptors (Glickstein*, et al.*, 2005). Here, the authors used an adaptation of another rat paradigm (Fox*, et al.*, 2003). In this form of the task, the number of reversal stages is reduced from three to one. This was to ensure that the mice could complete ID and ED stages of the task. Although the mice completed all stages presented and differences at various stages between wild type and mice lacking the D2 and D3 receptors were detected, there was no evidence of a significant ID/ED difference in any of the genotypes.

Garner and colleagues (Garner*, et al.*, 2006) did show an ID/ED difference, but training took place over a number of days in contrast with the one-day required in rats (Birrell and Brown, 2000a). The requirement for additional training suggests that mice may form attentional sets differently to rats and primates, but they nevertheless do form them (Garner*, et al.*, 2006).

The most convincing evidence that mice can indeed form attentional sets comes from a recent article, which studied the effects of medial and orbital prefrontal cortical lesions on attentional and learning set formation in mice (Bissonette*, et al.*, 2008). As with rats, learning and attentional set formation was disrupted by orbitofrontal and medial prefrontal neurotoxic lesions respectively. Interestingly, this study adapted the rat paradigm by introducing extra ID shifts. By increasing the number of ID shifts, a 4$^{th}$ ID/ED difference was found, thus suggesting the formation of attentional

sets. The authors failed to find evidence of formation of an attentional set following only one ID shift suggesting that formation of attentional set in the mouse is heavily dependent on the number of previously encountered discriminations.

Therefore, before investigating the potential cognitive enhancing effects of THIP, the optimisation of a mouse attentional set shifting task is carried out.

## *5.3 Optimisation of Attentional Set Shifting*

### 5.3.1 Equipment Changes

The testing box was a smaller version of the original design for rats. The box (Figure 5.1) consisted of a large waiting area (31.5 x 16cm) and two smaller areas (testing compartments; 7.5 x 8cm), which were separated by wooden doors which could be used to selectively occlude either or both of the two testing compartments. Within each of these testing compartments a digging bowl could be placed. The digging bowls (Figure 5.2) used were glass “t-light” holders (Morrisons, UK; 6 x 6cm, 4cm diameter). The advantages of these bowls were that they could be easily washed to prevent “cheating” by scent marking. They also had a flat surface for the mouse to stand on while digging and were of an ideal size.

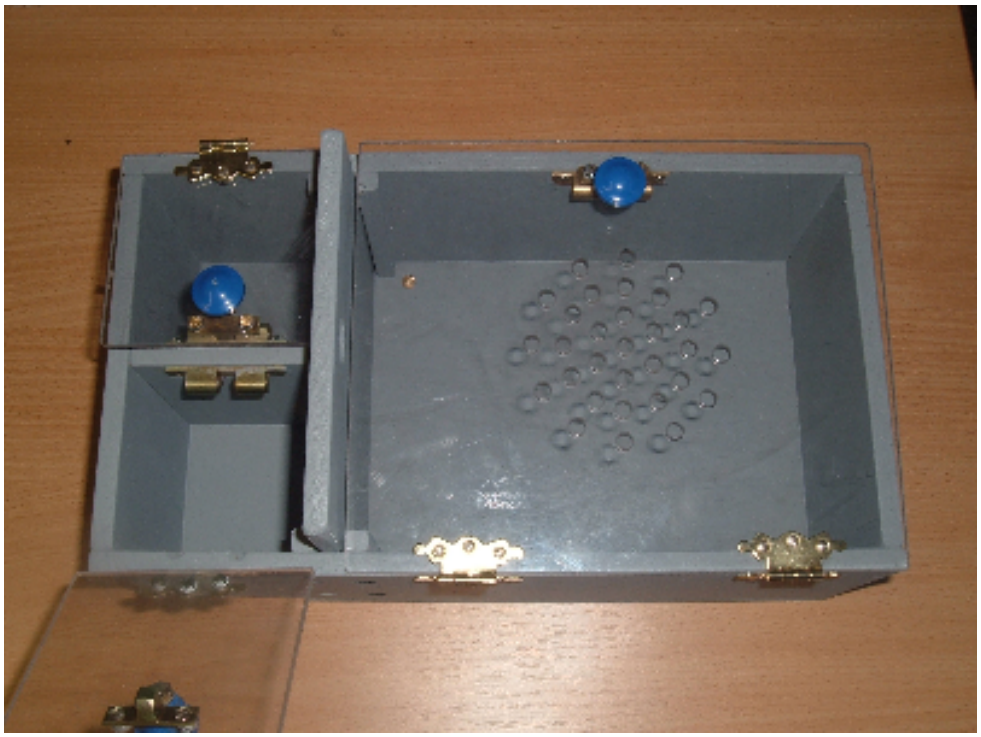

*Figure 5.1: Photograph of attentional set shifting box design for mice.*

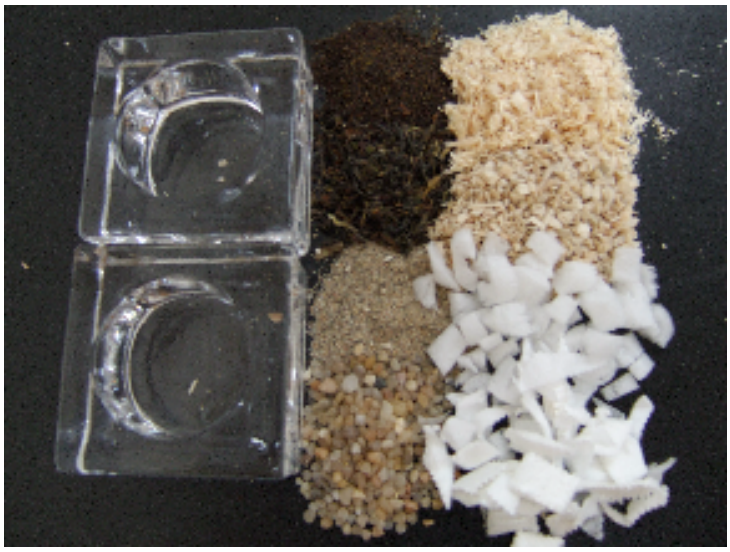

*Figure 5.2: Photograph of candleholders used as digging bowls and examples of digging media for attentional set shifting task.*

### 5.3.2 Exemplars

The dimensions, which were varied in this task, were digging medium (polystyrene, confetti, coarse or fine tea, gravel, pebbles, coarse or fine shavings) and odour (mint, oregano, cinnamon, cumin, thyme, paprika, nutmeg, cloves). These dimensions and exemplars are used in pairs that remain the same throughout testing (Table 5.1). These exemplars were the same as those used in the studies of rats.

### 5.3.3 Animals

To optimise the attentional set shifting task for mice, male C57BL/6J mice were used (n = 6). These animals were chosen because this is the background strain of the majority of the transgenically altered mice used in our laboratory. Food was removed from home cage 4 days before training. Mice were maintained ~85-90% of original free feeding body weight by feeding ~2-4g of standard laboratory chow each day with water available *ad libitum*. Mice then underwent a period of training before testing.

| Dimension | Training Pair | Pair 1 | Pair 2 | Pair 3 |
|---|---|---|---|---|
| **Medium** | Polystyrene<br>Confetti | Coarse Tea<br>Fine Tea | Gravel<br>Pebbles | Coarse Shavings<br>Fine Shavings |
| **Odour** | Mint<br>Oregano | Cinnamon<br>Cumin | Thyme<br>Paprika | Nutmeg<br>Cloves |

***Table 5.1: Exemplars used for attentional set shifting task***

*Exemplars (medium and odour) are always presented in pairs because full counter balancing was not possible. The order of exposure to pairs 1-3 was counterbalanced according to a Latin square.*

### 5.3.4 Experiment One

#### *5.3.4.1 Behavioural Training*

For the first 3 days, mice were placed in testing box for 10mins each to allow habituation to the box. Bowls used for testing were also placed over night in the home cage filled with home cage bedding and a quantity of the reward food (Noyes Food Pellets, 20mg). On the fourth day the mice were placed in the waiting area of the boxes and bowls filled with sawdust and containing a reward pellet were placed in the 2 testing compartments. The doors were removed in turn allowing the mice to dig and eat both rewards. The mice were required to carry this procedure a further 6 times before returning to the home cage. The next day mice were then exposed to 2 simple discriminations (SD) using the training pairs one based on odour the other based on digging medium. The mouse was required to learn which exemplar was associated with the reward. This stage of testing was terminated once the mouse reached criterion performance, which was set at 6 consecutive correct trials, and included the first 4 trials. The probability of making a correct choice 6 times consecutively by chance is 0.015.

#### *5.3.4.2 Behavioural Testing*

The following day, the mice were given a series of 7 discriminations. These included an SD, a compound discrimination (CD) in which the stimuli differed according to both their odour and digging medium, but with correct and incorrect exemplars remaining the same as for the

preceding SD; a reversal (CDRev) where the exemplars remain the same as the CD but the correct and incorrect stimuli are reversed; an intradimensional (ID) acquisition in which the mouse learned a novel discrimination with new stimuli but in which the new correct exemplar was of the same dimension as the previously correct stimulus exemplar; a ID reversal (IDRev) where the rules of CDRev are applied to the ID stimuli; an extradimensional-shift (ED) in which the mouse learned a second novel discrimination also with new stimuli but the new correct exemplar is from the other previously irrelevant dimension and finally a third reversal (EDRev) in which the rules of CDRev were applied to the ED stimuli. The mouse advanced to the next stage of the task when criterion was achieved.

If the mouse dug in the correct bowl and retrieved the reward a “correct” trial was recorded and the trial terminated by closing off the testing compartments once the mouse had returned to the waiting area. If the mouse dug in the incorrect bowl the mouse would then be allowed to explore the correct bowl to gain the reward for the first 4 trials only, from the fifth trial onwards the doors would be closed and the mouse would not gain a reward, in both cases an “incorrect” trial would be recorded.

#### *5.3.4.3 Analysis*

Analysis was carried out using one-way ANOVA with repeated measures (stage as the within subjects factor) and planned comparisons (paired t-tests) to test for differences between CD and CDrev as well as differences between ID and ED.

#### *5.3.4.4 Results*

Figure 5.4 shows that trials to criterion differs with stage of testing (main effect of stage, $F(5,25) = 97.4$, $p < 0.001$). As expected mice took significantly more trials to reach criterion at CDrev than when initially learning the CD (paired t-test, $t = -7.980$, $d.f = 5$, $p < 0.001$). However mice did not take significantly more trials to reach criterion at the ED than at the ID (paired t-test, $t = 1.0$, $d.f = 5$, $p > 0.05$) suggesting lack of attentional set formation. The lack of an ID/ED difference appears to be as a result of an unusually high number of errors at the ID acquisition.

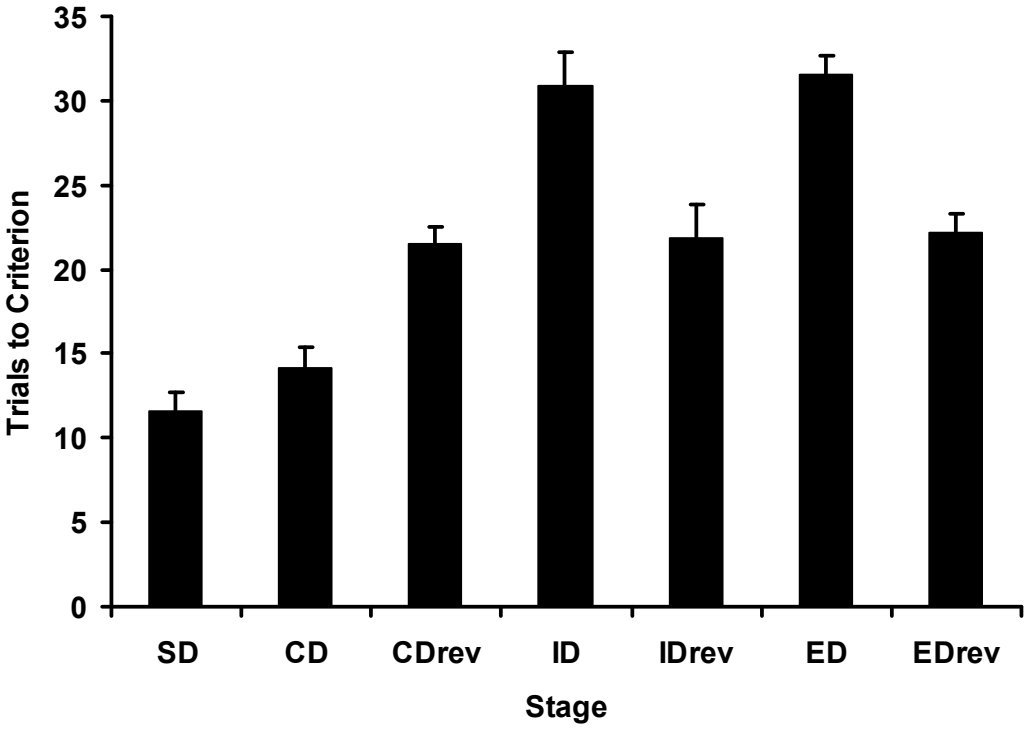


***Figure 5.3: No ID/ED difference was identified in C57BL/6 mice.***

*Histogram showing the trials to criterion (±SEM) of C57BL/6 mice (n = 6). Mice took more trials to reach criterion at the CDrev than at the CD ($p < 0.001$). There was no ID/ED difference ($p > 0.05$).*

### 5.3.5 Experiment Two

One possible explanation for the inflated number of trials required at the ID is the exposure to novel stimuli. To eliminate the effect of novel exemplars at the ID stage of behavioural testing, an extra day of behavioural training was introduced. After a 7 day rest period, mice were again trained on the task. On day 5, each mouse was required to dig for at least 2 rewards in the exemplars to be used in testing. Behavioural testing and analysis was carried out as per experiment 1.

#### *5.3.5.1 Results*

Figure 5.5 shows that trials to criterion differ with test stage (confirmed by a main effect of stage, $F(5,25) = 6.8$, $p < 0.01$). As expected, mice took significantly more trials to reach criterion at CDrev than when initially learning the CD (paired t-test, $t = -2.672$, d.f. = 5, $p < 0.01$). In contrast with the original training, mice also took a greater number of trials to reach the ED when compared with the ID (paired t-test, $t = -1.594$, d.f. = 5, $p < 0.05$), suggesting the formation of attentional set.

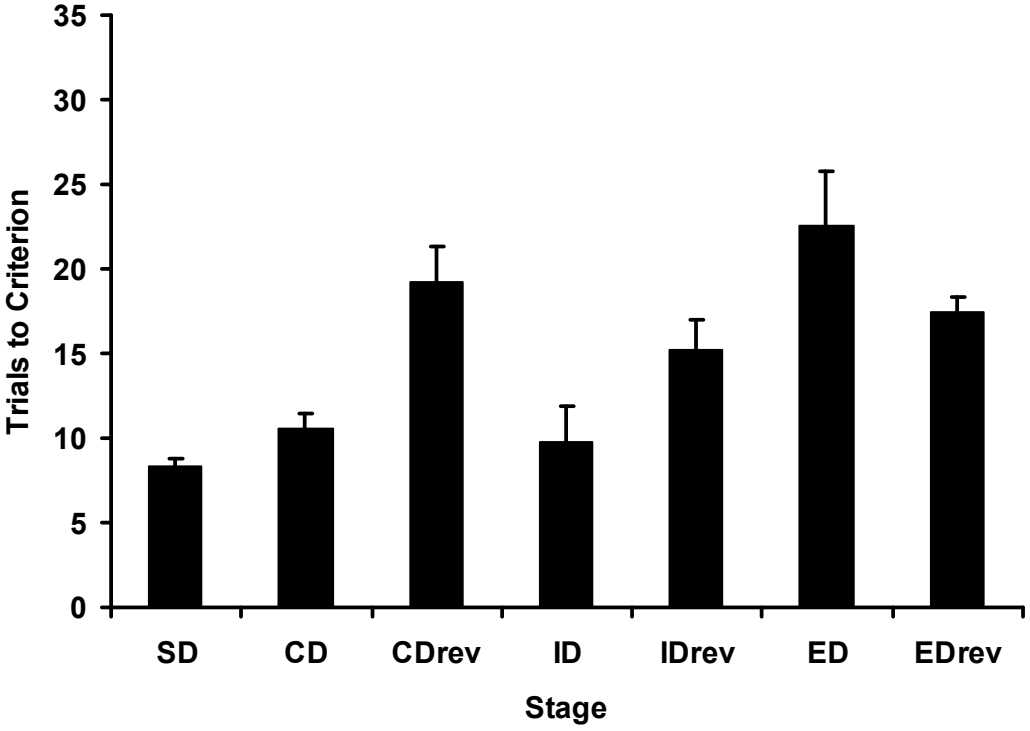


***Figure 5.4: An ID/ED difference was identified in C57BL/6 mice.***

*Histogram showing the trials to criterion (±SEM) of wild type mice (n = 6). Mice took more trials to reach criterion at the CDrev than at the CP ($p < 0.01$). In contrast to experiment one, there was a difference between ID and ED acquisitions ($p < 0.05$).*

### 5.3.6 Experiment Three: The effect of THIP on attentional set-shifting

The mice were retested following injection (i.p.) with 10mg/kg THIP or with saline vehicle at 24 or 36hrs before behavioural testing. Mice were tested repeatedly, 7 days apart in a counter balanced design. Planned comparisons of the trials to criteria and errors made on the ID and ED stages and reversal stages alone were performed, as this was where differences following THIP treatment were expected.

#### *5.3.6.1 Results*

##### *5.3.6.1.1 Trials to Criterion*

Figure 5.6 shows that mice took more trials to reach criterion at the ED stage versus the ID stage (confirmed by main effect of stage ED > ID; $F(1,5) = 144.9$, $p < 0.001$). There was no main effect of drug and no interaction (for both, $F(2,10) < 1.0$). Figure 5.6 also shows that 10mg/kg THIP appeared to reduce the trials to criterion, particularly at the first 2 reversal stages; however, the main effect of drug only approached significance ($F(2,10) = 3.85$, $p = 0.057$).

##### *5.3.6.1.2 Errors to Criterion*

Figure 5.7 shows the number of errors did not vary with reversal repeat ($F(2,10) = 0.018$, $p > 0.05$) or with type of error ($F(1,5) = 0.015$, $p > 0.05$). THIP had an effect on the number of overall errors (confirmed by main

effect of drug, $F(2,10) = 7.273$, $p < 0.05$). The total number of errors made during all three reversal stages 36hs post THIP injection were less than those made following injection with saline (paired t test, $t = 3.664$, d.f. = 5, $p = 0.015$).

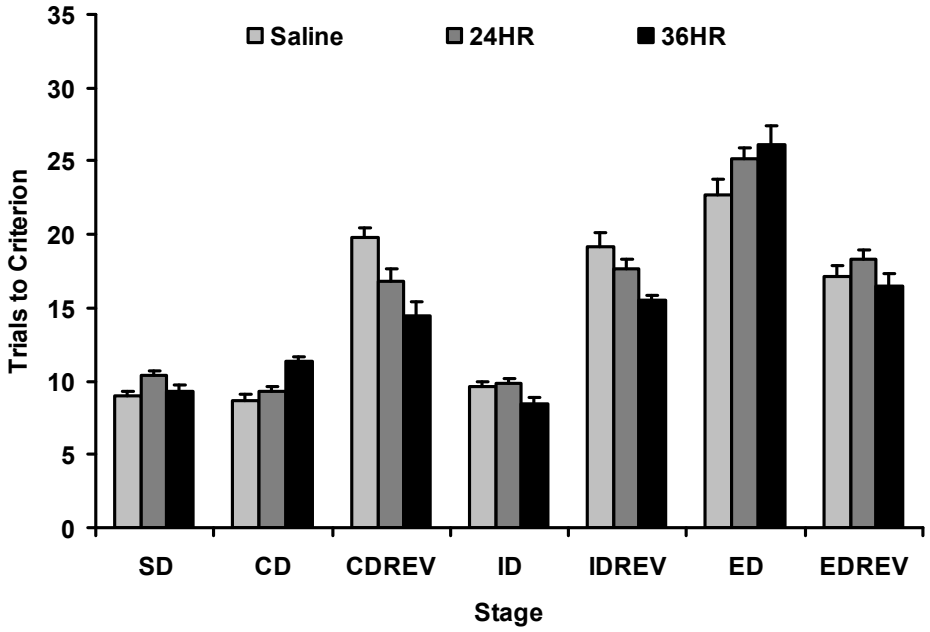


***Figure 5.5: THIP reduced the number of trials to criterion.***

*Histogram showing the trials to criterion (±SEM) of wild type mice (n = 6). Mice took more trials to reach criterion at the ED than at the ID ($p < 0.001$). A reduction in trials to criterion at the first 2 reversal stages only approaches significance ($p = 0.057$).*

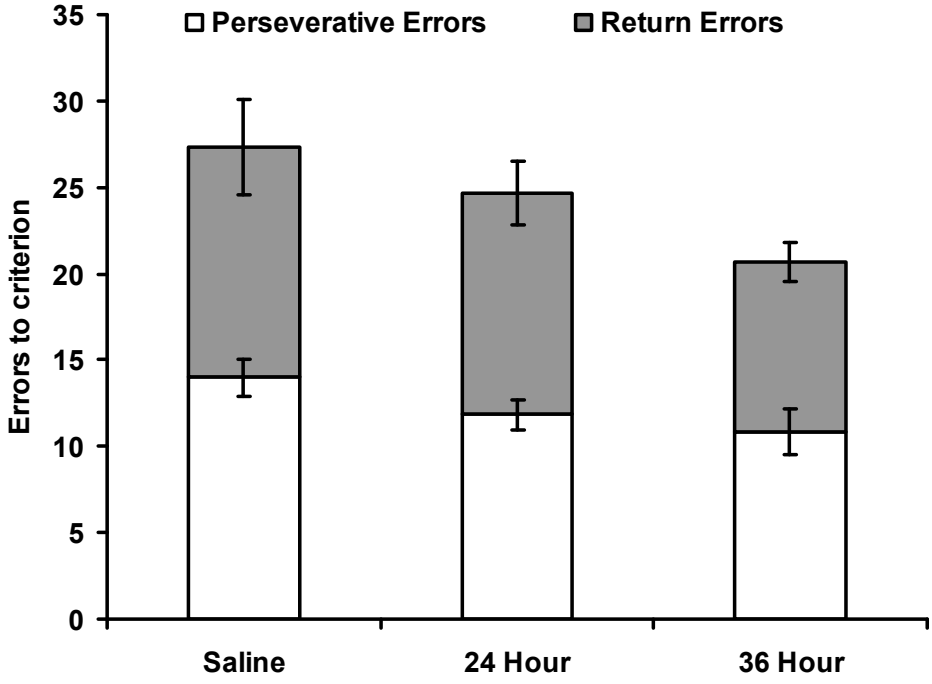


***Figure 5.6: THIP reduced the number of errors 36 hours after injection.***

*Histogram showing the errors to criterion (±SEM) of wild type mice (n = 6). THIP had an effect on the overall number of errors made at the reversal stages 36 hours after treatment ($p < 0.05$).*

## *5.4 Attentional Set Shifting in $\delta^{-/-}$ mice*

### 5.4.1 Subjects

Mice (n = 6) lacking the extrasynaptic δ subunit ($\delta^{-/-}$) and appropriate wild type ($\delta^{+/+}$) mice from the same colony were used for all experiments. For more details on genotypes used in these experiments, please refer to Chapter 2. All animals were maintained in similar environmental conditions, with nesting material and cardboard tubes as environmental enrichment.

### 5.4.2 Methods

Food was removed from home cage 4 days before training. Mice were maintained ~85-90% of original free feeding body weight by feeding ~2-4g of standard laboratory chow each day with water available *ad libitum*. Mice then underwent training and testing procedures as established above. Mice underwent training and testing in pairs so that one $\delta^{-/-}$ and one $\delta^{+/+}$ were tested concurrently.

### 5.4.3 Results

As described above (see Section 5.3.4), during training mice were required to dig for rewards from small bowls of bedding before testing begun. Unfortunately, $\delta^{-/-}$ mice did not readily dig in bowls and although extra training sessions were introduced, $\delta^{-/-}$ mice did not dig reliably to warrant further testing. Interestingly, during training, some personal observations were made which suggested an unusual behavioural

phenotype in these mice. This included reduced activity and exploration in comparison with simultaneously trained $\delta^{+/+}$ mice. The $\delta^{-/-}$ mice seemed to stay in one corner of the testing apparatus and not explore the digging bowls as readily as the $\delta^{+/+}$ mice. One potentially important behaviour worthy of note was the burying of bowls. Home cage bedding is placed in the testing apparatus, and on a few occasions during training $\delta^{-/-}$ would pile the bedding on top of the digging bowls. Due to this unusual behaviour and inability to train the mice this experiment was abandoned.

## *5.5 Discussion*

### 5.5.1 Optimisation of attentional set shifting task for mice

Wild type mice were tested in an attentional set shifting paradigm for mice to establish if mice were a suitable model for the further study of attention deficits. Using the established rat protocol, no ID/ED difference was found. However, following the addition of pre exposure to the testing exemplars, an ID/ED difference was detected. In experiment 1, an unusually large number of trials were required to reach criterion at the ID stage. By reducing the effect of neophobia at this stage, the data suggest that mice are indeed capable of forming attentional sets in a similar manner to rats. The role of anxiety in tasks involving learning processes is well documented; indeed, the performance of 129/SvEv mice (a strain known to display a higher anxiogenic profile in the elevated plus maze and open field {Homanics*, et al.*, 1999; Montkowski*, et al.*, 1997}) in the attentional set shifting task is below that of the C57BL/6 strain (Colacicco*,*

*et al.*, 2002). Furthermore, stress in humans has been shown to impair attentional set shifting (Orem*, et al.*, 2008) and, although there is still debate in the literature, stress has been linked with the onset of various cognitive deficits (for review, see de Kloet*, et al.*, 1999).

In contrast with our data, Colacicco*, et al.*, 2002 failed to show formation of attentional sets in either 129Sv/Ev or C57BL/6 strains. The authors suggest this may be a result of the lack of initial SD training. They found that SD performance predicted ED performance in both strains and suggest that selection of the relevant stimuli following the training SDs is an integral part of forming an attentional set. In the experiment, 2 mice were indeed pre trained with SDs, and this factor could account for the formation of attentional sets. However, no ID/ED difference was found in experiment 1, in which mice were also pre trained with SDs, although to a lesser extent.

Although this is the first example of mice forming attentional set shifting following a protocol very similar to that first used in rats (Birrell and Brown, 2000b), a recent publication has also demonstrated the formation of attentional set shifting in mice. Bissonette*, et al.*, 2008 showed that by increasing the number of ID shifts, a 4$^{th}$ ID/ED difference was found, thus suggesting the formation of attentional sets. The authors failed to find evidence of formation of an attentional set following only one ID shift suggesting that formation of attentional set in the mouse is heavily dependent on the number of previously encountered discriminations. In

contrast following pre exposure to the exemplars, we have also demonstrated formation of attentional sets in mice. This could however be a function of the repeated testing, but a recent study in rats using a repeated measures design to investigate the effects of asenapine on attentional set shifting found no effect of repeat testing (Tait*, et al.*, 2009).

There is now substantial literature linking lesions of the medial prefrontal cortex to deficits in attentional set shifting in primates (Nelson, 1976; Dias*, et al.*, 1996a; bPantelis et al, 1999; Goldstein et al, 2004) and rats (Birrell and Brown, 2000b) and lesions of the orbital frontal cortex have been linked to deficits in reversal learning (McAlonan and Brown, 2003; Schoenbaum*, et al.*, 2003). Recently, deficits in attentional set shifting following lesions of the medial prefrontal cortex and deficits in reversal learning following lesions of the orbital prefrontal cortex were shown in mice (Bissonette*, et al.*, 2008).

Both recently published work and the optimisation of the attentional set shifting protocol described here provides evidence that mice are indeed capable of forming attentional and affective sets and with the use of transgenic models provide a potential way of investigating the genetic basis of psychiatric disorders.

### 5.5.2 The effect of pre-treatment with THIP on attentional set shifting

Here, we investigated the potential “next morning” improvement of cognitive performance of pre-treatment with the sedative-hypnotic THIP. Although not significant, there was a trend for improved performance shown by a reduction in trials to criterion following THIP in the second and third reversal stages. We also analysed the errors made at the reversal stages and a significant reduction in total errors was observed 36hrs following injection with 10mg/kg THIP. Interestingly, the reduction in total errors was greater 36hrs post injection than 24hrs post injection. As this was a pilot study and low n numbers were used, it is possible that a significant effect may have been uncovered at 24hrs post injection with a greater sample size. However, if indeed this is a true representation of the effect of THIP, this is a somewhat surprising effect. One explanation is linked to the circadian rhythm and light/dark cycle. The mice were tested in the morning, therefore mice tested 24hrs post injection were injected at the beginning of the light cycle, the time at which mice are least active. However, mice tested 36hrs post injection were injected at the beginning of the dark cycle a time when activity would naturally be increasing. It is possible that THIP has somehow interfered with the natural circadian rhythm of the mice resulting in this greater effect 36hrs post injection. The circadian clock in mammals is largely mediated by the suprachiasmatic nucleus (SCN), which is reset daily by environmental cues. During the dark part of the cycle photic cues are particularly important but during the day other environmental cues play a role.

Evidence suggests that activation of $GABA_AR$s in the SCN (extrasynaptically mediated tonic inhibition) is responsible for phase shifting the circadian clock during the day and inhibits light input during the night. A recent study has shown that direct injection of THIP into the SCN abolished the ability of light to phase shift behavioural rhythms during the night but not midday (Ehlen and Paul, 2009). The effect of changes in circadian rhythm on attentional set shifting would require further investigation, and testing at different times post THIP injection and stages in cycle may help clarify this effect.

Although not significant, there is a trend in the data which suggests the reduction in total errors is largely due to a reduction in return errors. Return errors are errors made after making a correct choice in a reversal stage and can indicate impairment in learning a new response-reward association. Therefore, this decrease in return errors may indicate increased cognitive flexibility following THIP administration. Further testing is required to clarify this effect.

### 5.5.3 Attentional set shifting in $\delta^{-/-}$ mice

Unfortunately, it was not possible to train $\delta^{-/-}$ mice to take part in the attentional set-shifting task. During the training for this task, some unusual observations were made.

Reduced exploration and activity in the testing chamber were noted (although not recorded). This would reduce the chances of learning the

task as they would not explore and therefore dig in the bowls as readily as the $\delta^{+/+}$ counterparts. The use of the home cage bedding to "bury" the testing bowls seemed similar to behaviours observed in the marble burying test of anxiety used in mice. In this task, mice are placed individually into a novel environment. In this novel environment mice will bury glass marbles, which are present. This test has some predictive value for anti-depressant and/or anxiolytic drugs. Furthermore, mice treated with BDZs such as diazepam show reduced marble burying behaviour when compared to vehicle treated controls. Reduced exploration combined with the bowl burying could suggest heightened anxiety in $\delta^{-/-}$ mice. Indeed, the data produced while optimising this task for mice would suggest heightened anxiety would interfere with learning.

## *5.6 Summary*

In conclusion, the data shown here have established an attentional set shifting protocol for mice, which demonstrates that mice require only one intra dimensional shift to form an attentional set. We have also shown that we can manipulate the results of this test by pharmacological intervention. Furthermore, we have demonstrated that pre-treatment of THIP may have cognitive enhancing properties but further experiments are required. In the future, this test and various genetic and pharmacological interventions will be used to further our understanding of the role of GABAergic inhibition in the control of attentional processes.

The lack of the $\delta^{-/-}$ mice to participate in the attentional set shifting task suggests a previously undetected behavioural phenotype. Therefore, in the next chapter, the behavioural phenotype of the $\delta^{-/-}$ mouse is investigated further.

# 6 Behavioural phenotype of $\delta^{-/-}$ mice

## 6.1 Objective

To analyse the behavioural phenotype of $\delta^{-/-}$ to help elucidate reasons for the inability to carry out bowl digging task described in Chapter 5.

## 6.2 Introduction

As described in Chapter 5, it was not possible to train $\delta^{-/-}$ mice to carryout the bowl digging task. This chapter will investigate two possible causes for this.

To learn the task described in Chapter 5, mice must collect a sweetened reward from the bowls. If this sweetened pellet is not reinforcing, then the mice will not learn the task. Therefore, the first experiment will address the rewarding properties of a sweetened pellet using a lever pressing operant task.

The observations made in Chapter 5 suggest that neophobia and thus heightened levels of anxiety, can inhibit performance in this task. Therefore, the second experiment will measure the level of anxiety in $\delta^{-/-}$ mice using the EPM and the OF.

## 6.3 Animals

Mice lacking the extrasynaptic δ subunit ($\delta^{-/-}$) and wild type ($\delta^{+/+}$) mice from the same colony were used for operant training. For more details on the mice used in these experiments, please refer to Chapter 2. All animals were maintained in similar environmental conditions, with nesting material and cardboard tubes as environmental enrichment.

## 6.4 Responding for a sucrose reward

### 6.4.1 Method

Behavioural training and testing was carried out using an operant behaviour package acquired from Med Associates, USA. This consisted of a test chamber with a metal grid floor, which had two retractable response levers, a central pellet receptacle with head entry detector (photo beam), a house light and two stimulus lights, all contained within a sound-attenuating chamber (Figure 6.1). A pellet dispenser located outside the test chamber delivered sucrose reward pellets (Test Diet, 14mg) to the pellet receptacle following correct responses. The chambers were interfaced with a PC using DIG-716 SmartCtrl$^{TM}$ package and MED-PC IV software, which was used to record the number of lever presses and rewards gained. Programming was carried out as described in MED-PC IV programming manual.

Mice (n = 6) were food restricted to 85-90% of their free feeding body weight. Mice were habituated to the chambers over 3 days, 20min

sessions each day. During testing, the house light remain on and both levers were designated active (presses on either lever were rewarded). There was no time out period. Each session ended after 1 hour or when the mouse had gained 100 rewards, which ever occurred first. The number of both left and right lever presses was recorded. Mice were tested for 6 consecutive days on a fixed ratio of 1 reward for 1 lever press, on a continuous reinforcement paradigm with no time out period.

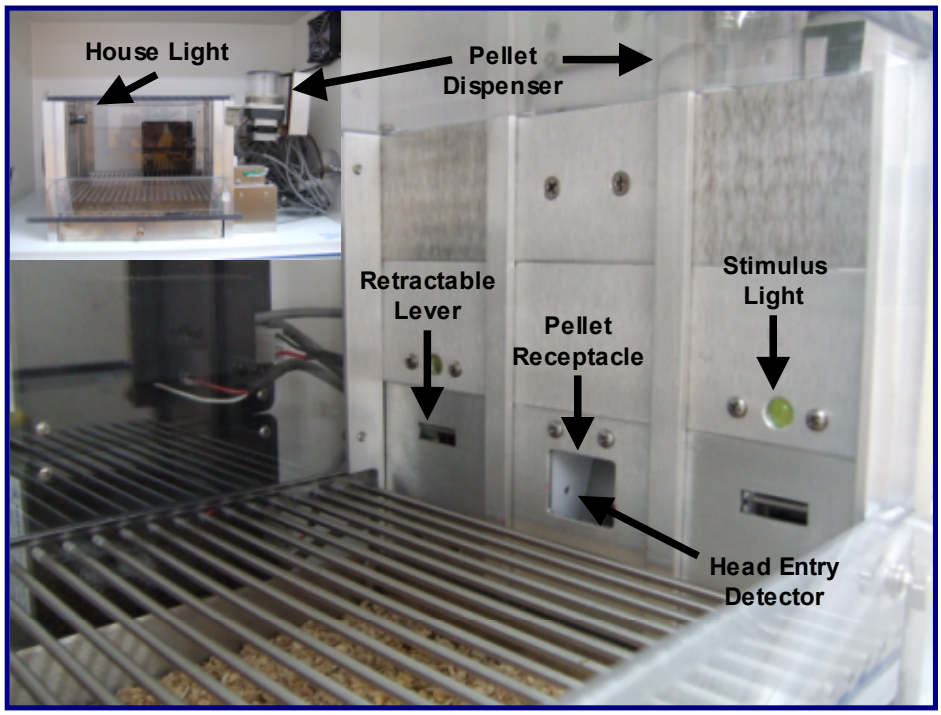


*Figure 6.1: Photograph of the operant chambers.*

### 6.4.2 Results

As shown in Figure 6.2 the number of lever presses made by $\delta^{+/+}$ and $\delta^{-/-}$ mice increases over days (confirmed by simple main effect of day: $F(5,70) = 21.1$, $p < 0.001$). Furthermore $\delta^{-/-}$ mice press less levers than $\delta^{+/+}$ mice and this difference occurred on all days (simple main effect of genotype: $F(1,14) = 97.3$, $p < 0.01$, day by genotype interaction not significant).

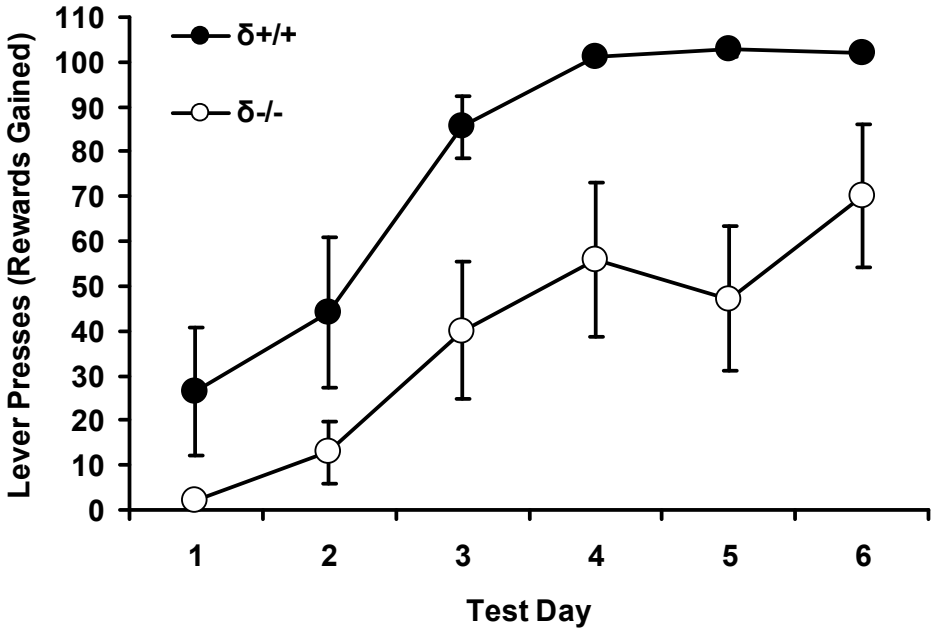


***Figure 6.2: $\delta^{-/-}$ mice respond less for a sweetened reward than $\delta^{+/+}$ mice.***

*Line graph showing the mean (±SEM) lever presses carried out by $\delta^{+/+}$ and $\delta^{-/-}$ mice ($n = 6$) on 6 consecutive days of operant testing. $\delta^{-/-}$ mice consistently pressed fewer levers than $\delta^{+/+}$ in the 1h session ($p < 0.01$).*

## *6.5 The anxiety profile of $\delta^{-/-}$ mice*

### 6.5.1 Elevated Plus Maze

Male (n = 8) and female (n = 4) $\delta^{-/-}$ and $\delta^{+/+}$ mice were tested on the EPM according to the methods described in Chapter 2. Due to the low number of arm entries in a number of mice these data were analysed using the non-parametric Mann Whitney U Test (2-tailed).

As shown in Figure 6.3A the total number of arm entries did not vary between genotype in male ($U = 26.5$, $p > 0.05$, mdn (wt) = 14, mdn (ko) = 12) or in female ($U = 5.5$, $p < 0.05$, mdn (wt) =16, mdn (ko) = 16) mice. This is consistent with the locomotor activity levels recorded previously (see Chapter 4). Male $\delta^{-/-}$ mice appear to make fewer open arm entries than $\delta^{+/+}$ mice ($U = 2$, $p < 0.01$, mdn (wt) = 4, mdn (ko) = 0); however, this does not appear to be the case in female mice ($U = 6.5$, $p > 0.05$, mdn (wt) = 1, mdn (ko) = 2). Furthermore, Figure 6.3B suggests that male $\delta^{-/-}$ spend less time on the open arms than male $\delta^{+/+}$ ($U = 8.5$, $p < 0.05$, mdn (wt) = 20, mdn (ko) = 2). Again this effect is absent in the female mice ($U = 7$, $p > 0.05$, mdn (wt) = 12, mdn (ko) = 6).

### 6.5.2 Open Field

Male (n = 4) $\delta^{-/-}$ and $\delta^{+/+}$ mice were tested in the OF according to the methods described in Chapter 2.

Figure 6.4A shows that the amount of time spent at the sides (thigmotaxis) does not differ with genotype (no main effect of genotype:

$F(1,5) = 3.9$, n.s.). Furthermore, the number of crossings to the centre (Figure 4.13B) does not differ with genotype (no main effect of genotype: $F(1,5) = 0.26$, n.s.).

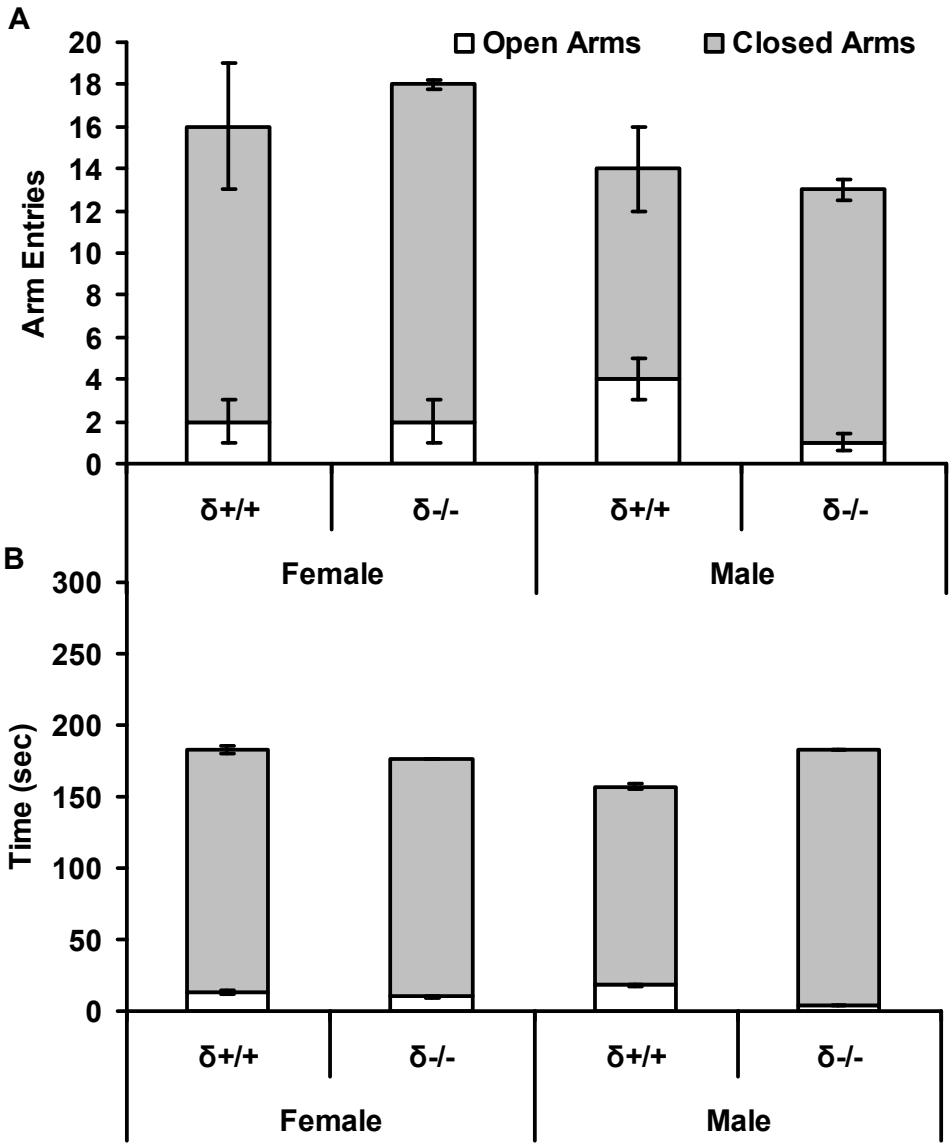


***Figure 6.3: Elevated plus maze behaviour of $\delta^{+/+}$ and $\delta^{-/-}$ mice.***

*Stacked histogram showing number (±SEM) of arm entries (A) and time in each arm (B) for $\delta^{+/+}$ and $\delta^{-/-}$ mice. Male $\delta^{-/-}$ mice showed significantly fewer open arm entries ($p < 0.01$) and less time on the open arms ($p < 0.05$) in comparison to $\delta^{+/+}$ counterparts ($n = 8$) however, there was no difference between $\delta^{+/+}$ and $\delta^{-/-}$ female mice (both $p > 0.05$, $n = 4$).*

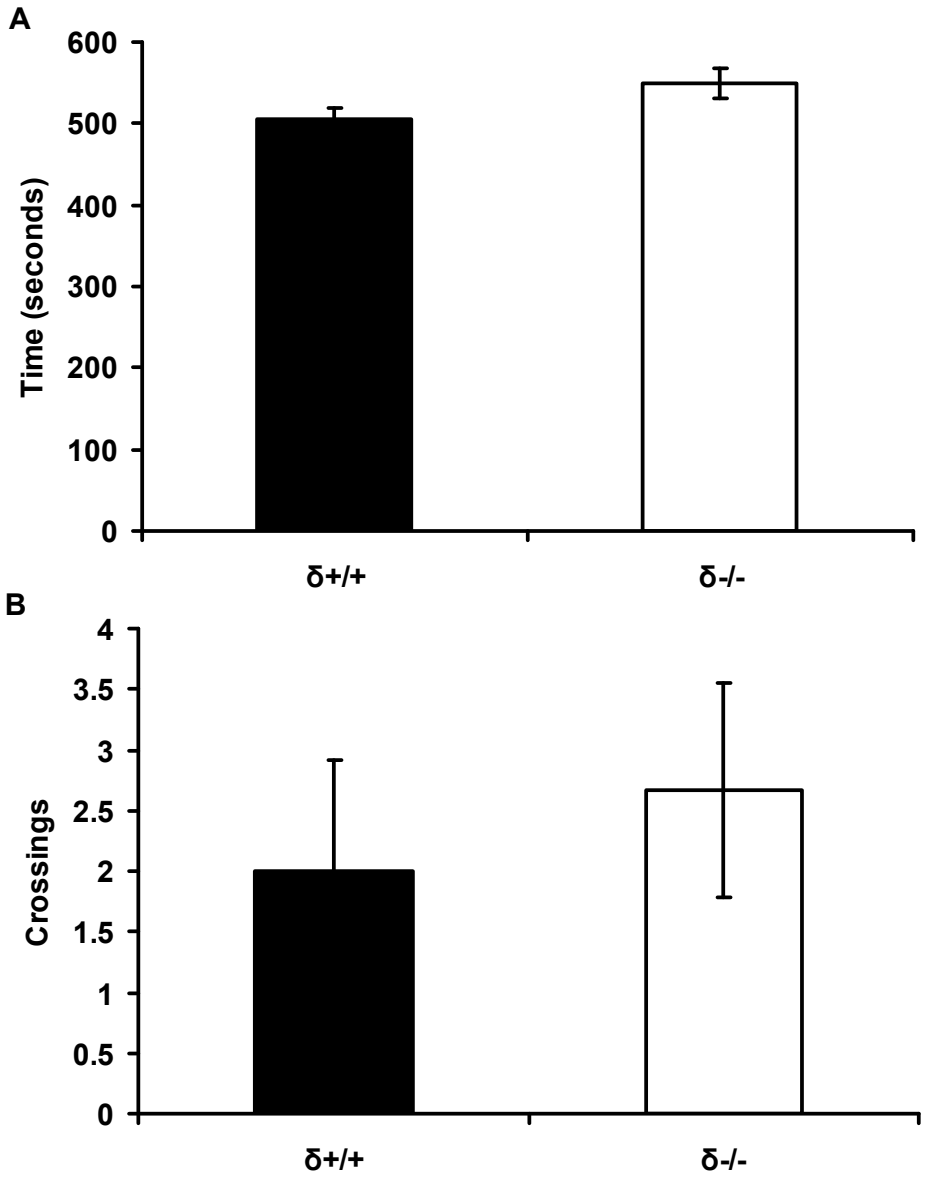


***Figure 6.4: Open field behaviour of $\delta^{+/+}$ and $\delta^{-/-}$ mice.***

*Histograms showing mean (±SEM) time spent at sides (A) and number of crossing to centre (B) of open field of $\delta^{+/+}$ and $\delta^{-/-}$ mice (n = 4). There were no differences between two genotypes in either measure ($p > 0.05$).*

## *6.6 Discussion*

### 6.6.1 Reduced responding for sweetened reward

$\delta^{-/-}$ mice made fewer lever presses than $\delta^{+/+}$ mice when rewarded with sweetened pellet. Reduced responding for a sucrose reward could be due to a number of reasons for example reduced locomotor activity, the inability to learn the lever/reward association, lack of motivation, reduced taste sensation or anxiety. It is likely due to the increased responding over days that $\delta^{-/-}$ mice are capable to learn the lever/reward association. Furthermore, data from Chapter 4 suggest there are no differences in baseline locomotor activity. The data produced here cannot rule out a lack of motivation for the reward or a reduced taste sensation. Future experiments to test for a lack of motivation and a lack of taste sensation could include progressive ratio operant experiments and sucrose/saccharine preference. Anxiety may also play a role in the disruption of lever pressing. Indeed, the testing of these mice in the light may affect lever pressing; therefore, testing with the house light off may help elucidate possible reasons for the differences observed here.

### 6.6.2 Heightened anxiety in $\delta^{-/-}$ mice

To test baseline anxiety levels, $\delta^{-/-}$ mice were tested in the EPM and OF. $\delta^{-/-}$ mice showed fewer open arm entries than $\delta^{+/+}$ mice in the EPM suggesting that $\delta^{-/-}$ mice have a higher anxiety like profile than $\delta^{+/+}$. In contrast, no difference was found in the OF test of anxiety where the number of crossings and thigomotaxic behaviour were no different

between the two genotypes. Although the number of subjects tested in the OF is relatively low and it may be worth repeating the observations from both tests, it is worth noting that the EPM and the OF measure different aspects of anxiety like behaviour and it is not improbable that different results can be obtained from the EPM and the OF. Different results have been observed between these two tests following pharmacological intervention (In Lewis rats chlordiazepoxide produces anxiolytic like effects in the EPM but not in the OF; Ramos, et al., 2002) and under baseline conditions. For a comprehensive review of the current theories in differences in tests of anxiety, see Ramos, 2008. The high anxiety like behaviour in the EPM of $\delta^{-/-}$ mice has not been previously reported (Mihalek*, et al.*, 1999).

Interestingly, there was no difference between female $\delta^{-/-}$ and $\delta^{+/+}$ mice. The most parsimonious explanation for this is a lack of power for statistical analysis (n = 4). However, it is worth noting the stage of oestrous cycle was not known in these females. Neurosteroids are known to be anxiolytic, and decreases in anxiety are correlated with increased levels of endogenous allopregnanolone (Frye*, et al.*, 2000). Importantly, the withdrawal of progesterone increases anxiety like behaviours in the light/dark box, marble burying test (Gallo and Smith, 1993) and the EPM (Smith*, et al.*, 1998). Levels of endogenous neurosteroids (namely allopregnanolone) fluctuate during the menstrual cycle, parallel to the parent compound progesterone. Progesterone levels peak during the estradiol phase. It has recently been shown that increases in the

expression of the δ subunit (and an increase in tonic current in the dentate gyrus) are evident during diestrous stage 1, which is associated with an increase in allopregnanolone (Maguire*, et al.*, 2005). Evidence suggests that δ subunit expression is increased following treatment with allopregnanolone (Shen*, et al.*, 2007; Shen*, et al.*, 2005). This evidence suggests that the anxiogenic effect of the δ subunit knockout may be dependent on the levels of neurosteroids and therefore it would be important to track the oestrous cycle of female mice when doing an experiment of this kind.

These data suggest that the $\delta^{-/-}$ mouse at the very least shows some signs of increased anxiety-like behaviour. This may be an inherent result of the deletion of the δ subunit; it could be hypothesised that dopamine transmission and the hypothalamic-pituitary-adrenal-axis (HPA-axis) may be intrinsically altered in these mice resulting in the anxiety and depression like behaviour observed in these mice. Indeed, it has recently been shown that acute stress caused an up regulation of δ subunit expression in the dentate gyrus (Maguire and Mody, 2007)

Recent evidence has suggested that $\delta^{-/-}$ (and respective +/-) postpartum mothers show higher levels of depression like behaviour as measured by the Porsolt forced swim and the 2 bottle sucrose preference tests (Maguire and Mody, 2008). These postpartum mothers also show abnormal maternal behaviour and the authors argue that these mice present a novel model of postpartum depression in humans. The effects

of maternal deprivation (modelled by maternal separation) on behaviour in adult life are still unclear; however, it has been shown that early life maternal separation of C57BL/6 postnatal pups increases anxiety like behaviour in the EPM and OF (Romeo*, et al.*, 2003) and increases depressive like behaviour in the forced swim test (McQuewen *et al*., 2003). It is also well known that rats that have undergone maternal separation in early life exhibit permanently altered HPA-axis function and exhibit reduced Adrenocorticotropic Hormone (ACTH) and corticosterone response to stress (for reviews of the effects of maternal separation see de Kloet et al, 2005 or Anisman & Matheson, 2005). If indeed $\delta^{-/-}$ mice exhibit maternal deprivation, it would not be improbable to expect offspring to demonstrate altered neurochemical and behavioural profiles. To investigate this further experiments examining the effects of maternal cross fostering of pups, analysis of corticosterone and ACTH levels in adult mice and analysis of the stress response could be utilised.

# 7 Thesis Summary and Further Work

*"The recognition of $GABA_A$ receptor subtype-specific functions has set the stage for the development of novel subtype-selective agents to provide, for example, anxiolysis without sedation, cognitive enhancement, and treatment of sensorimotor gating deficits in psychiatric disorders such as schizophrenia."*

Rudolph and Möhler, 2006

## *7.1 Thesis Summary*

The primary objective of this thesis was to behaviourally and pharmacologically characterise mice carrying mutations of $GABA_AR$s to further understand the functional significance of $GABA_AR$ heterogeneity and the role of GABAergic inhibition in behaviour.

### 7.1.1 Chapter Three

The primary aim of Chapter 3 was to investigate the role of the synaptic α1 subunit in the sedative actions of BDZs. To this purpose, the locomotor activity of $\alpha1^{-/-}$ and α1H101R mice was recorded under baseline conditions and after injection of the BDZ chlordiazepoxide. The results shown in Chapter 3 provided further evidence that the α1 subunit is involved in the mediation of the sedative actions of BDZs. It also highlighted that baseline investigations of both knockout and knock in mice are required before conclusions from pharmacological studies can be made. Although this study confirmed that $\alpha1^{-/-}$ mice are not more susceptible to the sedative effects of BDZs, it has not furthered the understanding of which receptor subunits are mediating the sedative actions of BDZs in the absence of the a1 subunit. Another weakness of this study is the lack of a range of doses used in the elevated plus maze experiments; it would have been ideal to investigate if the dose response curve of the anxiolytic actions of chlordiazepoxide has been shifted in the $\alpha1^{-/-}$ and α1H101R mice. However, due to the restrictions of repeat exposure to the elevated plus maze and the number of mice available, this is not something that was feasible at the time of the study.

### 7.1.2 Chapter Four

The primary aim of Chapter 4 was to investigate the role of the synaptic synaptic α1 and extrasynaptic δ subunits in the mediation of the behavioural effects of THIP. To this purpose, the ataxic and sedative actions of THIP were investigates in $\alpha1^{-/-}$ and $\delta^{-/-}$ mice. The data shown in Chapter 4 combined with that recently published (Herd et al, 2009) strongly suggests that the sedative actions of THIP are mediated by δ containing receptors. The association of these receptors with α4 in the thalamus and α6 in the cerebellum strongly suggests that these anatomical regions are involved in the mediation of the sedative and ataxic effects of THIP. Indeed selective knockouts of α4, α6 and δ would help clarify the roles of certain anatomical regions in the actions of THIP. The role of the δ subunit in the actions of BDZs, etomidate and neurosteroids was also investigated. There is no surprise that deletion of the δ subunit did not alter the sedative effect of chlordiazepoxide (BDZ binding is dependent on the γ subunit). The sedative and anaesthetic actions of neurosteroids appear to be mediated by the δ subunit, although the data provided here suggest that this may be strongly dependent on steroid type and dose of neurosteroids. Further work would include a wider variety of behavioural tests, including LORR, sleep time assay, locomotor activity, as well as tests of anxiety. A wide range of doses and neurosteroids (both endogenous and exogenous) would also be used to clarify the role of these receptors in the behavioural effects of neurosteroids.

### 7.1.3 Chapter Five

The primary aim of Chapter 5 was to develop an attentional set shifting task for mice with the view to use this task to investigate the putative cognitive enhancing effects of THIP and the role of GABAergic inhibition in the control of attention. The attentional set shifting task described here is the first evidence that mice can form attentional sets following only one ID exposure. The task was used to test for next day cognitive improvement following treatment with the $GABA_AR$ agonist THIP. The data suggested a time dependent improvement in reversal learning. Further testing would confirm this effect. To improve the experimental design, one could test under different light regimes and at more times points. To link this improvement in reversal learning with improved sleep quality, the EEG recordings of sleep patterns following THIP treatment and during the testing could be measured. Unfortunately, the direct effect of the removal of specific receptor subunits on the attentional set shifting abilities of mice was not investigated. Although this was originally planned as part of the project, the difficulties encountered with establishing a working protocol and training the $\delta^{-/-}$ mice meant that it was beyond the scope of this project. It would however be part of any future project now that a method is established. The role of both synaptic and extrasynaptic GABAergic inhibition could be investigated by using a variety of knockouts, including those used in this study. If the apparent anxiogenic profile of the $\delta^{-/-}$ mice explains the inability to learn the task, would treatment with BDZs or THIP (in $\delta^{+/-}$) mice reduce anxiety and result in the ability for mice to learn the task?

### 7.1.4 Chapter Six

The primary aim of Chapter 6 was to analyse the behavioural phenotype of $\delta^{-/-}$ mice to help elucidate reason for the reluctance to engage in attentional set shifting task. The data in this chapter suggests an anxiety like profile in these mice, together with reduced responding for a sweetened reward. Further analysis of the behavioural phenotype of these mice was beyond the scope of this project, however the data provided here hint at a behavioural phenotype that warrants further investigation.

Further work with the $\delta^{-/-}$ mice could investigate three main areas. The first will be the potential for an anhedonic-like phenotype and/or changes in motivation. Further operant work using progressive ratio to establish breaking point to measure motivation and other forms of operant task will be utilised to assess the motivation for a sweetened reward. In addition to this saccharine preference will be used to test preference for sweetened reward.

The second area will look at the response to stress in these animals – for example, operant work is already underway investigating the responding to a sucrose reward with the house light off thus making the testing environment less stressful.

Finally, further behavioural analysis of the anxiety state of $\delta^{-/-}$ mice will be carried out as well as analysis of the behavioural and neurochemical responses to stressful stimuli.

## *7.2 Silent mutations?*

Although not the original purpose of this thesis the research carried out here has uncovered some interesting behavioural phenotypes in the mice carrying mutations of the $GABA_AR$ that have not been reported previously.

In Chapter 3 a differential in baseline locomotor activity was found between $\alpha1^{-/-}$, α1H101R and wild type mice. Although a reduction in rotarod performance (Kralic*, et al.*, 2005) has been described previously, only in the current study has reduced locomotor activity been described in the $\alpha1^{-/-}$ mouse. In addition, in these studies, the apparently phenotypically silent α1H101R mouse (McKernan*, et al.*, 2000; Rudolph*, et al.*, 1999) has altered locomotor activity and patterns of habituation in comparison to wild type mice. No detectable behavioural phenotype in the $\delta^{-/-}$ mouse has been described previously (Boehm II*, et al.*, 2006; Mihalek*, et al.*, 1999); however, in the current studies (Chapters 5 & 6) data suggest that these mice are behaviourally different from their wild type counter parts (although the nature of these differences remain unclear). It is important to consider possible reasons for these different results.

The behavioural phenotype of rodents is complex and can be altered by many factors. These include the age of the subjects, animal house conditions (including number of cage changes per week, handling protocols etc.) and experimental design (timing, equipment, repeated vs between subjects design etc.). When possible the experiments carried out in this thesis have used animals of similar age/weight to those used in previously published works.

Differences between the work described here and previously published work may be due to experimental design.

For example, measurements of locomotor activity of $\alpha1^{-/-}$ mice were carried out at the beginning of the dark cycle with low level white background noise in a open field measuring 43.2 × 43.2 × 30.5cm with a white floor and clear Plexiglas walls (Kralic*, et al.*, 2002a) and no differences in baseline activity was observed. However, in our study, locomotor activity was recorded in a smaller arena, in the early part of the light cycle and with no background noise other than that normally experienced in animal house conditions. The locomotor activity of α1H101R mice was measured previously using cage crossings (McKernan*, et al.*, 2000) for 1 hour. In the cage crossing paradigm locomotor activity is only recorded when the animal makes 2 consecutive beam breaks at opposite ends of the open field, therefore the mouse has to travel further in this paradigm for the activity to count towards the activity count than in the measure of locomotor activity used in the studies in this thesis.

The results in this thesis further highlight the importance of experimental design in behavioural phenotyping and suggest the need for more details of experimental designs and conditions to be published in supplement to original articles to allow for direct comparisons of work.

Despite the comments made here (and those in Section 3.5.4 in regards to genetic background) it is worth noting that a given set of results from a

behavioural experiment are the results under those conditions and only using the same conditions can inferences be made.

## *7.3 Closing Statement*

Combined, this thesis has furthered knowledge of the functional aspects of the $GABA_AR$ heterogeneity and highlighted possible future avenues for research in this area. Further understanding of the roles of specific receptor subtypes and the brain regions associated with a given subtype depends largely on the development of regional specific knockout mice.

# 8 References


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