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# Novel Roles for the $GABA_B$ Receptor in Anxiety and Cocaine Addiction

*Thesis presented by*

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*under the supervision of*
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*for the degree of*
**Doctor of Philosophy**
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# *Declaration*

This submitted thesis is my own work and has not been submitted for another degree either at University College Cork or elsewhere.

*Author Contribution*

The author conducted all the work in this thesis independently with the exception of the following:

- Chapter 4 – Riccardo C. Pizzo and Daniela Felice helped with drug administration
- Chapter 5 – Original breeding pairs for the $GABA_{B(2)}$-S892A mouse were kindly provided by Martin Gassmann and Bernhard Bettler, University of Basel. The original development of the $GABA_{B(2)}$-S892A mice was carried out by Anders Jensen, Steven J. Moss, Martin Gassmann and Bernhard Bettler. Collette Manley (UCC Biological Service Unit) assisted with breeding and genotyping.
- Chapter 5 – Original breeding pairs for the $GABA_{B(2)}$-S892A mouse were kindly provided by Martin Gassmann and Bernhard Bettler, University of Basel. The original development of the $GABA_{B(2)}$-S892A mice was carried out by Anders Jensen, Steven J. Moss, Martin Gassmann and Bernhard Bettler. Collette Manley (UCC Biological Service Unit) assisted with breeding and genotyping. Gerard Moloney assisted with Western Blot analysis.
- Chapter 7 - Original breeding pairs for the $GABA_{B(1)}$ isoform null mice were kindly provided Bernhard Bettler. Daniele Felice, Riccardo C. Pizzo and Collette Manley (UCC Biological Service Unit) performed breeding and genotyping. Characterization of the acute locomotor effects of cocaine in the $GABA_{B(1)}$ isoform null mice was performed by Laura Jacobson at Novartis Institutes for Biomedical Research, Basel, Switzerland. Gerard Moloney assisted with Western Blot analysis.

Oliva F. O'Leary provided assistance regarding experimental planning, data analysis and provided comments on the text of all chapters.

# *Summary*

The $GABA_B$ receptor has been postulated as a possible drug target in the treatment of anxiety disorders and cocaine addiction. Indeed, a wealth of preclinical data is emerging that has shown that mice lacking functional $GABA_B$ receptors display a highly anxious behaviour across a range of behavioural models of anxiety. Additionally, novel compounds that act by altering the allosteric conformation of the $GABA_B$ receptor to a more active state; the $GABA_B$ receptor positive modulators, have been repeatedly demonstrated to have anxiolytic effects in animals. In addition to being a putative anxiolytic drug target, the $GABA_B$ receptor has been identified as a novel target for anti-addictive therapies. Indeed $GABA_B$ receptor positive modulators have been demonstrated to have anti-addictive properties across a broad variety of behavioural paradigms.

Despite these findings, several gaps in our knowledge of the role played by the $GABA_B$ receptor in both anxiety and drug abuse disorder exist. The aim of this thesis was to use preclinical animal models in an effort to further probe the role played by the $GABA_B$ receptor in anxiety and addiction.

Our studies initially examined the role played by the $GABA_B$ receptor in the neurodevelopmental processes underpinning of anxiety. Our studies demonstrated that treating mouse pups in early life with the $GABA_B$ receptor agonist baclofen produced an anxious phenotype in adult life, whereas treatment with the $GABA_B$ receptor antagonist CGP52432 produced no effects on adult behaviour. Further to this, we examined whether the anxious behaviour induced by early life blockade of the serotonin reuptake transporter

was dependant on alterations in $GABA_B$ receptor function. Our studies however revealed no effect of early life selective serotonin reuptake inhibitor treatment on adult life baclofen sensitivity.

The next issue addressed in this thesis is the characterization of the effects of a $GABA_B$ receptor positive modulator and a $GABA_B$ receptor antagonist in a behavioural model of conditioned fear behaviour. These novel classes of $GABA_B$ receptor ligands have been considerably less well characterized in this facet of preclinical anxiety behaviour than in terms of innate anxiety behaviour. Our study however revealed that the $GABA_B$ receptor positive modulator GS39783 and the $GABA_B$ receptor antagonist CGP52432 were without effect on the acquisition, expression or extinction of conditioned fear in our model.

The next element of this thesis dealt with the characterization of a novel mouse model, the $GABA_{B(2)}$-S892A mouse. This mouse has been engineered to express a form of the $GABA_{B(2)}$ receptor subunit wherein the function determining serine phosphorylation site cannot be phosphorylated. We initially tested this mouse in terms of its $GABA_B$ receptor function in adult life, followed by testing it in a battery of tests of unconditioned and learned anxiety behaviour. We also examined the behavioural and molecular responses of the $GABA_{B(2)}$-S892A mouse to cocaine. All of our studies appear to show that the $GABA_{B(2)}$-$GABA_B$2-S892A mouse is indistinguishable from wildtype controls.

The final aim of the thesis was to investigate the behavioural and molecular sensitivity of the $GABA_{B(1)}$ subunit isoform null mice, the $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice to cocaine.

Our studies revealed that these mice display differing behavioural responses to cocaine, with the $GABA_{B(1a)}^{-/-}$ mouse displaying a hypersensitivity to the acute locomotor effects of cocaine, while the $GABA_{B(1b)}^{-/-}$ displayed blunted locomotor sensitisation to cocaine.

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# *List of Publications*

## *Published Manuscripts*

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O'Mahony, C.M., ***Sweeney, F.F.,*** Daly, E., Dinan, T.G. & Cryan, J.F., 2010. Restraint stress-induced brain activation patterns in two strains of mice differing in their anxiety behaviour. *Behav. Brain Res.*, **213** (2), pp.148–154.

***Sweeney, FF***, O'Leary OF & Cryan JF - Activation but not Blockade of $GABA_B$ Receptors During Early–life Alters Anxiety in Adulthood – *Neuropharmacology*. 2013 Sep 17. doi:pii: S0028-3908(13)00407-3. 10.1016/j.neuropharm.2013.08.039.

### *Papers to be submitted*

***Sweeney, FF***, O'Leary OF & Cryan JF - Examination of the effects of early life selective serotonin reuptake inhibitor treatment on $GABA_B$ receptor expression and function in adult life – To be submitted to ***Behav. Brain Res.***

***Sweeney, FF***, Jensen A, Moss S.J, Gassmann M, O'Leary OF, Bettler B & Cryan JF - Ablation of the Ser892 Phosphorylation Site of the $GABA_{B(2)}$ Receptor does not modify Anxiety-related Behaviour - To be submitted to ***Neuroscience***

***Sweeney, FF***, Gassmann M, O'Leary OF, Bettler B & Cryan JF - Ablation of the S892 residue of the $GABA_{B(2)}$ subunit does not alter behavioural sensitivity to cocaine - To be submitted to ***Behav. Brain Res.***

***Sweeney, FF***, Jacobson LH, Gassmann M, O'Leary OF, Bettler B & Cryan JF - $GABA_B$ receptor subunits play different roles in mediating the acute locomotor effects of cocaine as well as cocaine locomotor sensitization - To be submitted to ***Psychopharm.***

***Abstracts***

***Sweeney, F.F***, O'Leary, O.F. & Cryan, J.F., $GABA_B$ receptor activation but not blockade during early-life affects adult anxiety behaviour – EBPS biannual meeting 2009 – Rome

# *List of Abbreviations*

GABA - γ-amino butyric acid

ISPC - Inhibitory post-synaptic current

KCTD - Potassium channel tetramerization domain-containing protein

PND – Postnatal days

CPP – Conditioned place preference

GAD – Generalised anxiety disorder

PTSD – Post-traumatic stress disorder

OCD – Obsessive compulsive disorder

EPM – Elevated plus maze

DMB – Defensive marble burying

FST – Forced swim test

SSRI – Selective serotonin reuptake inhibitor

CBT - Cognitive Behavioural Therapy

TCA - Tricyclic Antidepressant

5-HT – 5-hydroxy-tryptophan (serotonin)

SIH – Stress-induced hyperthermia

CS – Conditioned stimuli

US – Unconditioned stimuli

LA - Lateral amydala

EZM – Elevated zero maze

LD box - Light-dark box

# *Chapter 1*

# *Introduction*

## *1.1 The $GABA_B$ receptor*

γ-amino butyric acid (GABA) was established as the major inhibitory neurotransmitter in the brain in the 1950's (Bowery, 2010). However, our understanding of the pharmacology of GABA was, until the 1980's, limited to its activity at the ionotropic $GABA_A$ receptor (Curtis, 1978). This consensus was challenged in the early 1980's by the work of Norman Bowery and colleagues with the discovery of a receptor that displayed pharmacological properties markedly distinct from the GABA receptor as understood at the time. This novel receptor was initially characterised as being insensitive to the $GABA_A$ receptor agonist bicuculline (Bowery et al., 1981), and further characterised using radioligand binding experiments which demonstrated that these bicuculline insensitive receptors were bound by both 3H-baclofen and 3H-GABA (Hill & Bowery, 1981). The new receptor was coined the $GABA_B$ receptor in contrast to the classical bicuculline sensitive $GABA_A$ receptor (Bowery et al., 1981).

The $GABA_B$ receptor was then cloned in 1997 (Kaupmann et al., 1997), and it was subsequently discovered that the physiologically functional $GABA_B$ receptor is a heterodimer of two heptameric membrane spanning receptor subunits, the $GABA_{B(1)}$ and $GABA_{B(2)}$ subunits, which interact at their c-terminus and membrane spanning, and extracellular regions (Jones et al., 1998; White et al., 1998). The $GABA_B$ receptor is a G-protein coupled receptor and exerts its post-synaptic effects via inducing activity of the G-protein coupled inwardly rectifying potassium channel, or GIRK, which mediates the late phase of the inhibitory post-synaptic current (ISPC) via efflux of $K^+$ ions. Presynaptically the $GABA_B$ receptor is positively coupled to $Ca^{2+}$ receptors which cause an influx of $Ca^{2+}$

ions which inhibit the synaptic release of GABA or glutamate in the case of auto- and hetero- receptors respectively. Despite the existence of several splice variants, only two native functional isoforms of the $GABA_B$ receptor have been identified, comprising the $GABA_{B(2)}$ subunit in a heterodimeric assembly with one of two $GABA_{B(1)}$ subunit variants, the $GABA_{B(1a)}$ and the $GABA_{B(1b)}$ isoforms. These isoforms differ by the presence of two "sushi" domains at the N-terminal of the $GABA_{B(1)a}$ isoform and the absence thereof in the $GABA_{B(1)b}$ subunit (Couve et al., 2000) (Fig 1).

Although the $GABA_B$ agonist baclofen was synthesized 50 years ago and indeed played a crucial role in the initial discovery of the $GABA_B$ receptor (Froestl, 2010; Bowery, 2010), a parallel development to the genetic elucidation of the $GABA_B$ receptor has been the development of novel pharmacological agents that act at the $GABA_B$ receptor. One such class of compounds are the positive allosteric modulators of the $GABA_B$ receptor. Allosteric positive modulators are drugs that act on G-protein receptors at sites distinct from the orthosteric binding region where classical agonists and antagonists bind (Keov et al., 2011). In binding at this site positive allosteric modulators alter the pharmacological properties of the receptor making receptor activation by agonists more likely (Jensen & Spalding, 2004; Urwyler, 2011).

Positive allosteric modulators of the $GABA_B$ receptor act by enhancing the potency and maximal efficacy of GABA and other agonists at the $GABA_B$ receptor and their effects can be blocked by $GABA_B$ receptor antagonists (Urwyler et al., 2001a, 2003). They are devoid of effect in the absence of GABA (Urwyler et al., 2001a, 2003), although GS39783

can function as an allosteric agonist at desensitized $GABA_B$ receptors (Gjoni & Urwyler, 2009). Characterisation of these compounds *in vivo* has demonstrated that positive allosteric modulation of the $GABA_B$ receptor does not generate the same physiological effects as the $GABA_B$ receptor agonist baclofen. Indeed GS39783 is completely devoid of the ataxic, sedative and profound hypothermic effects of baclofen, suggesting these medicines may be of greater therapeutic utility than full agonists (Cryan et al., 2004).

An equally important development in $GABA_B$ receptor pharmacology has been the development of highly selective and potent $GABA_B$ receptor antagonists. The evolution of these compounds can be traced from the first generation $GABA_B$ receptor antagonists phaclofen and 2-hydroxy-saclofen, as well as the blood brain barrier penetrating compounds CGP35348 , CGP36742, CGP46381, CGP51176, all with potencies in the millimolar range (Froestl, 2010). Second generation of $GABA_B$ receptor antagonists display binding affinities in the nanomolar (nM) range and includes compounds such as CGP52432, CGP54626A, CGP55845A, CGP56433A, CGP56999A, CGP61334 (, CGP62349 and CGP63360A (Froestl, 2010). The most recent development in the chemistry of $GABA_B$ receptor antagonists has been the development of $^{125}I$ labelled high affinity antagonists of particular use in the crystallographic characterisation of the $GABA_B$ receptor (Froestl, 2010).

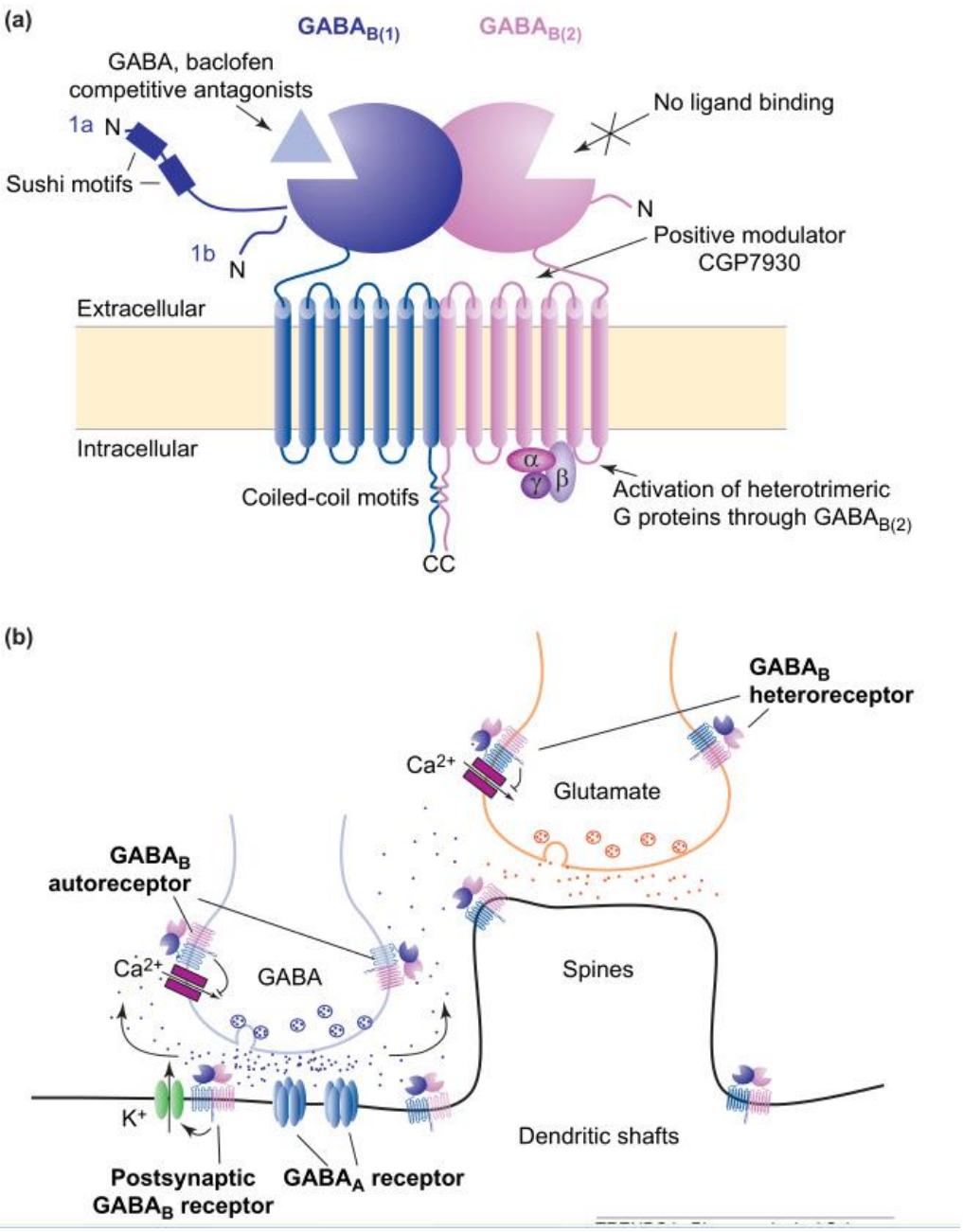


*Figure 1. Diagram of the $GABA_B$ receptor and its anatomical location in vivo. (a) The $GABA_B$ receptor is an obligate heterodimer of the $GABA_{B(1)}$ and the $GABA_{B(2)}$ subunits. The $GABA_{B(1)}$ subunit exists as two isoforms distinguished by the presence ($GABA_{B(1a)}$) and the absence ($GABA_{B(1b)}$) of two sushi domains at the n-terminus. The $GABA_{B(1)}$ subunit acts as a ligand binding domain whereas the $GABA_{B(2)}$ subunit acts as an effector domain. (b) In vivo $GABA_B$ receptors are located both post-synaptically triggering $K^+$ ion efflux in response to GABA release from the synaptic terminal as well as presynaptically, where they trigger $Ca^{2+}$ influx in response to activation by GABA spillover from nearby synapse. The $GABA_B$ receptor acts postsynaptically as both an autoreceptor on GABAergic terminals and as an autoreceptor on glutamatergic terminals (From Cryan and Kaupmann, 2005).*

Functionally, the $GABA_{B(1a)}$ isoform appears to predominantly act as a heteroreceptor modulating glutamate release in response to spill over of $GABA_B$ from GABAergic synapses, whereas the $GABA_{B(1b)}$ isoform appears to mediate the post-synaptic inhibitory effects of GABA. Both isoforms act as autoreceptors inhibiting GABA release (Vigot et al., 2006; Waldmeier et al., 2008). Recent evidence suggests that the sushi domain of the $GABA_{B(1a)}$ isoform may act as an axonal targeting signal (Biermann et al., 2010). The role the $GABA_B$ receptor plays in mediating the effects of GABA in the brain has only recently begun to be elucidated thanks to a novel, and ever-expanding array of genetic (transgenic and knockout mice) and pharmacological tools (selective and potent $GABA_B$ receptor antagonists and positive allosteric modulators) (Cryan & Kaupmann, 2005). Evidence suggests that the $GABA_B$ receptor may play an important role in regulating disease processes in a number of serious illnesses, including psychiatric disorders, and may indeed prove a valuable target for future drug development (Bowery, 2010).

### *1.1.1 Functional heterogeneity of $GABA_B$ receptors*

$GABA_B$ receptors display a large degree of heterogeneity *in vivo* in terms of agonist affinity; however, this functional heterogeneity is not determined by receptor subtype but by post translational modification of the receptor and protein-protein interactions resulting in the formation of complex multimeric structures (Pinard et al., 2010). Development of the isoform specific $GABA_{B(1a)}$ and $GABA_{B(1b)}$ knockout mouse has revealed a very limited impact on innate anxiety behaviour with effects mainly on cognitive and taste aversion paradigms (Jacobson et al., 2006b, 2007a, 2007b). One such mechanism of function and activity modulation is the association of the $GABA_B$ receptor, at the carboxyl

terminal of the $GABA_{B(2)}$ subunit with tetrameric KCTD (potassium channel tetramerization domain-containing) protein complexes, which increases agonist potency, accelerating G-protein signalling onset and promoting receptor desensitization in KCTD isoform specific manners (Schwenk et al., 2010).

Another important factor in regulating the activity of the $GABA_B$ receptor *in vivo* is that of receptor phosphorylation. N-methyl d-aspartate (NMDA) receptor-dependant phosphorylation of the $GABA_B$ receptor at various regulatory sites has recently been shown to markedly alter the function and desensitisation properties of the $GABA_B$ receptor (Guetg et al., 2010; Terunuma et al., 2010b). One such regulatory site on the $GABA_B$ receptor is the serine 892 residue of the $GABA_{B(2)}$ subunit. Cyclic AMP (cAMP) dependant phosphorylation of this residue can drastically modify the ligand potency, effector coupling and desensitisation behaviour of the $GABA_B$ receptor (Couve et al., 2002; Fairfax et al., 2004). To investigate the $GABA_B$ receptor phosphorylation on behaviour, a transgenic mouse has been developed with a serine to alanine mutation at the 892 position of the $GABA_{B(2)}$ subunit, rendering the protein impossible to phosphorylate at this site (Bettler et al. unpublished). Behavioural characterisation of this mouse will provide an important insight into the role this mode of $GABA_B$ receptor regulation plays in the processes underlying the pathophysiology of neuropsychiatric disorders. Furthermore, because positive modulators of the $GABA_B$ receptor potentially act in vivo by interfering with $GABA_B$ phosphorylation (Urwyler et al., 2001a), characterisation of the $GABA_{B(2)}$-S892A mouse may provide an insight into pharmacological effects of these drugs, both chronically and acutely.

## *1.2 Anxiety Disorders*

The anxiety response is an important mechanism by which we adapt and respond to real and perceived threats to homeostasis. Dysregulation of this healthy response leading to 'marked, persistent, and excessive or unreasonable fear' (American Psychiatric Association, 2000) and culminating in a significant interference in normal life can be described as an anxiety disorder. From a clinical perspective, anxiety disorders are described by DSM-IV in terms of subtypes distinguished by the nature of the anxiety provoking stimulus. Most common among these anxiety disorder subtypes are generalized anxiety disorder, panic disorder (diagnosed with or without agoraphobia), specific phobia, social phobia, obsessive-compulsive disorder, and posttraumatic stress disorder (see Table 1). It should be noted that DSM-V, published in May 2013 expands and modifies classification of the anxiety disorders and reclassifies obsessive compulsive disorder in a different diagnostic category (Holden, 2010; Miller & Holden, 2010).

*Table 1 – Anxiety disorders as described by DSM IV*

| Disorder | Trigger factors | Main Symptoms/ Characteristics | Treatment |
|---|---|---|---|
| Generalized Anxiety Disorder (GAD) | Anxiety in GAD is generalised to the point of becoming associated with life in general. Symptoms are typically gradual in onset. | General feelings of anxiety, heart palpitations, dizziness, and excessive worry. GAD is distinguished from PTSD by the lack of a specific triggering factor and from panic disorder by a lower level of symptom severity. | Behavioural and psychological therapies e.g. CBT, SSRIs , benzodiazepines (short term only) |
| Panic Disorder (diagnosed with or without agoraphobia) | Triggers for panic disorder tend to be subtle in nature. Patients tend to have a history of elevated anxiety. | Abrupt bouts of intense fear or anxiety, normally presenting with physical symptoms such as heart palpitations, rapid breathing, shortness of breath, blurred vision, dizziness, and racing thoughts. | Behavioural and psychological therapies e.g. CBT, SSRIs, TCAs |
| Social/Specific phobia | Anxiety is associated with specific stimuli or situation. | Anxiety and fear associated with the specific stimuli or situation or deliberate avoidance. | Behavioural and psychological therapies |
| Post traumatic stress disorder (PTSD) | PTSD exclusively occurs in the aftermath of a traumatic and fearful experience. Symptoms may develop between months and years of the triggering incident and are persistent in nature. | Re-experiencing the traumatic experience in the form of nightmares, flashbacks and obsessive thoughts. Patients may attempt to avoid stimuli associated with the traumatic event and may display generally elevated levels of anxiety. | Trauma focused psychological therapy, SSRIs, mirtazapine |
| Obsessive compulsive disorder (OCD) | | OCD is characterised by obsessions (persistent, often irrational, and seemingly uncontrollable thoughts) and compulsions (actions which are used to neutralize the obsessions). Symptoms must result in significant impairment in daily life. | Behavioural and psychological therapies, SSRIs, TCAs |

*CBT: Cognitive Behavioural Therapy, SSRI: Selective Serotonin Reuptake inhibitor, TCA: Tricyclic Antidepressant

Although these sub-disorders are to a degree epidemiologically comorbid they display differential responsiveness to the spectrum of anxiolytic drugs currently in clinical use. This suggests that divergent etiological factors may underlie the different disorders. Rating scales such as the Hamilton Rating Scale for Anxiety and the Clinical Global Impression Scale are used by clinicians both as tools to quantify symptom severity and as measures of treatment efficacy (Hoge et al., 2012). These disorders furthermore display distinct neurobiological and neuroendocrine characteristics, indicative of differing underlying pathology (Sramek et al., 2002).

Anxiety disorders are currently the most prevalent psychiatric diseases in Europe and the USA and as such represent, a grave and ever increasing strain on healthcare resources (Kessler, 2007; Nutt et al., 2007b; Kessler et al., 2005b; Alonso & Lépine, 2007) . Separate large scale epidemiological studies in both Europe (ESEMeD) (Alonso et al., 2004) and the USA (NCS-R) (Kessler & Merikangas, 2004) have demonstrated that anxiety disorders have the highest lifetime prevalence estimates (13.6 - 28.8%) and the earliest age of onset (11 years) among psychiatric disorders (Kessler et al., 2005a, 2005b; Kessler, 2007). Patients suffering from anxiety disorders also frequently present with other comorbid diseases including not only psychiatric disorders such as depression (Merikangas, 2003; Kessler et al., 2005b), but also medical conditions including functional gastrointestinal disease, asthma, cardiovascular disease, cancer and chronic pain, hypertension and migraine (Roy-Byrne et al., 2008; Härter et al., 2003). As such, anxiety disorders represent a huge burden in terms of both their social impact and their economic cost (Kessler, 2007; Nutt et al., 2007b) .

*1.2.1 Current drug treatment of anxiety*

For millennia humans have sought out chemical agents to modify the effects of stress and feelings of discomfort, tension, anxiety, and dysphoria; the oldest of these being ethanol. In the 19th century, alkaloids, bromide salts and choral hydrate were used for their sedative hypnotic effects. A major breakthrough came with the introduction of barbiturates into the clinical practice in the early part of the $20^{th}$ century (López-Muñoz et al., 2005). They induce their effects by facilitating the $Cl^-$ channel of the $GABA_A$ receptor to open, even in the absence of GABA tone. Animal models, especially canine-based paradigms, were particularly useful in identifying the sedative and anticonvulsant properties of such drugs although self-testing was also very popular in the early days of modern psychopharmacology. Although barbiturates were popular as major tranquillizers, their side effects, including sedation and behavioural changes, tolerance and dependence issues coupled with the fact that their therapeutic dose limit is dangerously close to its toxic level led the pharmaceutical industry to seek out safer alternatives (López-Muñoz et al., 2005).

It was in this context that the development of the benzodiazepines emerged and revolutionised the treatment of anxiety disorders. The first clinically available benzodiazepine was chlordiazepoxide, which was synthesised by Sternbach in the 1950s at the Hoffman La Roche Pharmaceutical Company (Sternbach, 1979). At a molecular level, benzodiazepines elicit their effects by allosterically activating the $GABA_A$ receptor channel at a site distinct from GABA itself and thus only induce effects in synapses where GABA is present. Key behavioural studies by Randall and colleagues (Hanson, 2005)

indicated that chlordiazepoxide might have a distinct pharmacological profile compared with that of barbiturates and other psychoactive drugs such as the antipsychotic chlorpromazine and anti-hypertensive reserpine. These initial tests were carried out in mice and cats and included the mouse inclined screen test indicative of muscle relaxation and sedation, a foot shock test showing "taming effects", the anaesthetized cat model of muscle relaxation, seizure-based pentylenetetrazole and electroshock tests (Randall, 1960). Later more sophisticated tests included Skinner-box based Sidman avoidance task (Sidman, 1953; Boren et al., 1959) in rats and monkeys which provided a sensitive and reliable, measure of depressant action on behaviour.

The clinical realisation that anxiety and depression are comorbid has led to the clinical observation that selective serotonin reuptake inhibitors (SSRIs) are effective in treating anxiety disorders following on from observations regarding the efficacy of tricyclic antidepressants in anxiety (Rickels et al., 1974, 1993). Indeed, today SSRIs are first-line therapy for many anxiety disorders (Hoffman & Mathew, 2008). The development of SSRIs for depression and subsequently anxiety was firmly driven by mechanistic studies focusing on modulation of monoamine neurotransmission *in vitro* and *in vivo* with little input of behavioural models initially (Wong et al., 2005). Indeed, the reliance on traditional animal models of anxiety shows little positive effects of SSRIs and indeed anxiogenic effects are often observed (Borsini et al., 2002; Sánchez & Meier, 1997). It should however, be borne in mind that a transient period of increased anxiety is often reported in patients initiated onto SSRI therapy (Vaswani et al., 2003; Baldwin et al., 2010). This has led to much criticism of the models used. Likewise, there has been a

growing discussion focused on whether anxiety and depression should be isolated from a drug development perspective (Shorter & Tyrer, 2003). Moreover, given the relative success of SSRIs it is becoming clear that many pharmaceutical companies are compelled to develop a "one pill fits all" approach to anxiety and mood disorders. This propelled research in the area of neuropeptides, such as CRF receptor antagonists, $NK_1$ receptor antagonists and melanocortin antagonists which to date have yet to fulfil its initial promise (Takahashi, 2001; Ebner et al., 2009; Shimazaki et al., 2006). Recent drug discovery efforts have additionally focused on ligands acting at G-protein coupled receptors for the non-monoaminergic neurotransmitters GABA and glutamate (Chojnacka-Wójcik et al., 2001; Cryan & Kaupmann, 2005). The current status of several promising putative drug classes for Anxiety Disorders is given in Table 2.

*Table 2 - Novel anxiolytic targets*

| Brain Target | Drug Action | Sample Compound | References |
|---|---|---|---|
| ***GABA*** | | | |
| **$GABA_A$ Receptors** | α2 subunit specific agonists | MRK-409 | (Atack et al., 2011) |
| **$GABA_B$ Receptors** | Positive Allosteric Modulators | GS39783, CGP7930, BHF177, rac-BHFF | (Cryan et al., 2004; Cryan & Kaupmann, 2005) |
| ***Glutamate*** | | | |
| **NMDA receptor** | Partial agonists | d-cycloserine | (Amaral & Roesler, 2008; Ressler et al., 2004) |
| **$mGluR_{2/3}$** | Agonists | LY354740, LY314582, APDC | (Monn et al., 1997; Helton et al., 1998; Kłodzińska et al., 1999; Benvenga et al., 1999; Spooren et al., 2002; Dunayevich et al., 2008) |
| **$mGluR_2$** | Positive Allosteric Modulators | 4-MPTTS, CBiPES, ADX71149 | (Johnson et al., 2005) Addex company website) |
| **mGluR4** | Agonists | LSP1-2111 | (Wierońska et al., 2010) |
| **mGluR5** | Antagonists | MPEP, MTEP | (Kuhn et al., 2002; Spooren & Gasparini, 2004; Ballard et al., 2005; Varty et al., 2005) |
| **mGluR7** | Agonists | AMN082 | (Fendt et al., 2008; Stachowicz et al., 2008) |
| **mGluR8** | Agonist<br>positive allosteric modulator | DCPG<br>AZ12216052 | (Duvoisin et al., 2010) |
| ***Serotonin*** | | | |
| **5-$HT_7$ receptor** | Antagonist | SB269970 | (Wesolowska et al., 2006) |
| **5-$HT_{1A}$ receptor** | Antagonists | WAY100635, p-MPP, ISL88.0338 | (Griebel et al., 1999) |
| **5-$HT_{2C}$ receptor** | Antagonists | S32006 | (Dekeyne et al., 2008; Wacker & Miller, 2008) |
| ***Neuropeptide Systems*** | | | |
| **$CRH_1$ receptor** | Antagonists | Antalarmin, Pexacerfont, GSK561679, CP154-526 | (Kehne & De Lombaert, 2002; Takahashi, 2001; Coric et al., 2010) |
| **$CRH_2$ receptor** | Antagonists | Anti-savagine-30 | (Takahashi, 2001) |
| **$NK_1$ receptor** | Antagonists | Vestipitant | (Ebner & Singewald, 2006; Brocco et al., |

| | | | |
|---|---|---|---|
| | | | 2008; Ebner et al., 2009) (www.clinicaltrials,gov) |
| **Translocator protein (18kD)** | Neurosteroid production enhancement | XBD173 | (Rupprecht et al., 2009, 2010) |
| **$MCH_1$** | Antagonists | SNAP -7941, TPI 1361-17 | (Doggrell, 2003; Shimazaki et al., 2006; Lee et al., 2010) |

## *1.3 Measuring Anxiety-related Behaviour in Rodents*

Many of the symptoms of anxiety disorders are dependent on the processing of complex psychological and cognitive concepts that clearly cannot be measured in animals such as "fear of losing control or going crazy" or a "sense of a foreshortened future". It is thus clear from the clinical presentation of anxiety disorders that they can never be fully emulated as a syndrome in animals (Arguello & Gogos, 2006; Cryan & Holmes, 2005; Crawley, 2007). If however, we consider the substantial conservation of genetic, neurochemical and neuroanatomical features seen across mammals (Jones, 2002; Tecott, 2003; Arguello & Gogos, 2006) as well as Darwin's observations regarding the conservation of many fundamental behavioural and pharmacological responses between species (Darwin, 1871, 1872), theoretically, by studying the neural and genetic determinants of animal behavioural response we can, by inference, develop our understanding of the neural and genetic basis of human behaviour under both normal and pathological states (Geyer & Markou, 2002; Cryan & Holmes, 2005; Crawley, 2007). A necessary extension of this theory is that the validity of any animal model of psychiatric disease is determined by the robustness of the diagnostic techniques used to describe the disease state in the clinic. Translational interspecies comparisons are dependent on combined advances in the fields of both human diagnostics and animal modelling as well as developments in our understanding of behavioural, genetic and neurobiological function in healthy humans and animals (Geyer & Markou, 2002; Markou et al., 2009). Likewise novel reverse translational approaches such as measuring human exploratory behaviour (Perry et al., 2009) may provide novel ways to model anxiety disorder endophenotypes in animals.

To determine the validity of an experimental model of a neuropsychiatric endophenotype, standardised criteria such as those proposed by McKinney and Bunney (1969) for depression, and which are equally applicable to anxiety disorders, can be used. These authors suggest that animal models should bear a reasonable analogy to the human disorder in either manifestation or symptoms, induce a behavioural change that can be objectively monitored, display sensitivity to effective clinical treatments and display inter-researcher reproducibility in order to be considered valid (McKinney & Bunney, 1969). Current thinking on the validity of animal models acknowledges the existence of several types of validity including face validity (similar symptom manifestation to the clinical condition); construct validity (similar underlying biology); predictive validity (responsiveness to clinically effective therapeutic agents); etiological validity (induced by similar stimuli as the clinical condition); convergent validity (convergent measures with other construct based models) and divergent validity (divergent measures from other construct based models) while maintaining that the reliability and predictive validity are the most important criteria in determining the overall validity of the system (Geyer & Markou, 2002).

In the context of anxiety it has been argued by Treit (2010) that the validity of behavioural tests of anxiety should be based on three principles arising from the evolutionarily conserved roles the fear response plays in normal survival behaviour. Firstly, a correspondence between the behavioural fear expressions in the animal model biochemical or physiological correlates of these behaviours, and the expression of isomorphic

behavioural responses in humans; Secondly, if no isomorphism is present, biological function should be conserved between the anxiety-like behaviour in the animal model and the human fear response; and thirdly, conservation of the neural mechanisms, engaged during the fear response, that underlie anxiety-related behaviour in both animals and humans (Treit et al., 2010).

In preclinical psychiatry research there remains some confusion on the distinction between an animal model versus a test (see Cryan & Slattery 2007). When describing preclinical anxiety research, it is important to try and draw a distinction between animal models of anxiety and experimental tests of anxiety (Rodgers 2010). In general, when the term "animal model of anxiety" is used it refers to an animal that exhibits a phenotype behaviourally relevant to clinical anxiety disorders. When we use the term "test of anxiety" we refer to a behavioural paradigm that induces a quantifiable fear related behaviour related to the normal adaptive fear response (Young & Liberzon, 2002; Rodgers, 2010). We can thus say that a model comprises both an independent variable i.e. the inducing manipulation; and a dependent variable; i.e. the behavioural/neurochemical readout (Geyer & Markou, 2000), whereas a test simply comprises a dependent variable. Thorough clinical understanding of the underlying pathophysiology of anxiety disorders is vital to determining appropriate independent variables in preclinical research. Identification of appropriate anxiety endophenotypes has been useful in this regard (Cryan & Slattery, 2007). Tests of anxiety are often described as "models of anxiety" based on the translationally questionable premise that anxiety disorders represent an exaggerated activation of the normal fear response, when in fact they more accurately represent models

of particular behavioural endophenotypes present in anxiety disorders and indeed models of anxiolytic drug activity (Young & Liberzon, 2002; Cryan & Holmes, 2005; Holmes & Cryan, 2006). Rodgers (2010) points to the fact that the distinction between animal test and animal model in anxiety research highlights the crucial difference in the knowledge we can garner from their use in understanding the neural circuitry of anxiety. Studying the induction of fear in an animal test in a normal animal can provide insight into the neurobiology of the adaptive fear response but may not necessarily be appropriate for investigating the dysregulated fear responses observed in anxiety disorder patients (Rodgers, 2010). It is thus important to remember that symptoms could conceivably arise from pathological processes upstream of the fear response and not from an abnormal fear response *per se*. Knowledge of the dysregulated anxiety response in humans is thus best derived from animals with a translationally relevant dysregulation of their anxiety response, evidenced by greater levels of anxiety in etiologically valid behavioural tests. This may explain why, although our knowledge of the basic fear response has significantly advanced over the past number of decades, the pathophysiology of anxiety disorders remains to be fully undestood (Rodgers, 2010).

By far the most commonly used species in preclinical anxiety research are the mouse (*Mus musculus*) and the rat (*Rattus norvegicus*) although as noted early studies in dogs and pigeons have played an important role in anxiety research historically. Traditionally rats have been the species of choice for behavioural pharmacology due to the practical considerations of their size and amenity to surgical intervention as well as superior cognitive ability and superior performance in operant and cognitive tasks. Many commonly

used behavioural paradigms were initially developed and validated as screens of anxiolytic activity in the rat before adaptation to use with other species (Cryan & Holmes, 2005). The development of novel genetic modification techniques, developed most extensively in the murine models has led to a surge in the popularity of the mouse in neuropsychiatric research. The mouse additionally has the advantages as regards ease of breeding, low cost, short generation turnover and smaller size from a drug dosing perspective (Joyner & Sedivy, 2000; Tarantino & Bucan, 2000; Phillips et al., 2007; Tecott, 2003; Cryan & Holmes, 2005; Jacobson & Cryan, 2007; Crawley, 2007). However, this has brought with it its own logistical problems in terms of difficulty in combining blood collection for pharmacokinetic – pharmacodynamic studies or biomarker analysis. Moreover, the enormous inter-strain difference in mouse behaviour across many anxiety tests both under baseline conditions and in response to pharmacological manipulation (Jacobson & Cryan, 2010) can make the interpretation of data difficult. The question which invariably arises as to which mouse strain is most like human is not an easy question to try and answer. Thus, it is becoming clear that testing of putative anxiolytic drugs requires testing across multiple strains (and species if possible) to ensure the risk of a false negative is minimised. It should be also noted that the manner in which a rodent responds to an anxiety-provoking situation may be qualitatively different to that of humans but it is becoming clear that many of the same neuronal circuits are recruited (Singewald, 2007). Often efforts at developing translational models of anxiety are interpreted as forming completely homologous models in both humans and rodents, although this may be possible in certain domains (e.g. startle response, stress-induced hyperthermia), it also may be a very narrow approach and disregards the ethological and species-specific aspects of mouse behaviour (Rodgers et al.,

1997). The most commonly used experimental paradigms used in the assessment of anxiety behaviour in mice are outline in Table 2 and several common tests are depicted in Figure 2.

*Table 2 – Commonly used animal models of anxiety*

| **Test** | **Brief Description** | **Principles** | **Animals** | **References** |
|---|---|---|---|---|
| ***Ethological Tests*** | | | | |
| **Elevated-plus Maze** | Animals are placed on an elevated, plus shaped maze consisting of two open arms and two enclosed arms connected by a central connecting square | The open arms of the maze are considered to be more aversive than the closed arms. Anxiolytic drug treatment results increased entries to and time spent in the open arms | Mice, Rats, Gerbils | (Handley & Mithani, 1984; Lister, 1987; Wilks & File, 1988; Moser et al., 1990; Filip et al., 1992; Hogg, 1996; Braun et al., 2011) |
| **Elevated Zero Maze** | Animals are placed on an elevated circular maze consisting of four segments; two exposed segments and two walled segments of equal length | The open segments of the maze are considered to be more aversive than the closed segments. Anxiolytic drug treatment results increased entries to and time spent in the open segments | Mice | (Shepherd et al., 1994; Mombereau et al., 2007; Braun et al., 2011) |
| **Light-dark Box** | Mice are placed in an apparatus consisting of two compartments; an illuminated "light" compartment and a dark compartment. The compartments are connected by a small opening at floor level. Animals are allowed to freely explore the apparatus | The light compartment is believed to be more aversive to the mouse than the dark compartment. Anxiolytic drug administration increases the number of entries to the light compartment; time spent in the light compartment and reduces freezing behaviour. | Mice | (Crawley & Goodwin, 1980; Jones et al., 1988; Onaivi & Martin, 1989; Bourin et al., 1996; Hascoët et al., 2000b; De Angelis & Furlan, 2000; Bourin & Hascoët, 2003) |
| **Open Field** | Mice are placed in a brightly lit arena and allowed to freely explore | The brightly lit exposed arena is a highly anxiogenic environment. Anxiety levels are assessed by a variety of ethological parameters as well as by the amount of time the animal spends in the more anxiety provoking central compartment | Mice, Rats | (Crawley, 1981; Lucki et al., 1989; Stefański et al., 1992; Sherif & Oreland, 1995; de Angelis, 1996; Schmitt & Hiemke, 1998; Prut & Belzung, 2003) |
| **Defensive Marble Burying/ Shock Probe** | Animals are placed in a novel cage containing bedding and a number of novel marbles or an | Burying of the marbles is interpreted as anxiety-like behaviour. Anxiolytic drug | Mice, Rats | (Treit et al., 1981; Craft et al., 1988; Njung'e & |

| | | | | |
|---|---|---|---|---|
| **burying Test** | electrical shock probe. | treatment reduces the number of marbles buried | | Handley, 1991; Czech & Quock, 1993; Picazo & Fernández-Guasti, 1995; De Boer & Koolhaas, 2003; Joel, 2006) |
| **Ultrasonic Vocalisations** | Mouse pups are separated from their mothers and the frequency of ultrasonic distress calls is recorded | A reduction in the number of USVs emitted from separated pups is regarded as an anxiolytic effect | Mice, Rats, Guinea Pigs | (Nastiti et al., 1991b, 1991a; Groenink et al., 2008; Scattoni et al., 2009) |
| **Staircase test** | Animals are placed in an enclosed staircase, with the number of steps climbed and the number of rearing behaviours measured. | The climbing of steps in this test is regarded as a measure of exploratory behaviour, with the number of rearing behaviours being regarded as an anxiety-like behaviour | Mice, Rats | (Molinengo & Ricci-Gamalero, 1970; Hughes, 1972; Cunha & Masur, 1978; Simiand et al., 1984), |
| **Social Interaction Test** | Animals are introduced to a novel animal of the same species and social behaviour is monitored | Anxiolytic drugs increase levels of social interaction with the novel mouse | Mice | (File, 1980) |
| **Suok Ropewalking/ Elevated alley test** | Animals are placed in the centre of an elevated beam or alley and allowed to freely explore the apparatus. Can be modified to the light-dark Suok test by illuminating one half of the beam and leaving the other half in darkness | The elevated beam induces anxiety by virtue of its novelty and elevation. Increased horizontal exploration in this test is regarded as a decrease in anxious behaviour. In the light-dark test increased time spent in the illuminated area is regarded as a decrease in anxiety. The Suok test is also able to detect non-specific drug effects e.g. ataxia and is of specific use in examining anxiety-vestibular system interactions. | Mice, Rats | (Kalueff & Tuohimaa, 2005; Kalueff et al., 2007, 2008), |
| **T-Maze** | The elevated T-maze consists of an elevated platform consisting of two opposing exposed arms and one enclosed arm perpendicular to the open arms. Animals are placed at the distal end of the enclosed arm and are allowed to explore the | Learned fear (inhibitory avoidance) can be assessed by measuring the time taken for the animal to leave the enclosed arm subsequent to initial exposure. Anxiolytic agents decrease the amount | Mice, Rats | (Graeff et al., 1998; Carvalho-Netto, 2004) |

| | | | | |
|---|---|---|---|---|
| | apparatus. After several trials, the animal is placed on the distal end of an open arm and allowed to explore the apparatus | of time taken by the animal to leave the open arm. Unconditioned fear is measured using the latency of the animal to escape into the closed arm from the open arm | | |
| **Seed-finding test** | Hamsters are fasted overnight and are then removed from their home cages to a novel environment. Sunflower seeds are then placed under the bedding of the home cage | Latency to find the seed when returned to the cage is taken as a marker of anxious behaviour | Hamster | (King et al., 2002) |
| **Anxiety/Defence Test Battery, Mouse Test Defence Battery (MTDB)** | Rats or mice are confronted with a dead or heavily anaesthetised predator (rat) and the defensive behaviour exhibited by the mouse is measured | Risk assessment, flight responses and defensive attack behaviours are measured as well as the development of context dependant defensive behaviours i.e. in the absence of threat are ethologically examined | Mice, Rats, Non-Human Primates | (Griebel et al., 1995a, 1995b, 1999; Barros et al., 2008) |
| **(Modified) Hole Board test** | Rats or mice are placed in an open field containing an opaque holed board. The board contains several holes with removable plastic covers | The number of entries onto the board and duration spent on the board are measures of anxiolyitc activity. Exploratory behaviours (no. of holes visited) and locomotor activity as well as a number of ethological parameters can be measured | Mice, Rats | (Ohl et al., 2001b, 2001a; Sillaber et al., 2008; Thoeringer et al., 2010) |
| ***Conflict Tests*** | | | | |
| **Geller–Seifter task** | Food deprived animals are trained to activate a lever to receive food (unpunished period). Once trained the delivery of the food reward coincides with an electrical shock (punished period) | The paradigm generates a conflict between hunger stimulated approach behaviour and fear of the shock. Anxiolytic drugs increase approach behaviour during the punished period | Rats | (Geller & Seifter, 1960, 1962; Geller et al., 1962; Wiley et al., 1993; Millan & Brocco, 2003; Paterson & Hanania, 2010), |
| **Vogel punished Drinking** | Water deprived rats are given access to water. Drinking behaviour is "punished" with electrical shocks | The paradigm generates a conflict between thirst stimulated approach behaviour and fear of the shock. Increases in "punished" water consumption is viewed as an anxiolytic | Rats | (Vogel et al., 1971; Depoortere et al., 1986; Shibata et al., 1989; Filip et al., 1992; Matsuo et al., 1997; Dekeyne et al., |

| | | | | |
|---|---|---|---|---|
| | | effect | | 2000; Millan & Brocco, 2003) |
| **Four-plate test** | Apparatus consists of a cage with a floor comprised of four metal plates. The animal is allowed to freely explore the novel apparatus but crossing between floor plates results in a mild electrical shock | In the four plate test, the aversive shock can only be avoided by immobility. The number of crossings between quadrants in this paradigm is inversely related to the levels of passive avoidance behaviour. | Mice | (Aron et al., 1971; Hascoët et al., 2000a) |
| ***Hyponeophagia Tests*** | | | | |
| **Novelty Suppressed Feeding** | Food deprived animals are introduced to a novel anxiogenic environment where food is presented. | Thus paradigm generated a conflict between the anxiogenic stimulus and hunger induced approach behaviour. Anxiolytic drugs reduce the latency of the animal to approach the food | Mice, Rats | (Bodnoff et al., 1988, 1989) |
| **Novelty Induced Hypophagia** | Animals are trained to consume a desirable food, e.g. sweetened milk before being presented with the milk in a novel environment | Hesitation on the part of the animal to consume the highly palatable food in the novel environment is regarded as a measure of both anxiety and of anhedonia | Mice, Rats | (Rex et al., 1998; Merali et al., 2003; Santarelli et al., 2003; Dulawa & Hen, 2005) |
| ***Cognitive-Based Tests*** | | | | |
| **Pavlovian fear conditioning** | Animals are trained to associate a particular contextual or cue related stimulus (CS) with an unpleasant stimuli such as an electric foot shock, acoustic stimulus or an aversive odour (US). The animal then learns to display an observable startle or freezing response to the CS independent of the presence of the US | Increased levels of freezing behaviour and enhanced startle responses when the CS is presented in the absence of the US are used as measures of fearful behaviour | Mice, Rats | (Davis, 1990; Fendt & Fanselow, 1999; Borsini et al., 2002) |
| **Conditioned emotional response** | Animals are trained to associate a specific operant, feeding or drinking behaviour (CS) with unpleasant stimuli (US). | Association of the US behaviour results with the CS results in a decrease in the US behaviour. Anxiolytic drugs reduce this effect. | Mice, Rats | (Davis, 1990; Goddyn et al., 2008) |
| **Conditioned taste aversion** | Consumption of sweetened milk is paired with the injection of the malaise inducing compound Lithium | Association of the sweetened milk with the LiCl induced malaise results in a | Rats | (Ervin & Cooper, 1988; Yasoshima & Yamamoto, |

| | | | | |
|---|---|---|---|---|
| | Chloride (LiCl). Animals are then offered a free choice between consumption of water and the same sweetened milk | decrease in consumption of the sweetened milk. Anxiolytic drugs reduce this effect | | 2005) |
| ***Physiological tests*** | | | | |
| **Stress-Induced Hyperthermia** | Mice are singly housed over night prior to rectal temperature measurement. Temperature is measured twice at an interval of 10-15 minutes. | Rectal temperature measurement is used as a stressor in this procedure. The increase in temperature seen between the first and second measurements is taken regarded as a physiological anxiety response. Drugs regarded as having an anxiolytic effect in this test reduce the magnitude of this temperature increase. | Mice, Rats. | (Bouwknecht et al., 2007; Van Bogaert et al., 2006; Conley & Hutson, 2007; Vinkers et al., 2008) |
| **Autonomic Telemetry** | Electrocardiogram transmitters are surgically attached to the mouse allowing remote monitoring of heart rate, body temperature and locomotor activity | The autonomic response to stressful stimuli, such as heart rate, blood pressure, body temperature and locomotor activity can be measured and recorded in freely behaving animals | Mice, Rats | (van Bogaert et al., 2006) |

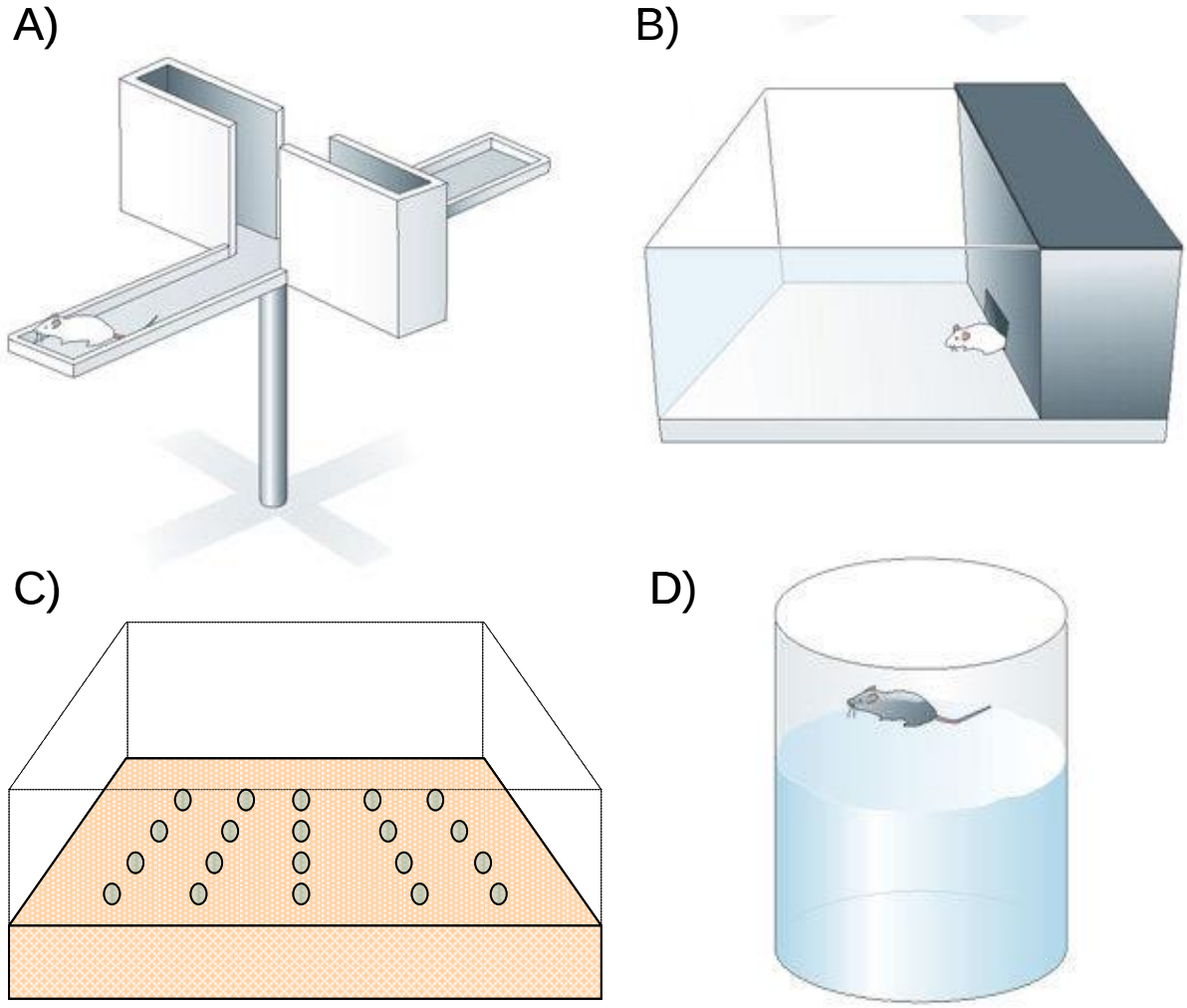


*Figure 2 – Diagrams of commonly used tests of anxiety-like behaviour as well as an image of the forced swim test; a commonly used screen for antidepressant activity. A) The elevated plus maze, B) the light dark box, C) the apparatus for defensive marble burying and D) the forced swim test. Adapted from Cryan and Holmes, 2005.*

Many tests have an ethological foundation based on the conflict that exists in small rodents such as rats and mice between the natural exploratory drive in these animals and aversion to exposed brightly lit environments (Rodgers, 1997). These models emerged over the past 40 years or so and relied on an ethological approach to understanding anxiety as opposed to the pharmacological approaches used in the development of drugs such as the benzodiazepines (see above). Behavioural paradigms based on approach-avoidance conflict include the elevated plus maze (Handley & Mithani, 1984; Pellow et al., 1985; Lister, 1987; Rodgers, 1997; Holmes, 2001; Crawley, 2007), elevated zero maze (Lee & Rodgers, 1990; Shepherd et al., 1994) the open field test , the light-dark box test (Crawley, 2007), the staircase test (Simiand et al., 1984), and the mirrored arena (Rodgers, 1997; Rodgers et al., 1997; Belzung & Griebel, 2001; Crawley, 2007) where avoidance of exposed, brightly lit or elevated areas is measured. The modified hole-board test combines the approach-avoidance aspects of the open field with the addition of board containing several holes which allows for the direct measurement of exploratory behaviour (Ohl et al., 2001a, 2001b). Within these approach avoidance procedures, several species-specific behaviours and postures are quantified and used as behavioural readouts. Reductions in these "ethological parameters" such as head dipping over the edges of elevated apparatuses, rearing and stretch-attend postures regarded as a manifestation of increased anxiety (Shepherd et al., 1994; Rodgers, 1997; Rodgers et al., 1997; Belzung & Griebel, 2001). Ethological analysis is taken to its extreme in the measurement of mouse risk-assessment, flight and defensive attack behaviour following threat cue exposure in the Mouse Defence Test Battery (MDTB) (Blanchard, 2003). Apprehension and heightened levels of vigilance are frequently a component of anxiety disorders and measurement of risk assessment behaviour, indexed by relevant ethological parameters, are regarded as a model of this endophenotype (Rodgers, 1997; Blanchard, 2003; Cryan & Holmes, 2005).

Although the constructs underlying each of these approach avoidance tests is similar, it is important to emphasise that the pharmacology and underlying neurobiology is not necessarily identical. To add to the complexity large species and strain differences occur. Thus it is very difficult to define which test is the best to model human anxiety responses. This necessitates the use of a battery style approach for assessing novel pharmacological agents. However, questions always emerge on whether a compound that is showing an anxiolytic effect in more tests is it going to be more effective in the clinic? The reciprocal experiences of researchers with SSRIs [very little activity, (Borsini et al., 2002)] and NK1 receptor antagonists [activity in a number of tests (Heldt et al., 2009; Varty et al., 2002; Vendruscolo et al., 2003)] would suggest not.

### *1.3.1 Conflict-based anxiety tests in laboratory animals*

Conflict situations, in which a subject experiences two opposing impulses, are a common and clinically relevant feature of anxiety and therefore employed in many models employed for the detection of anxiolytic agents in rodents. Conflict-based models have been among the most sensitive to GABAergic manipulation and have play an important role in assessing anxiolytic potential. In conflict-based tests of anxiety in laboratory animals, subjects receive a punishment (mild electric shock) leading to suppression of a conditioned (learned) response for reinforcement (food or water) (Rodgers, 1997). Punishment-based conflict procedures have been employed for over 50 years in the identification and characterisation of anxiolytic agents (Geller & Seifter, 1960). Studies demonstrated that when rats are trained to lever press for a food reward, during the "conflict" component, responses are inhibited by concomitant, mild electric shocks. This paradigm is known as the Geller-Seifter test. Anxiolytic properties are deduced for drugs

which selectively enhance punished responses in the presence of shock as compared to unpunished responses emitted in its absence. Benzodiazepines and barbiturates were initially demonstrated to exert specific anxiolytic properties active in the Geller–Seifter test and, subsequently, many classes of potential anxiolytic agents have been characterized employing this procedure. However, major disadvantages remain: (1) the necessity for long-term (months) and daily training of subjects and (2) their repeated utilization. That is, exposure to drugs may modify the actions of those subsequently evaluated.

In an effort to overcome these problems Vogel and colleagues (Vogel et al., 1971) developed a novel conflict procedure in which male rats were water-deprived for 48h and, during a test session of 3 min, drinking was punished by a mild, but aversive, shock delivered via the spout of the bottle every 20 licks. Accordingly, a specific, drug-induced increase in the number of shocks taken was considered to reflect anxiolytic properties. Today Vogel's test is one of the most widely used tests for assessing anxiolytic activity in rodents (Millan & Brocco, 2003).

A similar conflict procedure is the four-plate test, where the drive to explore a novel environment is conflicted with the drive to avoid floor-delivered foot shocks (Ripoll et al., 2006). Defensive marble and shock-probe burying tests, where animals bury novel or aversive items, differ from other tests of anxious behaviour in that an active behaviour i.e. burying is used as an index of anxiety as opposed to other tests relying on passive avoidance behaviour. It should however be noted that controversy exists as to the precise nature of the behaviour elicited in the defensive marble burying assay in mice. It has been argued that this assay may be more ethologically relevant to obsessive compulsive disorder

than to the rest of the anxiety disorders (Witkin, 2008) or may represent a species specific repetitive and perseverative behaviour with little correlation to levels of anxiety-like behaviour (Thomas et al., 2009). As such they make valuable additions to anxiety test batteries (Broekkamp et al., 1986; Sluyter et al., 1996, 1999; Spooren et al., 2000; Jacobson et al., 2007a).

### *1.3.2 Other unconditioned anxiety tests in laboratory animals*

Anxiolytic activity in many of the abovementioned tests can be confounded by aspects of altered locomotor activity induced by genetic or pharmacological manipulations (Cryan & Holmes, 2005; Holmes & Cryan, 2006; Jacobson & Cryan, 2010). Thus, it is important to consider other tests in battery style approaches that are less dependent on motor outputs. The following are some of the more widely used:

*Hyponeophagia*

Hyponeophagia, the suppression of eating due to anxiety-related states caused by novelty, can be assessed by measuring the latency to begin eating in a variety of potentially anxiogenic situations. Once again, it is a conflict-based model which has a long history in the assessment of emotionality and anxiety with Hall (Hall, 1934) observing an inverse relationship between feeding and defecation in animals exposed to a novel environment. In hyponeophagia tests the level of anxiety-related stimuli is manipulated by using novel food and by conducting the experiment in novel, potentially anxiogenic environments (Bannerman et al., 2002, 2003; Dulawa & Hen, 2005; Deacon & Rawlins, 2005; Finger et al., 2010). These paradigms are ethologically relevant and therefore do not require complex training procedures, are not confounded by painful stimuli, are simple to conduct, and are relatively cost-effective. Hyponeophagia-based models are conducted either by presenting

chow to food-deprived animals, or by presenting a highly palatable and familiar food to satiated animals, and measuring the latency to feed and/or the amount eaten in a novel environment. The same dependent measures should also be assessed in the home environment to control for effects of the independent variable on appetite. As in all anxiety assays inbred mouse strains show baseline differences in levels of hyponeophagia (Trullas & Skolnick, 1993). A number of genetic manipulations resulting in anxious phenotypes including leptin-deficient mice (Finger et al., 2010); 5-$HT_{1A}$ receptor (Gross et al., 2000) and the NK1 receptor (Santarelli et al., 2002) have also increases in hyponeophagia.

Several variations of hyponeophagia-based behavioural paradigms exist. These include the novelty suppressed feeding paradigm where animals are presented with normal food in a novel anxiogenic environment and the latency to begin eating is taken as an index of the anxiety state of the animal (Bodnoff et al., 1988, 1989; Gross et al., 2002). Repeated exposures to hyponeophagia paradigms in different environments and to different food stuffs can be used to modulate the levels of anxiety generated in these paradigms in order to optimise the sensitivity of the behavioural output in this test (Deacon & Rawlins, 2005; Finger et al., 2010). Another variant is the novelty-induced-hypophagia model in which animals are trained to consume a highly palatable food such as sweetened milk and then later presented this food in a novel aversive environment (Soubríe et al., 1975; Santarelli et al., 2003; Gross et al., 2002).

*Separation-induced ultrasonic vocalisations (USVs)*

Rodent pups produce vocalisations in the ultrasonic range when separated from their mother and littermates. This distress behaviour is intended to elicit maternal attention and retrieval as well as to modulate maternal care behaviour by stimulating prolactin

production (Noirot, 1972; Farrell & Alberts, 2002; Hashimoto et al., 2001). These distress behaviours can be recorded and analysed both quantitatively and qualitatively in order to measure levels of distress like behaviour in both infant mice and rats (Scattoni et al., 2009; Groenink et al., 2008). Suppression of ultrasonic vocalisation emission is an ethologically valid marker of anxiolytic drug efficacy (Groenink et al., 2008). Anxiolytic effects in this test can be elicited with administration of benzodiazepines, 5-$HT_{1A}$ receptor agonists and SSRIs. Agents acting on the noradrenergic system such as tricyclic antidepressants, are however, less consistent in their anxiolytic effects (Borsini et al., 2002). USV reduction is also seen with administration of a range of putative anxiolytic agents including NK1 receptor antagonists (Groenink et al., 2008).

Measurement of USV production in response to painful or stressful stimuli has also been proposed as a potential measure of anxiety behaviour in adult rodents. 5-$HT_{1A}$ receptor antagonists and SSRI antidepressants are effective at suppressing USV emission in this model although benzodiazepines have limited effects (Sánchez, 2003).

*Stress-Induced Hyperthermia (SIH)*

A key element of the adaptive anxiety response is activation of the autonomic nervous system and subsequent physiological responses including an increase in body temperature. This process is conserved across mammalian species including rodents and humans. Measurement of the hypothermic response generated subsequent to stressful stimuli represents a translationally valid and useful approach to modelling anxiety disorders (Bouwknecht et al., 2007; Vinkers et al., 2008). The hyperthermic response to stress can be attenuated using benzodiazepines as well as buspirone and ethanol (Spooren et al., 2002) and chronic but not acute SSRI treatment (Conley & Hutson, 2007). The SIH paradigm is

additionally sensitive to the effects of numerous putative anxiolytic agents (Spooren et al., 2002) as well providing a useful technique for exploring the role of individual neurotransmitter systems in the anxiety response (Vinkers et al., 2010).

### *1.3.3 Fear conditioning-based models of anxiety*

Alterations in conditioned fear-learning and cognitive defects form an important facet of the clinical manifestation of anxiety disorders (American Psychiatric Association, 2000; Lang et al., 2000). These include inappropriate processing of potentially threatening stimuli in GAD, panic disorder and phobias as well as the long-term salience of traumatic memories seen in PTSD. In order to model these aspects of anxiety disorders several conditioned tests of anxiety such as Pavlovian fear conditioning have been developed and validated (Cryan & Holmes, 2005; Ledgerwood et al., 2005; Delgado et al., 2006; O'Connor et al., 2010). More recently the discovery that insular cortex dysfunction may play a role in anxiety disorders (Paulus & Stein, 2006) has led to the insular-cortex dependent, conditioned taste aversion paradigm becoming more widely used (Bermúdez-Rattoni et al., 2004; Guitton & Dudai, 2004; Mickley et al., 2004; Yasoshima & Yamamoto, 2005; Jacobson et al., 2006b; Hefner et al., 2008) .

Conditioned fear paradigms revolve around the association of innocuous stimuli such as a tone or palatable taste (conditioned stimulus) with a painful or stressful stimulus such as a foot-shock or chemically induced malaise (unconditioned stimulus). Levels of conditioned fear generated in these paradigms are indexed by a number of behavioural outputs including conditioned freezing, fear potentiated startle, active defensive behaviours, vocalisations, physiological responses as well as alterations in sucrose preference in the conditioned taste aversion paradigm (Fendt & Fanselow, 1999; Cryan & Holmes, 2005).

Sleep disturbances form part of the diagnostic criteria for several forms of anxiety disorder (Prut & Belzung, 2003; American Psychiatric Association, 2000) and also represent a sensitive output in select fear conditioning paradigms (Sanford et al., 2003b, 2003a).

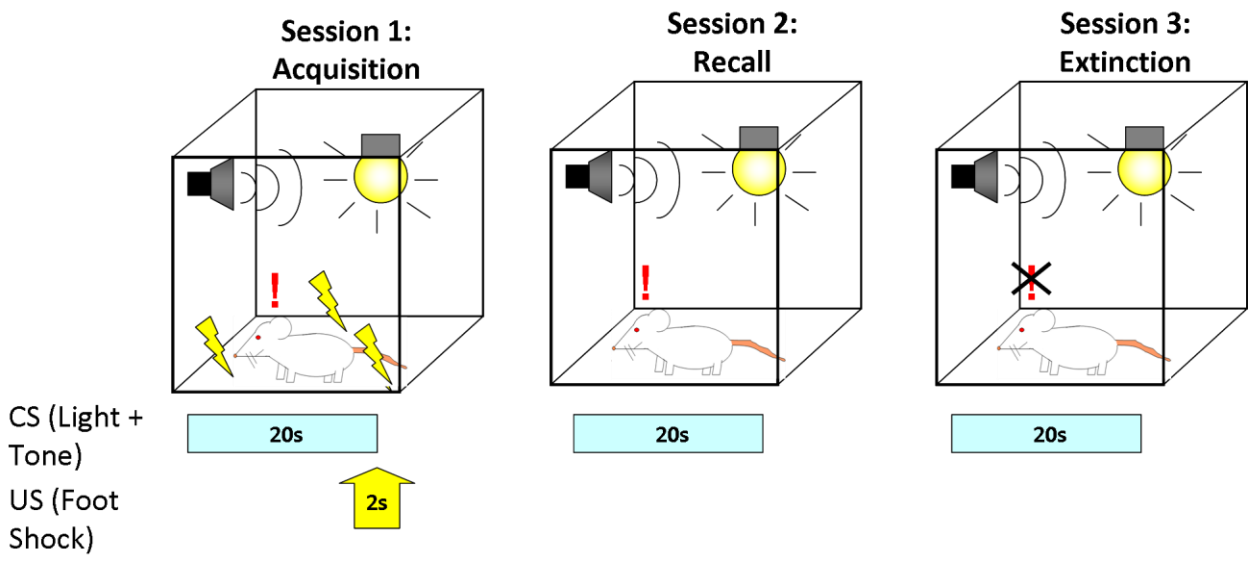


*Figure 2 - Diagram of a model of learned fear behaviour in rodents. Using a classical Pavlovian model of fear conditioning, rodents are trained to associate an innocuous conditioned stimuli (CS), with a painful unconditioned stimuli (US). Animals will display a fear response e.g. freezing in response to both the context of the conditioning and to the CS. With repeated exposure to the CS in the absence of the US, animals will gradually extinguish the conditioned fear. In this example the three stages of fear conditioning are shown. In session one, the acquisition session animals are presented with a painful US (auditiory tone and light) presented in combination with the innocuous CS (footshocks). In the second session animals display fear behaviour in response to the previously innocuous stimuli. Extinction is a process that occurs after several presentations of the CS in the absence of US whereby the CS-induced fear behaviour is no longer expressed.*

The brain circuitry that underpins Pavlovian fear conditioning essentially revolves around the convergence of auditory (CS) and nociceptive (US) sensory pathways from thalamic and cortical regions in the lateral amydala (LA). The LA both directly and indirectly innervates the central nucleus of the amygdala; a key region in activating the behavioural, autonomic and endocrine stress responses (Phelps & LeDoux, 2005). Molecular plasticity in the LA results in increased neuronal activation in response to the CS and thus increased central nucleus mediated responses (Johansen et al., 2011). Emerging data suggests that another key component of the brain's fear conditioning circuitry, particularly in the extinction of conditioned fear, is the prefrontal cortex which likely mediates its effects via

indirect input to the central nucleus of the amygdala or by direct input to the LA (Sotres-Bayon et al., 2006). The hippocampus is another important region the neurocircuitry of fear conditioning, with its function appearing to be related to the acquisition and consolidation of contextual stimuli related to the US (Sanders et al., 2003). The neuronal pathways that underlie auditory fear conditioning and extinction are outlined in Figure 3.

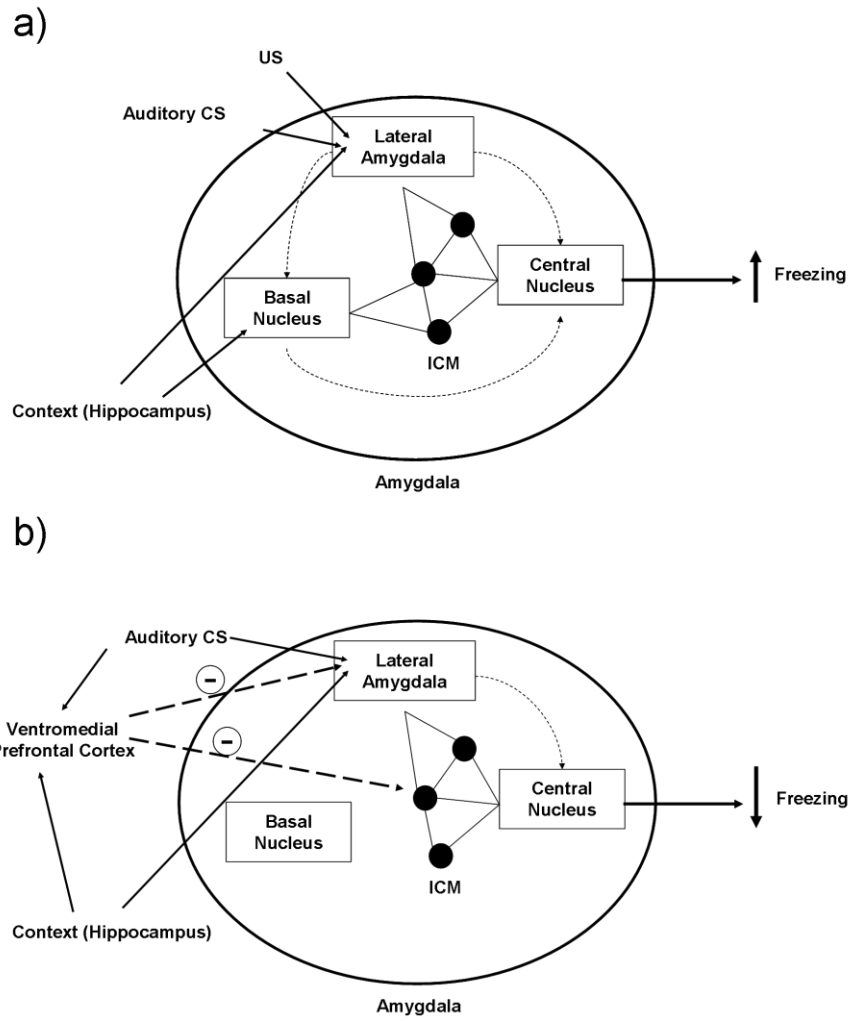


*Figure 3 –a) The neuronal pathways that underlie the acquisition and expression of auditory fear conditioning. Inputs related to CS and US converge in the lateral amygdala. Neuronal plasticity in the lateral amydala (LA) resuling in re-exposure to the conditioned stimuli (CS) activating the LA. The LA activates the central nucleus of the amydala directly and also through the intercalated cell masses (ICM) and the basal nucleus of the amydala. The basal nucleus is processes information related to spacial context from the hippocampus. The central nucleus controls the expression of fear behaviours such as freezing b) The neuronal pathways that underlie auditory fear extinction. Initial*

*presentations of CS in the absence of US result in decreased firing rates in some inhibitory amygdala neurons. Later, input from the ventromedial prefrontal cortex to LA and ICM, as well as hippocampal input to the LA directly suppress amugdala activity in response to both CS and contextual cues. Adapted from Sotres-Bayon et al., 2006.*

The behavioural process demonstrated in the Pavlovian conditioning model can be broken down into three distinct phases; acquisition, consolidation and retrieval. A consensus is growing to the effect that synaptic plasticity in the basolateral amygdala is a central molecular mechanism across all three of these processes (Fanselow & LeDoux, 1999). A central finding regarding the molecular mechanisms of fear conditioning, was the discovery that the acquisition of conditioned fear memory could be inhibited by intra-amygdala administration of NMDA receptor antagonists (Miserendino et al., 1990). Another crucial discovery were the *in vivo* findings that changes in neural activity in the lateral amygdala are very similar to those observed in LTP and that these changes indeed parallel the emergence of conditioned fear responses (Rogan et al., 1997). Activation of NMDA receptors and in cases of more robust neuronal activation voltage gated Ca2+ channels results in an increase in levels of intracellular calcium and a corresponding increase in activity of the protein kinase CaMKII. The isoform αCaMKII is specifically implicated in the development of synaptic plasticity (Wayman et al., 2008). Activation of protein kinase pathways via NMDA receptor activation marks the beginning of a molecular cascade of alterations in intracellular protein trafficking, as well as transcriptional, post-transcriptional and translation control of protein expression, culminating in the development of structural plasticity (Pape & Pare, 2010).

Extinction of the fear response generated in fear conditioning and conditioned taste aversion paradigms is of particular use in modelling the persistence of traumatic memories associated with PTSD and panic disorder (Ressler et al., 2004; Barad, 2005; Cryan &

Holmes, 2005; Ledgerwood et al., 2005; Delgado et al., 2006; Jacobson et al., 2006b). The efficacy of the NMDA receptor antagonist D-cycloserine in facilitating fear extinction in both the rat fear-potentiated startle paradigm and in human acrophobic patients indicate a predictive validity for this approach (Ressler et al., 2004; Davis et al., 2006; Ledgerwood et al., 2005). Other drugs that have been shown to alter fear conditioning processes in animals and are currently proposed as adjunctive therapeutic agents are propanolol, benzodiazepines, SSRIs, yohimbine and glucocorticoids (Graham et al., 2010). In addition several novel classes of drug have been put forward as putative anxiolytic strategies including fibroblast growth factor 2, brain derived neurotrophic factor (BDNF), histone deacetylase inhibitors, oestrogen, the metabolic enhancer methylene blue as well as drugs affecting the endocannibinoid (eCB) system such as CB1 agonists and eCB uptake inhibitors (Graham et al., 2010).

In order to discuss the extinction of conditioned fear it is imperative to define the qualities that define extinction as a behavioural process as opposed to "forgetting". It is the loss of a conditioned behaviour response that is produced through a specific training paradigm that is robust and cue specific although not permanent i.e. the extinguished behaviour can become re-established by presentation of the US or CS context with spontaneous recovery also being observed in some studies (Myers & Davis, 2007). Much like the learning of conditioned fear behaviour, the extinction of these behaviours can be broken down into the three phases of acquisition, consolidation and retrieval (Quirk & Mueller, 2008). The neural processes underpinning fear extinction have a distinct neuroanatomical basis, based upon inhibitory input from the ventromedial prefrontal cortex (vmPFC) and the hippocampus to the lateral amygdala. Extinction formation is underpinned by NMDA receptor dependant cellular cascades that result in the development of synaptic plasticity

(Orsini & Maren, 2012). Interestingly, evidence also suggests a crucial role for synaptic plasticity in the GABAergic interneurons of the amygdala in the extinction of conditioned fear (Pape & Pare, 2010).

## *1.4 The $GABA_B$ Receptor in Anxiety*

Behavioural experiments using $GABA_B$ receptor subunit-deficient mice and pharmacological manipulation of the $GABA_B$ receptor have shown that the $GABA_B$ receptor clearly plays a vital role in the control of anxiety behaviour (Cryan & Kaupmann, 2005; Gassmann & Bettler, 2012). The $GABA_{B(1)}$ knockout mouse has a phenotype characterized by hyperalgesia, impaired memory and a susceptibility to epileptiform seizures in adult life (Schuler et al., 2001) and behaviourally, displays an increased level of anxious behaviour in the light dark box, staircase test and displays a panic type response to the elevated-zero maze (Mombereau et al., 2004a), additionally the $GABA_{B(2)}$ knockout mouse displays increased anxiety in the light dark box (Mombereau et al., 2005).

Analysing the effects of pharmacological manipulation of the $GABA_B$ receptor on anxiety behaviour in rodents produces a complex picture. In later life the $GABA_B$ receptor antagonist CGP36742 has an anxiolytic effect in rats in both the elevated plus maze and conflict drinking paradigm and in mice in the four-plate test (Partyka et al., 2007). Furthermore in the elevated plus maze in the rat, the $GABA_B$ receptor antagonist SCH50911 (CGP35024) has shown an anxiolytic effect (Frankowska et al., 2007). It must however be said that the $GABA_B$ receptor antagonist CGP51176 has no effect in the mouse four-plate test (Partyka et al., 2007), chronic treatment with the $GABA_B$ receptor antagonist CGP56433A has no effect on behaviour in the light dark box paradigm (Mombereau et al.,

2004a) and that the $GABA_B$ receptor antagonist CGP35348 has no effect on anxiety behaviour in the elevated-plus maze (Dalvi & Rodgers, 1996).

On the clinical side, the potent selective $GABA_B$ agonist baclofen has been shown to be effective in treating anxiety symptoms associated with alcohol withdrawal (Addolorato et al., 2006) and post traumatic stress disorder (Drake et al., 2003), a more complex picture has been observed in rodent models. Baclofen has previously been shown to reduce ultrasonic separation vocalisations in mouse pups (Nastiti et al., 1991a), and display anxiolytic effects in both the Vogel conflict test and in animal models of drug-withdrawal induced anxiety (Cryan & Kaupmann, 2005). More recent evidence suggests that direct pharmacological activation of the $GABA_B$ receptor by agonists has a limited anxiolytic effect in behavioural tests of innate anxiety. Acute treatment with the $GABA_B$ receptor agonists baclofen and SKF 97541 (CGP 35024) produces no effect on anxiety behaviour in the elevated-zero maze in rats (Frankowska et al., 2007) and the agonist CGP 44532 even has an anxiogenic effect in the mouse four-plate test (Partyka et al., 2007).

The newly developed $GABA_B$ receptor allosteric positive modulators however, are not confounded by many of the behavioural side-effects of baclofen and have shown an anxiolytic profile in rodents. These compounds act at the $GABA_{B(2)}$ subunit of the $GABA_B$ receptor causing a conformational change in the receptor increasing the potency and affinity of GABA for the receptor (Cryan & Kaupmann, 2005). The positive allosteric modulator of the $GABA_B$ receptor GS39783 has anxiolytic effects in the in passive avoidance test, elevated zero maze and SIH paradigm in mice and in the passive avoidance test, elevated-plus maze and elevated-zero maze in rats with no effects on locomotion, memory, temperature or cognition. Other positive modulators have shown anxiolytic

activity in a host of rodent tests of anxiety behaviour such as GS7930 in the SIH paradigm and staircase test in mice as well as the elevated plus maze and elevated zero maze in both rats and mice (Partyka et al., 2007; Jacobson & Cryan, 2008)' and the positive modulator rac-BHFF in the SIH paradigm (Malherbe et al., 2008).

GABA is well-characterised as playing a crucial role in modulating the acquisition and extinction of conditioned fear. GABAergic interneurons within the amygdala have been shown to regulate neuronal activity and the induction of LTP, while GAD65 levels are notably reduced subsequent to fear conditioning (Johansen et al., 2011). Indeed, pharmacological studies have shown that intra-LA infusions of the $GABA_A$ agonist muscimol can inhibit the acquisition of conditioned fear (Muller et al., 1997; Wilensky et al., 1999). More recent studies however have pointed to a role for the $GABA_B$ receptor in this process. Studies using $GABA_{B(1a)}$ subunit isoform knockout mice have revealed that the receptor functions as a brake on the development of synaptic plasticity at presynaptic sites on cortico-amygdala afferents. Indeed genetic ablation of the $GABA_{B(1a)}$ receptor isoform results in generalisation of LTP in the amygdala, with animals generalising conditioned fear in behavioural assays (Shaban et al., 2006). It has furthermore been shown that $GABA_B$ receptors specifically inhibit glutamatergic input to primary neurons of the amygdala and not to the GABAergic interneurons thus playing a crucial role in the maintenance of normal fear-learning behaviour (Pan et al., 2009; Bolshakov, 2009).

The varying nature of these findings points to a complex pharmacological profile for the $GABA_B$ receptor as a modulator of anxiety behaviour. Different $GABA_B$ receptor agonists either lack effect, or produce either anxiogenic or anxiolytic effects in different behavioural paradigms. $GABA_B$ antagonism produces some degree of anxiolytic activity in some

behavioural tests. Only positive allosteric modulation of the receptor appears to produce a robust anxiolytic effect. The diverse effects elicited by ligands of varying potency may suggest that several populations of $GABA_B$ receptors with varying pharmacological properties are involved in the control of different aspects of anxiety behaviour. The current body of evidence surrounding the role of the $GABA_B$ receptor in the modulation of anxiety states in animal models is summarised in Table 5.

*Table 5 - The $GABA_B$ receptor in anxiety – behavioural evidence.*

| Compound | Behavioural Test(s) | Effect | Reference |
|---|---|---|---|
| ***Agonists*** | | | |
| Baclofen | Separations induced vocalisations | Anxiolytic effect | (Nastiti et al., 1991a) |
| | Social interaction test following social defeat | 0.5, 1 mg/kg anxiolytic. 2mg/kg confounding sedative effects | (File et al., 1991) |
| | EPM | No behavioural effect. 3.0 mg/kg caused non specific behavioural disruption | (Dalvi & Rodgers, 1996) |
| | EZM | No behavioural effect | (Frankowska et al., 2007) |
| SKF 97541 | EZM | No behavioural effect | (Frankowska et al., 2007) |
| CGP 4453 | Four plate test | 0.125 mg/kg has anxiogenic effects | (Partyka et al., 2007) |
| ***Positive Allosteric Modulators*** | | | |
| GS39783 | EPM (rats only), EZM (mice and rats), SIH (mice only) | Anxiolytic effects in all tests at all doses | (Cryan et al., 2004) |
| | LD box (acute + chronic), EZM | Anxiolytic activity in all tests at all doses | (Mombereau et al., 2004a) |
| | Geller Seifter test | No behavioural effect | (Paterson & Hanania, 2010) |
| CGP 7930 | EZM | Anxiolytic effect at 1mg/kg | (Frankowska et al., 2007) |
| | SIH, staircase test (mouse), EZM, EPM (rat) | Anxiolytic in mouse SIH (100 mg/kg), staircase (100 and 300 mg/kg) and EZM (3–100 mg/kg). No anxiolytic effects in the rat EPM | (Jacobson & Cryan, 2008) |
| rac-BHFF | SIH | Anxiolytic effects at 100mg/kg | (Malherbe et al., 2008) |
| ***Antagonists*** | | | |
| CGP 35348 | EPM | No effect | (Dalvi & Rodgers, 1996) |
| CGP 56433A | EPM | No effect | (Mombereau et al., 2004a) |
| CGP 35024 (SCH50911) | EZM | Anxiolytic effects at 1-3 mg/kg | (Frankowska et al., 2007) |

| | | | |
|---|---|---|---|
| CGP 36742 | EPM, conflict drinking test and four-plate test | Anxiolytic effects at 30 mg/kg in all tests | (Partyka et al., 2007) |
| CGP 51176 | Four plate test | No behavioural effect | (Partyka et al., 2007) |
| CGP 46381 | Geller Seifter test | No behavioural effect | Paterson & Hanania, 2010) |
| ***Knockout mice*** | | | |
| $GABA_{B(1)}$ KO | LD box, staircase test, EZM | Enhanced anxiety in LD box, staircase test. Panic response to EZM. Reduced benzodiazepine response in LD box | (Mombereau et al., 2004a, 2004b) |
| $GABA_{B(2)}$ KO | LD box | Enhanced anxiety | (Mombereau et al., 2005) |
| $GABA_{B(1a)}$ null | LD box, DMB, EPM, EZM. Conditioned taste aversion, Pavlovian fear conditioning | No behavioural phenotype in tests of innate anxiety. Fail to acquire CTA. Generalise conditioned fear | (Jacobson et al., 2006b, 2007b; Shaban et al., 2006) |
| $GABA_{B(1b)}$ null | LD box, DMB, EPM, EZM. Conditioned taste aversion | No behavioural phenotype in tests of innate anxiety. Fail to extinguish CTA. Normal sensitivity to early life stress in terms of visceral hypersensitivity. | (Jacobson et al., 2006b, 2007a; Moloney et al., 2012) |

**EPM: Elevated Plus Maze, EZM: Elevated Zero Maze, SIH: Stress-Induced Hyperthermia paradigm, LD box: Light/Dark box*

### *1.4.1 Anxiety as a developmental disorder: Role of serotonin*

Evidence is accumulating that an individual's risk for developing neuropsychiatric disorders are strongly determined by early life developmental problems. Central to the emergence of this theory are two landmark studies by Avshalom Caspi and colleagues demonstrating an interaction between early life stress and a polymorphism of the 5-HTT gene, which predisposed to both aggressive behaviour and depression (Caspi et al., 2002, 2003). Further studies have confirmed and developed upon these studies (for review see (Nugent et al., 2011). Recent developmental studies in rodents, using both genetic and pharmacological techniques have further elucidated the vital role played by the serotonergic system in the development of normal anxiety-related behaviour, particularly with regard to the serotonin reuptake transporter and the 5-$HT_{1A}$ receptor (Leonardo & Hen, 2008).

Pharmacological studies have indicated that blockade of the serotonin reuptake transporter using the SSRI antidepressant fluoxetine during a postnatal days P4-21 results in an anxiety phenotype in later life (Ansorge et al., 2004). This effect appears to be mediated solely by serotonergic agents and not by noradrenergic acting antidepressants and furthermore appears to be dependant on the pharmacokinetic profile of the SSRI used with citalopram having a less pronounced effect on anxiety behaviour in adult life than fluoxetine due to its shorter elimination half life (Ansorge et al., 2008). Fluoxetine treatment at later periods in adolescence, in either the C57BL/6J or BALB/cJ mice, has little effect on conditioned or innate anxiety in later life (Norcross et al., 2008).

5-$HT_{1A}$ receptor knockout mice display increased anxiety behaviour in models of both conditioned and innate anxiety (Gross et al., 2000). Use of a conditional 5-$HT_{1A}$ receptor knockout mouse, where 5-$HT_{1A}$ receptor expression can be modulated in a temporal and region specific manner has shown that receptor expression rescue, restricted to the post-synaptic receptors in the forebrain in early postnatal life (prior to P21), is sufficient to reverse this behavioural phenotype (Gross et al., 2002). Treatment with a 5-$HT_{1A}$ receptor antagonist from P13-34 also produces an increase in anxiety-related behaviour in adult life (Lo Iacono & Gross, 2008). Similar treatment with the 5-$HT_{1A}$ receptor antagonist WAY 100,635 from P0-21 also increases levels of anxiety-like behaviour in both the adolescent mouse in the EPM test and in adult mice in the open-field test (Vinkers et al., 2009). Transient 5-$HT_{1A}$ receptor antagonist treatment in the third and fourth postnatal week is sufficient to induce aberrant conditioned fear responses in the mouse akin to that of a knockout phenotype, which can be reversed by the *in vivo* silencing of the hippocampal dentate gyrus cells (Tsetsenis et al., 2007) These findings, taken together, have led to the postulation of a period in early adolescent life in the rodent, from P15 to P21, during which 5-$HT_{1A}$ receptor-mediated signalling and the activity of the serotonin reuptake transporter play a vital role in determining levels of anxious behaviour in adult life (Leonardo & Hen, 2008) (Fig 4).

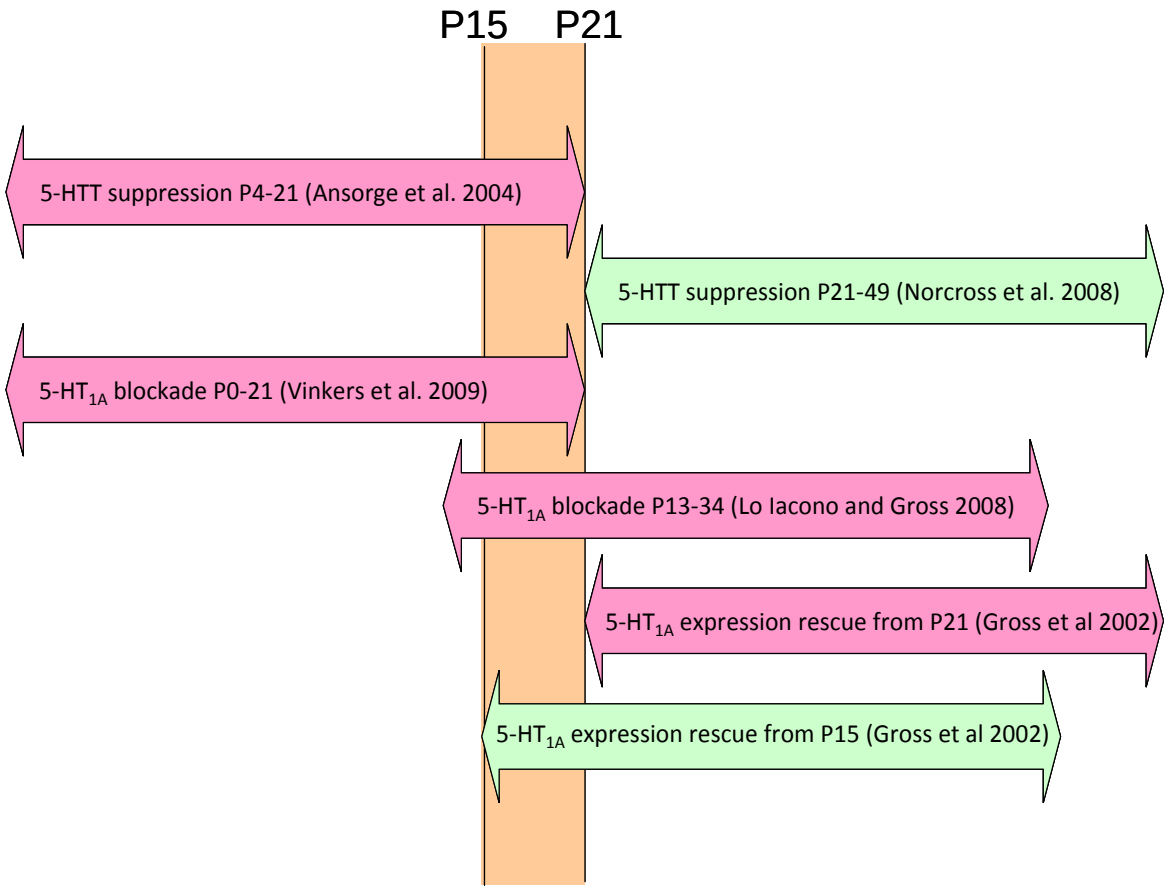


*Figure 4 - The effects of serotoninergic modulation on the early life programming of anxiety behaviour. Interventions that result in enhanced anxiety in adult life are depicted in pink; those that do not affect anxiety behaviour are shown in green. The literature clearly suggests a period of critical period from P15-21 where serotonergic function determines adult life anxiety behaviour (Modified from Leonardo and Hen, 2008).*

### *1.4.2 The $GABA_B$ receptor – serotonin interaction*

Evidence is growing to suggest a strong reciprocal relationship between the $GABA_B$ receptor and the serotonergic system at both a behavioural and molecular level. The behavioural effects of positive allosteric modulators of the $GABA_B$ receptor are dependant on serotonergic activity, being abolished where serotonin is depleted in the mouse brain (Slattery et al., 2005a). Further evidence for this interaction has been demonstrated with in vitro studies showing that application of GABA or the clinically used $GABA_B$ receptor

agonist baclofen to the neurons from the dorsal raphe nucleus (DRN), the main location of serotonergic cell bodies in the brain, strongly implicated in the behavioural regulation, results in an inhibition of serotonin release. These results are suggestive two distinct receptor $GABA_B$ receptor subgroups in the DRN at both serotonergic neurons and GABAergic interneurons (Abellán et al., 2000). This is supported by immunostaining co-localisation studies which identified $GABA_B$ receptors in both serotonergic and GABAergic cells of the DRN (Serrats et al., 2003). Interestingly, recent findings have shown that $GABA_B$ receptor activation, but not blockade in the DRN can increase levels of aggressive behaviour. Crucially this increase in aggression can be blunted by activation of $5\text{-}HT_{1A}$ receptors on serotonergic neurons in this brain region (Takahashi et al., 2010). These results both point to a complex mechanism by which the $GABA_B$ receptor is involved in the regulation of the function of the serotonergic system.

Current evidence suggests that the $GABA_B$ receptor and the $5\text{-}HT_{1A}$ receptor share a common transduction pathway utilizing G-protein coupled inwardly rectifying potassium channels (GIRKs). The capacity of both the $5\text{-}HT_{1A}$ and $GABA_B$ receptors to inhibit neuronal firing in both the dorsal raphe nucleus and hippocampus are both reduced in the 5-HTT knockout mouse (Mannoury la Cour et al., 2004). This is further supported and indeed illuminated by pharmacological studies where chronic treatment with the serotonin transporter inhibitor, fluoxetine decreases the capacity of both these receptors to recruit GIRK channels (Cornelisse et al., 2007). The hypothermic response to both the $5\text{-}HT_{1A}$ receptor agonist 8-OH-DPAT and the $GABA_B$ agonist baclofen is markedly reduced in the GIRK2 subunit null mouse, indicating the GIRK as a key mediator of the anxiety modulating effects of drugs which alter the function of these receptors (Costa et al., 2005). This suggests a shared transduction pathway for the two receptors, converging on a

downstream target, the activity of which can be modulated by fluoxetine. The $GABA_B$ receptor thus interacts on a molecular level with pharmacological targets heavily implicated in the development of the anxiety circuits of the brain. Although the implications of this interaction are yet to be elucidated, it seems possible that the $GABA_B$ receptor plays a functional role in the development of the anxiety circuits by a shared mechanism to 5-$HT_{1A}$ receptors. This possible mechanistic convergence in the development of anxiety disorders is further supported when we consider that $GABA_B$ and 5-$HT_{1A}$ receptor knockout mice share not only a behavioural phenotype, but both appear to have lowered levels of forebrain benzodiazepine sensitivity. The $GABA_B$ receptor knockout mouse appears to exhibit resistance to the behavioural effects of benzodiazepines, while the 5-$HT_{1A}$ receptor knockout mouse appears to have a reduction in $GABA_A$ receptor expression and benzodiazepine binding when generated on certain background strains (Sibille et al., 2000).

## *1.5 Cocaine Addiction*

From a clinical perspective, substance use disorders are described by the diagnostic terms "substance abuse" and "substance dependence". Substance abuse refers to a pattern of substance consumption leading to impairment or distress associated with specific social, legal and interpersonal problems. Substance dependence differs slightly in that it specifies tolerance i.e. (increased doses of drug are required to achieve a rewarding response) and presence of withdrawal syndrome in the absence of the substance, a lack of control over intake levels to the cost of other activities as well as persistent substance use despite obvious deleterious effects on the patients health (American Psychiatric Association, 2000). In the literature, the more commonly used term "drug addiction" is synonymous with the DSM-IV classification "drug dependence" (Koob & Volkow, 2010). It should

however be noted that with the publication of DSM-V the diagnostic classification of substance use related disorders is likely to be altered to a classification system based on the individual drugs being abused by the patient. Additionally the drug abuse/dependence dichotomy as described in DSM-IV will likely be removed, with major additions being the description of drug craving as a symptom of drug dependence (O'Brien, 2011).

Cocaine is a highly addictive drug and is strongly associated with physical harm, i.e. serious cardiovascular disorders, psychiatric illness and increased risk of violence and accidents as well as other health risks; with both chronic and acute use (Nutt et al., 2007a; EMCDDA, 2010). The last decade has seen a trend for an increase in levels of cocaine abuse in Europe, which represents a major emerging public health issue (EMCDDA, 2010). There is a growing recognition that medical interventions are crucial in the recovery of patients from addiction (Volkow et al., 2011). However, initiation and maintenance of treatment for problem cocaine users is intrinsically challenging for clinicians due to social and lifestyle factors surrounding cocaine abuse (Penberthy et al., 2010). These issues are exacerbated in a context where effective pharmacotherapy for reducing use and maintaining abstinence is unavailable (Penberthy et al., 2010).

An alkaloid, derived from the coca plant, cocaine has long been recognised as having sympathomimetic effects and acting as a catecholamine reuptake inhibitor. Although, data from knockout mice have been controversial (Rocha et al., 1998; Caine et al., 2012) the primary pharmacological action, and indeed the mechanism underlying the addictive effects of cocaine, is thought to be the blockade of the dopamine transporter. The consequence of this is enhancement of both tonic and phasic dopaminergic neurotransmission, effectively blurring the distinction between the two (Sulzer, 2011).

The behavioural effects of all drugs of abuse can be parsed into three phases; the acute binge / intoxication phase, the preoccupation / anticipation phase and the the withdrawal / craving phase with each stage being mediated by different brain circuits and structures (Koob & Volkow, 2010). The primary rewarding effects of cocaine are primarily mediated by the mesolimbic dopaminergic system. This system comprises the dopaminergic neurons emanating from the ventral tegemental area (VTA) and innervating basal forebrain structures such as the nucleus accumbens and dorsal striatum. The acute rewarding effects of cocaine are due to the strength of this dopaminergic signal being enhanced at the nucleus accumbens (Koob & Volkow, 2010). Several molecular targets have been identified as mediating the structural and transcriptional changes that underlie the development of drug addiction. These include elements of the cAMP/Creb signalling pathway (Chao & Nestler, 2004), ΔFosB, a transcription factor whose expression is markedly increased in the reward-related structures of the brain in response to exposure to almost all drugs of abuse (Nestler, 2008) as well as molecules related to these pathways such as DARPP-32 (Goodman, 2008). ΔFosB is of particular interest as a mediator of the effects of long term exposure to drugs of abuse. It is a splice variant of the FosB, a key element of the AP-1 transcription factor, and has a particularly long half-life in the brain, persisting for several weeks after drug administration has ceased (Nestler, 2008). ΔFosB then mediates addictive behaviours via alterations the expression of genes such as dynorphina and mGluR2 reward-related brain regions such as the nucleus accumbens (Nestler, 2008). The molecular changes induced by chronic cocaine exposure are outlined in Figure 5.

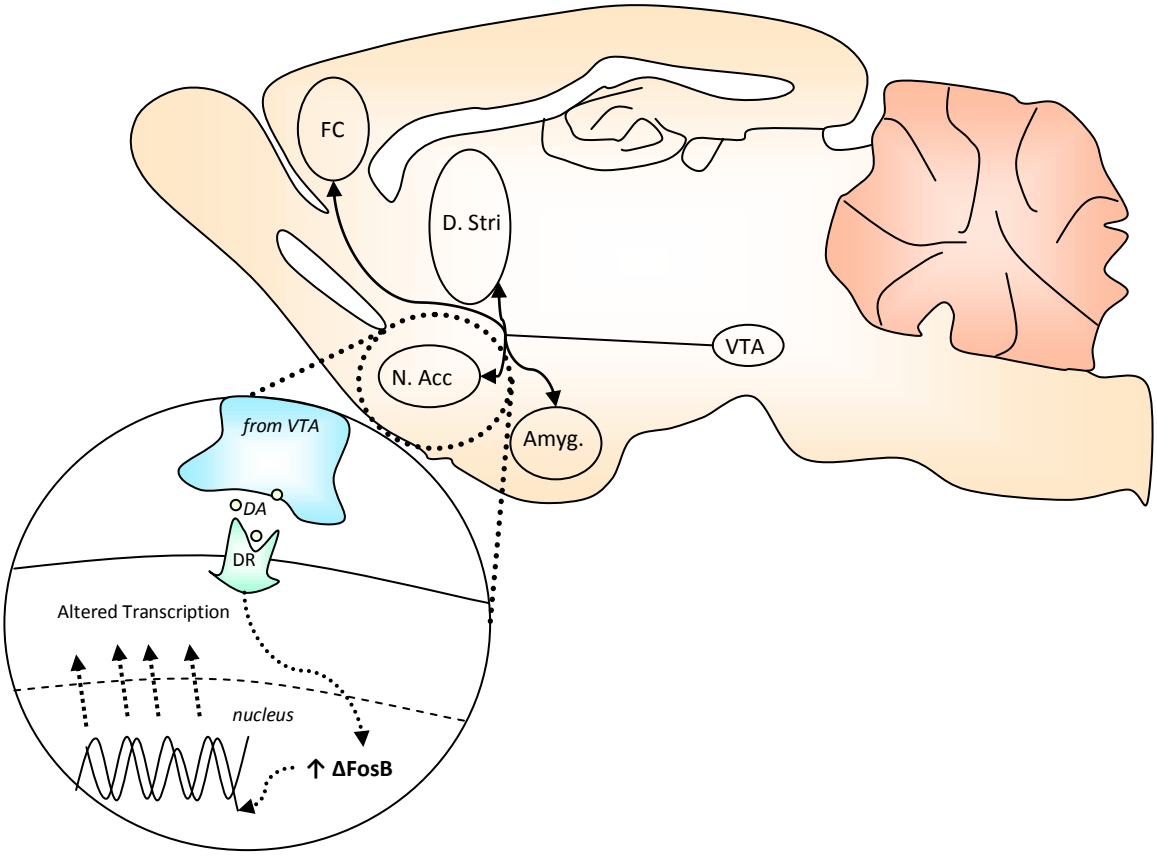


*Figure 5 – Simplified diagram of a proposed neurobiology of drug addiction. Drugs of abuse such as cocaine exert their behavioural effects via enhancement of dopaminergic neurotransmission from the VTA to forebrain structures such as the frontal cortex, dorsal striatum, nucleus accumbens and the amygdala. The transition from habitual drug use to addiction may be based on the induction of transcription factors, in particular ΔFosB, a stable truncated splice variant of FosB, in these reward-related brain areas. This induces long lasting transcriptional changes that result in alterations to neuronal function and ultimately to behaviour. VTA: Ventral tegemental area, Amyg.: Amygdala, N.Acc: Nucleus accumbens, D.Stri: Dorsal Striatum, FC: Frontal cortex, DA: Dopamine, DR: Dopamine receptor. Adapted from Koob and Volkow, 2010; Nestler, 2008.*

Current medical interventions for addiction consist of both behavioural and pharmacological techniques. Generally speaking, current behavioural techniques are most effective at altering the pre-frontal cortex mediated cognitive elements of addiction whereas pharmacological treatments tend to target subcortical reward- and mood-regulating pathways (Potenza et al., 2011). Pharmacological strategies currently licensed for use in the clinic are methadone (μ opioid receptor agonist), buprenorphine (μ opioid receptor partial

agonist, κ opioid receptor antagonist) and naltrexone (opioid receptor agonist, also used clinically in alcohol addiction) for treatment of opioid addiction; disulfiram (aldehyde dehydrogenase inhibitor) and acamprosate (NMDA receptor modulator) for alcohol addiction; as well as nicotine replacement therapy, buproprion (dopamine and noradrenaline reuptake inhibitor, nAch receptor agonist) and varenicline (α4β2 nAch receptor partial agonist and α7 nAch receptor agonist) for nicotine addiction (Potenza et al., 2011). Currently licensed treatments for drug addiction are listed in Table 6

*Table 6 – Currently licensed drugs for the treatment of addiction*

| ***Drug*** | ***Clinical Use*** | ***Drug Class*** | ***Mechanism of Action*** | ***References*** |
|---|---|---|---|---|
| ***Methadone*** | Treatment of opioid addiction | μ opioid receptor agonist, NMDA antagonist. | μ opioid receptor activation results in relief of craving and withdrawal symptoms. Blocks the euphoric and sedating effects of opiates at steady state | (Bart, 2012) |
| ***Buprenorphine*** | Treatment of opioid addiction | μ opioid receptor partial agonist, κ opioid receptor antagonist | Not fully understood. Buprenorphine acts as an antagonist at the μ opioid receptor in the presence of full agonists (addictive opioids), but has a low dependency liability. | (Lutfy & Cowan, 2004) |
| ***Naltrexone*** | Treatment of opioid addiction, alchoholism | Opioid receptor antagonist | Antagonises the behavioural effects of opioid administration. Naltrexone reduces the urge to drink, antagonises the rewarding effects of alcohol intake and causes adverse effects in combination with alcohol | (Gonzalez & Brogden, 1988; Hillemacher et al., 2011) |
| ***Disulfiram*** | Alcoholism | Aldehyde dehydrogenase inhibitor | Produces unpleasant physical effects in the presence of alcohol due to acetaldehyde accumulation. | (Barth & Malcolm, 2010) |
| ***Acamprosate*** | Alcoholism | NMDA receptor modulator | Thought to prevent development of a hyper-glutamatergic state subsequent to alcohol withdrawal, reducing craving and preventing relapse | (Mann et al., 2008) |
| ***Nicotine Replacement Therapy*** | Tobacco dependence | | Nicotine from tobacco is replaced with other forms of nicotine, (dermal, sublingual administration) easing the transition to abstinence. | (Stead et al., 2012) |
| ***Buproprion*** | Tobacco dependence | Dopamine/noradrenaline reuptake inhibitor | Reduces symptoms of acute nicotine withdrawal, inhibits nicotine reward, may decrease cue-induced nicotine craving | (Paterson, 2009) |
| ***Varenicline*** | Tobacco dependence | α4β2 nAch receptor partial agonist ( also α7 nAch receptor agonist) | Binds to the α4β2 nAch receptor with a greater affinity than nicotine but produces a lesser receptor response, thus acting as an agonist when alone (alleviating withdrawal and symptom craving levels) while acting as an antagonist in the presence of nicotine (blocking | (Foulds, 2006) |

| | | | | |
|---|---|---|---|---|
| | | | the rewarding effects of the drug.) | |

## *1.6 Animal Models of Drug Abuse*

Like anxiety disorders, in order to explore the pathogenesis of drug addiction and to assess novel therapeutic modalities, animal models and tests have been developed. These animal models are all based on the clinical features of addicted patients and relate to the three proposed stages of the addiction cycle with different models relating to different facets of the clinical disorder (Koob, 1995). When modelling drug addiction, as with all psychiatric disorders, assessment of the validity of a particular model is based on multiple factors described by Geyer and Markou (Geyer & Markou, 2000). Transgenic mice have played a crucial role in this process (Sora et al., 2010). The various behavioural paradigms used to model drug addiction in rodents are outlined in table 7.

*Table 7 – Commonly used behavioural models of addiction. Adapted from Koob and Volkow, 2010.*

| | Element(s) of Addiction Modelled | Test Description | Reference |
|---|---|---|---|
| *Drug self-adminstration* | Acute Binge/ Intoxication | Animals are trained to self administer drugs of abuse via an operant task | (Koob, 1995) |
| *Conditioned Place Preference (CPP)* | Acute Binge/ Intoxication | Animals are trained to associate the rewarding effects of a drug of abuse with a previously neutral administration context. Preference of the drug associated context is regarded as a measure of drug reward sensitivity | (Tzschentke, 1998, 2007) |
| *ICSS reward threshold* | Acute Binge/ Intoxication, Drug Withdrawal | Animals are trained to deliver rewarding electrical stimuli to the brain associated with an operant task. Administration of drugs of abuse results in increased thresholds for brain stimulation, drug withdrawal results in a decrease. | (Koob & Volkow, 2010) |
| *Hyperlocomotor response* | Acute Binge/ Intoxication | Administration of psychostimulant drugs of abuse results in increased levels of locomotor activity | (Morgan et al., 2012) |
| *Drug withdrawal induced anxiety* | Withdrawal associated negative affect | Drug withdrawal results in an anxiety-like state measurable using common animal models of anxiety-like behaviour | |
| *Conditioned Place Aversion (CPA)* | Withdrawal associated negative affect | Animals are trained to associate the aversive experience of drug withdrawal with a previously neutral context. Animals will avoid this context in a free choice situation. | (Tzschentke, 1998, 2007) |
| *Reinstatement of Drug self-adminstration* | Drug preoccupation and anticipation | Animals previously trained to self administer a drug of abuse extinguish the behaviour in the absence of drug. Drug administration can be reinstated by exposure to the drug, exposure to stress or re-exposure to a drug associated cue. | (Koob, 1995) |
| *Locomotor sensitisation* | Incentive sensitisation | Repeated administration of psychostimulant drugs of abuse results in progressively enhanced levels of locomotor response. Regarded as analogous to the human phenomenon of incentive sensitisation. | (Robinson & Berridge, 2008) |

### *16.1.1 Self-adminstration studies*

Beginning with the experiments of James Weeks, who demonstrated that morphine could act as a positive reinforcer in rats in an operant task (Weeks, 1962), self-administration studies, both in primates and rats, have formed a cornerstone of the preclinical study of addiction. It is now known that animals will readily self-administer drugs of abuse to the extent of animal self-administration being regarded as a screen for abuse potential of novel compounds in man (Koob, 1995). Drug self-adminstration studies take the form of operant tasks, whereby animals in particular rats are shown expected to perform set tasks e.g. lever presses which are associated with intravenous drug administration. Several variations of drug self-adminstration paradigms exist which vary in terms of the pattern of responding necessary to achieve the reward. Such response patterns are based on a fixed-ratio schedule i.e. drug reward after a fixed number of lever presses; second order schedules, multiple order schedules and progressive-ratio schedules where the number of responses required to receive the drug reward progressively increases to the point where the animal will no longer continue to respond, a so-called breakpoint (Koob, 1995).

Self-administration paradigms primarily model the acute binge intoxication stage of drug addiction and are a measure of the acute rewarding potential of the drug; however they can also be used to model other aspects of addiction. When performed in drug dependant animals, self-adminstration studies can also be used to explore the negative reinforcing effects of drug withdrawal (Koob, 1995) or the chronic abuse potential of drugs/conditions humans.

A major effect of almost all drugs of abuse is the lowering of the brain reward threshold (Koob & Volkow, 2010). In animals this effect can be modelled using intracranial self stimulation (ICSS) paradigms, where reward associated brain regions are directly activated by electrical stimulation in response to an operant response from an animal. These responses required to receive electrical brain stimulation are, like in drug self-administration responses, organised along several different operant response schedules. Just as drug administration decreases ICSS thresholds, drug withdrawal can indeed increase ICSS thresholds. As such ICSS paradigms can be used to model drug withdrawal states.

### *1.6.2 Conditioned place preference*

Conditioned place preference (CPP) is a Pavlovian training procedure where animals are trained to associate different physical environments with the presence or absence of drug reward. Then, in a free choice situation, the animal will display a preference for the drug associated context, presumably due to association with the positive reinforcing effects of the drug. Similarly, animals trained to associate the negative reinforcing effects of drug withdrawal with a particular place will develop place aversion. Thus, CPP and conditioned place aversion (CPA) can be used to model both the acute rewarding and the negative withdrawal effects of drugs of abuse (Koob, 1995; Shippenberg & Koob, 2002, Tzschentke, 1998, 2007 ). A typical CPP apparatus is depicted in Fig 5.

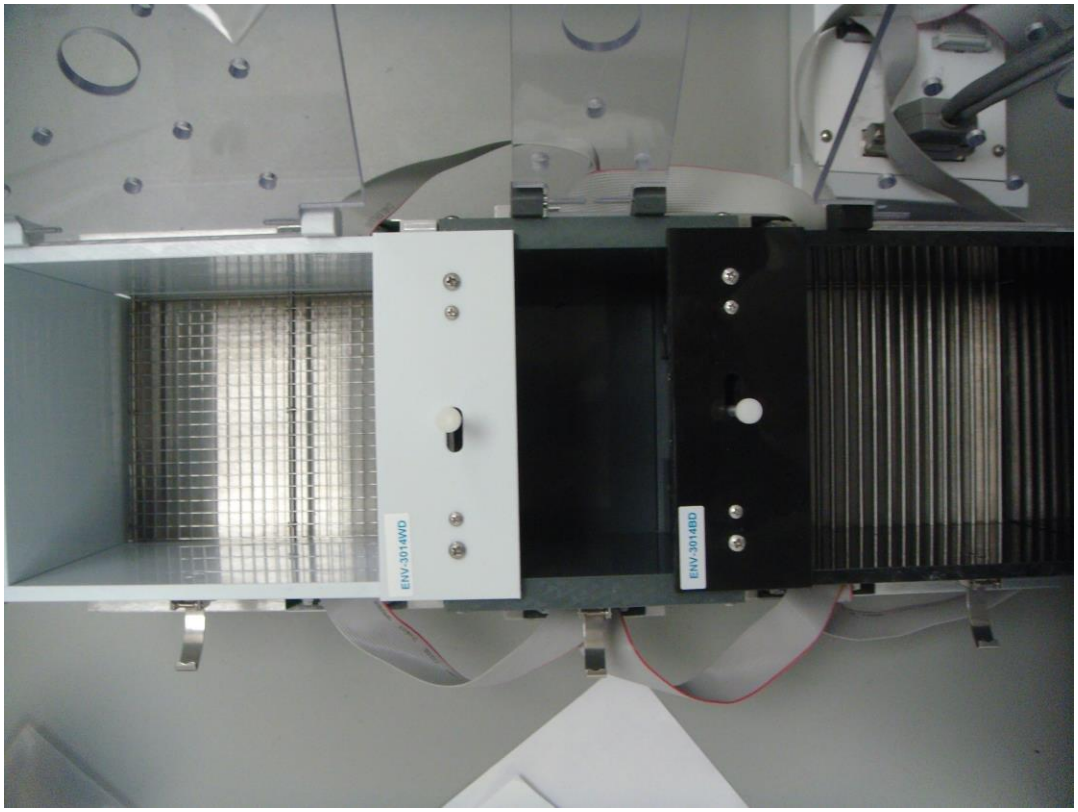


*Figure 5 – 3-compartment CPP apparatus consisting of two choice compartments with a central connecting compartment. The choice compartments are distinguished from both a visual (Black vs white) and a tactile (Barred floor vs grid floor) perspective with the potential for the addition of differential olfactory stimuli. Doors connecting the chambers can be closed for conditioning and left open for preference testing. This model additionally features a beam-break system for the tracking the movement and location of mice.*

CPP paradigms present a wide variety of advantages to researchers such as their sensitivity to a broad array of drugs of abuse, their capacity for adaptations allowing the study of preference reinstatement and drug tolerance as well as their utility in assessing alterations in the reward circuitry of transgenic mice (Tzschentke, 2007). The pharmacology of CPP has been comprehensively reviewed by Thomas Tzchentke in both 1998 (Tzschentke, 1998) and 2007 (Tzschentke, 2007). Conditioned place preference can be generated by drugs from across the range of those commonly abused including amphetamines, cocaine,

nicotine, caffeine, opiates, ethanol and benzodiazepines (Tzschentke, 1998, 2007; Bardo & Bevins, 2000).

Several methodological issues surround CPP. The most obvious choice is that of whether to use a two-chamber or three-chamber apparatus design. Another crucial methodological decision is that of biased vs. non-biased study and apparatus design, that is the design of a protocol wherein non drug-treated animals display either a clear preference towards one chamber (biased design) or spend as much time in all chambers as one would predict via probability, i.e. 50%, of total time in each chamber for a two-choice apparatus (unbiased) (Cunningham et al., 2006). Other equally important parameters are the time between drug exposure and context exposure, the time for which an animal is exposed to the apparatus and the duration of time between conditioning sessions (Cunningham et al., 2006). When using inbred mice in a CPP experiment, mouse strain also becomes a crucial factor in study design, particularly given the manner in which temporal aspects of the experimental procedure can alter behaviour in a strain dependent manner (Cunningham et al., 1999).

Bardo and Bevin (2000) succinctly outline the main advantages and disadvantages of CPP as a method for studying the pharmacology of drug reward. In addition to the ease of conduct and relatively high throughput of CPP, the fact that animals are tested in a drug-free state, its capacity to detect both reward and aversion, that most protocols also yield data on the locomotor responses to drugs, its utility across a broad species range, the monophasic dose-response curves typically generated as well as its use in studying the neuronal basis of drug reward are particular advantages of the CPP procedure (Bardo &

Bevins, 2000; Cunningham et al., 2006). Conditioned place preference testing does, however, carry the downsides of difficulty in assessing data where animals display a baseline preference, limitations in its utility for generating graded dose-response curves, a potential for novelty-seeking behaviour to influence results (particularly where drug treatment interferes with context familiarisation), as well as an inherent lack of face validity (Bardo & Bevins, 2000).

### *1.6.3 Locomotor activity*

One of the most commonly used behavioural techniques used in assessing the behavioural effects of stimulant drugs of abuse, such as cocaine, is measurement of the hyperlocomotor effects of the drug. The popularity of this approach is due to a relative ease of measurement and rapidity of output (Morgan et al., 2012). Furthermore, lesion studies have demonstrated that the hyperlocomotor effects of both d-amphetamine and cocaine are blunted when the mesolimbic dopaminergic system is ablated suggesting that hyperlocomotor behaviour may represent a measurable index of dopaminergic activity (Kelly & Iversen, 1976). Based on this finding, many studies couple examination of locomotor activity with examination of neuronal activation in frontal regions innervated by mesolimbic dopaminergic neurons (Lhuillier et al., 2007).

A distinctive feature of the hyperlocomotor effect of cocaine administration is how the effect is modulated with repeated dosing, and indeed how the regimen of administration and cocaine dose can influence this. As an example of this, when animals are dosed with cocaine (40 mg/kg), a tolerance to the locomotor effects is observed, whereas with lower

doses a sensitisation effect is seen (Reith, 1986). The context of drug administration also appears to be important with animals developing sensitisation to cocaine only when delivered in a novel environment (Badiani et al., 1995).

Psychomotor senstisation, the process where psychostimulant drugs generate an enhanced locomotor response with repeated administration, is regarded as being a particularly useful for modelling the clinical phenomenon of incentive sensitisation (Robinson & Berridge, 2008). Incentive sensitisation is a proposed theory of drug addiction whereby initially neutral stimuli are attributed with increasing levels of incentive salience in response to repeated drug administration. This is suggested as a possible mechanism by which drug abuse can develop into a drug addiction (Robinson & Berridge, 2008). Although the construct validity of these models is in question due to the lack of free choice in the administration paradigm, locomotor sensitisation and tolerance are still useful indices of the molecular changes that occur in the brain in response to chronic exposure to drugs of abuse (Nestler & Aghajanian, 1997).

## *1.7 The $GABA_B$ Receptor and Addiction*

The $GABA_B$ receptor exerts a strong influence *in vivo* on the reward circuitry of the brain and has been suggested as a potentially valuable drug target in the clinical treatment of drug addiction (Tyacke et al., 2010; Vlachou & Markou, 2010). Indeed, the prototypical $GABA_B$ receptor agonist baclofen has been well-characterised as a potentially useful drug in the management of cocaine abuse across a wide range of behavioural paradigms in animals. In particular the effects of baclofen on cocaine self-adminstration are well-

characterised. Baclofen dose dependently attenuates cocaine self-adminstration delivered in both progressive-ratio (Roberts et al., 1996; Brebner et al., 2000a) and fixed-ratio schedules (Shoaib et al., 1998; Brebner et al., 2000a), in addition to attenuating self-adminstration in discreet trials based on a predictable circadian pattern of cocaine consumption (Roberts & Andrews, 1997). Baclofen furthermore can prevent the reinstatement of self-adminstration behaviour (Campbell et al., 1999) and reduce responding to drug associated cues in a second-order schedule based paradigm (Di Ciano & Everitt, 2003). An attenuation of reinstatement of cocaine self-administration in response to baclofen has also been reported in primates (Weerts et al., 2007). Recent evidence from a novel animal model of drug self-adminstration, where a fixed response results in a decreasing amount of drug, suggests that baclofen selectively decreases the amount of effort the animal is prepared to exert for cocaine reward (Oleson et al., 2011). The effects of baclofen in progressive-ratio based and discreet-trial paradigms are also generated by administration of the $GABA_B$ agonist CGP 44532 (Brebner et al., 1999) and the effects of baclofen on cocaine, but not heroin, self-adminstration can be blocked by concurrent administration of a $GABA_B$ receptor antagonist (Brebner et al., 2002) confirming that $GABA_B$ receptor activation underlies these effects.

$GABA_B$ receptor agonist administration can also antagonise several other behavioural effects of cocaine in addition to self-adminstration. Baclofen reduces the locomotor response of mice to drug-associated cues (Hotsenpiller & Wolf, 2003). Baclofen is also effective in preventing the onset of cocaine induced seizures (Gasior et al., 2004). $GABA_B$ receptor agonism additionally attenuates locomotor sensitisation following

chronic cocaine administration (Frankowska et al., 2009). In addition, both blockade and stimulation of $GABA_B$ receptors can attenuate increases in depression-like behaviour in rats following discontinuation of cocaine (Frankowska et al., 2010). Baclofen, and indeed other $GABA_B$ receptor-active compounds including antagonists and positive modulators have however been demonstrated to have little effect on drug discrimination paradigms (Munzar et al., 2000; Filip et al., 2007a).

Several trials have attempted to assess whether baclofen administration is an effective intervention in the clinic. Despite the fact that baclofen, at clinically relevant doses, does not inhibit the subjective or physiological effects of acute intranasal cocaine consumption (Lile et al., 2004), in a pilot randomised placebo controlled trial, cocaine-dependent patients maintained on (-)baclofen, combined with cognitive behavioural therapy (CBT) used less cocaine than placebo-treated patients (Shoptaw et al., 2003). Subsequent studies have shown that baclofen can reduce cocaine self-adminstration in the absense of co-morbid opioid dependancy (Haney et al., 2006) and that baclofen in combination with amantadine can indeed reduce self-reported cocaine craving (Rotheram-Fuller et al., 2007). However, when tested in a multi-centre double-blind clinical trial, baclofen proved ineffective in reducing cocaine consumption among heavily dependent users (Kahn et al., 2009).

The development of potent selective $GABA_B$ receptor antagonists and positive allosteric modulators has further elucidated the role the $GABA_B$ receptor plays in mediating the behavioural effects of cocaine. The positive modulators CGP7930 and GS39783 have been

demonstrated to reduce cocaine responding on both fixed and progressive-ratio schedules of reinforcement (Smith et al., 2004). In addition, the $GABA_B$ receptor positive modulator CGP 7930 prevents the reinstatement of cocaine seeking behaviour in response to both cocaine- and cue-priming without affecting food-seeking behaviour (Filip & Frankowska, 2007). GS39783 also reduces the acute hyperlocomotor responses generated by cocaine administration and cocaine induced biochemical changes in key reward structures in the brain such as the nucleus accumbens and the dorsal striatum (Lhuillier et al., 2007). GS39783 also inhibits cocaine induced reductions of intracranial self-stimulation threshold, a measure of the rewarding effect of drugs of abuse, in rats (Slattery et al., 2005b).

Chronic administration of $GABA_B$ receptor positive modulators reduces nicotine self-adminstration, but not food-seeking behaviour in rats and can inhibit reinstatement of extinguished nicotine-seeking behaviour (Paterson et al., 2008; Vlachou et al., 2011a). Furthermore, treatment with GS39783 in a CPP paradigm can reduce the rewarding effects of nicotine as well as inhibit ΔFosB accumulation in the nucleus accumbens of nicotine-treated rats (Mombereau et al., 2007). Preliminary evidence thus suggests a potential role for $GABA_B$ positive allosteric modulators in the treatment of cocaine abuse and other drug addictions.

The $GABA_B$ receptor antagonist SCH 50911 has also been shown to selectively prevent (compared to food) the reinstatement of cocaine seeking behaviour in rats (Filip & Frankowska, 2007), however other studies have found $GABA_B$ receptor antagonism to be

ineffective in altering cocaine self-adminstration on a fixed-ratio schedule (Filip et al., 2007a), suggesting that tonic $GABA_B$ receptor signalling may play a role in the reinstatement but not the maintenance of cocaine self-adminstration.

It must however be borne in mind that $GABA_B$ receptor positive modulators, as well as agonists and antagonists have been shown to further increase elevated ICSS thresholds during nicotine withdrawal, suggesting an increase in anhedonic behaviour (Vlachou et al., 2011b) and, consistently with the above study, that both $GABA_B$ receptor agonists and antagonists have been shown to lower ICSS reward in the absence of a drug of abuse (Macey et al., 2001). Controversy also exists as to the selectivity of $GABA_B$ receptor agonism for cocaine self-adminstration as opposed to general reward circuitry disruption. Both baclofen and SKF 97541 prevent the reinstatement of both cocaine- and importantly food-seeking behaviour in rats (Filip & Frankowska, 2007). Similarly in primates, $GABA_B$ receptor agonist administration generates lower rates of drug self-adminstration coupled with lower rates of food-dependant task-responding (Weerts et al., 2005).

$GABA_B$ receptors are expressed both pre- and postsynaptically in the VTA (Calver et al., 2002). The demonstration that $GABA_B$ receptors can modulate the activity of mesolimbic dopaminergic afferents (Kalivas et al., 1990; Westerink et al., 1996, 1998) strongly suggests that the anti-addictive effects of $GABA_B$ receptor activation are mediated by this pathway. Microinjection of baclofen of directly into the VTA, but not to the striatum and nucleus accumbens produces a specific reduction in cocaine administration on a progressive-ratio response (Brebner et al., 2000b; Backes & Hemby, 2008). The

demonstration that baclofen dose dependently reduces cocaine (as well as other drugs of abuse) induced dopamine release in the nucleus accumbens (Fadda et al., 2003) further suggested that baclofen exerts its anti-addictive effects by exerting an inhibitory influence on the mesolimbic dopamine neurons.

The role of the $GABA_B$ receptor in the VTA is complicated when we consider that the low potency $GABA_B$ agonist γ-hydroxybutyric acid (GHB) is actually an addictive drug of abuse (Nicholson & Balster, 2001). The reason for this may lie in the fact that $GABA_B$ receptors expressed in dopaminergic neurons of the VTA couple to effector molecules with a much lower affinity than those expressed in GABAergic neurons. This finding suggests that low affinity agonists such as GHB would primarily activate $GABA_B$ receptors on GABAergic neurons, resulting in disinhibition of the dopaminergic neurons in the VTA, whereas baclofen, a high affinity agonist, acts preferentially at $GABA_B$ receptors on dopaminergic neurons (Cruz et al., 2004). $GABA_B$ receptors in the VTA are expressed both presynaptically on GABAergic interneurons and postsynaptically on dopaminergic neurons projecting to reward structures in the forebrain. The two populations of $GABA_B$ receptors are pharmacologically distinct, with the $GABA_B$ receptors located on dopaminergic neurons having a lower EC50 than those located presynaptically on GABAergic interneurons (Bettler et al., unpublished observations). The manner in which $GABA_B$ receptors can thus bidirectionally modulate the reward circuitry of the brain in the VTA is depicted in Figure 6.

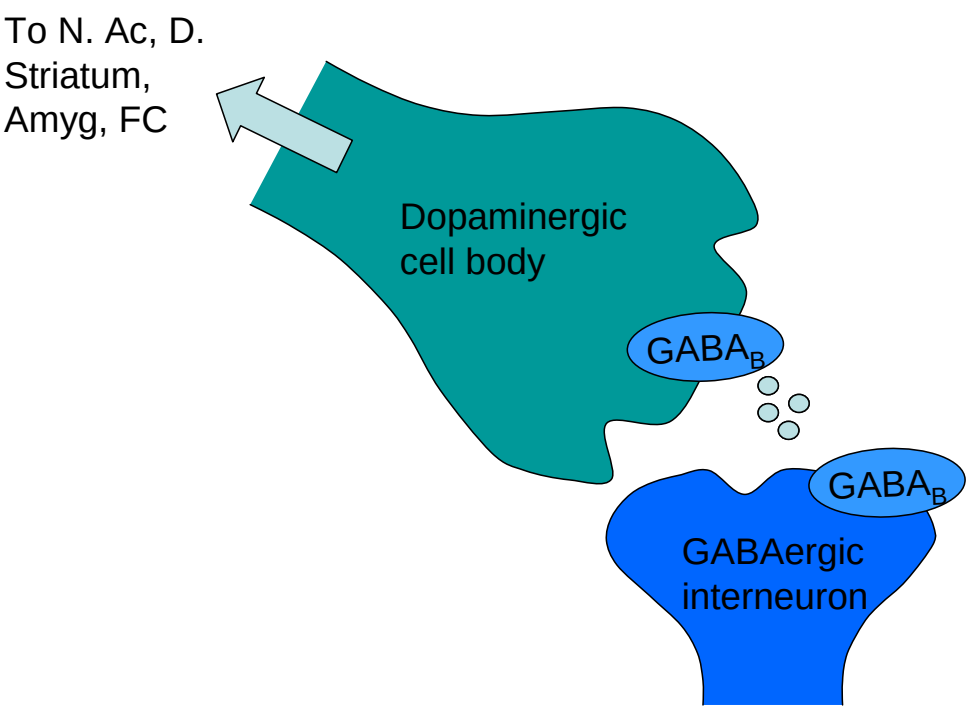


*Figure 6 - The dual effects of the GABA$_B$ receptor in the VTA. GABA$_B$ receptors in the ventral tegemental area (VTA) appear to act in two opposing manners. One population of GABA$_B$ receptors act as post-synaptic receptors, responding to GABAergic input to VTA and inhibiting activation of dopaminergic neuronal pathways leading to reward-related brain centres such as the nucleus accumbens, dorsal striatum, amygdala and frontal cortex. Another population of GABA$_B$ receptors act as auto-receptors on GABAergic interneurons within the VTA. Activation of these receptors inhibits GABA release from these interneurons exerting a net excitatory effect on the forebrain projecting dopaminergic neurons. (N. Ac = Nucleus accumbens, D. Striatum = Dorsal Striatum, Amyg = Amygdala, FC = Frontal cortex)*

It must also be noted that just as GABA$_B$ receptors can modulate the behavioural and pharmacological effects of cocaine, administration of cocaine can influence the functionality of GABA$_B$ receptors *in vivo*. Chronic cocaine treatment in rats can reduce the functionality of presynaptic GABA$_B$ receptors in several brain regions, including the VTA (Shoji et al., 1997; Kushner & Unterwald, 2001; Jayaram & Steketee, 2004); this is associated with alteration in Ser –phosphorylation of the GABA$_{B(2)}$ receptor subunit (Xi et

al., 2003). The behavioural consequence of this is that the locomotor effects of cocaine cannot be attenuated by baclofen in cocaine-sensitised animals (Steketee & Beyer, 2005).

## *1.8 Thesis Goals*

Both anxiety disorders and cocaine abuse both represent emerging crises in health care for which current pharmacological care interventions are inadequate. As previously said a prerequisite to the development of novel care interventions is dependant on a deeper understanding of the processes in the brain underlying the pathophysiology of these conditions. The $GABA_B$ receptor has been shown to be a key player in modulating both the anxiety circuits and the reward pathways that dictate the behavioural response to drugs of abuse as being a promising drug target (Cryan & Kaupmann, 2005; Vlachou & Markou, 2010). However, the mechanisms underlying this remain unclear. The overall aim of this thesis is therefore to elaborate on the role of $GABA_B$ receptors in both anxiety behaviour and brain reward/addiction behaviour using both novel and established pharmacological and genetic methods.

***Aim 1 -To determine the role played by the $GABA_B$ receptor on the development of anxiety-related behaviour.***

Given the established role for the serotonergic system in developmentally priming adult anxiety behaviour, and the well-established interactions observed between the serotonergic system and the $GABA_B$ receptor, the question arises as to the role the $GABA_B$ receptor plays in the early life programming of adult anxiety behaviour and the role of $GABA_B$-serotonin interactions in this process. This will be examined by administering both $GABA_B$ receptor agonists and antagonists to mouse pups and assessing their effect on anxiety-like behaviour in adult life. In addition, the effects of early life blockade of the

serotonin transporter, using SSRIs, on $GABA_B$ receptor function in adult life will be examined.

***Aim 2 – To determine the role of the $GABA_B$ receptor in modulating the effects of conditioned fear***

Although the effects of $GABA_B$ receptor ligands have been well-characterised in animal models of unconditioned fear, the effects of these ligands in conditioned fear are less well established. Using Pavlovian fear conditioning techniques, the effects of positive modulators of the $GABA_B$ receptor and $GABA_B$ receptor antagonists on both hippocampus and amygdala dependant conditioned fear behaviour will be assessed.

***Aim 3 -To characterise the anxiety phenotype of the novel $GABA_{B(2)}$-S892A transgenic mouse.***

We have developed a novel transgenic mouse where the serine 892 residue of the $GABA_{B(2)}$ subunit has been changed to an alanine residue, markedly altering the pharmacological properties of the receptor. This mouse will be characterised in terms of its behaviour across a battery of tests for anxiety-related behaviour as well as in terms of its $GABA_B$ receptor agonist sensitivity.

***Aim 4 – To characterise the sensitivity of models of altered $GABA_B$ receptor function to the behavioural and molecular effects of both acute and chronic cocaine administration.***

As we have seen, an accumulating body of evidence suggests an influential role for the $GABA_B$ receptor in regulating the behavioural and molecular effects of drugs of abuse. In

order to expand on the literature in this field the $GABA_{B(2)}$-S892A mouse, as well as the $GABA_{B(1a)}$ and $GABA_{B(1b)}$ receptor subunit knockout mice will be assessed for alterations in sensitivity to the acute rewarding and locomotor effects of cocaine. This will involve use of a conditioned place preference paradigm and locomotor activity paradigms in addition to biochemical assessment of striatal and accumbens activation in response to acute and repeated cocaine administration. We will specifically quantify cFos levels subsequent to acute cocaine treatment as a marker of neuronal activation and examine ΔFosB levels subsequent to repeated cocaine administration.

# *Chapter 2*

# *Activation but not Blockade of $GABA_B$ Receptors During Early–life Alters Anxiety in Adulthood in BALB/c mice*

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Revision submitted to: ***Neuropharmacology***

## *Abstract*

Although the underlying pathophysiology of anxiety disorders is unknown, it is clear that a combination of genetic and environmental factors in early life predispose to disease risk. Preclinical research increasingly suggests an important role for the $GABA_B$ receptor in modulating anxiety behaviour, with $GABA_B$ receptor-deficient mice having increased anxiety behaviour. Previous studies have highlighted critical windows during development where adult anxiety behaviour is primed. However, little is known regarding the role played by the $GABA_B$ receptors in the developmental processes that underlie adult anxiety behaviour. To this end, we treated male BALB/c mouse pups with the either the selective $GABA_B$ receptor agonist, R-baclofen (2 mg/kg, s.c), the $GABA_B$ receptor antagonist CGP 52432 (10 mg/kg and 30 mg/kg) or vehicle from postnatal days (PND) 14 to 28. The anxiety behaviour of these mice was then assessed in adulthood (PND 62 onwards) in a battery of behavioural tests comprising; the Stress-Induced Hyperthermia (SIH) test, the defensive marble burying test (DMB), the elevated-plus maze test (EPM) and the forced swim test (FST). Postnatal R-baclofen treatment resulted increased anxiety-like behaviour in the EPM as shown by approach-avoidance and ethological measures. Other behavioural measures were not significantly altered. Interestingl,y blockade of $GABA_B$ receptors with the $GABA_B$ receptor antagonist CGP52432 in early life caused no alterations in emotional behaviour. These data suggest that during early life $GABA_B$ receptor signalling can play a functional role in programming anxiety behaviour in adulthood. The underlying neurodevelopmental processes underlying these effects remain to be discovered.

## *2.1. Introduction*

Emerging evidence suggests that anxiety disorders in the adult may be heavily influenced by stress during early life as well as various genetic risk factors (Gross & Hen, 2004). Longitudinal studies in humans have presented a clear link between polymorphisms in genes governing serotonergic function, adverse experiences in childhood and behavioural disturbances in adult life (Caspi et al., 2002, 2003). Moreover, chronic treatment with drugs that increase synaptic concentrations of serotonin (selective serotonin reuptake inhibitors) are frequently used in the treatment of some anxiety disorders (Hoffman & Mathew, 2008). A role for the serotonergic system in developmentally priming anxiety circuits has been further bolstered by the demonstration that pharmacological inhibition of either the serotonin transporter (Ansorge et al., 2004) or blockade of the 5-$HT_{1A}$ receptor (Vinkers et al., 2009) from PND15-21 results in increased anxiety in adulthood in mice (Leonardo & Hen, 2008). Moreover, it has been demonstrated that a transient genetic inactivation of 5-$HT_{1A}$ receptors specifically in the forebrain during early-life (until PND 21) is sufficient to increase anxiety levels in adulthood (Gross et al., 2002).

One of the most effective strategies for the acute treatment of anxiety is the positive modulation of the $GABA_A$ receptor by benzodiazepines such as diazepam (Hoffman & Mathew, 2008; Cryan & Sweeney, 2011). However, it has been demonstrated that transient pharmacological positive modulation of the $GABA_A$ receptor in mice during early-life from both P14-28 and P10-16 results in an anxious phenotype in adulthood (Depino et al., 2008; Shen et al., 2012). It has also been shown that partial genetic inactivation of the γ2 GABAA receptor subunit on P13-14 also results in enhanced anxiety behaviour in adult life (Shen et

al., 2012). Together with the findings from the serotonergic studies, these data suggest that there is a period of vulnerability during early life in mice, ranging from P15-21, during which alterations in serotonergic and GABAergic neurotransmission can significantly alter anxiety behaviour in adulthood (Leonardo & Hen, 2008; Depino et al., 2008; Shen et al., 2012).

Despite being well-established therapies for the treatment of anxiety disorders, benzodiazepines are limited clinically in terms of tolerability (Hoffman & Mathew, 2008). This has led to an increasing focus on the $GABA_B$ receptor as a drug target in anxiety (Cryan & Kaupmann, 2005). The $GABA_B$ receptor acts via G-protein coupling to induce slow postsynaptic $K^+$ mediated neuronal hyperpolarisation and inhibiting Ca2+ channels presynaptically, thus inhibiting neurotransmitter release. The recent synthesis of novel $GABA_B$ receptor-active ligands coupled with the advent of $GABA_B$ subunit KO mice has revealed a key role for the $GABA_B$ receptor in the regulation of anxiety in adult life (Cryan & Kaupmann, 2005). Specifically, genetic ablation of either of the $GABA_B$ receptor subunits throughout life results in an increased anxiety-like behaviour in adulthood (Mombereau et al., 2004b, 2005). Interestingly, pharmacological blockade of the $GABA_B$ receptor in adulthood failed to recapitulate these findings from knockout mice suggesting a developmental genesis for the elevated levels of anxiety behaviour seen (Mombereau et al., 2004a). However, there is a paucity of evidence regarding the role the $GABA_B$ receptor plays, if any, in the developmental programming of anxiety behaviour.

Thus, we hypothesised that pharmacological modulation of $GABA_B$ receptor function during adolescence using the $GABA_B$ receptor antagonist (CGP52432) or agonist (baclofen) may result in alterations in anxiety-related behaviour in adulthood.

## *2.2 Methods*

### *2.2.1 Animals*

BALB/cOlaHsd mice were used for all studies (Harlan, UK). This strain was chosen as $GABA_B$ receptor knockout mice that have been tested previously in our laboratory come from a BALB/c background.

Mice, aged 6-8 weeks were mated after 1 week acclimatisation to the laboratory. Fathers were removed before parturition after which mothers were singly housed with their pups. Male pups were weaned at P23 and were housed in groups of 2-5 per cage. Drug treatments were distributed randomly between littermates. Mice were maintained on a 12h light/dark cycle with lights on at 7.00am. All experiments were conducted in accordance with the European Directive 86/609/EEC, the Recommendation 2007/526/65/EC and approved by the Animal Experimentation Ethics Committee of University College Cork. All efforts were made to minimize animal suffering and to reduce the number of animals used.

### *2.2.2 Drug treatment*

Two separate cohorts of male pups were used for the study. One cohort was treated with either R(+)baclofen HCl (2 mg/kg; Sigma; n=10) or with vehicle (phosphate buffered saline, PBS; n=13). The second cohort was treated with the $GABA_B$ receptor antagonist CGP52432 (Tocris; 10, 30 mg/kg; n=10) or vehicle (PBS; n=10). Drugs were freshly prepared for injection each day, by dissolution in PBS with vortexing and brief sonication at room temperature. Doses of R(+)baclofen and CGP52432 were chosen based on those

previously shown to be well tolerated in adult mice (Colombo et al., 2001; Voigt et al., 2011). All drug treatments were given via subcutaneous injection, once daily from PND14-28 in a volume of 0.05ml. This treatment regime was chosen as PND14-28 has been demonstrated to be a period of vulnerability to the developmental effects of drugs acting on the GABAergic system (Depino et al., 2008).

### *2.2.3 Behavioural testing*

Subsequent to drug treatment, animals were allowed to mature to adulthood, prior to being tested in a battery of behavioural tests, consisting of SIH (PND62), DMB (PND64), EPM(P76) and FST (PND83). Elevated plus maze and forced swim test experiments were recorded and subsequently scored by a trained observer who was blind to the treatment group.

### *2.2.4 Stress-induced hyperthermia*

The Stress-Induced Hyperthermia paradigm is a well validated technique used to measure the physiological response to an acute stressor in mice as well as a valid measure of levels of innate anxiety (Vinkers et al., 2008) and is particularly useful for determining the effects of modulating the GABAergic system on anxiety behaviour (Vinkers et al., 2010). Testing was conducted in a similar manner to that previously described (Jacobson & Cryan, 2008). Briefly, mice were singly housed 24 hours prior to testing. The rectal temperatures were then taken using a thermistor probe ca. 2mm (Model DM852, Ellab, Denmark) in diameter inserted 20mm into the mice rectum (T1) for 15 seconds. The temperature was measured again 15 minutes later (T2). The increase in temperature due to the stress of the initial temperature measurement (T2 value minus T1 value) was taken as a

measure of the physiological response to an acute stressor. Mice were returned to their home cage immediately after testing.

### *2.2.5 Defensive marble burying*

The defensive marble burying paradigm provides a measure of active anxiety behaviour following an acute stressful challenge. The test was adapted from a protocol previously described (Jacobson et al., 2007a). Briefly, mice were placed in the centre of a cage (38x25x18cm) containing 4cm of bedding with 20 blue glass marbles (1.5cm diameter) on top, arranged in 4 rows of 5 marbles (2cm apart). The lid of the cage was used to prevent mice from escaping the test chamber. Mice were allowed to explore the chamber for 30 minutes and then returned to their home cage. Following removal of the mouse, the number of marbles that were covered in bedding by at least two thirds were counted.

### *2.2.6 Elevated plus maze*

The elevated plus maze was conducted as previously described (Savignac et al., 2011). The apparatus consists of a plus-shaped maze that is elevated by 91cm from the ground. It consists of two enclosed arms and two arms exposed, joined by an intersecting square area. This test is a behavioural assay designed to test levels of passive anxiety-like behaviour, based on the conflict between the exploratory instinct of the mouse and its aversion to the exposed open arms of the maze. Mice were introduced to the centre of the maze facing into the open arm and were allowed to explore the maze for a period of 6 minutes. The experiment was performed under dim light (circa 30 lux) Parameters measured were time

in the open arms, time in the closed arms, entries to the open arms and time in the open arms. An animal was judged to have entered an arm of the maze only when all four paws were inside the arm in question. In addition to these measures the behaviour of animals in the elevated plus maze was assessed in terms of ethological parameters. The behaviours analysed were open and protected stretch-attend postures, head-dips and grooming behaviours. These were scored based on criteria previously described in the literature (Rodgers et al., 2002).

### *2.2.7 Forced swim test*

The forced swim test is a behavioural test commonly used to assess levels of antidepressant-like behavioural activity in mice (Cryan & Mombereau, 2004). Mice are placed in glass cylindrical tank containing 17cm of water at 23-25°C for 6 minutes and their behaviour is recorded. The length of time the mouse spends immobile, i.e. not actively swimming, during the last 4 minutes of the test is scored manually by a trained observer blinded to the treatment group.

### *2.2.8 Statistical analyses*

Data from the R(+)baclofen and CGP52432 experiments were analysed separately. Behavioural parameters in the R(+)baclofen experiment were analysed by Student's t-test. Behavioural analysis in the CGP52432 experiment were analysed via one-way ANOVA followed by Bonferonni post-hoc tests where appropriate. Statistical analyses were carried out using the Statistical Package for Social Sciences (SPSS) 20.0. P values of less than 0.05 were considered significant.

## *2.3. Results*

### *2.3.1 Elevated plus maze*

The effects of early-life treatment with R(+)baclofen or CGP52432 on behaviour on the elevated-plus maze are shown in Figure 1. Although the behaviour of control animals indicates an inherent innate anxiety this is in line with previous data from our lab and others using the BALB/c mouse (Browne et al., 2012). Mice treated with the $GABA_B$ receptor agonist R(+)baclofen spent significantly less time in the open arm of the elevated plus maze ($P<0.05$; Panel A) and spent significantly more time in the closed arms ($p<0.05$; Panel B). When the ratio of open arm entries to total entries, a measure of anxiety behaviour independent of locomotor activity, was calculated, mice treated postnatally with R(+)baclofen trended towards a decreased ratio of open arm entries to closed arm entries ($p=0.07$; Panel C). The total number of arm entries was not affected by drug treatment ($p= 0.66$; Panel D). Treatment with CGP52432 had no effect on time spent in the open arm ($F_{2,23}=0.3101$, $p=0.74$; Panel E), time spent in the closed arm ($F_{2,23}=0.5160$, $p=0.60$; Panel F) or on the ratio of open arm entries to closed arm entries ($F_{2,23}=0.6413$, $p=0.54$; Panel G). Total arm entries were not affected by drug treatment ($F_{2,23}=0.3391$, $p=0.72$; Panel H).

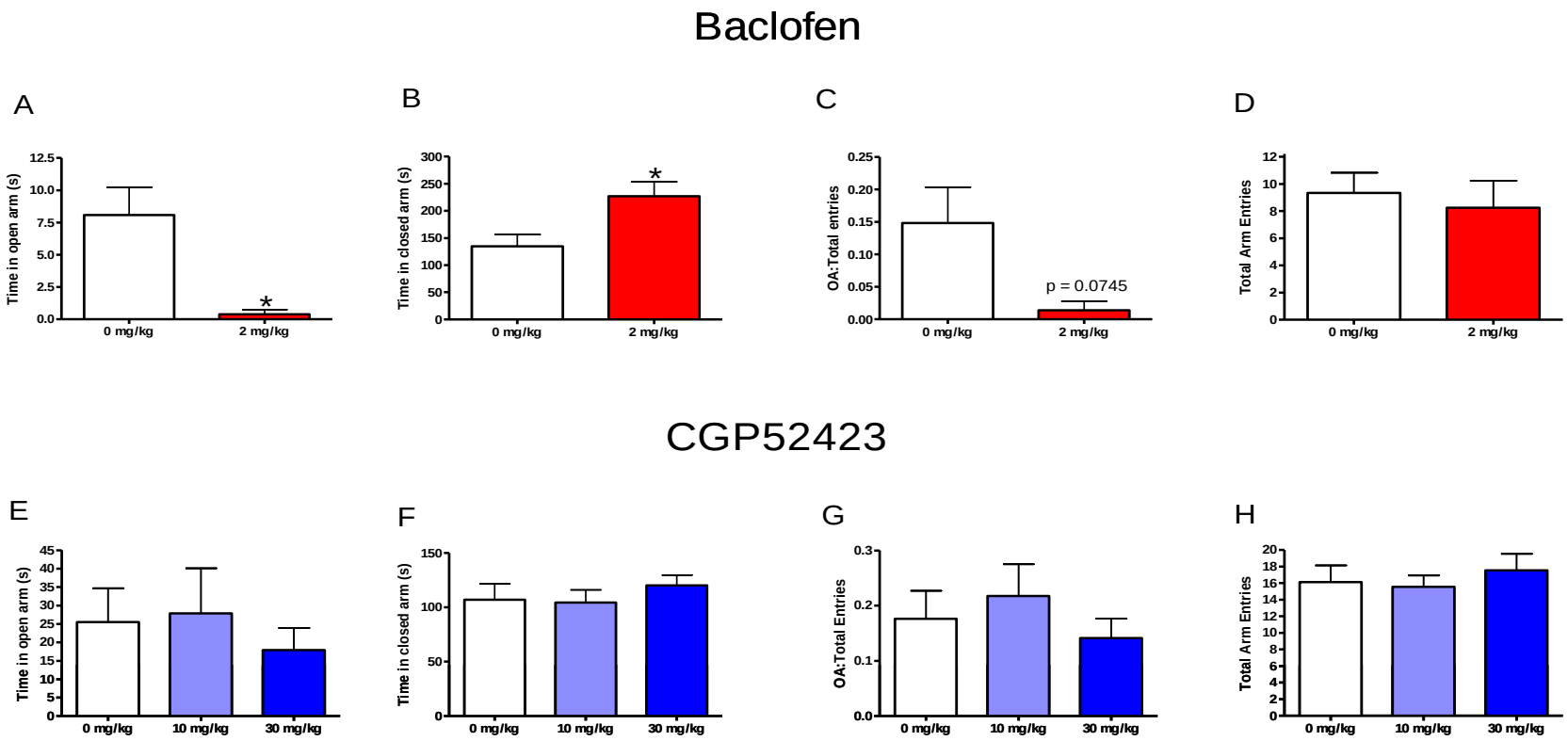


*Figure 1 - (A)Time in open arm, (B) time in closed arm and (C) ratio of open arm/ total entries in the elevated plus maze by mice treated with baclofen and (D) time in open arm, (E) time in closed arm and (F) ratio of open arm/ total entries in the elevated plus maze by mice treated CGP52432 early during postnatal development Data are expressed as mean±SEM. Statistical significance of $p<0.05$ in students t-test versus relevant vehicle treated control represented as *.*

Ethological analysis of the behaviour exhibited by baclofen and CGP52432-treated mice is exhibited in Figure 2. Mice treated with baclofen in early life displayed a significantly decreased total number of head dips (Panel A, $p<0.05$), and an increased percentage of stretch-attend postures (SAPs) in these animals was performed in a protected space (Panel D). The percentage of protected head dips showed a trend towards being decreased but did not reach significance ($p=0.05$); Panel B). Total SAPs ($p=0.61$;Panel C), and grooming behaviours were unchanged ($p=0.82$, ;Panels E) . Early life treatment with CGP52432 had no effect on total head dips ($F_{2,23}=0.41$, $p=0.67$), percentage of protected head dips ($F_{2,23}=0.36$, $p=0.70$) total SAPs ($F_{2,23}=0.41$, $p=0.67$), percentage of protected SAPs ($F_{2,23}=1.24$, $p=0.31$) or grooming behaviours ($F_{2,23}=0.07$, $p=0.93$) (Panels F-J).

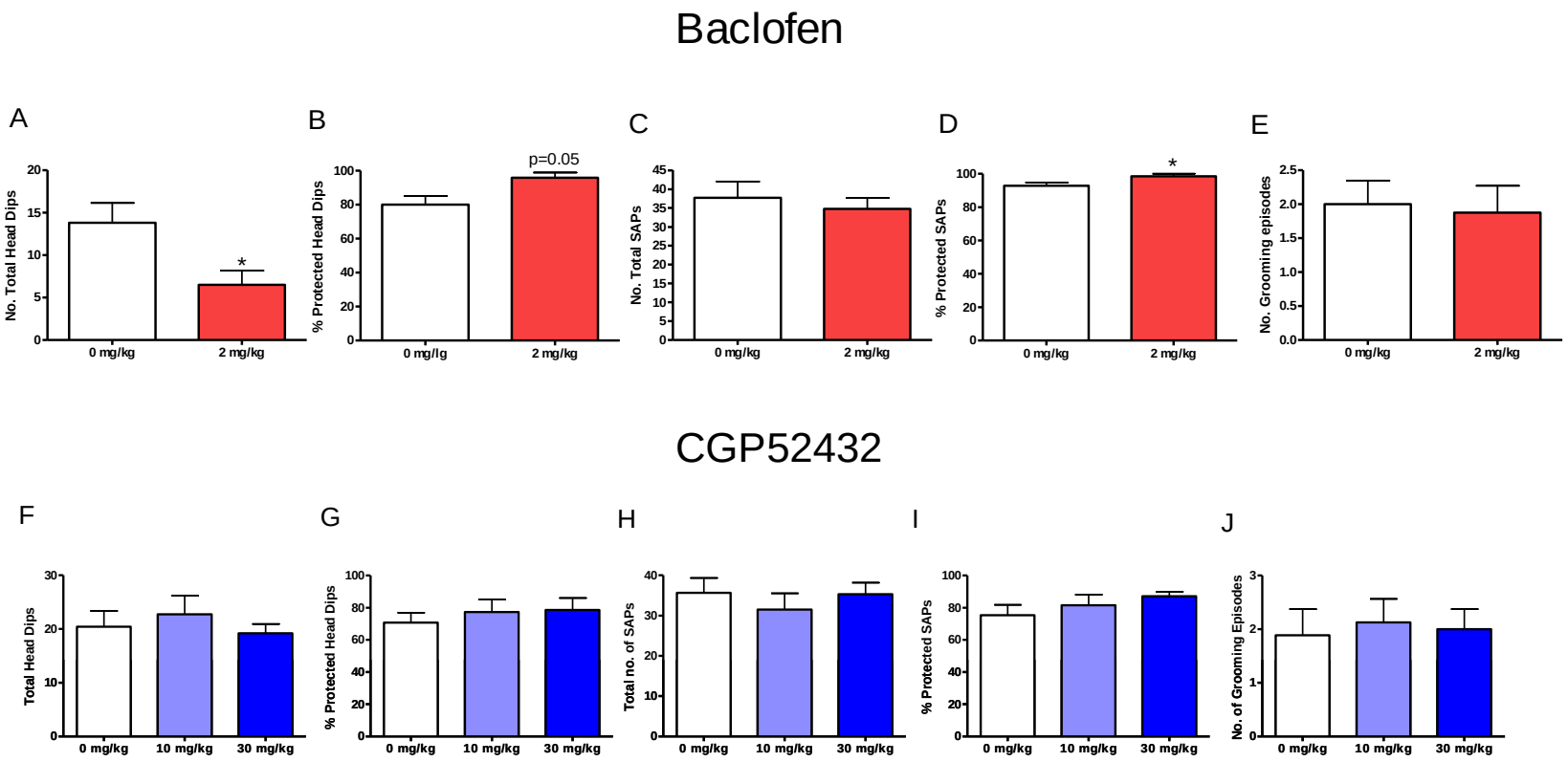


*Figure 2 - (A) total head dips, (B) percentage of protected head dips, (C) total stretch attend postures SAPs, (D) percentage of protected SAPs and (E) grooming behaviours in the elevated plus maze by mice treated with baclofen in early life as well as (F) total head dips, (G) percentage of protected head dips, (H) total SAPs, (I) percentage of protected SAPs and (J) grooming behaviours in the elevated plus maze by mice treated with CGP52432 in early life. Data are expressed as mean±SEM. Statistical significance of $p<0.05$ in students t-test versus relevant vehicle treated control represented as *.*

### *2.3.2 Stress-induced hyperthermia*

The effects of early-life treatment with R(+)baclofen or CGP52432 in the stress-induced hyperthermia paradigm are shown in Figure 3. Neither postnatal treatment with R(+)baclofen (P=0.263; Panel A) nor CGP52432 ($F_{2,27}$= 2.197, P = 0.131; Panel C) resulted in changes in baseline body temperature (T1) in adult life. However, early life treatment with R(+)baclofen tended to increase the hyperthermic response to acute stress but this effect did reach not statistical significance (P=0.07; Panel B). In contrast, CGP52432 did not affect the SIH response ($F_{2,27}$=0.006, P=0.99; Panel D)

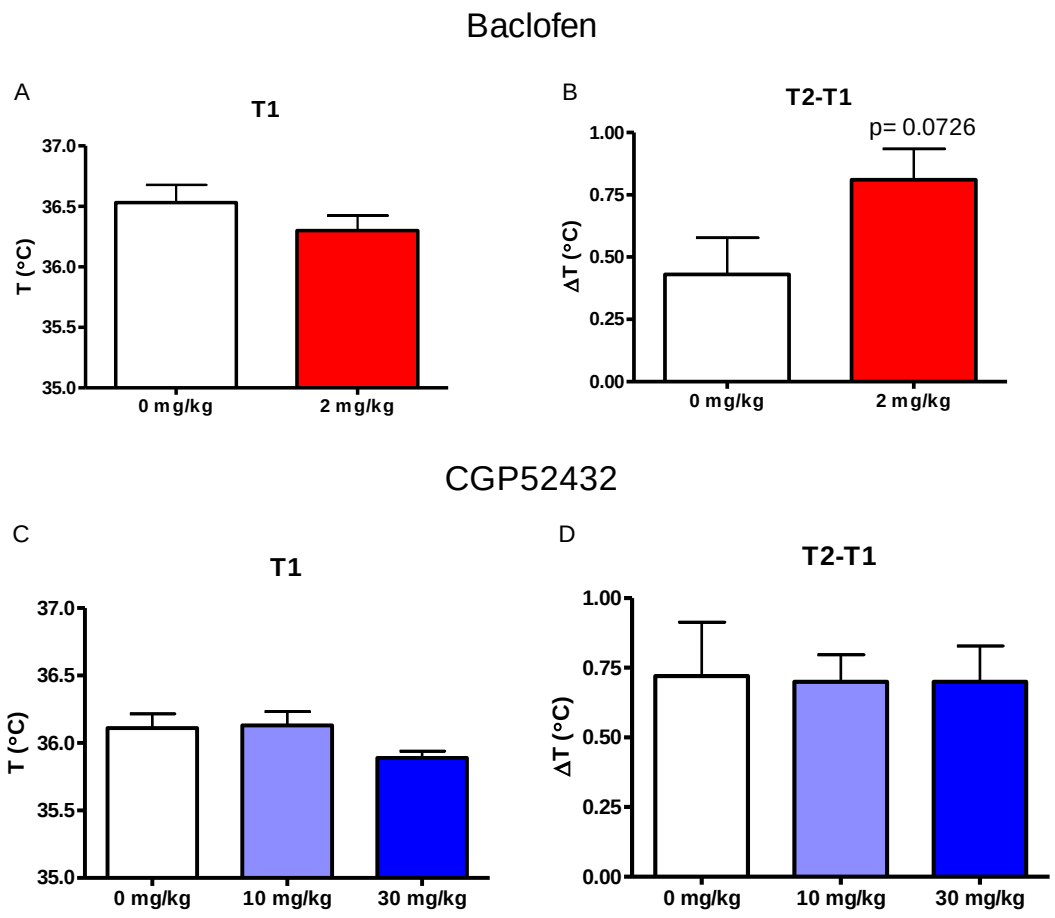


*Figure 3 - (A) Baseline temperatures and (B)stress induced increases in temperature in mice treated during early life with baclofen and (C) baseline temperatures and (D) stress-induced increases in temperature in mice treated during early life with CGP52432. Data are expressed as mean±SEM. Significance is expressed as p value from Student's T-test vs vehicle treated control.*

### *2.3.3 Defensive marble burying*

The effects of early-life treatment with R(+)baclofen or CGP52432 on behaviour in the defensive marble burying paradigm are shown in Figure 4. Neither R(+)baclofen (P=0.75; Panel A) nor CGP52432 ($F_{2,26}$=0.5153, P=0.60; Panel B) had no effect on the number of marbles buried by mice in the defensive marble burying paradigm.

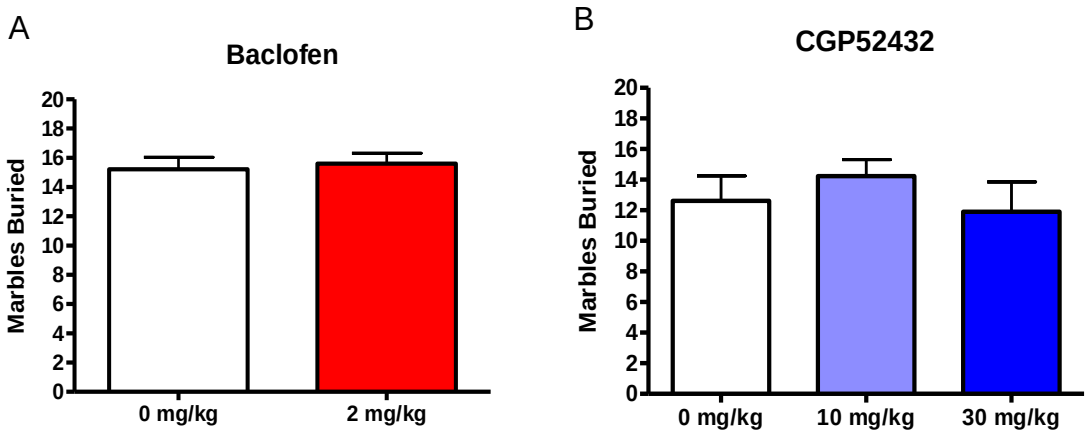


*Figure 4 - (A)Number of marbles buried in the defensive marble burying paradigm by mice treated in early life with baclofen and (B) number of marbles buried in the defensive marble burying paradigm by mice treated in early life with CGP52432. Data are expressed as mean±SEM.*

### *2.3.4 Forced swim test*

The effects of early-life treatment with R(+)baclofen or CGP52432 on behaviour in the forced swim test are shown in Figure 5. Postnatal treatment with either R(+)Baclofen

(P=0.95; Panel A) or CGP52432 ($F_{2,23}$=1.208,P=0.31; Panel B) had no effect on the levels of immobility seen in the forced swim test.

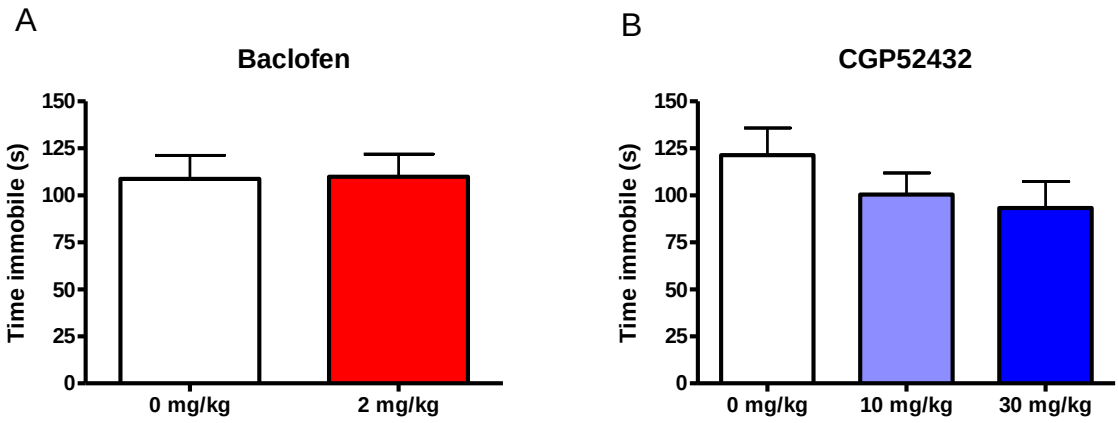


*Figure 5 - (A)Time spent immobile during the forced swim test by mice treated in early life with baclofen and (B) time spent immobile during the forced swim test by mice treated in early life with CGP52432. Data are expressed as mean±SEM.*

## *2.4 Discussion*

The aim of the present study was to investigate the role played by the $GABA_B$ receptor in the early life processes that govern adult anxiety behaviour. The main finding of the present study is that $GABA_B$ receptor plays an important role in programming anxiety levels in adulthood. Transient treatment with R(+)baclofen from postnatal days 14-28 resulted in an increase in anxiety behaviour in the elevated plus maze paradigm in adult life with a trend towards increased anxiety behaviour in the stress-induced hyperthermia paradigm. Interestingly, an anxiogenic effect was not detected in the defensive marble burying test. In further contrast, treatment with the $GABA_B$ receptor antagonist CGP52432 during this period did not produce any distinct anxiety phenotype. Neither treatment with the agonist nor the antagonist altered levels of immobility in the forced swim test, an index of antidepressant-like drug activity.

Clinically, there have been some reports of the selective $GABA_B$ receptor agonist baclofen being effective in treating anxiety symptoms in adults during alcohol withdrawal (Addolorato et al., 2006) and post traumatic stress disorder (Drake et al., 2003), a more complex picture has been observed in rodent models. Although baclofen has previously been shown to reduce ultrasonic separation vocalisations in mouse pups (Nastiti et al., 1991a), and display anxiolytic effects in both the Vogel conflict test (Ketelaars et al., 1988) and animal models of drug-withdrawal induced anxiety (File et al., 1992), more recent studies have shown that acute treatment with the $GABA_B$ receptor agonists baclofen or SKF 97541 (CGP 35024) produces no effect on anxiety behaviour in the elevated-zero maze in rats (Frankowska et al., 2007) and the $GABA_B$ receptor agonist CGP 44532 even

has an anxiogenic effect in the mouse four-plate test (Partyka et al., 2007). Most recent pharmacological efforts have been directed towards developing positive allosteric modulators of the receptor which have a more robust anxiolytic profile in animal while not generating the undesirable side-effects of either benzodiazepines or baclofen (Urwyler et al., 2001a; Cryan et al., 2004; Cryan & Kaupmann, 2005; Urwyler et al., 2005; Frankowska et al., 2007; Partyka et al., 2007; Malherbe et al., 2008) The generally anxiolytic effects of $GABA_B$ receptor activation in adult life contrast sharply with the anxiolytic effects of early life $GABA_B$ receptor agonist treatment.

Early-life $GABA_B$ receptor blockade did not generate behavioural effects in the current study. Similarly, chronic treatment with the $GABA_B$ receptor antagonist CGP56433A has been shown to have no effect on behaviour in the light dark box paradigm (Mombereau et al., 2004a) and that the $GABA_B$ receptor antagonist CGP 35348 has no effect on anxiety behaviour in the elevated-plus maze (Dalvi & Rodgers, 1996). However, more recently the $GABA_B$ receptor antagonist CGP36742 has been shown to have an anxiolytic effect in rats in both the elevated plus maze and conflict drinking paradigm and, in mice, in the four-plate test (Partyka et al., 2007). Likewise, the $GABA_B$ receptor antagonist SCH 50911 (CGP35024) has shown an anxiolytic effect in the rat elevated plus maze (Frankowska et al., 2007). Furthermore, when $GABA_B$ receptor function is genetically ablated the resulting phenotype is characterised by heightened anxiety behaviour as well as by antidepressant-like behaviour (Mombereau et al., 2004b, 2005). While the bidirectional behavioural effects generated by adult pharmacological blockade of the $GABA_B$ receptor and lifelong loss of $GABA_B$ receptor function suggest a developmental basis for the phenotype of the

$GABA_B$ subunit knockout mouse, the current data suggests that impaired $GABA_B$ receptor function in early life is not sufficient to generate an anxiogenic phenotype in adulthood. Recent data has suggested that anxiety and depression like behaviours in rodents are critically controlled by GABAergic neurotransmission at discreet neurodevelopmental periods. Partial genetic inactivation of the γ2 subunit of the $GABA_A$ receptor has been shown to induce deficits in anxiety behaviour in adult life when induced from PND13-14, and deficits in antidepressant like behaviour when induced from PND27-28 (Shen et al., 2012). Likewise, $GABA_A$ receptor potentiation with benzodiazepines appears to generate enhanced anxiety behaviour in adult life when administered from P10-16 and pro-depressant behaviour when administered from PND29-32 (Shen et al., 2012). The treatment period used in our experiment appears thus to extend into a critical neurodevelopmental period that governs forced swim test behaviour (PND27-P32), however neither baclofen nor CGP52432 altered adult life behaviour in the forced swim test. This suggests that this critical period is influenced by $GABA_A$ mediated neurotransmission, but not by $GABA_B$ receptor function.

To our knowledge, only one study has previously looked at the effects of $GABA_B$ receptor antagonism early during postnatal development on behaviour in adulthood. This study examined the short and long term effects of a seizure generating dose of the $GABA_B$ receptor antagonist CGP56999A to rat pups at P15. The seizures generated were primarily hippocampal in nature. These rats furthermore exhibited long term reductions in hippocampal $GABA_B$ receptor activity coupled with an increased susceptibility to seizures induced by hippocampal kindling (Tsai et al., 2008). This may suggest that treatment with

$GABA_B$ receptor antagonists in early life may alter hippocampal dependant fear behaviours, such as context specific conditioned anxiety, in contrast to the unconditioned anxiety testing in the current study.

The neurobiological circuits, pathways and substrates that are altered by early-life treatment with $GABA_B$ receptor agonists and thereby generating an anxious phenotype in adulthood remain to be elucidated. However, given the role of the developing serotonergic system in programming anxiety in adulthood (Leonardo & Hen, 2008), it is possible that alterations in serotonergic neurotransmission may underlie some of the behavioural changes observed in the present study. Indeed, activation of $GABA_B$ receptors generated by systemic administration of baclofen can exert bi-directional effects on serotonin release from DRN serotonergic neurons (Abellán et al., 2000). Based on these findings it is plausible to suggest that baclofen may be acting preferentially on the presynaptic $GABA_B$ autoreceptors, resulting in a decrease in GABAergic inhibition of the serotonergic neurons of the DRN and a resultant increase in serotonin release during this sensitive period in the development of the anxiety circuits. This is supported by recent work demonstrating that direct application of baclofen, but not $GABA_B$ receptor antagonists to the DRN can increase aggression in mice, which can be blunted by 5-$HT_{1A}$ receptor-mediated inhibition of serotonergic neurons in the DRN (Takahashi et al., 2010). Furthermore, the failure of $GABA_B$ antagonists to alter serotonergic tone and modify aggressive behaviour in this study may explain the lack of effect of early-life exposure to CGP52432 on adult anxiety behaviour, even at doses which have been shown previously to produce a behavioural response in mice (Colombo et al., 2001).

Another mechanism underlying the developmental effects of baclofen treatment seen in the current study may be interactions between the $GABA_B$ receptor and glutamatergic neurotransmission. Glutamate receptors play a crucial role in regulating anxiety behaviour (O'Connor et al., 2010) and the $GABA_B$ receptor has been demonstrated to be a regulator of glutamatergic neurotransmission via its function as both an autoreceptor and post-synapitc receptor on glutamatergic neurons (Waldmeier et al., 2008). It is thus conceivable that alterations in glutamatergic neurotransmission in early life may have long-lasting effects on anxiety behaviour. Similarly robust interactions have been demonstrated between the $GABA_B$ receptor and the dopaminergic system (Cruz et al., 2004; Vlachou & Markou, 2010). These interactions may also influence the neurodevelopmental processes that underlie adult anxiety behaviour.

*In vitro* studies on cultured hippocampal neurons have revealed that baclofen can alter expression of mRNA transcripts for a variety of genes which play a key role in neural migration, proliferation, survival and synaptogenesis such as BDNF, butylcholinesterase and Rho-GTPase activating protein 4 (Ghorbel et al., 2005) and that protein and mRNA levels of the neurotrophins brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) in the spinal cord are elevated by $GABA_B$ receptor antagonists (Heese et al., 2000). Alteration of these important factors in determining neural growth and proliferation may represent a pathway by which the $GABA_B$ receptor mediates its early life effects.

An important caveat in the current study is the use of the inbred BALB/cJ mouse strain. Mouse strain plays an important role in determining the sensitivity of mice to the acute effects of treatment with baclofen (Jacobson & Cryan, 2005) suggesting the possibility that the effects of early life baclofen may not be identical in different strains. Furthermore, BALB/cJ mice, despite being resistant to the lethal effects of $GABA_B$ receptor subunit knock-out (Schuler et al., 2001) are also associated with increased levels of anxious behaviour in tests of anxiety relative to other mice strains (Jacobson & Cryan, 2007). This may result in a "ceiling" effect where potentially anxiogenic interventions may induce little effect on due to already maximal levels of anxiety.

The treatment period used in this study, P14-28 is equivalent to the perinatal and early childhood period in humans (Khazipov & Luhmann, 2006). The study thus raises a concern that clinical use of baclofen during pregnancy and in paediatric patients may carry a risk of long term vulnerability to emotional disorders. Although baclofen use in pregnancy is reported in the literature, administered both intrathecally and orally (Moran et al., 2004; Morton et al., 2009) as well as in paediatric patients (Borowski et al., 2010), little is known about the long term effects on emotional behaviour of early life baclofen treatment in humans. However, alterations in $GABA_B$ receptor activity may also be involved in the pathogenesis of foetal alcohol syndrome (Lee et al., 2008), a syndrome associated with maternal alcohol consumption during pregnancy which is, in part, characterised by neuropsychiatric disorders including an increased incidence of emotional disorders (O'Connor & Paley, 2009). The treatment period used in the current study also extends into periadolescence, a period hypothesised to be associated with increased

vulnerability to stress induced behavioural disorders (Sturman & Moghaddam, 2011; Buwalda et al., 2011). The current data raise the possibility that the $GABA_B$ receptor may be an important substrate in modulating the impact of environmental and social stress in this period.

The present study provides further evidence for the involvement of altered $GABA_B$ receptor function in the development of neuropsychiatric illness, however, it must be remembered that the neurodevelopmental processes that determine adult levels of anxiety behaviour are dependant on a number of different neurotransmitter systems and the precise manner in which these systems interact is unclear. Likewise the manner in which environmental and genetic factors influence these processes remain to be elucidated. A deeper understanding of these processes and their neurochemical underpinning may allow for a more clear insight into the pathophysiology of anxiety disorders and indeed allow for more effective clinical interventions.

# *Chapter 3*

# *Examination of the Effects of Early Life Selective Serotonin Reuptake Inhibitor Treatment on $GABA_B$ Receptor Expression and Function in Adulthood*

**Fabian F. Sweeney, Olivia F. O'Leary, and John F. Cryan**

*For submission to:* ***Eur J. Pharmacol***

## *Abstract*

A significant body of data from both population based studies and from behavioural studies in animals has demonstrated that early life serotonergic function is a crucial determining factor in adult anxiety behaviour. In particular it has been demonstrated that perturbation of serotonergic neuorotransmission in early life via treatment with selective serotonin reuptake inhibitors (SSRI) antidepressants or transient genetic inactivation of serotonergic receptors, results in marked increases in adult anxiety behaviour in mice. In parallel with these findings has been the realisation that the $GABA_B$ receptor has a major role in regulating anxiety behaviour, with $GABA_B$ receptor knockout mice displaying pronounced increases in anxiety-like behaviour. Furthermore, the $GABA_B$ receptor has been shown to closely interact with the serotonergic system. Indeed, the antidepressant-like effects of $GABA_B$ receptor antagonists seen in animal models are ablated when animals are depleted of serotonin. We therefore sought to examine if the anxiolytic effects of early life SSRI treatment were associated with alterations in $GABA_B$ receptor function.

To investigate this we first treated C57/BL6 mice pups from PND 14-28 with the SSRI antidepressant fluoxetine (0, 5, 10 mg/kg via s.c. injection). Once these animals reached adulthood we examined the anxiety behaviour of these mice in the light-dark box and novelty-suppressed feeding paradigms. We also examined $GABA_B$ receptor function by measuring the hypothermic and ataxic effects of baclofen as well as the hypothermic effects of the $5\text{-}HT_{1A}$ agonist 8-OH-DPAT in these mice. The mice displayed a mildly anxious phenotype in the light-dark paradigm, but displayed no alterations in baclofen sensitivity. The mice did, however, display enhanced sensitivity to the hypothermic effects

of 8-OH-DPAT. Overall, our data suggest that early life fluoxetine treatment does not alter $GABA_B$ receptor function in adult life.

## *3.1 Introduction*

Interactions between stress and genes governing serotonergic function in early life appear to be predisposing factors for psychiatric disease in adulthood (Caspi et al., 2002, 2003). This finding has been expanded upon by animal models which have demonstrated clearly a pivotal early life period in mice (P15-21), where serotonergic tone exerts a strong influence on adult anxiety behaviour (Leonardo & Hen, 2008). In mice, transient genetic inactivation or pharmacological blockade of the $5HT_{1A}$ receptor during this period results in increased anxiety in adulthood in a variety of behavioural tests (Gross et al., 2002; Vinkers et al., 2009). Treatment of mice in early life with the SSRI antidepressant fluoxetine from P4-21 has been shown to robustly enhance anxiety-like behavioural responses in a host of behavioural tests (Ansorge et al., 2004; Karpova et al., 2009). However, although some studies have shown enhancements in brain derived neurotrophic factor (BDNF) mRNA expression in adult life subsequent to early life fluoxetine treatment (Karpova et al., 2009), the mechanisms that underlie the changes in behaviour resulting from early life alterations in serotonergic signalling and the molecular sequelae of early life SSRI treatment remain to be fully elucidated.

The $GABA_B$ receptor has recently been identified as a key substrate in regulating anxiety-like behaviour in animals and as a potential target for novel anxiolytic drugs (Cryan & Kaupmann, 2005). Molecules that act to enhance the activity of $GABA_B$ receptor activity, positive allosteric modulators have been characterised as having a broadly anxiolytic effect in animal models of anxiety (Cryan et al., 2004; Mombereau et al., 2004a; Frankowska et al., 2007; Jacobson & Cryan, 2008; Malherbe et al., 2008), whereas genetic models wherein

either $GABA_B$ receptor subunit is knocked out, display enhanced levels of anxiety (Mombereau et al., 2004a, 2005).

Behavioural data suggests that the behavioural effects of the $GABA_B$ receptor antagonists are mediated to some degree via interactions with the serotonergic system with the anti-depressant like effects of $GABA_B$ receptor antagonist being abolished when serotonin is depleted in the brain (Slattery et al., 2005a). Furthermore, it has been shown that the increase in aggressive behaviour in mice induced by $GABA_B$ receptor agonists can be reduced by 5-$HT_{1A}$ receptor blockade in the dorsal raphe nucleus (Takahashi et al., 2010). $GABA_B$ receptor mRNA is indeed expressed in both serotonergic neurons and GABAergic interneurons in the DRN (Serrats et al., 2003), and activation of these receptors *in vitro* can influence serotonin release (Abellán et al., 2000).

Of particular interest in the $GABA_B$-serotonin interaction is the relationship between the $GABA_B$ receptor, the 5-$HT_{1A}$ receptor and the serotonin transporter (5-HTT). Both the $GABA_B$ receptor and the 5-$HT_{1A}$ receptor act via recruitment of G-protein coupled inwardly rectifying potassium channels (GIRKs). Indeed the GIRK knockout mouse displays a reduced sensitivity to the hypothermic effects of both $GABA_B$ and 5-$HT_{1A}$ receptor agonists (Costa et al., 2005). More interestingly, genetic knockout of 5-HTT results in a reduced function for both of these receptors (Mannoury la Cour et al., 2004) and chronic pharmacological inhibition of 5-HTT function with fluoxetine results in both receptors displaying a reduced capacity to recruit GIRKs (Cornelisse et al., 2007). Both the $GABA_B$ receptor and the 5-$HT_{1A}$ receptor thus appear to share a transduction pathway, the

activity of which can be modulated by chronic fluoxetine treatment. However the effect of early life fluoxetine treatment on the functional properties of these two recep,tors in adult life is not currently known.

Given that $GABA_B$ receptor subunit expression has been shown to be altered in a mouse model of altered anxiety behaviour (Bravo et al., 2011) and that the serotonergic system and the $GABA_B$ receptor display these strong reciprocal interactions, it is possible that $GABA_B$ receptor function may be altered in adult life subsequent to early life SSRI treatment. We intend, using the pronounced hypothermic and ataxic effects of the $GABA_B$ receptor agonist baclofen (Jacobson and Cryan, 2005) and the 5-$HT_{1A}$ receptor agonist 8-OH-DPAT (Vinkers et al., 2008), as outputs of receptor function in adulthood, to examine whether the anxiogenic effects of early life SSRI treatment are coupled with alterations in $GABA_B$ and 5-$HT_{1A}$ receptor function.

## *3.2 Methods*

### *3.2.1 Animals*

Male and female C57/BL6 aged 6-8 weeks were acquired from Harlan laboratories, UK. After 1 week acclimatisation to the laboratory mice were mated. Fathers were removed before parturition after which mothers were singly housed with their pups. Male pups were weaned at P23 and were housed in groups of 2-5 per cage. Drug treatments were distributed randomly between littermates. Mice were maintained on a 12h light/dark cycle with lights on at 7.00am. All experiments were conducted in accordance with the European Directive 86/609/EEC, the Recommendation 2007/526/65/EC and approved by the Animal Experimentation Ethics Committee of University College Cork. All efforts were made to minimize animal suffering and to reduce the number of animals used.

### *3.2.2 Drug treatment*

Male pups were treated with fluoxetine (5,10 mg/kg; Sigma; n=10/11) or with vehicle (0.9% saline; n=11). Drugs were freshly prepared for injection each day, by dissolution in saline with vortexing. Fluoxetine or vehicle was given via subcutaneous injection, once daily from PND14-28 in a volume of 0.05ml. The treatment regime was chosen to include the period from PND15-21, that appears to be the critical period in which serotonergic perturbations result in altered adult anxiety behaviour (Leonardo & Hen, 2008).

### *3.2.3 Behavioural testing schedule*

Subsequent to drug treatment, animals were allowed to mature to adulthood before being tested, beginning from ages of 9-10 weeks, in a battery of assays consisting of a light-dark box, rotarod performance and temperature sensitivity to baclofen and then 8-OH-DPAT followed by a novelty suppressed feeding assay. Animals were given at least 4 days between each test to recover and allow for wash out of drugs.

### *3.2.4 Light dark box*

The light dark box is a behavioural test used to assess levels of unconditioned anxiety in rodents based on levels of passive avoidance behaviour. The apparatus consisted of a Plexiglas enclosure ($44 \times 21 \times 21$ cm) comprised of two adjoining compartments (one light and one dark). The light compartment had transparent Plexiglas walls and had an open top. Bright lighting (approximately 1,000 lx) was used to generate aversive conditions in this compartment. The dark compartment (14 cm width) consisted of a black Plexiglas wall and roof. The compartments were connected by a small opening ($12 \times 5$ cm) at floor level.

Animals were placed in the light compartment facing away from the dark compartment and were allowed to freely explore the apparatus for 10 minutes. During this period the behaviour of the animals was recorded. The latency of the animal to enter the dark compartment of the apparatus, the number of transitions between the two compartments and the total time spent in the light compartment were measured. An animal was adjudged to have entered a compartment when all four paws had crossed the threshold. The number of

faecal pellets produced by each animal during the test period was also recorded as a measure of the physiological response to a stressful stimulus.

### *3.2.5 8-OH-DPAT and baclofen sensitivity measurements*

Mice were singly housed over night prior and acclimatised to the testing room for at least one hour prior to testing. Temperature was measured using a thermistor probe of 2mm in diameter (Ellab, Denmark – Model DM852). The thermistor probe was lubricated with petroleum jelly and inserted roughly 20mm into the rectum until a stable temperature was achieved (10-15s). Measurement of the ataxic effects of baclofen was assessed via rotarod endurance. The rotarod (Ugo Basile, Italy - Model 47600) was set at 12RPM and each trial lasted for a maximum of 300 seconds. Endurance was determined by the time taken before mice fell off the rotarod. Mice clinging to the rotarod for more than 80% of the 300 seconds test time were given a score of zero. Mice clinging to the rotarod for less than 80% of the 300 seconds only had their time spent actively walking on the rotarod measured.

### *3.2.6 Novelty suppressed feeding test*

The novelty suppressed feeding test is a measure of anxiety-like behaviour wherein animals that have been food deprived are presented with a novel food in the context of a novel environment. A conflict thus between the anxiogenic stimulus and hunger-induced approach behaviour. Clinically used anxiolytics reduce the latency of the animal to approach the food (Bodnoff et al., 1988, 1989). Animals were singly housed and food deprived overnight before being transported to the testing room for at least one hour of

acclimatisation. The apparatus consisted of a plexiglas cylinder (diameter - 24 × 21 cm) placed on white paper with a small well (1.2cm diameter x 0.9cm height) secured in the middle. The novel food used for the experiment was sweetened condensed milk mixed with equal amounts of water. Light levels were maintained at 100 lux. Animals were placed in the apparatus facing away from the well. Latency until the mice had commenced drinking for at least two seconds was measured. A cut off of 120 seconds was observed for each individual test session.

#### *3.2.6 Statistical analyses*

Behavioural results from the light dark box and novelty suppressed feeding test were analysed via one-way ANOVA followed by Bonferonni post-hoc tests where appropriate. The locomotor and hypothermic responses of mice to OH-DPAT and baclofen were analyses by repeated measures two-way ANOVA, followed by Bonferonni post-hoc tests where appropriate. Statistical analyses were carried out using the Statistical Package for Social Sciences (SPSS) 20.0. P values of less than 0.05 were considered significant.

## *3.3 Results*

#### *3.3.1 Light dark box*

The effects of early-life treatment with fluoxetine on behaviour in the light-dark box are shown in Figure 1. Mice treated with fluoxetine produced significantly greater numbers of faecal pellets in the apparatus compared to non-treated animals as revealed by one way ANOVA ($F_{2,29}$=3.55, p=0.042; Panel A). Bonferroni post-test revealed no significant difference between individual treatments. One-way ANOVA revealed no significant effects

of drug treatment on latency to enter the dark chamber ($F_{2,29}$=1.96, p=0.159; Panel B), time spent in the light ($F_{2,29}$=0.11, p=0.89; Panel C) or total number of light-dark transitions ($F_{2,29}$=0.86, p=0.43; Panel D).

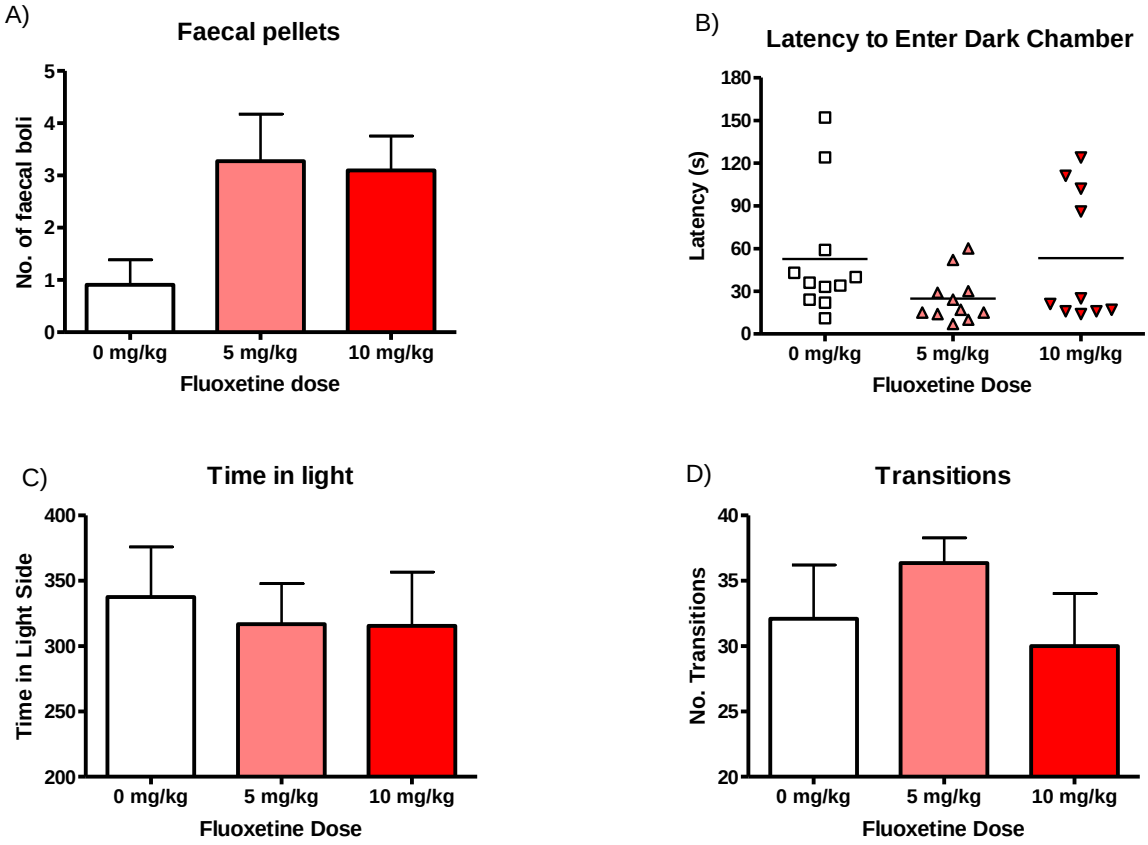


*Figure 1 - Behavioural phenotype of mice treated in early life with fluoxetine on behaviour in the light-dark box paradigm. A) Faecal pellet production, B) latency from entry into the box until entry into the dark compartment, C) time spent in the light side of the compartment and D) number of transitions from light to dark chamber and vise-versa. Data displayed in panels A,B and D as mean +/- SEM. Data displayed in panel B as mean with individual data points.*

### *3.3.2 Baclofen challenge*

The hypothermic and ataxic effects of baclofen in mice treated in early life with fluoxetine are shown in Figure 2. Mice treated with baclofen from all groups displayed transient hypothermia (Panel A) coupled with transient ataxia (Panel B) subsequent to baclofen administration. Two-way ANOVA revealed no effect of early life fluoxetine treatment on sensitivity to the hypothermic ($F_{2,29}$= 0.2703, p= 0.76) or ataxic ($F_{2,29}$=0.11, p=0.89) effects of baclofen.

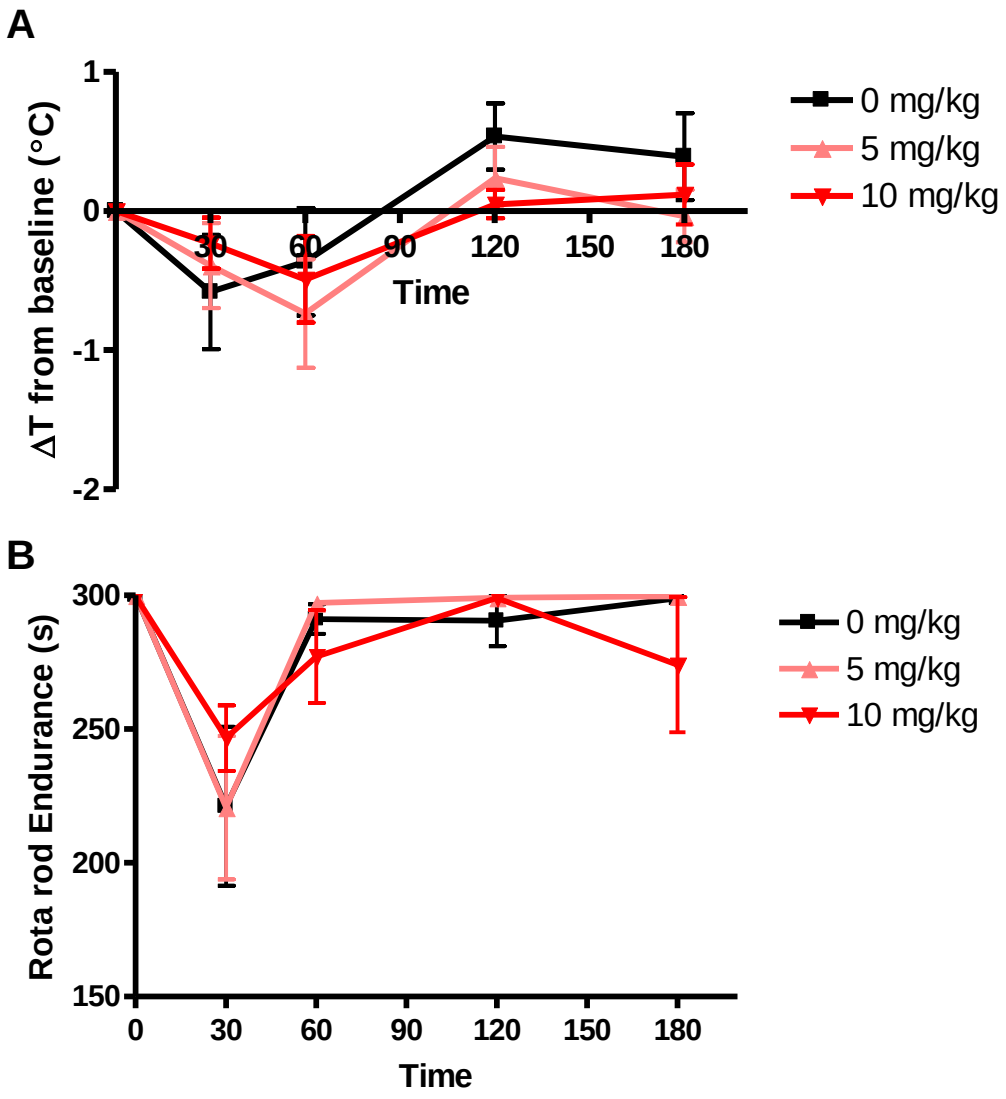


*Figure 2- Physiological sensitivity of mice treated in early life with fluoxetine. A) Effects of baclofen (4mg/kg IP) on rectal temperature of mice. Data is displayed as difference in temperature from baseline (T0). Data represented as mean +/-SEM. B) Effects of 4 mg/kg baclofen on rotarod endurance in mice over 300 seconds. Data represented as mean +/-SEM.*

### *3.3.3 OH-DPAT challenge*

The hypothermic effects of 8-OH-DPAT in mice treated in early life with fluoxetine are shown in Figure 3. Mice treated with 8-OH-DPAT all displayed transient decreases in body temperatures from baseline subsequent to 8-OH-DPAT treatment. A trend was observed towards a significant effect of treatment on sensitivity to the hypothermic effects of 8-OH-DPAT ($F_{2,29}$= 3.10, p= 0.06) suggesting that animals treated with fluoxetine in early life were more sensitive to the hypothermic effects of 8-OH-DPAT.

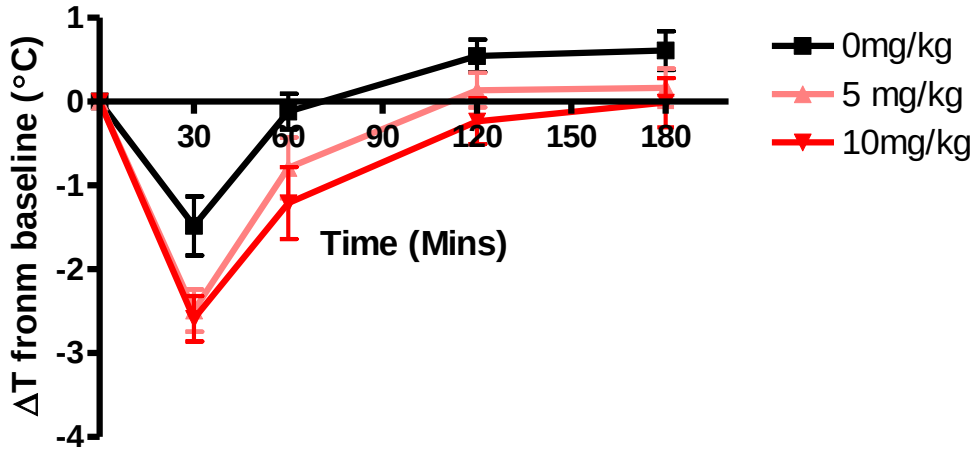


*Figure 3 -Effects of 8-OH-DPAT (1 mg/kg, IP) on rectal temperature of mice treated in early life with fluoxetine. Data is displayed as difference in temperature from baseline (T0). Data represented as mean +/-SEM.*

### *3.3.4 Novelty suppressed feeding (NSF) test*

Latency for animals to eat in the novelty suppressed feeding test is shown in Figure 4. Treatment in early life with fluoxetine had no effect of latency to eat in the novelty suppressed feeding test ($F_{2,23}$=0.24, p=0.79).

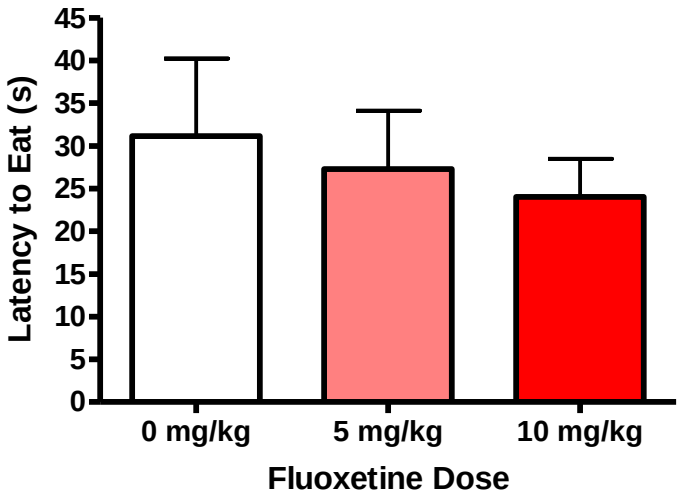


*Figure 4- Latency to eat of mice treated with fluoxetine in early life in a novelty suppressed feeding paradigm. Data is expressed as mean +/-SEM.*

## *3.4 Discussion*

While treatment with fluoxetine from P14-28 produced mild anxiogenic effects in the light-dark box paradigm, but not in the novelty suppressed feeding test, no alterations in sensitivity to the hypothermic effects of baclofen were observed. Animals treated with fluoxetine in early life did, however, display enhanced sensitivity to the hypothermic effects of the $5HT_{1A}$ receptor agonist 8-OH-DPAT. Our results, thus, suggest that the anxiogenic effects of early life SSRI treatment are not due to alterations in $GABA_B$ receptor function, but that enhanced $5\text{-}HT_{1A}$ receptor function may play a role in this phenotype.

The lack of alterations in $GABA_B$ receptor sensitivity seen in this study is in some ways unsurprising given the crucial role played by the $GABA_B$ receptor in maintaining general inhibitory control in the brain. Indeed in the vast majority of inbred mouse strains, knockout of functional $GABA_B$ receptors results in inevitable mortality due to uncontrollable seizures (Prosser et al., 2001; Schuler et al., 2001). It is also of note that $GABA_B$ receptor function is regulated by a vast number of regulatory pathways including kinase mediated phosphorylation and varying patterns of subunit composition (Terunuma et al., 2010a; Pinard et al., 2010; Gassmann & Bettler, 2012). This plethora of regulatory mechanisms, taken with the crucial importance of the $GABA_B$ receptor to normal brain function suggests a high level of redundancy leading to relatively stable levels of function *in vivo.*

While measurement of the ataxic and hypothermic effects of baclofen is a useful tool for demonstrating broad disparities $GABA_B$ receptor sensitivity (Jacobson & Cryan, 2005), it ultimately remains a crude tool. Evidence is growing to suggest that $GABA_B$ receptor function differs throughout the brain, with different regions displaying markedly different receptor kinetics (Pinard et al., 2010). Investigation of levels of baclofen induced γGTP recruitment in various brain regions *in vitro* may give a more detailed insight into the effects of early life SSRI treatment on adult life $GABA_B$ receptor function. Moreover, the discovery that $GABA_B$ receptor function is markedly altered by not only phosphorylation states but by association with a broad range of effector components (Gassmann & Bettler, 2012) suggests that in order to thoroughly investigate the effects of any intervention on $GABA_B$ receptor function, detailed analysis of all these factor is required.

The inbred mouse strain used in our experiment may also have influenced our results. C57BL/6 mice were chosen for our study, as other groups have demonstrated repeatedly that this strain is vulnerable to the anxiety provoking effects of early life SSRI treatment (Karpova et al., 2009), however given that baclofen sensitivity differs markedly between inbred mouse strains (Jacobson & Cryan, 2005), it is impossible to rule out the possibility that were other strains treated with fluoxetine in early life, alterations in baclofen sensitivity would be observed.

A longstanding hypothesis regarding the actions of SSRI antidepressants at a molecular level, is that they induce a downregulation of 5-$HT_{1A}$ receptor function (Stahl, 1998). Furthermore, the 5-$HT_{1A}$ receptor has been shown to critically modulate the development

of normal anxiety behaviour in mice (Gross et al., 2002). It is thus intriguing that in our study, animals treated with fluoxetine in early life displayed enhanced sensitivity to the hypothermic effects of 8-OH-DPAT, which suggests enhanced levels of 5-$HT_{1A}$ receptor function. Reduced levels of 5-$HT_{1A}$ receptor expression has been observed in animals treated in early life with clomipramine (Kim et al., 2012), interestingly, this alteration was reversed by social defeat stress in this study. Animals with constitutionally downregulated 5-HTT expression display no alterations to $5HT_{1A}$receptor function (Bordukalo-Niksic et al., 2010).

The interactions observed between the serotonin transporter, $5HT_{1A}$ receptor and the $GABA_B$ receptor make it interesting to speculate as to the potential molecular links that underlie the changes in $5HT_{1A}$ receptor function. Although the $GABA_B$ receptor and 5-$HT_{1A}$ receptor share a transduction pathway that can be regulated by SSRI administration (Cornelisse et al., 2007), in our study fluoxetine treatment had specific effects of fluoxetine on OH-DPAT as opposed to baclofen sensitivity. This suggests that early life fluoxetine treatment affects $5HT_{1A}$ receptor function at the level of receptor expression or serotonergic tone rather than at the level of an effector pathway.

Early life treatment with fluoxetine produced a mild anxiogenic effect in the light dark box in terms of faecal pellet production, but not in terms of any of the exploratory outputs measured. Treatment did not produce any alterations in behaviour in the hyponeophagia paradigm. These mild effects are somewhat unexpected given the robustly anxiogenic effects of early life fluoxetine treatment seen in the literature. Previously deficits in

exploratory behaviour in mice subsequent to early life fluoxetine treatment have been observed in the open field test, the elevated plus maze and the light-dark box (Ansorge et al., 2004, 2008; Karpova et al., 2009). Enhanced latency to eat in a novel environment has also been observed in animals treated with fluoxetine in early life (Ansorge et al., 2004, 2008), but was not observed in our study. It must be borne in mind here that alterations in the nature of the food used (novel palatable food vs normal chow) and environment can alter the levels of stress and anxiety generated in a novelty suppressed feeding paradigm (Cryan & Sweeney, 2011). Subtle changes to our protocol from those used by other groups may have contributed to our failure to see changes. It is also of note that our treatment was administered daily via IP injection as opposed to administration orally via water supply (Ansorge et al., 2004, 2008; Karpova et al., 2009). Pharmacokinetic variation between the two administration protocols may have thus resulted in a less robust phenotype being generated. Taken together our data suggests that early life SSRI treatment does not exert its anxiogenic effectsm via alterations in $GABA_B$ receptor function.

# *Chapter 4*

## *$GABA_B$ Receptor Ligands Do Not Modify Conditioned Fear Responses in BALB/c Mice*

**Fabian F. Sweeney, Olivia F. O'Leary and John F. Cryan**

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## *Abstract*

The $GABA_B$ receptor has been well-characterised as a substrate of unconditioned anxiety behaviour. Indeed, the anxiolytic effects of positive modulators of the $GABA_B$ receptor have been demonstrated across a range of behavioural tests of innate anxiety, whereas functional deactivation of the $GABA_B$ receptor using genetic techniques generates a phenotype of pronounced anxiety behaviour. However, the role of the $GABA_B$ receptor in regulating conditioned anxiety behaviour, an important facet of the preclinical study of anxiety disorders such as post-traumatic stress disorder, is less well understood. *In vitro* data suggests that the $GABA_B$ receptor plays an important role in regulating the neural circuitry that underpins conditioned fear-learning and extinction, but whether these effects translate into alterations in conditioned anxiety behaviour has not been widely investigated. This represents a crucial deficit in the preclinical characterisation of these drugs as putative anxiolytic agents. Using the highly anxious mouse strain, BALB/C and an auditory fear conditioning protocol, we sought to characterise the $GABA_B$ receptor positive modulator GS39783 and $GABA_B$ receptor antagonist CGP52432, two compounds not previously evaluated for their effects on conditioned fear in this regard. Neither GS39783 nor CGP52432 altered freezing behaviour irrespective of whether drugs were administered before the acquisition, recall or extinction training sessions. These findings suggest limitations to the potential role of $GABA_B$ receptor-active drugs as clinical agents in the treatment of anxiety.

## *4.1 Introduction*

Current data has suggested a role for the $GABA_B$ receptor as a crucial regulator of anxiety-like behaviour in rodents and highlighted the $GABA_B$ receptor as potential therapeutic target in anxiety disorders and depression (Cryan & Kaupmann, 2005).
Indeed, animals deficient in functional $GABA_B$ receptors display marked increases in anxiety behaviour across a range of tests of unconditioned anxiety such as the light-dark box, the staircase test and the elevated zero maze with an apparent resistance the behavioural anxiolytic effects of benzodiazepines (Mombereau et al., 2004a, 2004b, 2005). The anxiolytic effects of $GABA_B$ receptor positive modulators in tests of unconditioned anxiety have also been well-characterised (Cryan & Kaupmann, 2005) with some studies suggesting that $GABA_B$ receptor antagonists have anxiolytic effects in some exploratory and conflict based tests of anxiety (Partyka et al., 2007; Frankowska et al., 2007).

Although behavioural tests of unconditioned anxiety play a crucial role in the screening and development of novel anxiolytic compounds, it is increasingly clear that models of conditioned and learned fear also represent an important tool in this process (Cryan & Holmes, 2005; Cryan & Sweeney, 2011). The persistent salience of traumatic cue and memories in post-traumatic stress disorder and disordered processing of potentially threatening stimuli seen in generalised anxiety disorder patients suggest that cognitive deficits and in particular altered conditioned fear-learning is a key facet of the pathology of anxiety disorders (American Psychiatric Association, 2000; Lang et al., 2000).
Behavioural tests designed to model these aspects of anxiety disorders are based on the Pavlovian principles of associating a neutral cue, such as a tone or light, (conditioned

stimulus) with a painful or aversive stimulus such as an electrical foot-shock (conditioned stimulus). Conditioned fear responses can then be indexed through a wide variety of outputs such as conditioned freezing (Fendt & Fanselow, 1999; Cryan & Holmes, 2005).

The neuronal basis for the development of conditioned fear revolves around the onset of molecular plasticity in the lateral amygdala (LA). Auditory and nociceptive sensory afferents converge in the LA, which in turn innervates the central nucleus of the amygdala. Molecular plasticity in the LA results in increased input to the central nucleus in response to auditory conditioned stimuli, and corresponding increases in central nuclei mediated fear responses (Phelps & LeDoux, 2005; Johansen et al., 2011). GABA plays a crucial role in this process, with GABAergic interneurons in the amydala regulating neuronal activity and LTP generation (Johansen et al., 2011). In particular $GABA_A$ receptor antagonists, when applied to the LA can inhibit the acquisition of conditioned fear (Muller et al., 1997; Wilensky et al., 1999). The $GABA_B$ receptor agonist baclofen has been demonstrated to inhibit the extinction of conditioned fear when given before fear training or before recall testing (Heaney et al., 2012). This study also demonstrated that the $GABA_B$ receptor antagonist phaclofen had no effect on fear-learning or extinction (Heaney et al., 2012). However, recent years have seen a proliferation of novel $GABA_B$ receptor ligands, most importantly $GABA_B$ receptor positive allosteric modulators such as GS39783 and highly selective and potent $GABA_B$ receptor antagonists such as CGP52432 (Froestl, 2010). Drugs from these novel pharmacological classes have yet to be thoroughly evaluated in terms of their effects in conditioned fear paradigms and in particular the effects of the aforementioned compounds have not yet been assessed in conditioned fear. Therefore, we

examined whether GS39783 or CGP52432 impact the acquisition, expression and extinction of such fear responses using an auditory conditioned fear paradigm and the highly anxious BALB/c mouse (Belzung & Griebel, 2001).

## *4.2 Methods*

### *4.2.1 Animals*

Male BALB/cOlaHsd aged 6-8 weeks were acquired from Harlan and given at least seven days acclimatisation to laboratory conditions prior to testing. Mice were housed in cages of 10 with food and water available *ad-libitum.* Animals were maintained on a 12h light/dark cycle with lights on at 7.00am. All experiments were conducted in accordance with the European Directive 86/609/EEC, the Recommendation 2007/526/65/EC and approved by the Animal Experimentation Ethics Committee of University College Cork. All efforts were made to minimize animal suffering and to reduce the number of animals used.

### *4.2.2 Auditory fear-conditioning protocol*

Auditory fear conditioning was conducted as previously described by our lab (Bravo et al., 2011) based on a protocol developed by Brinks and colleagues (Brinks et al., 2009). On day 1, mice were placed in conditioning chambers (21.6 cm x 17.8 cm x 12.7 cm), with transparent walls in the front and back, stainless-steel bars, and a metal-grid floor connected to a shock scrambled and generator in sound-attenuating box. After180 s acclimatisation mice received 6 pairings (60s interpairing interval) of a conditioned stimulus (CS; 20 s, light, 10 dB, 10kHz tone) and a unconditioned stimulus (US; 2 s, scrambled foot shock; 60 mA). The US was presented during the last 2 s of the CS. After a 60 s no-stimulus consolidation period after the final CS–US pairing, mice were returned to the home cage. Chambers were cleaned with 70% ethanol between each mouse. On days 2 and 3 mice were returned to the same chamber as day one and the procedure repeated, with the absence of

electrical footshocks. Activity of mice was monitored by Video Freeze (Med Associates, USA). Animals were filmed at rate of 15 frames per second, with a freezing behaviour represented by movement of less than 255 pixels (to accommodate breathing) over 7 frames. This paradigm allows for the measurement of the acquisition and extinction of conditioned fear. The design of the procedure additionally allows us to measure both context and cue induced freezing which are measures of hippocampus and amygdala dependent fear behaviours, respectively. An outline of the experimental procedure is given in Figure 1.

The $GABA_B$ receptor positive modulator GS39783 (Discovery Fine Chemicals, Dorset, UK) was administered via oral gavage suspended in 0.5% methylcellulose (0, 10, 30 mg/kg). The $GABA_B$ receptor antagonist CGP52432 (Tocris Bioscience, Bristol, UK) was delivered via intraperitoneal injection in phosphate buffered saline (0, 10, 30 mg/kg). Doses and routes of administration were based on those shown to produce behavioural effects in the literature (Mombereau et al., 2004a; Felice et al., 2012b). In order to dissect specific effects of the $GABA_B$ receptor-active drugs on the acquisition, expression and extinction of conditioned fear, animals were received drugs either prior to acquisition, recall testing or extinction training. GS39783 or its vehicle were administered 60 minutes prior to the start of the trial for the specific day, with CGP52432 being administered 30 minutes prior to each trial. To obviate the effects of injection stress all groups received an injection with drug or vehicle on each day.

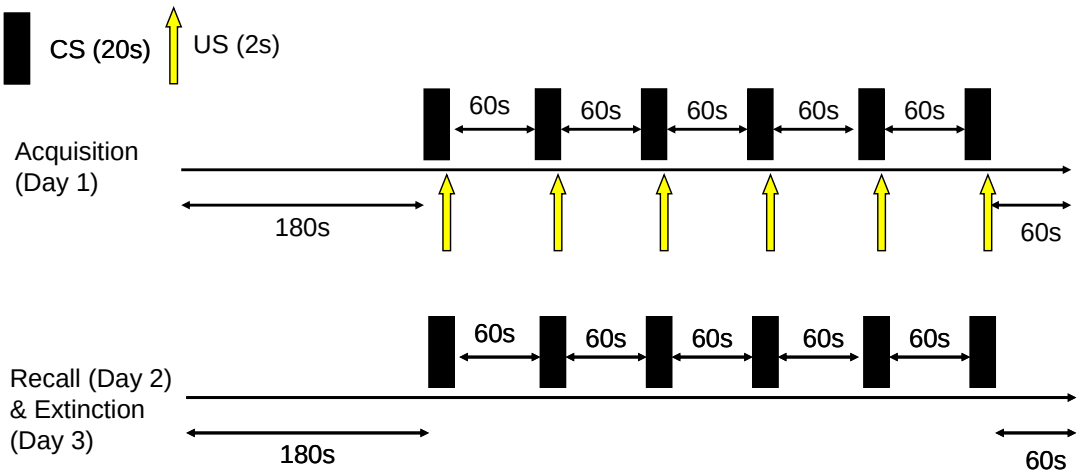


*Figure 1 –Schematic of the auditory fear conditioning protocol. On day one (acquisition training) mice are given 180 seconds acclimatisation to the novel test chamber before presentation of the CS (light, 10 dB, 10kHz tone) for 20 seconds (Black rectangles) with the US (60 mAmp scrambled footshock) presented for the final 2 seconds (yellow arrow). The US-CS pairing was repeated six times with a 60 second inter-pairing interval and a final 60 second consolidation period. On day two (recall testing) and three (extinction testing) an identical protocol in the same contextual environment, was used with the absence of the footshock.*

### *4.2.3 Statistical analyses*

The effect of drug administration on each day was examined by two-way repeated measures ANOVA followed by Bonferonni post-hoc tests. Statistical analyses were carried out using the Statistical Package for Social Sciences (SPSS) 20. P values of less than 0.05 were considered significant.

## *4.3 Results*

### *4.3.1 The effects of GS39783 on conditioned fear in BALB/c mice*

The effects of GS39783 administration on conditioned fear behaviour in BALB/c mice are shown in Figure 1. All groups were observed to acquire, retain and extinguish conditioned fear as measured by freezing responses to both the conditioning chamber context and the light/sound cue. When drugs were administered prior to acquisition training, two-way ANOVA revealed no effect of drug treatment on freezing behaviour on the acquisition ($F_{2,25}$=0.07, p=0.93), recall ($F_{2,25}$=0.84, p=0.44) or extinction of conditioned fear ($F_{2,25}$=0.55, p=0.58).

When GS39783 was administered prior the recall phase, no difference in the acquisition of conditioned fear was observed between treatment groups ($F_{2,27}$=0.001, p=0.99). Two-way ANOVA revealed no effect of drug treatment on the recall ($F_{2,27}$=0.99, p=0.39) or extinction ($F_{2,27}$=0.25, p=0.78) of conditioned fear.

When GS39783 was administered prior to the extinction phase no overall effect of group was observed on fear acquisition ($F_{2,27}$=0.22 p=0.81) or fear recall ($F_{2,27}$=2.09, p=0.14). Likewise, drug treatment had no significant effect on the extinction of conditioned fear ($F_{2,27}$=1.9, p=0.17).

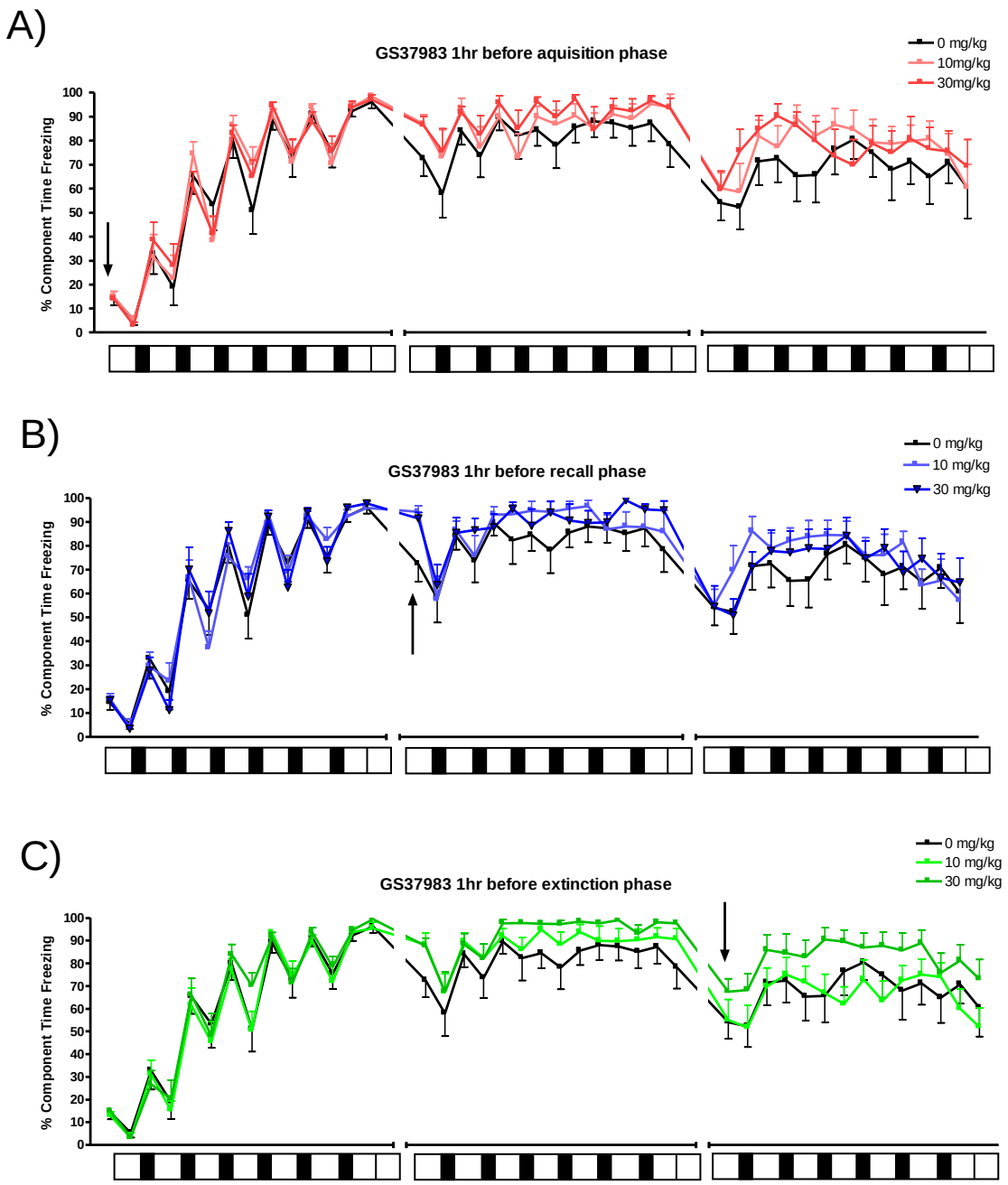


*Figure 2 – Effects of GS39783 on freezing behaviour in a paradigm of conditioned fear. Black arrows indicate the point of drug dosing (30 minutes prior to testing. White boxes on the x-axis represent context components and black boxes signify cue. Drug was administered at three different timepoints A) before the acquisition phase, B) before the recall phase and C) before the extinction phase*

### *4.3.2 The effects of CGP52432 on fear conditioning behaviour in BALB/c mice*

The effects of CGP52432 administration on conditioned fear behaviour in BALB/c mice are shown in Figure 2. All groups were observed to acquire, retain and extinguish conditioned fear as measured by freezing responses to both the conditioning chamber context and the light/sound cue. When drugs were administered prior to acquisition training, two-way ANOVA revealed no effect of drug treatment on freezing behaviour on the acquisition ($F_{2,54}$=1.13, p= 0.33), recall ($F_{2,54}$=0.21, p=0.8) or extinction of conditioned fear ($F_{2,56}$=0.86, p=0.43).

When CGP52432 was administered prior the recall phase, no difference in the acquisition of conditioned fear was observed between treatment groups ($F_{2,54}$=0.61, p=0.68). Two-way ANOVA revealed no effect of drug treatment on the recall ($F_{2,54}$=1.5, p=0.89) or extinction ($F_{2,54}$=1.2, p=0.33) of conditioned fear.

When CGP52432 was administered prior to the extinction phase no effect of group was observed on fear acquisition ($F_{2,54}$=1.4, p=0.26) or fear recall ($F_{2,54}$=0.07, p=0.93). Drug treatment had no significant effect on the extinction of conditioned fear ($F_{2,54}$=1.08, p=0.35).

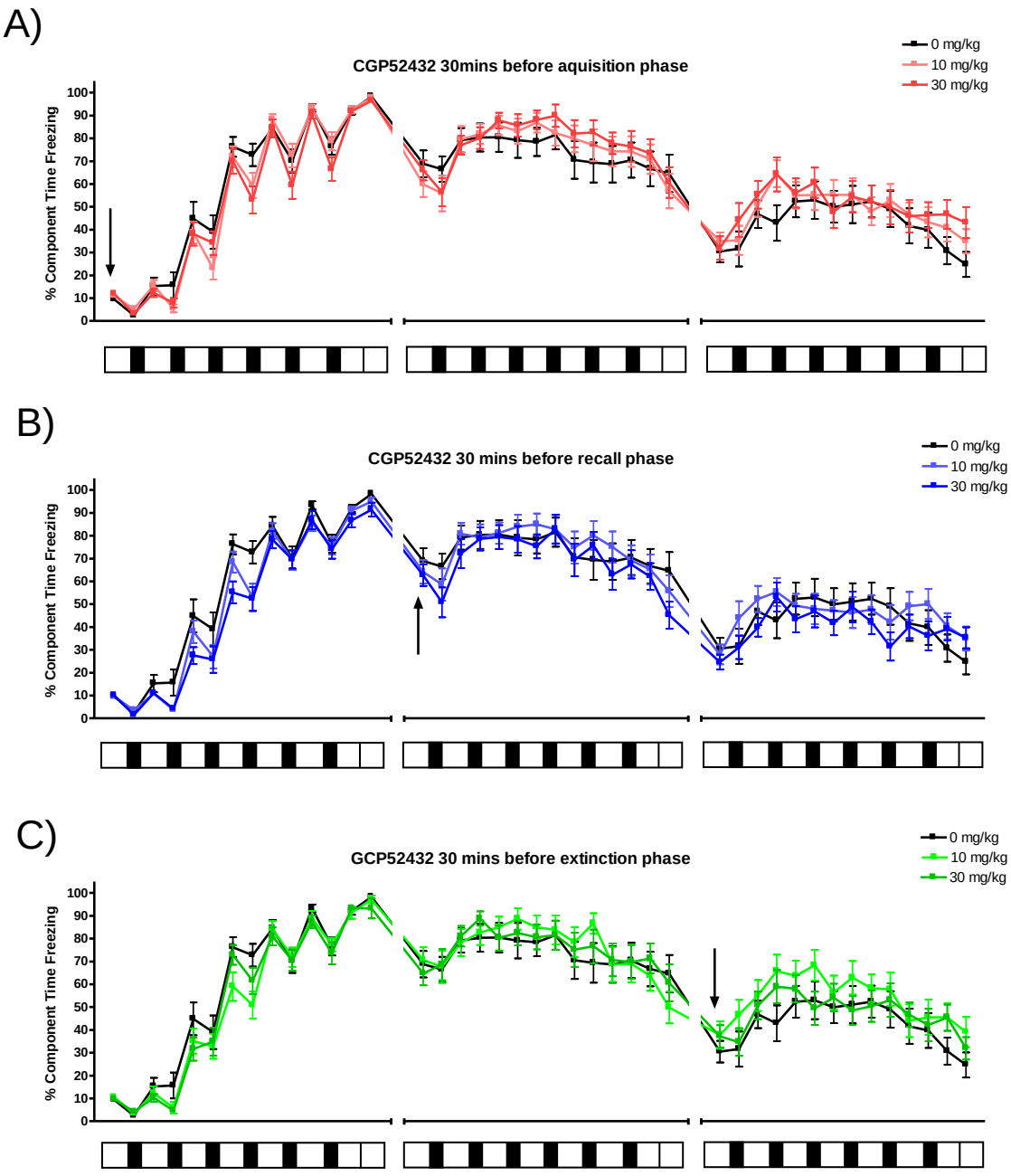


*Figure 3 – Effects of CGP52432 on freezing behaviour in a paradigm of conditioned fear. Black arrows indicate the point of drug dosing (30 minutes prior to testing. White boxes on the x-axis represent context components and black boxes signify cue. Drug was administered at three difference time points, A) before the acquisition phase, B) before the recall phase and C) before the extinction phase*

## *4.4 Discussion*

The current results demonstrate that $GABA_B$ receptor positive modulators and antagonists have no effect on the acquisition, recall or extinction of conditioned fear. This is somewhat surprising given that current data suggests the $GABA_B$ receptor is a substrate in the molecular pathways that underlie conditioned fear. Genetic ablation of the $GABA_{B(1A)}$ subunit results in generalisation of LTP in the amygdala and results in generalisation of conditioned fear in mice (Shaban et al., 2006). $GABA_B$ receptors furthermore inhibit excitatory input to the primary neurons of the amygdala suggesting a crucial role in the regulation of normal fear-learning behaviour (Pan et al., 2009). The absence of effects on auditory fear conditioning associated with GS39783 and CGP52432 administration suggests that the role the $GABA_B$ receptor plays in conditioned fear is not absolutely crucial.

Similar studies to ours have recently examined the effects of other $GABA_B$ receptor ligands in tests of contextual and cued conditioned fear. In these studies neither the PAM BHF117 nor the antagonist phaclofen were shown to alter the processes of acquiring, retaining or extinguishing conditioned fear (Heaney et al., 2012; Li et al., 2013). In contrast, conflicting reports of the effects of the agonist baclofen, on fear conditioning have been reported. Specifically, one study reported that baclofen impaired the extinction of trace cue and context fear conditioning (Heaney et al., 2012) while another reported no effect on fear behaviour (Li et al., 2013). The experimental design of the aforementioned studies differed from the present study in that difference species, strains and fear conditioning protocols were used specifically, Li and colleagues (Li et al., 2013) used

C57/BL6 mice while Heaney and colleagues (Heaney et al., 2012) used Sprague-Dawley rats. Another major methodological difference was that those studies the used of a fear-conditioning protocol which examines contextual and cue-induced fear conditioning in different sessions, i.e. cue exposure in a novel environment, context exposure in the absence of cue, as opposed to our procedure that tests both contextual and cued fear recall/extinction in the same session (Brinks et al., 2009). The fact that the results from our experiment using a PAM and and antagonist are in agreement with the aforementioned studies despite methodological differences, strongly suggest that neither $GABA_B$ receptor positive modulators nor antagonists affect conditioned anxiety.

Auditory fear conditioning is a cognitive process underpinned by neuronal circuitry overlapping to other aspects of learning and memory (Fendt & Fanselow, 1999). Thus, the lack of effect of PAMs and antagonists on learned fear is somewhat surprising given the role of the $GABA_B$ receptor in cognitive function. For example, $GABA_B$ receptor antagonists have been shown to enhance cognitive function across several species in a broad range of functional tests (Mondadori et al., 1993) and enhance hippocampus dependant memory function in particular (Helm et al., 2005). Interestingly, the $GABA_B$ receptor agonist baclofen has recently been shown to ameliorate cognitive deficits seen in methamphetamine-treated mice (Arai et al., 2009). In contrast, positive allosteric modulators of the $GABA_B$ receptor appear to be devoid of effects on cognitive function (Cryan et al., 2004), while evidence from genetic models suggest that $GABA_{B1}$ receptor subunit isoforms play different yet important roles in regulating cognition (Jacobson et al., 2007b). The fact that $GABA_B$ receptor ligands can have such an influence over cognitive

function, but not over auditory fear conditioning, suggests that $GABA_B$ receptors do play a role in cognition but via neuronal populations other than those that underpin auditory fear conditioning.

Despite the status of positive modulators of the $GABA_B$ receptor as promising anxiolytic drugs, GS39783 failed to alter learned fear behaviour in the present study. GS39783 has previously been demonstrated to generate anxiolytic effects in mice in many tests of innate anxiety including the elevated plus maze, elevated zero maze, stress induced hypothermia paradigm and the light dark box (Cryan & Kaupmann, 2005), as have other positive modulators (Cryan & Kaupmann, 2005; Frankowska et al., 2007) although it has been shown to lack anxiolytic activity in the modified Geller-Seifter test (Paterson & Hanania, 2010). Taken with our current data, a pre-clinical profile of the $GABA_B$ receptor positive allosteric modulators as having anxiolytic effects in exploratory and autonomic measures of anxiety behaviour, but lacking these effects in learning based models of anxiety emerges. This suggests a potential clinical limitation for these compounds when attempting to modify certain symptoms of anxiety disorders especially those of a cognitive nature.

The current literature has provided limited behavioural evidence for $GABA_B$ receptor antagonists as anxiolytic agents. Although some studies have demonstrated anxiolytic effects for the $GABA_B$ receptor antagonists in the elevated plus maze, elevated zero maze, conflict drinking test and the four plate test (Partyka et al., 2007; Frankowska et al., 2007) others have shown $GABA_B$ receptor antagonists to have no effects on anxiety behaviour in

these tests as well as the Geller-Seifter test (Partyka et al., 2007; Mombereau et al., 2004a; Paterson & Hanania, 2010). Our data appears to further suggest that the anxiolytic effects of $GABA_B$ receptor antagonist are limited. The antidepressant like effects of the $GABA_B$ receptor antagonists are well-characterised (Cryan & Kaupmann, 2005), however given the frequent comorbidity of anxiety and depression, a failure to generate anxiolytic effects in these tests makes the $GABA_B$ receptor a less promising therapeutic avenue. Taken together this data does not support a specific role of $GABA_B$ receptor allosteric modulators and antagonists in the modulation of conditioned fear which is a cognitive facet of some anxiety disorders.

# *Chapter 5*

# *Ablation of the Ser892 Phosphorylation Site of the $GABA_{B(2)}$ Receptor subunit does not Modify Anxiety-related Behaviour*

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## *Abstract*

Although the $GABA_B$ receptor has been suggested as a potential drug target in the treatment of both anxiety disorders and depression, development of novel $GABA_B$ receptor-active therapeutic drugs is hampered by our poor understanding of the molecular basis of the heterogeneity of this receptor *in vivo*. In vitro studies have suggested that a key process underlying the functional modulation of the receptor is phosphorylation of the Ser892 residue of the $GABA_{B(2)}$ receptor subunit. In an effort to assess the role played by this phosphorylation process *in vivo* we have developed a mouse model wherein a point mutation in the $GABA_{B(2)}$ subunit has converted the Ser892 residue to an alanine residue, thus ablating phosphorylation. This mouse was assessed in terms of its sensitivity to both acute and chronic baclofen treatment as well as its behaviour in tests of anxiety and antidepressant like activity. Our results revealed that the $GABA_{B(2)}$-S892A mouse displays a sensitivity to acute and chronic baclofen treatment which is indistinguishable from wildtype controls. We also demonstrated that the $GABA_{B(2)}$-S892A mouse behaves identically to wildtype controls in a battery of commonly used behavioural tests of anxiety and anti-depressant like behaviour. The overall lack of alteration in behaviour displayed by this mouse suggests that Ser892 phosphorylation plays a limited role in regulating $GABA_B$ receptor function in vivo. A variety of factors including the presence of a plethora of other $GABA_B$ receptor regulatory mechanisms as well as the unique $GABA_B$ receptor sensitivity profile of the BALB/c background strain may underlie these results.

## *5.1 Introduction*

The $GABA_B$ receptor is currently postulated as a potential therapeutic target in a variety of psychiatric disorders including depression, anxiety and drug addiction (Cryan & Kaupmann, 2005; Tyacke et al., 2010). However, a major barrier to our understanding the function of the $GABA_B$ receptor *in vivo* is heterogeneity of agonist potency observed between different neuronal populations (Pinard et al., 2010). Among other processes, $GABA_B$ receptor functionality is critically controlled by the phosphorylation of the receptor heterodimer complex by protein kinases *in vivo*, with both the $GABA_{B(1)}$ and $GABA_{B(2)}$ receptor subunits possessing multiple phosphorylation sites (Terunuma et al., 2010a).

cAMP-dependent protein kinase (PKA) was first suggested to have a role in regulating $GABA_B$ receptor function when it was demonstrated that administration of dibutyric cAMP could suppress $GABA_B$ receptor function in active receptor expressing *Xenopus* oocytes (Yoshimura et al., 1995). This role for PKA in the modulation of the $GABA_B$ receptor was confirmed with the discovery of the sole site for PKA mediated phosphorylation of the $GABA_B$ receptor at the serine 892 (S892) residue of the $GABA_{B(2)}$ receptor subunit (Couve et al., 2002). Using phospho-$GABA_B$ specific antibodies it has been shown that the S892 residue undergoes significant basal PKA dependent phosphorylation in vivo. PKA dependent phosphorylation of the S892 residue plays a crucial role in inhibiting agonist induced receptor desensitisation in both HEK cells and cultured hippocampal neurons (Couve et al., 2002).

Although chronic exposure to the $GABA_B$ receptor agonist baclofen promotes $GABA_B$ receptor degradation, this effect can be attenuated by concurrent activation of PKA or treatment with a β-adrenergic receptor agonist. Furthermore, the rate of $GABA_B$ receptor degradation is directly correlated to S892 residue phosphorylation (Fairfax et al., 2004). This data strongly suggests that S892 phosphorylation plays a crucial role in maintaining the cell surface stability of the $GABA_B$ receptor and promoting receptor function. It must also be noted that data obtained from *Xenopus* oocytes conversely suggests that PKA mediated phosphorylation can effectively desensitise the receptor (Yoshimura et al., 1995). The precise role played by S892 phosphorylation in the regulation of $GABA_B$ receptor function thus remains elusive.

As an investigational tool designed to further characterise the effect of PKA dependent phosphorylation on $GABA_B$ receptor function, a mouse model has been developed wherein the serine 892 residue in the $GABA_{B(2)}$ subunit is substituted for an alanine residue which is not subject to PKA- mediated phosphorylation; the $GABA_{B(2)}$-S892A mouse. This mutation appears to results in cell and region specific enhancements of ligand potency and membrane stability suggestive of receptors being in a tonically active state (Bettler et al. unpublished data). The development of this model offers the opportunity to determine the role of PKA mediated phosphorylation of the $GABA_B$ receptor *in vivo*, in particular on the sensitivity of the animal to the effects of both acute and chronic $GABA_B$ receptor agonist treatment. In addition, it is of interest to note that the *in vitro* effects of serine 892 to alanine substitution on both ligand potency and receptor membrane stability appear to be analogous to the molecular effects of positive modulators of the $GABA_B$ receptor (Urwyler et al., 2001a).

Thus, given the pronounced anxiolytic activity of $GABA_B$ receptor modulators in a host of behavioural measures of unconditioned anxiety in rodents (Cryan & Kaupmann, 2005), it is possible that the $GABA_{B(2)}$-S892A mutation may modulate anxiety behaviour in animal models. To this end, we compared the response of $GABA_{B(2)}$-S892A mice with their wildtype littermate controls in the physiological responses to $GABA_B$ receptor agonist baclofen. Moreover, the behaviour of the animals in tests of anxiety and antidepressant action was also assessed.

## *5.2 Methods*

### *5.2.1 Generation and analysis of $GABA_{B(2)}$-S892A knock-in mice*

The gene targeting construct contained a neomycin resistance cassette (pRay-2; Genbank accession number U63120) flanked by two arms of BALB/c $GABA_{B(2)}$ genomic DNA. The S892A mutation in exon 19 was introduced into the 3' arm along with a diagnostic silent NheI restriction site (Fig. 1A). The targeting construct was electroporated into Balb/c embryonic stem cells. Neomycin resistant colonies were screened for homologous recombination by PCR, and positive clones confirmed by Southern blot (Fig. 1b). Positive ES cells were microinjected into C57BL/6 blastocysts. Chimeric males were crossed with female BALB/c mice resulting in a F1 of inbred BALB/c mice heterozygous for the S892A+neo allele. The neomycin cassette in the S892A+neo allele was excised by mating heterozygous F1 mice with BALB/c Cre-deleter mice (Vigot et al., 2006). Deletion of the neomycin cassette in the S892A allele was confirmed by Southern blot analysis. $GABA_{B(2)}$ immunoblot analysis was performed as described (Gassmann et al., 2004). All animal experiments were subjected to institutional review and approved by the Veterinary Office of Basel-Stadt.

$GABA_{B(2)}$-S892A mice were bred from heterozygous breeding pairs. Genotyping was performed on offspring on tail clip samples taken from animals as described in Appendix 1. Male mice homozygous for the $GABA_{B(2)}$-S892A mutation were used for all experiments, with age matched homozygous wildtype littermates used for controls.

All experiments were conducted in accordance with the European Directive 86/609/EEC, the Recommendation 2007/526/65/EC and approved by the Animal Experimentation Ethics Committee of University College Cork and in accordance with methods approved by the Veterinary Authority of the City of Basel. All efforts were made to minimize animal suffering and to reduce the number of animals used.

### *5.2.2 Sensitivity of the $GABA_{B(2)}$-S892A mouse to the physiological effects of baclofen*

In order to determine the effects of the $GABA_{B(2)}$-S892A mutation on $GABA_B$ receptor activity *in vivo* a cohort of $GABA_{B(2)}$-S892A mice were assessed for their sensitivity to the ataxic and hypothermic effects of the $GABA_B$ receptor agonist baclofen.

*Baclofen treatment and measurement of physiological effects*

Wildtype and $GABA_{B(2)}$-S892A mice were dosed once daily with baclofen [(10mg/kg, IP enantiomeric baclofen (Tocris)] for 14 days. The sensitivity of the mice to the hypothermic and ataxic effects of the baclofen dose was assessed on days 1, 7, and 14. The baclofen doses and experimental procedures were selected based on work previously conducted in our lab (Jacobson & Cryan, 2005)

*Apparatus*

Temperature was measured using a thermistor probe of 2mm in diameter (Ellab, Denmark – Model DM852). The thermistor probe was lubricated with petroleum jelly and inserted roughly 20mm into the rectum until a stable temperature was achieved (10-15s).

Measurement of the ataxic effects of baclofen was assessed via rotarod endurance. The rotarod (Ugo Basile, Italy - Model 47600) was set at 12RPM and each trial lasted for a maximum of 300 seconds. Endurance was determined by the time taken before mice fell off the rotarod. Mice clinging to the rotarod for more than 80% of the 300 seconds test time were given a score of 0. Mice clinging to the rotarod for less than 80% of the 300 seconds only had their time spent actively walking on the rotarod measured.

*Procedure*

One day prior to the first baclofen dose, mice were injected with saline and rectal measurements of temperature were taken in order to ensure that mice were acclimatised to both injection and temperature measurement stress. Animals were then trained to walk on the rotarod apparatus (Ugo Basile, Italy - Model 47600) for 300s over a series of 8 trials each 30 minutes apart. The number of falls during training was recorded for each mouse.

Prior to each testing day, mice were singly housed overnight to limit levels of variability in rectal temperature between animals. The morning of testing, animals were moved to the experimental room 2 hours prior to the baclofen dose, with the first measurement of rotarod endurance and temperature measurement being taken 1 hour later (T-60) in order to re-establish endurance. A second measurement was taken immediately prior to the baclofen injection (T0), with subsequent measurements 30, 60, 120, 180 and 240 minutes after the baclofen dose. Animals remained singly housed in their holding cages between each temperature/endurance measurement and were returned to their home cages subsequent to the final measurement.

*Statistical analyses*

Wildtype and $GABA_{B(2)}$-S892A endurance on the rotarod and rectal temperature subsequent to baclofen treatment was compared for each day by repeated measures two-way ANOVA. Each timepoint was then assessed by Bonferonni's post-hoc test. All statistical analyses were conducted in statistical package for social sciences (SPSS, ver 20.1).

### *5.2.3 Anxiety behaviour of the $GABA_{B(2)}$-S892A mouse*

A separate cohort of mice (n=11) were tested in a battery of tests of anxiety behaviour consisting of the light dark box, defensive marble burying, stress-induced hyperthermia, a conditioned fear acquisition/extinction paradigm and a forced swim test. Three recovery days were given between each of the first four tests with a seven day break between the conditioned fear paradigm and the forced swim test. A schematic depicting the timeline of behavioural experiments is shown in Figure 2.

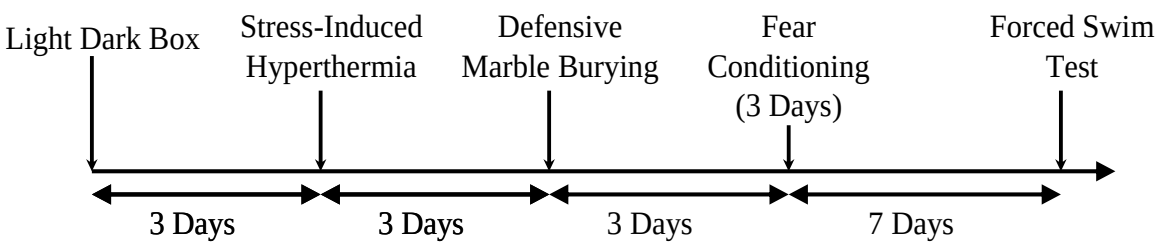


Figure 1 –Timeline illustrating the sequence of behavioural testing

*Light-Dark box*

The light dark box is a behavioural test used to assess levels of unconditioned anxiety in rodents based on levels of passive avoidance behaviour and was conducted a previously described (Finger et al., 2011). The apparatus consisted of a Plexiglas enclosure (44×21×21 cm) comprised of two adjoining compartments (one light and one dark). The light compartment had transparent Plexiglas walls and had an open top. Bright lighting (approximately 1,000 lx) was used to generate aversive conditions in this compartment. The dark compartment (14 cm width) consisted of a black Plexiglas wall and roof. The compartments were connected by a small opening (12×5 cm) at floor level. Animals were placed in the light compartment facing away from the dark compartment and were allowed to freely explore the apparatus for 10 minutes. During this period the behaviour of the animals was recorded. The latency of the animal to enter the dark compartment of the apparatus, the number of transitions between the two compartments and the total time spent in the light compartment were measured. An animal was adjudged to have entered a compartment when all four paws had crossed the threshold. The number of faecal pellets produced by each animal during the test period was also recorded as a measure of the physiological response to a stressful stimulus.

*Defensive Marble Burying*

The test was adapted from a protocol previously described (Jacobson et al., 2007a). Briefly, mice were placed in the centre of a cage (38x25x18cm) containing 4cm of bedding with 20 blue glass marbles (1.5cm diameter) on top, arranged in 4 rows of 5 marbles (2cm apart).

The lid of the cage was used to prevent mice from escaping the test chamber. Mice were allowed to explore the chamber for 30 minutes and then returned to their home cage. Following removal of the mouse, the number of marbles that were covered in bedding by at least two thirds was counted.

*Stress-Induced Hyperthermia (SIH)*

Testing was conducted in a similar manner to that previously described (Jacobson & Cryan, 2008). Briefly, mice were singly housed 24 hours prior to testing. The rectal temperatures were then taken using a thermistor probe ca. 2mm in diameter inserted 20mm into the rectum (T1) for 15 seconds. The temperature was measured again 15 minutes later (T2). The increase in temperature due to the stress of the initial temperature measurement (T2 value minus T1 value) was taken as a measure of the physiological response to an acute stressor. Mice were returned to their home cage immediately after testing.

*Conditioned fear/extinction paradigm*

Conditioned fear testing was conducted according to a protocol previously described by our lab (Bravo et al., 2011; Sweeney et al., 2013). On day 1, mice were placed in conditioning chambers (21.6 cm x 17.8 cm x 12.7 cm), with transparent walls in the front and back, stainless-steel bars, and a metal-grid floor connected to a shock scrambled and generator in sound-attenuating box. After180 s acclimatisation mice received 6 pairings (60s interpairing interval) of a conditioned stimulus (CS; 20 s, 10 dB, 10kHz tone) and a unconditioned stimulus (US; 2 s, scrambled footshock; 60 mA). The US was presented during the last 2 s of the CS. After a 60 s no-stimulus consolidation period after the final CS–US pairing, mice

were returned to the home cage. Chambers were cleaned with 70% ethanol between each mouse. On days 2 and 3 mice were returned to the same chamber as day one and the procedure repeated, with the absence of electrical footshocks. Activity of mice was monitored by Video Freeze (Med Associates, USA). Animals were filmed at rate of 15 frames per second, with a freezing behaviour represented by movement of less than 255 pixels (to accommodate breathing) over 7 frames. This paradigm allows for the measurement of the acquisition and extinction of conditioned fear. The design of the procedure additionally allows us to measure both context and cue induced freezing which are measures of hippocampus and amygdala dependent fear behaviours respectively.

*Forced Swim Test*

The forced swim test is a behavioural test commonly used to assess levels of antidepressant-like behavioural activity in mice (Cryan & Mombereau, 2004). Mice are placed in glass cylindrical tank containing 17cm of water at 23-25°C for 6 minutes and their behaviour is recorded. The length of time the mouse spends immobile, i.e. not actively swimming, during the last 4 minutes of the test is scored manually by a trained observer blinded to the treatment group.

*Statistical analyses*

Data from the light-dark box, defensive marble burying, and Stress-Induced Hyperthermia paradigm were analysed by student's t-test. Data from the conditioned fear experiment was analysed by two-way ANOVA followed by Bonferonni's post-hoc test where appropriate.

Significance was accepted at $p>0.05$. Data were analysed using the statistical package for social sciences (SPSS v.20.1).

## *5.3 Results*

### *5.3.1 Generation and analysis of S892A knock-in mice*

S892A knock-in mice carrying a S892 to alanine mutation in the $GABA_{B(2)}$ gene were generated using standard gene targeting techniques (Fig. 1a,b). Homozygous S892A mice were kept on a pure inbred Balb/c genetic background and exhibited no overt phenotypic abnormalities. Immunoblot analysis revealed similar levels of $GABA_{B1a}$, $GABA_{B1b}$ and $GABA_{B2}$ protein in S892A and wildtype (WT) brain extracts (Fig. 1c). Antibodies specific for pS892 detected basal phosphorylation in WT but not in S892A brain extracts (Fig. 1c), confirming the absence of S892 phosphorylation in the knock-in mice.

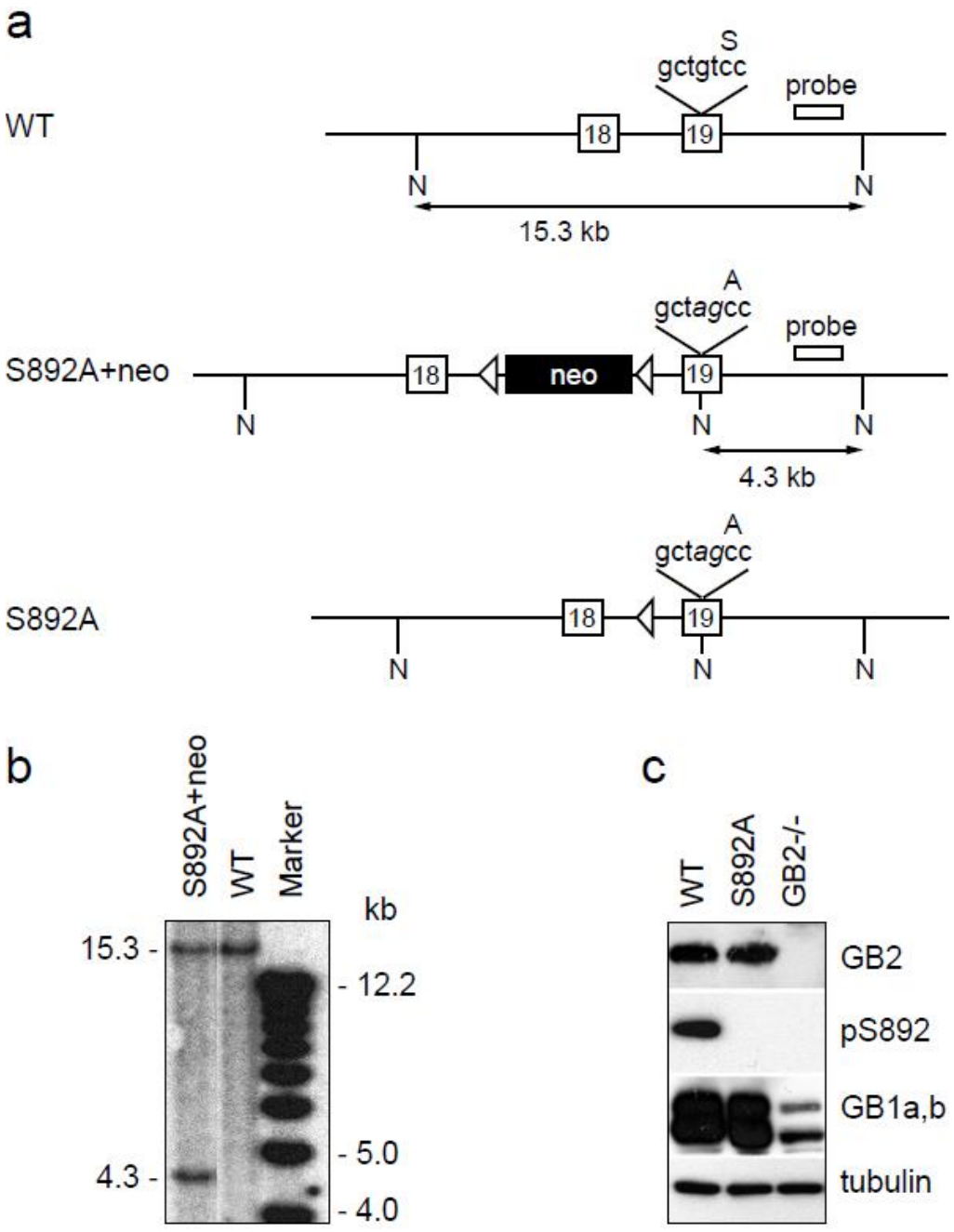


*Figure 1. Generation of S892A knock-in mice. (a) WT and mutated $GABA_{B(2)}$ alleles. The S892 to alanine mutation (tcc→gcc) and a silent diagnostic NheI restriction site (gctagc) were introduced into exon 19 using homologous recombination in Balb/c embryonic stem (ES) cells[37]. Mutated nucleotides are shown in italic. A neomycin marker (neo) flanked by loxP sites (arrowheads) was used for selection of ES cells. Correctly targeted ES cells (S892A+neo allele) were injected into C57BL/6 blastocysts. A founder mouse was crossed with a Balb/c mouse expressing Cre-recombinase[35] to excise the neomycin cassette, leaving one loxP site behind (S892A allele). The hybridization probe used in the Southern blot in (b) is indicated. N, NheI restriction sites. (b) Southern blot of NheI cut genomic*

*DNA from correctly targeted ES cells. The probe labels a 15.3 kb fragment for the WT allele and a 4.3 kb fragment for the S892A+neo allele. (c) Immunoblot analysis of brain extracts showing that S892A mice express normal levels of $GABA_{B(2)}$ (GB2), $GABA_{B(1)a}$ (GB1a) and $GABA_{B(1)b}$ (GB1b) proteins. S892 was phosphorylated in brain extracts of WT but not S892A mice, as shown with an antibody specific for phosphorylated S892 (pS892)[9]. Brain extracts of $GABA_{B2}$-deficient mice (GB2-/-)[36] confirm the specificity of the GB2 and pS892 antibodies. An antibody specific for tubulin controls for equal loading of samples.*

### *5.3.2 Baclofen sensitivity*

*Hypothermic response to baclofen*

The hypothermic effects of 10mg/kg baclofen are shown in Figure 2 (Panels A-D). Both $GABA_{B(2)}$-S892A and WT mice displayed a pronounced and transient drop in body temperature in response to treatment with baclofen; these effects were greatly diminished on days 7 and 14 in both groups. One-way repeated measures ANOVA following different durations of treatment revealed no significant effect of genotype on treatment day 1 ($F_{1,15}$=0.97, p=0.34), day 7 ($F_{1,15}$= 0.28, p= 0.61) or day 14 ($F_{1,15}$=0.002, p= 0.96). Comparing the cumulative changes in temperature from baseline across the 240 minute trial period for each test session revealed a significant effect of test day ($F_{2,15}$=22.61, p>0.001). Bonferroni's posthoc test revealed significant differences between the cumulative changes in temperature in wildtype animals from day 1 to day 7 ((p<0.001) and from day 1 to day 14 (p<0.01). Bonferroni's posthoc test also revealed significant differences between the cumulative changes in temperature in wildtype animals from day 1 to day 7 (p<0.001) and from day 1 to day 14(p<0.001).

*Ataxic response to baclofen*

The ataxic effects of 10mg/kg baclofen are shown in Figure 2 (Panel E-H). Both $GABA_{B(2)}$-S892A and WT mice displayed a pronounced and transient reduction rotatod endurance in response to treatment with baclofen, these effects were greatly diminished on days 7 and 14 in both groups. Two-way ANOVA revealed no significant effect of genotype on treatment day 1 ($F_{1,9}$=0.17, p=0.69), day 7 ($F_{1,9}$=0.95, p=0.35) or day 14 ($F_{1,9}$=0.005, p=0.94).

Comparing the cumulative changes in temperature from baseline across the 240 minute trial period for each test session revealed a significant effect of test day ($F_{2,9}$=22.9, p>0.001). Bonferroni's posthoc test revealed significant differences between the cumulative changes in temperature in wildtype animals from day 1 to day 7 (p<0.001) and from day 1 to day 14 (p<0.01). Bonferroni's posthoc test also revealed significant differences between the cumulative changes in endurance in $GABA_{B(2)}$-S892A mice from dose 1 to dose 7 (p<0.001) and from dose 1 to day 14 (p<0.01).

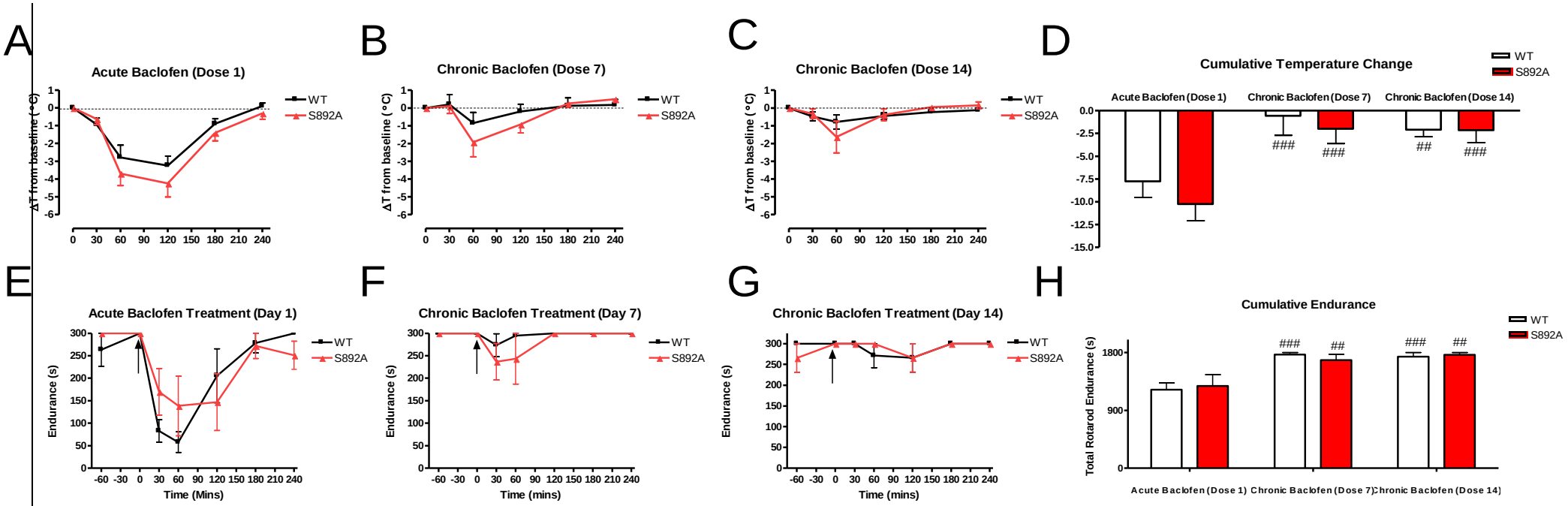


*Figure 1- Desensitisation to the physiological effects of baclofen in wildtype and GABA$_{B(2)}$-S892A mice. A) The hypothermic response of WT and GABA$_{B(2)}$-S892A on day 1 of baclofen treatment, B) the hypothermic response of WT and GABA$_{B(2)}$-S892A on day 7 of baclofen treatment, C) the hypothermic response of WT and GABA$_{B(2)}$-S892A on day 14 of baclofen treatmen (baclofen doses given at T0) and D) the cumulative change in temperature from baseline across all five temperature measurements. Data is expressed as change in temperature from T0. B) E) The rotarod endurance of WT and GABA$_{B(2)}$-S892A on day 1 of baclofen treatment, F) the rotarod endurance of WT and GABA$_{B(2)}$-S892A on day 7 of baclofen treatment, G) the rotarod endurance of WT and GABA$_{B(2)}$-S892A on day 14 of baclofen treatment (arrows indicate timing of Baclofen dose (10mg/kg IP) and H) the cumulative endurance across all measurements on each test day. ## represents p<0.01, ### represents p<0.001 from acute baclofen response.*

### *5.3.3 Anxiety behaviour*

*Light dark box*

Figure 3 shows the behaviour of wildtype and $GABA_{B(2)}$-S892A mice in the light dark box. Students T test revealed no significant effect of the $GABA_{B(2)}$-S892A mutation on latency to enter the dark side of the apparatus (p=0.869, Panel A), total time spent in the light side of the box (p=0.753, Panel B), number of transitions between the two chambers (p= 0.313, Panel C) or number of faecal boli produced during the test session (p=0.741, Panel D).

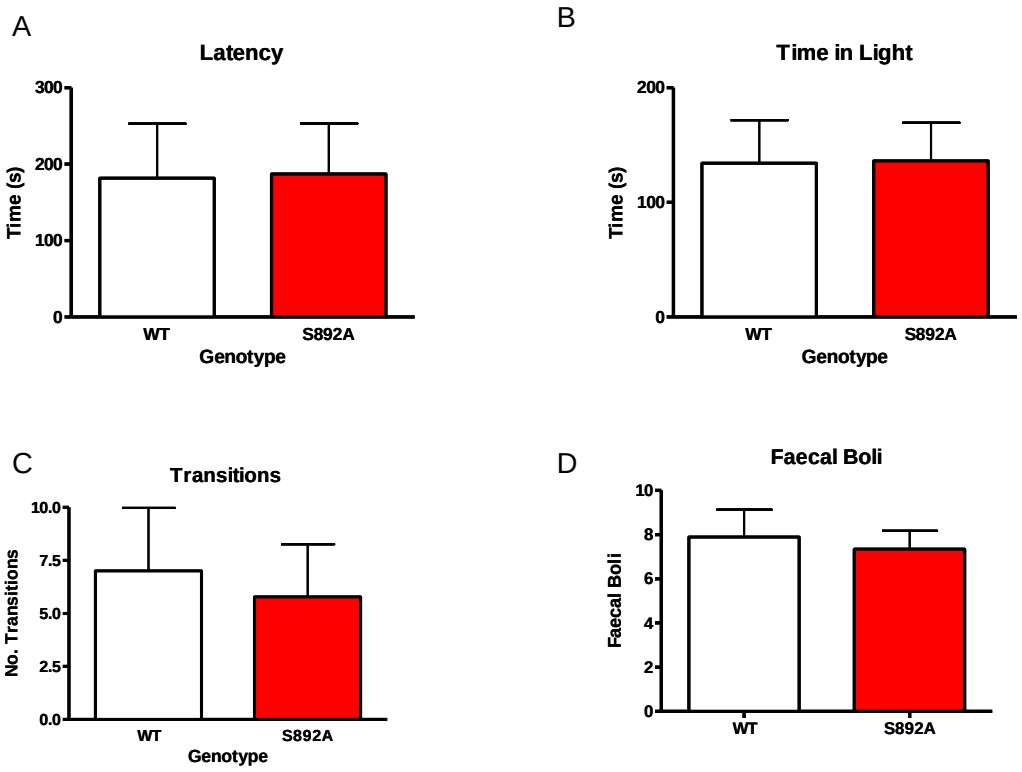


*Figure 3- A)Latency to enter the dark compartment, B) time in light compartment, C) transitions between the light and dark compartments and D) number of faecal boli produced in the light-dark box by wildtype and $GABA_B2$-S892A mice. Data are expressed as mean±SEM.*

*Defensive marble burying*

Figure 4 shows the behaviour of wildtype and $GABA_{B(2)}$-S892A mice in the defensive marble burying test. No difference in the number of marbles buried in the defensive marble burying test were seen between wildtype and $GABA_{B(2)}$-S892A mice (p=0.328).

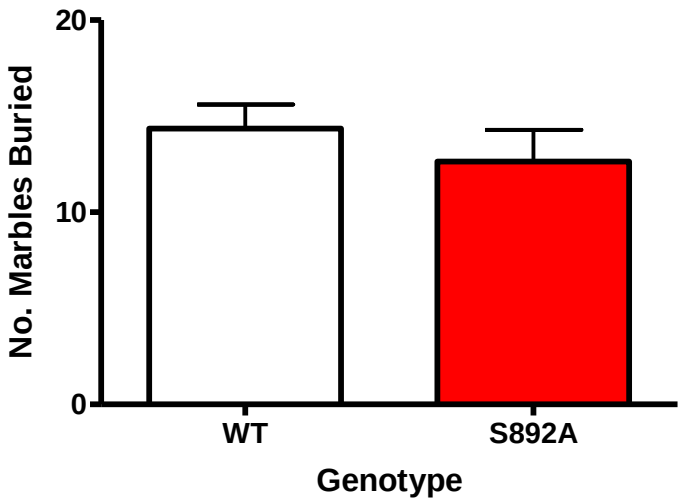


*Figure 4 -Number of marbles buried by wildtype and $GABA_B2$-S892A mice in the defensive marble burying test. Data are expressed as mean±SEM.*

*SIH*

Figure 5 shows the behaviour of wildtype and $GABA_{B(2)}$-S892A mice in the SIH paradigm. No difference in either basal temperature (p=0.893) or in the increase in temperature in response to stress (p=0.577) was observed between wildtype and $GABA_{B(2)}$-S892A mice.

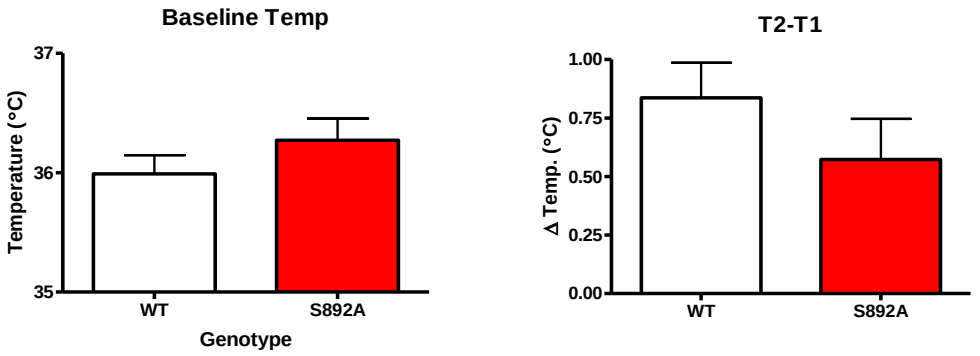


*Figure 5 - The baseline temperatures and stress induced increases in temperature (T2-T1) of wildtype and GABA$_{B(2)}$-S892A mice observed in the Stress-Induced Hyperthermia paradigm. Data are expressed as mean±SEM.*

*Conditioned fear paradigm*

Figure 6 shows the behaviour of wildtype and GABA$_{B(2)}$-S892A mice in the fear conditioning paradigm. Both GABA$_{B(2)}$-S892A and wildtype animals acquired, expressed and extinguished conditioned fear as measured by freezing responses to both the conditioning chamber context and the light/sound cue. When data was analysed in terms of % freezing during each component of the three testing sessions (cue and context), no significant effect of genotype, likewise was observed ($F_{1,18}$=0.01, p=0.92) (Fig 5).

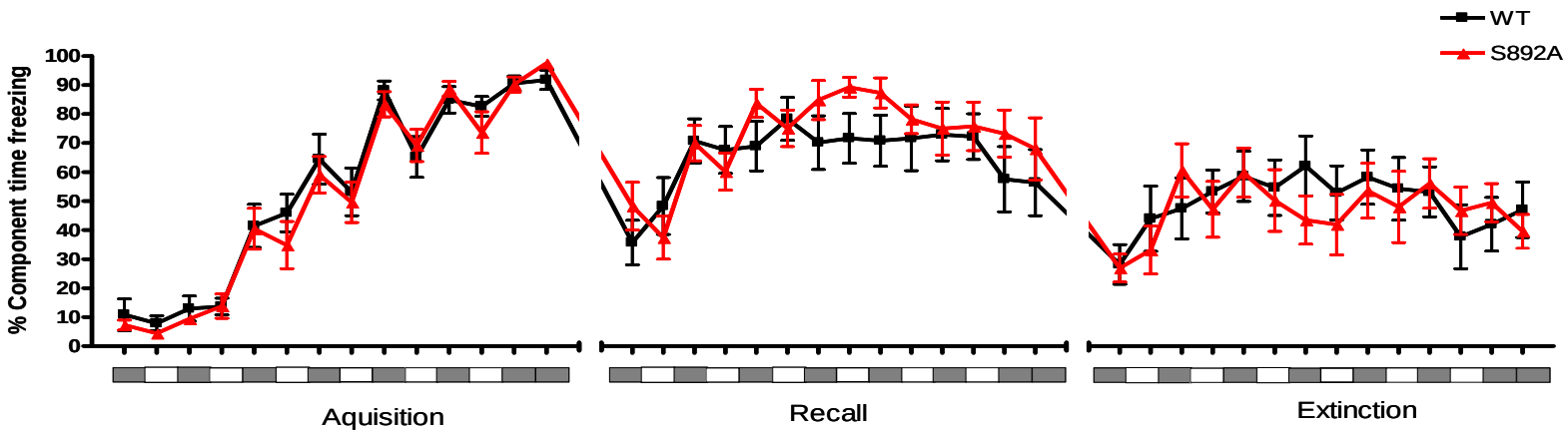


*Figure 6 - Freezing behaviour of wildtype and GABA$_{B(2)}$-S892A mice observed in the conditioned fear paradigm.White boxes on the x-axis represent **context** components and black boxes signify **cue** components. Data are expressed as % time spent immobile (freezing) during each stage of the procedure. Data are expressed as mean±SEM.*

*Forced swim test*

Figure 7 shows the behaviour of wildtype and $GABA_{B(2)}$-S892A mice in the forced swim test. No difference in immobility in the forced swim test was observed between wildtype and $GABA_{B(2)}$-S892A mice in the forced swim test (p=0.885)

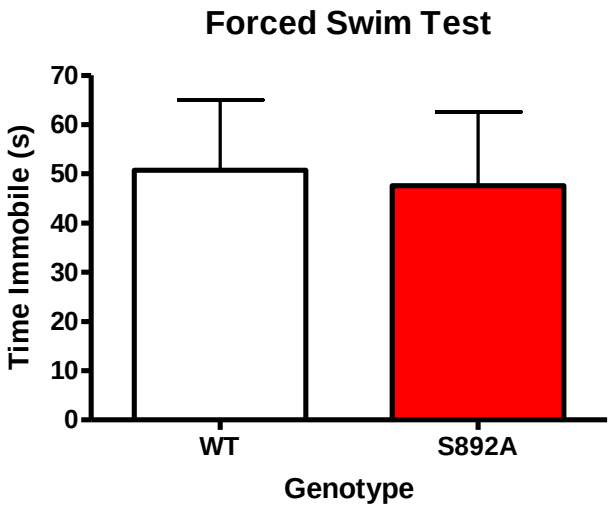


*Figure 7 - Time spent immobile in the final 4 minutes of the forced swim test by WT and $GABA_B2$-S892A mice. Data are expressed as mean±SEM.*

## *5.4 Discussion*

Treatment with baclofen produces pronounced ataxic effects in both $GABA_{B(2)}$-S892A and wildtype mice, although after seven and fourteen days of treatment, both groups displayed almost complete tolerance to these effects. This is in line with reported data in the literature.

$GABA_{B(2)}$-S892A mice did not differ in either their acute response or in their desensitisation rate to chronic baclofen. Given the crucial role played by PKA dependent phosphorylation in regulating $GABA_B$ receptor expression at the cell surface, the lack of difference seen between the $GABA_{B(2)}$-S892A mouse and wildtype controls is interesting. Our data would suggest that sensitisation to the physiological effects of baclofen is independent of this process. It is worth noting that given the crucial role of the $GABA_B$ receptor in maintaining inhibitory control of the brain, it is likely that other phosphorylation processes may compensate in maintaining stable levels of $GABA_B$ receptor activity the absence of S892 phosphorylation.

The current results also strongly suggest that $GABA_{B(2)}$-S892A mice display a phenotype indistinguishable from wildtype littermates across a broad range of tests of anxiety, fear conditioning and depression like behaviours. This stands in stark contrasts to the administration of $GABA_B$ positive allosteric modulator (PAM), which has robust anxiolytic effects in a diverse range of behavioural tests. Indeed, the prototypical modulator CGP 7930 and the more potent GS39783 and rac-BHFF (Froestl, 2010) have been shown to produce anxiolytic effects in the light dark box test and the SIH paradigm as used in this

experiment (Cryan et al., 2004; Mombereau et al., 2004a; Jacobson & Cryan, 2008; Malherbe et al., 2008), as well as in other behavioural tests such as the elevated zero maze, the elevated plus maze and the staircase test (Cryan et al., 2004; Mombereau et al., 2004a; Frankowska et al., 2007; Jacobson & Cryan, 2008). The fact that no behavioural phenotype is observed in the $GABA_{B(2)}$-S892A mouse suggests that the $GABA_{B(2)}$-S892A point mutation does not produce the same pharmacological effects as $GABA_B$ PAMs.

$GABA_B$ receptor PAMs were initially hypothesised to interact with the helical domain of the $GABA_{B(2)}$ subunit based on discoveries relating to the action of positive modulators of metabotropic glutamate receptors and known interactions between the $GABA_{B(2)}$ subunit and G-proteins (Pin et al., 2001). This was confirmed using *in vitro* studies assessing the activity of the $GABA_B$ PAM CGP7930, on both wildtype and a series of chimeric $GABA_B$ receptor subunit dimers co-expressed with G-protein effector proteins in the HEK-293 cell line. These studies clearly demonstrated that CGP7930 could exert pharmacological effects on chimeric dimers lacking either or both extracelluar domains and the helical domains of the $GABA_{B(1)}$ subunit, but required the presence of a $GABA_{B(2)}$ HD (Binet et al., 2004a). These studies went on to demonstrate that CGP7930 is not only a partial $GABA_B$ receptor agonist, but that it can directly activate $GABA_{B(2)}$ HD domains expressed by themselves, with data suggesting that the compound acts by stabilising an active conformation of the $GABA_{B(2)}$ subunit (Binet et al., 2004a, 2004b). Further work on establishing the precise site where $GABA_B$ PAMs bind to the $GABA_{B(2)}$ helical domain established that GS39783 has its functionality radically altered by the point mutations G706T and A708P, suggesting a binding site in the sixth transmembrane domain of the $GABA_{B(2)}$ subunit (Dupuis et al.,

2006). The current data suggests that the behavioural effects of $GABA_B$ positive allosteric modulators are not analogous to the ablation of PKA mediated $GABA_B$ receptor phosphorylation.

Although phosphorylation of the $GABA_{B(2)}$ subunit at the S892 site has pronounced effects on $GABA_B$ receptor function, indeed being the only PKA dependent phosphorylation site on the $GABA_B$ receptor (Couve et al., 2002), other regulatory phosphorylation mechanism exist to regulate receptor function (Terunuma et al., 2010a; Gassmann & Bettler, 2012). The $GABA_B$ receptor undergoes Protein-kinase C (PKC) mediated regulation of its signalling efficiency (Pontier et al., 2006) as well as AMPK mediated regulatory phosphorylation at sites on both the $GABA_{B1}$ and $GABA_{B2}$ subunits (Kuramoto et al., 2007). Furthermore CamKII mediated phosphorylation of the S867 residue of the $GABA_{B(1)}$ has been shown to regulate cell surface receptor levels (Guetg et al., 2010). It is of note that more recent discoveries have revealed even more complex mechanisms by which $GABA_B$ receptor function is regulated. One example is the discovery that potassium channel tetramerisation domains (KCTD) proteins form heteromultimers with functional $GABA_B$ receptors and strongly influence fast receptor desensitization and agonist potency (Schwenk et al., 2010). Indeed the distribution of these proteins varies greatly across brain regions, neuronal subpopulations and even across different periods of brain development, suggesting that the heterogeneity of $GABA_B$ receptor function may be heavily dependent on formation of different heteromultimers across the brain (Metz et al., 2011). It is thus increasingly clear that the functional properties of $GABA_B$ receptors are regulated by multiple pathways and protein interactions and as such it is possible that the effects of the

$GABA_{B(2)}$-S892A mutation could be compensated for by changes in one or more of the other regulatory mechanisms. Given the high levels of cell surface stability of the $GABA_B$ receptor heterodimer (Fairfax et al., 2004) it seems likely that these mechanisms may exist.

One caveat to the study is the use of the BALB/c mouse as a background strain for the $GABA_{B(2)}$-S892A mutation. Mouse strains vary significantly in their sensitivity to baclofen (Jacobson & Cryan, 2005), with the BALB/c mouse displaying complete insensitivity to lower doses. It is also of note that the BALB/c mouse is the only mouse strain that appears to be capable of surviving GABAB receptor knockout into adulthood (Prosser et al., 2001; Schuler et al., 2001). This suggests that the BALB/c mouse displays slightly altered $GABA_B$ receptor signalling properties that may explain its insensitivity to lack of S892 phosphorylation.

In conclusion, our data appear to suggest that S892A substitution does not markedly alter the baclofen sensitivity in vivo, nor does it alter anxiety or antidepressant-like behaviour. This may be the result of compensatory changes in other phosphorylation pathways or of alterations in one or more of the many complement proteins that influence $GABA_B$ receptor activity.

# *Chapter 6*

# *Ablation of the S892 Residue of the $GABA_{B(2)}$ Subunit Does Not Alter Behavioural Sensitivity to Cocaine*

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## *Abstract*

The $GABA_B$ receptor has long been implicated as a potential drug target for the treatment of substance abuse disorders. In particular preclinical data has suggested that positive allosteric modulators of the $GABA_B$ receptor possess anti-addictive effects and lack the side effect profile of full $GABA_B$ receptor agonists such as baclofen. *In vitro* data has suggested that $GABA_B$ receptor function in the ventral tegemental area (VTA) may play a crucial role in regulating the dopaminergic innervation of forebrain structures such as the nucleus accumbens and the dorsal striatum. We have recently developed a mouse model wherein phosphorylation of the S892 residue of the $GABA_{B(2)}$ subunit is eliminated resulting in a tonically active receptor. This has been shown to reduce dopaminergic neurotransmission to reward-related structures in the forebrain *in vitro.* We thus attempted to assess *in vivo* the sensitivity of these mice to the acute rewarding and locomotor effects of cocaine using a conditioned place preference (CPP) paradigm. We then sought to examine the levels of ΔFosB, a molecular correlate of chronic cocaine exposure, in reward-related brain structures subsequent to CPP testing. We found that the $GABA_{B(2)}$-S892A mouse displayed behaviour identical to those of its wildtype littermates in the CPP paradigm as well as displaying an identical pattern of locomotor sensitisation to cocaine as wildtype animals. $GABA_{B(2)}$-S892A substitution furthermore had no effect on levels of cocaine-induced ΔFosB accumulation in reward-related forebrain regions. Together these data suggests that $GABA_{B(2)}$-S892A substitution does not modify the behavioural or molecular consequences of repeated cocaine *in vivo*.

## *6.1 Introduction*

Preclinical research has shown that the $GABA_B$ receptor plays a crucial role in mediating the behavioural and molecular effects of drugs of abuse, with activation of the $GABA_B$ receptor being identified as a potential anti-addictive therapeutic strategy (Roberts, 2005; Vlachou & Markou, 2010). Human studies have shown that the $GABA_B$ receptor agonist baclofen can reduce cue-associated cocaine craving as well as reduce cocaine use in a double blind placebo controlled trial (Vocci & Elkashef, 2005). Over the past decade, positive allosteric modulators of the $GABA_B$ receptor have emerged as a therapeutic strategy for targeting the $GABA_B$ receptor. Positive allosteric modulators stabilise the $GABA_B$ receptor in a conformation which increases the affinity of GABA for the receptor (Urwyler et al., 2001b, 2005). They have been shown to share the behavioural effects of $GABA_B$ receptor agonists without inducing the sedation, impairment in cognitive function and hypothermic response of full agonists (Cryan & Kaupmann, 2005). Furthermore, positive allosteric modulators of the $GABA_B$ receptor have been shown to reduce the sensitivity of rats to the hyperlocomotor effects of acute cocaine administration and reduce the expression of the neuronal marker of cfos in the dorsal striatum and nucleus accumbens of these animals (Lhuillier et al., 2007). Similarly, the $GABA_B$ receptor positive allosteric modulator GS39783 prevents the acquisition of nicotine-induced conditioned place preference (CPP), coupled with a reduction in ΔfosB accumulation in the striatum (Mombereau et al., 2007). In addition, other positive allosteric modulators including CGP7930 and GS39783 have been shown to decrease cocaine self-administration (Smith et al., 2004). Positive allosteric modulators of the $GABA_B$ receptors have futhermore been repeatedly shown to decrease the expression of cocaine

reinforced behaviours (Filip et al., 2007a, 2007; Filip & Frankowska, 2007; Halbout et al., 2011).

The behavioural effects of drugs of abuse such as cocaine are strongly associated with enhanced activation of dopaminergic projections from the ventral tegemental area (VTA) to forebrain targets such as the dorsal striatum and the nucleus accumbens. $GABA_B$ receptors have been demonstrated to influence dopaminergic neuron activity in the VTA (Kalivas et al., 1990; Westerink et al., 1996, 1998). Indeed, genetic ablation of the $GABA_{B(1)}$ subunit results in elevated dopamine levels in striatal regions and reduced availability of dopamine (Vacher et al., 2006). $GABA_B$ receptor effector coupling in the VTA is lower in dopamine neurons than in GABA neurons resulting in $GABA_B$ receptors exerting bidirectional control of dopaminergic signalling i.e. higher potency agonists decrease dopaminergic signalling, whereas lower potency $GABA_B$ agonists such as GHB increase dopaminergic tone (Cruz et al., 2004).

A process vital to the regulation of $GABA_B$ receptor stability and ligand affinity is phosphorylation of the serine 892 (S892) residue of the $GABA_{B2}$ subunit (Fairfax et al., 2004). In order to further characterise the role of this phosphorylation process on $GABA_B$ receptor function *in vivo*, a knock-in mouse where the serine 892 residue in the $GABA_{B(2)}$ subunit is replaced with an alanine residue; the S892A knock-in mouse has been developed. The result of this modification is removal of this regulatory phosphorylation site. Preliminary research indicates that this mutation results in $GABA_B$ receptors producing high potency, non-desensitising responses to GABA, specifically on dopaminergic, but not GABAergic

neurons in the ventral tegemental area (VTA) suggesting that these $GABA_B$ receptors are active at tonic levels of GABA (Bettler et al., unpublished). These effects are mimicked *in vitro* by administration of positive modulators of $GABA_B$ receptors (Bettler et al. unpublished data). Dopaminergic projections to forebrain structures from the VTA form a key component of the neuronal circuitry of reward and underpins the rewarding effects of drugs of abuse such as cocaine (Koob & Volkow, 2010). Alterations in inhibitory signalling in this region can thus be hypothesised to result in alterations in sensitivity to the rewarding effects of drugs of abuse.

The current study aims to detect whether the $GABA_B$ receptor subtypes play differing roles in the regulation of cocaine reward and whether the alterations in GABAergic signalling in the VTA of the $GABA_{B(2)}$-S892A mouse can lead to changes in the behavioural and molecular response to cocaine. In order to examine this, we first examined the behaviour of the $GABA_{B(2)}$-S892A mouse in a CPP paradigm. CPP is a widely employed Pavlovian technique wherein animals are trained to associate the rewarding effects of a drug of abuse such as cocaine with a specific physical context , thus developing a preference for this context and is regarded as an effective way of quantifying the rewarding effects of a drug of abuse (Cunningham et al., 2006; Tzschentke, 1998). In addition to this, we assessed the locomotor sensitisation profile of the $GABA_{B(2)}$-S892A mouse. Locomotor sensitisation is a behavioural phenomenon whereby the acute hyperlocomotor effect of cocaine increases in magnitude with repeated cocaine dosing and is regarded as a potential model of incentive sensitisation seen in addicted patients (Robinson & Berridge, 2008). Subsequently, we examined levels of ΔFosB in the reward-related brain regions of $GABA_{B(2)}$-S892A mice.

ΔFosB is a protein that is increased in forebrain regions subsequent to chronic cocaine exposure and may underpin the neuronal changes that govern the transition from habitual drug use to addiction (Nestler, 2008). Finally, we assessed whether the molecular consequences of acute cocaine administration were altered by quantifying the levels of the immediate early gene cFos, a marker of neuronal activity (Lhuillier et al., 2007; O'Mahony et al., 2010), in reward-related brain regions in a separate cohort of $GABA_{B(2)}$-S892A mice.

## *6.2 Methods*

### *6.2.1 Mice*

The gene targeting construct contained a neomycin resistance cassette (pRay-2; Genbank accession number U63120) flanked by two arms of BALB/c $GABA_{B(2)}$ genomic DNA. The S892A mutation in exon 19 was introduced into the 3' arm along with a diagnostic silent *Nhe*I restriction site. The targeting construct was electroporated into BALB/c embryonic stem cells. Neomycin resistant colonies were screened for homologous recombination by PCR, and positive clones confirmed by Southern blot. Positive ES cells were microinjected into C57BL/6 blastocysts. Chimeric males were crossed with female BALB/c mice resulting in an F1 of inbred Balb/c mice heterozygous for the S892A+neo allele. The neomycin cassette in the S892A+neo allele was excised by mating heterozygous F1 mice with BALB/c Cre-deleter mice (Vigot et al., 2006). Deletion of the neomycin cassette in the S892A allele was confirmed by Southern blot analysis. $GABA_{B(2)}$ immunoblot analysis was performed as described previously in the literature (Gassmann et al., 2004).

Male mice homozygous for the $GABA_{B2}$-S892A mutation were used for all experiments, with age match homozygous wildtype littermates used for controls. Genotyping was performed on offspring on tail clip samples taken from animals as described in Appendix 1. Mice homozygous for the $GABA_{B2}$-S892A mutation were used for the experiment, with wildtype aged match homozygous wildtype mice obtained from the same breeding pairs used for controls.

Animals were maintained on a 12 hour light dark cycle with lights on 7AM. Food pellets and tap water were available ad-libitum.

All experiments were conducted in accordance with the European Directive 86/609/EEC, the Recommendation 2007/526/65/EC and approved by the Animal Experimentation Ethics Committee of University College Cork and in accordance with methods approved by the Veterinary Authority of the City of Basel. All efforts were made to minimize animal suffering and to reduce the number of animals used.

### *6.2.2 CPP*

Drug induced CPP is a behavioural paradigm used to determine the acute rewarding effects of drugs of abuse in rodents. It consists of an initial habituation phase, a conditioning phase where administration of a drug of abuse is repeatedly paired with a specific tactile, visual and olfactory context and finally a test phase where the animal is given a choice between the drug-paired (CS+) and a non-drug paired (CS-) context. The acute rewarding effects of the drug can then be assessed in terms of animal's preference for the CS+ context (Cunningham et al., 2006). The acute rewarding effects of cocaine in this paradigm are well established, with cocaine producing robust conditioned place preference across a number of species and experimental conditions (Tzschentke, 1998).

To ensure the development of place preference in control mice a protocol based on one previously described in the literature (Medvedev et al., 2005) was utilised with slight

modifications to the apparatus and an extended period of conditioning to accommodate the apparent resistance of the BALB/c strain to the acquisition of place preference. Place preference was assessed using a 3-compartment place preference apparatus for mouse (Med Associates, VA, USA). The apparatus consisted of a central compartment (7.2cm x 12.7cm x 12.7cm) flanked on either side by two "choice" compartments (16.8cm x 12.7cm x 12.7cm). Compartments were connected via manual guillotine doors. The two choice compartments were differentiated by visual, tactile and olfactory cues. One chamber had white walls, a fine stainless steel mesh floor (6.35 mm x 6.35 mm grid) and a lemon and ginger teabag (Lipton) placed in a stainless steel pan beneath the floor out of physical contact. The other chamber had black walls, a floor consisting of 3.2mm diameter bars at intervals of 7.8mm and no specific olfactory stimulus. Preliminary experiments were performed on normal BALB/c mice to determine conditions (light intensity/ olfactory stimuli) which did not elicit any baseline preference (Appendix 2). All chambers were wiped down with 70% ethanol and dried with paper towels between each mouse.

Animals were group-housed throughout the experiment and returned to their home cages subsequent to each testing session. All sessions were carried out at the same time each day for each animal during the light part of the light dark cycle. Behaviour was monitored using beam-break detection (13 beams) and analysed using Med-PC® IV (Med Associates, USA) software.

The conditioned place preference procedure consisted of three phases; an initial habituation/ pre-test phase, a conditioning phase and a test phase. The conditioning and test phases were then repeated.

*Habituation and Pretest*

In the initial habituation phase mice received a single IP injection of 0.9% saline prior to being introduced to the central compartment the apparatus and being allowed to explore the apparatus freely for 30 minutes. 24 hours later this was repeated and the time spent in each chamber was recorded to determine baseline preference.

*Conditioning phase*

Mice were randomly allocated a cocaine-context pairing. Conditioning consisted of 4 alternating pairings of drug with the CS+ chamber and saline with the CS- chamber. Conditioning sessions consisted of intraperitoneal injections of either vehicle or cocaine (10 mg/kg) and then placed in the appropriate chamber for 30 minutes. Locomotor activity was recorded for each conditioning session.

*Test Phase*

Animals were allowed to explore the apparatus freely for 30 minutes, with the amount of time spent in each compartment recorded.

*Tissue Collection*

Animals were killed by cervical dislocation followed by decapitation 24 hours subsequent to the second testing session. Brains were removed, briefly immersed in ice cold phosphate-buffered saline and 1mm slices taken using a mouse brain matrix (RBM 2000C, Asi Instruments). Dorsal striata and nuclei accumbens were then dissected out over ice as previously described in our lab (Lhuillier et al., 2007).

### *6.2.3 Acute cocaine treatment*

Mice were singly housed overnight to minimise variability between animals before recieviing a single IP injection of either 0.9% saline or 10 mg/kg cocaine Animals were killed by cervical dislocation followed by decapitation 2 hours subsequent to cocaine injection to ensure maximal expression of the immediate early gene cFos. Brains were removed, briefly immersed in ice cold phosphate buffered saline and 1mm slices taken using a mouse brain matrix (RBM 2000C, Asi Instruments). Dorsal striata and nuclei accumbens were then dissected out over ice.

### *6.2.4 Western blot*

Western blotting for cFos and ΔFosB were performed using a technique modified from one previously described by our lab (Lhuillier et al., 2007). Nuclear fractions were extracted from the dorsal striatum and nucleus accumbens of mice from both the locomotor monitoring and CPP experiments using a commercially available extraction kit (Active Motif, 40010). Extracts were then separated in via SDS-PAGE, with proteins then transferred to PVDF membranes. In order to confirm that our extraction had generated a

pure nuclear extract, expression levels of histone deacetylase (HDAC-1) was also measured with samples not expressing single band expression of HDAC were excluded from analyses (Galbán et al., 2003). Briefly, 16-18mg of protein from each sample was separated via SDS-PAGE, before proteins were transferred to a polyvinylidene fluoride membrane. Membranes were blocked in 5% milk in 1% TBS-Tween 20, prior to overnight incubation with primary antibody. Primary antibodies used were sc-253 rabbit anti-cFos (sc-253, Santa Cruz, CA, USA, 1:1000), rabbit anti-ΔFosB (sc-48, Santa Cruz, CA, USA, 1:1000) and rabbit anti-HDAC-1 (#2062, Cell Signalling, MA, USA, 1:1000) . Membranes were subsequently incubated with horseradish peroxidase–conjugated goat anti-rabbit (111-035-003, Jackson Immunoresearch, Suffolk, UK 1:5000) prior to visualisation using Westernbright quantum HRP substrate (K-12042, Advansta, CA, USA). (Densiometric analysis of blot images was performed using MultiGauge (v.2.2) software (Fujifilm). Expression levels of cFos were measured in extracts from animals treated acutely with cocaine, whereas ΔFosB levels were examined in extracts from animals in the CPP study.

### *6.2.5 Drug treatment*

Animals were treated with 10mg/kg of cocaine (Sigma, UK) in sterile 0.9% (10ml/kg) saline. A 10mg/kg dose of cocaine was selected as this is a dose shown in the literature to robustly generate behavioural effects and molecular responses in mice (Lhuillier et al., 2007; Medvedev et al., 2005).

### *6.2.6 Statistical analyses*

*Conditioned place preference*

Preference for the CS+ chamber i.e. Time in CS+ chamber minus Time in CS- chamber) at the pretest session and the two preference testing sessions, was analysed via two-way repeated measures ANOVA with the two factors being genotype and time. The Fisher Least Significant Difference (LSD) method was used for post hoc comparisons where indicated by significant ANOVA factors.

The number of beam breaks made by animals during each conditioning session was recorded across the conditioned place preference experiment i.e. 8 cocaine paired and 8 saline paired sessions. A two-way repeated measures ANOVA was performed to examine the effects of genotype and time. Data from cocaine paired and saline paired sessions was analysed separately. The Fisher Least Significant Difference (LSD) method was used for post hoc comparisons where indicated by significant ANOVA factors.

*Molecular Data*

Relative levels of ΔFosB expression were analyses via students t-test Relative levels of cFos expression subsequent to acute cocaine treatment were analysed via two-way ANOVA with genotype and cocaine dose as factors, followed by a bonferroni post-hoc test where appropriate.

## *6.3 Results*

### *6.3.1 Conditioned place preference in GABA$_{B(2)}$ -S892A mice*

Both WT and GABA$_{B(2)}$-S892A mice displayed no preference for either the CS+ or CS- chambers of the CPP apparatus at baseline, apart from one WT animal that displayed a preference to the drug paired chamber at baseline (greater than 2 standard deviations from mean preference) and was excluded from the study. Both WT and GABA$_{B(2)}$-S892A mice developed a clear preference for the drug paired chamber at trials one and two, with Two-way ANOVA detecting a significant effect of time on levels of preference ($F_{(2,52)} = 9.67$, $p < 0.005$). Two-way ANOVA revealed no effect genotype on preference ($F_{(1,52)} = 0.01$, $p = 0.92$) nor a time vs genotype interaction ($F_{(2,52)} = 1.16$, $p = 0.3205$) (Fig 1.).

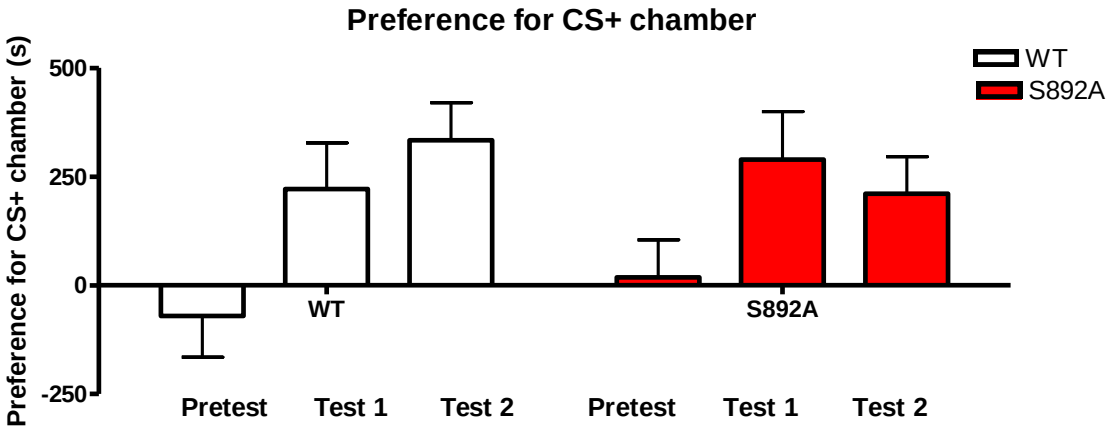


*Figure 1 – Preference of Wildtype and GABA$_{B2}$- S892A mice across the CPP paradigm. Preference data is measured as the time in seconds spent in the drug paired chamber minus time spent in the saline paired chamber. Data is expressed as mean +/- SEM.*

### *6.3.2 Locomotor sensitization to cocaine in $GABA_{B(2)}$ -S892A mice*

The locomotor response of WT and $GABA_{B2}$-S892A mice in response to cocaine is shown in Figure 2. Interestingly, when the locomotor activity of the animals in the CPP paradigm was analysed it was revealed that cocaine administration did not cause any locomotor activity following the first doses. Both strains however displayed a locomotor sensitisation to cocaine, displaying markedly increased locomotor activity after the $8^{th}$ cocaine dose. Two-way ANOVA of the total number of beam breaks revealed no effect of time ($F_{7,126}$=0.96, p=0.46)or of genotype ($F_{1,126}$=0.004, p=0.95) on locomotor activity across saline paired training sessions as well as detecting no genotype-time interaction ($F_{7,126}$=0.79, p=0.60) (Fig 2A). Analyses of cocaine paired training sessions revealed a significant effect of time on beam break frequency indicating locomotor sensitisation effect ($F_{7,112}$=13.81, p<0.0001). No effect of genotype ($F_{1,112}$=0.30, p=0.59) and no genotype x time interaction ($F_{7,112}$=0.38, p=0.91)was observed (Fig 2B).

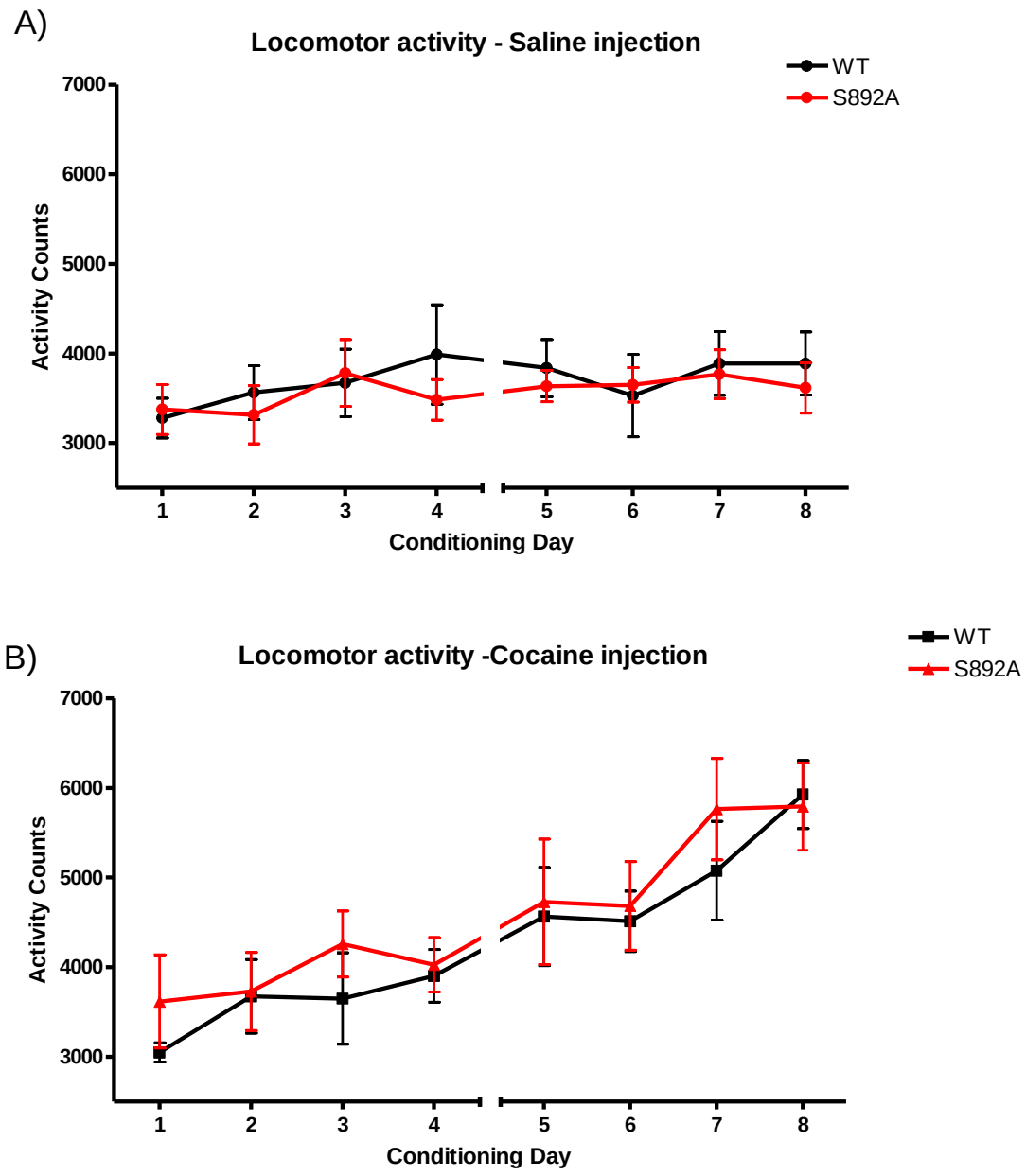


*Figure 2 – Cocaine induced hyperlocomotor response of Wildtype and $GABA_{B2}$- S892A mice across the conditioning days of the CPP experiment. A) Locomotor response to cocaine across the conditioning period. B) Locomotor response to saline across the conditioning period. Data is measured as no. of beam breaks per 30 minute conditioning session and expressed as mean +/- SEM*

### *6.3.3 Western blot analyses*

Once samples failing to express pure HDAC-1 and samples displaying low levels of overall protein content were excluded from our study an *n* of 7-9 samples remained in each group for the dorsal striatum and nucleus accumbens. A Student's T-test of our western blot analyses revealed no significant difference was observed between $GABA_{B2}$-S892A and WT animals in terms of dorsal striatum ΔFosB accumulation (p= 0.47) (Fig 3A) or in terms of nucleus accumbens ΔFosB accumulation (p=0.46) (n=7-9 samples per group) (Fig 3B).

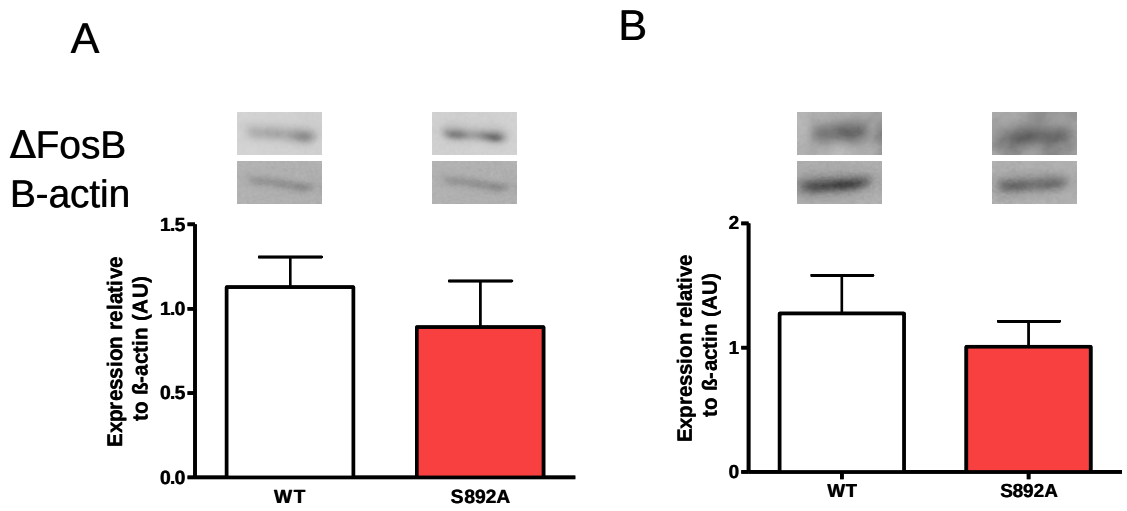


*Figure 3 – Levels of ΔFosB protein in the A) dorsal striatum and B) nucleus accumbens subsequent to the CPP experiment as measured by western blot. Protein levels are expressed in terms of densitometry of band relative to β-actin expression. Data is expressed in terms of mean +/- SEM.*

Once samples failing to express pure HDAC-1 and samples displaying low levels of overall protein content were excluded from our study an *n* of 5-6 samples remained in each group for the dorsal striatum and nucleus accumbens. In samples from the dorsal striatum of animals treated acutely with cocaine (n=5-6), two-way ANOVA revealed no significant

effects of cocaine treatment ($F_{1,18}$=0.77, p=0.39) or of genotype ($F_{1,18}$=0.43, p=0.52) on cFos expression. No interaction was detected between cocaine and genotype ($F_{1,18}$=0.09, p=0.76). An inadequate number of samples from the nucleus accumbens were shown to contain pure HDAC protein via western blot for any meaningful statistical analysis. This may be due to difficulties in obtaining a pure nuclear fraction due to difficulties in extracting protein from a small sample of tissue such as the tissue collected for our studies.

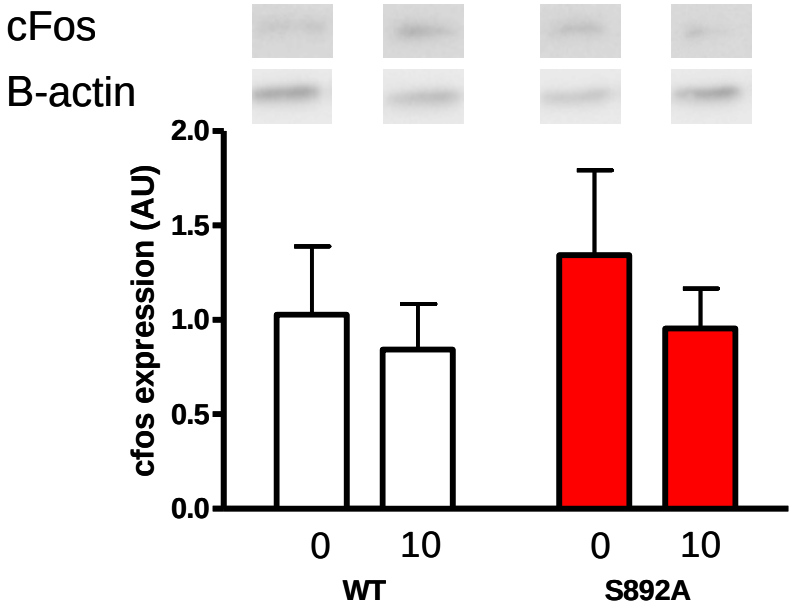


*Figure 4 – Levels of cFos protein in the dorsal striatum 2 hours subsequent to acute cocaine injection (0/10 mg/kg) as measured by western blot. Protein levels are expressed in terms of densitometry of band relative to β-actin expression. Data is expressed in terms of mean +/- SEM.*

## *6.4 Discussion*

Increasing evidence points to a role for the $GABA_B$ receptor in the pathophysiology and treatment of cocaine dependence. In these studies, to the best of our knowledge for the first time, investigated the role of the S892 residue phosphorylation site of the $GABA_{B(2)}$ subunit in the manifestation of the acute and chronic behavioural and molecular effects of cocaine using a transgenic mouse model whereby the phosphorylation of the S892 residue of the $GABA_{B(2)}$ subunit is eliminated resulting in a tonically active receptor. Interestingly, the $GABA_{B(2)}$-S892A mouse did not display any altered preference behaviour compared to wildtype and no difference in locomotor activity compared to wildtype mouse at baseline as well as with exposure to acute and chronic cocaine. This appears to conflict with preliminary evidence that the $GABA_{B(2)}$-S892A mouse displays considerably enhanced $GABA_B$ receptor function in dopaminergic neurons projecting from the VTA to forebrain (Bettler et al. unpublished data). Furthermore, several studies have clearly shown that like other GPCRs, $GABA_B$ receptor activity is potently modulated by the phosphorylation status of the receptor (Terunuma et al., 2010a), with the S892 site on the $GABA_{B(2)}$ subunit being a target of particular importance (Couve et al., 2002; Fairfax et al., 2004). In addition, we did not observe reduced levels of ΔFosB expression in the nucleus accumbens or dorsal striatum of $GABA_{B(2)}$-S892A mice subsequent to CPP treatment which suggests that these mice do not differ in susceptibility to the molecular effects of chronic cocaine. Overall, current data on the $GABA_{B(2)}$-S892A mouse suggest that although they display reduced sensitivity to cocaine at a neuronal level, this does not translate to the levels of reward system function or behavioural sensitivity.

Recent findings have also shown that $GABA_{B(1)}$-S867 phosphorylation dependant receptor internalisation of $GABA_B$ receptors is governed by NMDA receptor activation and subsequent CaMKII activity (Guetg et al., 2010). Indeed, evidence has suggested a much more complex regulation of $GABA_B$ receptor activity dependant not only on phosphorylation status but on the formation of complexes with potassium channel tetramer domains (KCTDs) (Schwenk et al., 2010). Thus, the picture that emerges is that of a complex situation involving numerous intracellular signalling pathways and molecules and indeed several phosphorylation sites on the $GABA_B$ receptor itself. Compensatory changes in the expression or function of other elements of these regulatory mechanisms cannot be excluded. It should also be noted that current unpublished *in vitro* data from the $GABA_{B(2)}$-S892A mouse has solely examined levels of S892 phosphorylation and $GABA_B$ receptor function in a subset of neurons in the VTA. Compensatory changes or indeed a lack of change in function in other reward-related brain regions such as the nucleus accumbens or dorsal striatum may underlie the maintenance of a wildtype behavioural phenotype in these animals.

It is also important to bear in mind that cocaine administration has been demonstrated to alter $GABA_B$ receptor function in reward-related brain regions. Baclofen administration to cocaine-sensitised animals results in increased levels of glutamate in the mPFC, VTA and nucleus accumbens, an effect not seen in cocaine naïve animals (Jayaram & Steketee, 2004) and that repeated cocaine treatment results in decreased levels of baclofen-induced 35S-GTPγS binding in the VTA, suggestive of reduced levels of receptor effector coupling (Kushner & Unterwald, 2001). Most importantly of all, it has also been shown that $GABA_B$

receptor function is decreased in the nucleus accumbens of rats subsequent to 3 weeks of cocaine treatment with no reductions in $GABA_{B(1)}$ or $GABA_{B(2)}$ protein levels, suggesting alterations to functionality (Xi et al., 2003). The functional changes in $GABA_B$ receptor function seen in response to repeated cocaine administration thus appear to be dependant on post-translational alterations to the receptor such as S892 phosporylation or protein-protein interactions. Indeed, changes to cocaine-dependant regulation of $GABA_B$ receptor function throughout the brain may further complicate interpretation of the behavioural responses of the $GABA_{B(2)}$-S892A mouse to cocaine. Recent data has furthermore demonstrated that dopaminergic neurons projecting to striatal regions release $GABA_A$ receptor activating ligands, likely GABA, as a co-transporter with dopamine (Tritsch et al., 2012). In light of this data, the simple model of GABA mediating addiction solely in the VTA becomes redundant with GABA receptor activity in forebrain targets such as the nucleus accumbens, striatum and prefrontal cortex playing equally important role in regulating the behavioural effects of drugs of abuse.

Our western blot experiments, in support of our behavioural data, demonstrated no significant differences between $GABA_{B(2)}$- S892A and WT mice in terms of ΔFosB accumulation subsequent to the CPP paradigm, and displayed no differences in cFos accumulation after acute cocaine treatment. This data stands, like our behavioural data, in contrast to reports from our lab showing that $GABA_B$ receptor positive modulator administration can reduce the accumulation of ΔFosB in response to repeated cocaine treatment and attenuate increases in levels of cFos in response to acute cocaine treatment (Lhuillier et al., 2007). Our data further suggests that $GABA_{B(2)}$- S892A mice are

indistinguishable from their WT littermates in terms of cocaine sensitivity. It must be stated however that the small tissue samples used in this experiment resulted in difficulties in generating a pure nuclear extract from our samples. As such, our molecular data must be interpreted with caution.

The background strain of a transgenic mouse can play a significant influence on behaviour (Jacobson & Cryan, 2007) and a significant caveat to the use of conditioned place preference in examining the $GABA_{B(2)}$-S892A mouse is the lack of robust place preference to cocaine seen in the BALB/c mouse strain (Belzung & Barreau, 2000). BALB/c mice have also been shown to be relatively insensitive to the locomotor effects of acute cocaine treatment as well as the reinforcing effects of cocaine reward (Thomsen & Caine, 2011; Eisener-Dorman et al., 2011). Indeed in the present study, no robust increases in cFos expression, a marker of neuronal activation were seen in the dorsal striatum of either wildtype or $GABA_{B2}$-S892A mice. However, it must be said that $GABA_B$ receptor knockout do not survive to adulthood when generated on mouse strains other than the BALB/c (Schuler et al., 2001; Prosser et al., 2001). Given the vastly different levels of sensitivity to $GABA_B$ agonist treatment displayed by different mouse strains (Jacobson & Cryan, 2005), it would be unsurprising if differing effects of $GABA_B$ receptor mutation were seen across the spectrum of background strains.

It must also be borne in mind that we have only used a few of the many animal behavioural models of drug abuse and indeed used techniques that model only certain facets of drug abuse disorders in the clinic. Further characterisation across the spectrum of behavioural

techniques including self-administration and intracranial self stimulation based paradigms (Koob & Volkow, 2010) remains to be carried out, and may indeed reveal a role for S892 phosphorylation in determining drug of abuse sensitivity. Other molecular markers of drug exposure such as DARRP-32 and pCREB/CREB ratio (Lhuillier et al., 2007) should perhaps also be investigated. Indeed, study of the $GABA_{B(2)}$-S892A mouse in terms of other drugs of abuse such as nicotine and alcohol may represent a route for further research.

In conclusion, our data suggests that the $GABA_{B(2)}$-S892A mouse is indistinguishable from its wildtype in terms of its cocaine sensitivity. These findings highlight how the regulation of $GABA_B$ receptor function *in vivo* is a highly complex system involving multiple receptor phosphorylation sites and indeed several interactions with complementary proteins (Pinard et al., 2010). It remains difficult to predict how pharmacological and genetic interventions aimed at the $GABA_B$ receptor system will affect behaviour or other brain functions. The need for detailed *in vitro* studies to unravel the molecular bases of the heterogeneity of $GABA_B$ receptor is paramount to the development of novel $GABA_B$ directed therapeutic approaches.

# *Chapter 7*

# *Differential Roles of the $GABA_{B(1)}$ Receptor Subunit Isoforms on the Locomotor Responses to Acute and Repeated Cocaine*

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## *Abstract*

The $GABA_B$ receptor are made up of heterodimers of $GABA_{B(1)}$ and $GABA_{B(2)}$ receptor subunits. In addition $GABA_{B(1)}$ receptors exist as two distinct isoforms based on the presence or absence of several sushi domains of the $GABA_{B(1)}$ subunit of the receptor heterodimer. These variants are referred to as the $GABA_{B(1a)}$ and $GABA_{B(1b)}$ receptor subtypes. Development of mice wherein either subunit is selectively ablated, $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice, has revealed that the two receptor subunit isoforms differentially regulate several behavioural responses, including fear behaviour, learning, memory and stress sensitivity. However, despite the $GABA_B$ receptor being shown act as a crucial modulator of the behavioural effects of drugs of abuse, the $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice have not been characterised in regard to their sensitivity to drugs of abuse. We thus sought to characterise the behaviour of the $GABA_{B(1)}$ specific isoform null mice in a cocaine-induced conditioned place preference (CPP) paradigm as well as in terms of their locomotor sensitivity to acute and repeated cocaine administration. We furthermore sought to examine levels of neuronal activation in reward-related forebrain structures subsequent to acute cocaine administration and quantify levels of the FosB splice variant, ΔFosB, in similar regions subsequent to our CPP paradigm. ΔFosB is a neuronal marker of prolonged cocaine exposure and is believed to underpin the development of drug addiction. Our data demonstrated enhanced locomotor activation in response to acute cocaine as well as enhanced locomotor sensitisation to cocaine in $GABA_{B(1a)}^{-/-}$ mice compared to wildtype (WT) mice, whereas $GABA_{B(1b)}^{-/-}$ mice failed to develop locomotor sensitisation despite displaying higher levels of basal locomotor activity. Our data furthermore suggests that $GABA_{B(1a)}^{-/-}$ mice display a heightened locomotor response to acute cocaine administration.

## *7.1 Introduction*

Native $GABA_B$ receptors are obligate heterodimers of the $GABA_{B(1)}$ and $GABA_{B(2)}$ receptor subunits and can be divided into two specific receptor subtypes characterised by differing $GABA_{B(1)}$ isoforms; $GABA_{B(1a)}$ and $GABA_{B(1b)}$ (Möhler & Fritschy, 1999; Bettler et al., 2004) These isoforms are distinguished by the presence, in $GABA_{B(1a)}$, or the absence, in $GABA_{B(1b)}$ of two sushi domains at the n-terminus (Couve et al., 2000). With the generation of knockout mice with either isoform selectively ablated (Jacobson et al., 2006a) the role played by these isoforms in mediating fear behaviour, learning, memory and stress sensitivity have been extensively characterised (Jacobson et al., 2007b, 2006b, 2007a; Shaban et al., 2006; Moloney et al., 2012). Characterisation of the $GABA_{B(1a)}$ and $GABA_{B(1b)}$ containing isoforms have revealed distinctive anatomical and functional differences. Although both isoforms appear to behave as presynaptic autoreceptors, inhibiting GABA release, the $GABA_{B(1a)}$ isoform also acts as a heteroreceptor modulating glutamate release in response to spill over of $GABA_B$ from GABAergic synapses. The $GABA_{B(1b)}$ isoform mediates inhibitory effects of GABA at post-synaptic sites (Vigot et al., 2006; Waldmeier et al., 2008).

The $GABA_B$ receptor is an emerging candidate drug target in the treatment of addiction (Roberts, 2005; Vlachou & Markou, 2010). Clinical data suggests that the $GABA_B$ receptor agonist baclofen may be an effective treatment for alcohol and cocaine addiction (Vocci & Elkashef, 2005; Brennan et al., 2013). Moreover, a plethora of preclinical data has shown that baclofen inhibits self-administration of a spectrum of drugs of abuse, including cocaine, as well as preventing reinstatement of drug craving in rodents (Brebner et al., 2005; Walker

and Koob, 2007; Spano et al., 2007; Filip et al., 2007; Fattore et al., 2009). More recently positive modulators of the $GABA_B$ receptors have emerged and been shown to inhibit the behavioural and molecular effects of cocaine (Slattery et al., 2005b; Lhuillier et al., 2007; Filip et al., 2007a; Filip & Frankowska, 2007; Halbout et al., 2011)

The behavioural effects of drugs of abuse such as cocaine are strongly associated with enhanced activation of dopaminergic projections from the ventral tegemental area (VTA) to forebrain targets such as the dorsal striatum and the nucleus accumbens (Koob & Volkow, 2010). The $GABA_B$ receptor acts as an important modulator of dopaminergic innervation from the VTA to the forebrain, with enhanced dopaminergic transmission along this pathway observed in $GABA_B$ receptor-deficient mice (Vacher et al., 2006). Further research has revealed a diversity in the $GABA_B$ receptor population in the VTA, with a low effector coupling population expressed in dopaminergic neurons and a population with higher effector coupling expressed in GABAergic neurons (Cruz et al., 2004). Given the differential role of $GABA_{B(1)}$ receptor subunit isoforms in regulating neurotransmitter release it is possible that they play distinct roles in mediating the rewarding effects of drugs of abuse.

Interestingly, $GABA_{B(1b)}^{-/-}$ mice display a hyperlocomotor phenotype in response to novelty and during the circadian dark phase (Jacobson et al., 2006a). Given the well established role played by dopamine in regulating levels of locomotor activity (Kelly & Iversen, 1976), it may be that the $GABA_B$ receptor subtypes play differing roles on dopaminergic tone. However, the role played by these isoforms in regulating reward behaviour and specifically drug of abuse sensitivity have yet to be characterised. Using $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$

mice previously characterised in our lab (Jacobson et al., 2006a, 2006b, 2007a, 2007b), the current study aimed to determine whether $GABA_B$ receptor subtypes play differing roles in the regulation of cocaine and if ablation of either subunit can lead to changes in the behavioural and molecular response to cocaine. To this end, we determined the levels of hyperlocomotor response to acute cocaine administration, the sensitivity of these mice to the rewarding effects of cocaine in a conditioned place preference paradigm, and the psychomotor sensitisation effect of cocaine in these mice. These behaviours are models of the acute rewarding effects of cocaine (locomotor activity, CPP) (Morgan et al., 2012; Tzschentke, 2007) and incentive sensitisation (psychomotor sensitisation) (Robinson & Berridge, 2008). We furthermore sought to characterise the molecular responses of WT, $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice to repeated and acute cocaine administration. In particular we examined the effects of repeated cocaine injection on expression levels of ΔFosB in reward-related brain areas. This splice variant of FosB has been repeatedly shown to accumulate and persisit in forebrain regions such as the dorsal striatum subsequent to repeated cocaine administration and may underlie the neuronal changes that precipitate the development of drug addiction (Chao & Nestler, 2004; Nestler, 2008). Additionally we examined levels of cFos expression in the dorsal striatum and nucleus accumbens of $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice subsequent to acute cocaine administration as a surrogate measurement of acute neuronal activation (Singewald, 2007; Lhuillier et al., 2007).

## *7.2 Methods*

### *7.2.1 Mice*

$GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice were generated as previously described (Vigot et al., 2006). Briefly, $GABA_{B(1a)}$ and $GABA_{B(1b)}$ initiation codons were converted to stop codons by targeted insertion of a floxed neo-cassette. All the mutant and WT mice were maintained on a pure inbred BALB/c genetic background. $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice were derived from homozygous breeding of siblings originating from the founding heterozygotic mice, while homozygous wildtype controls for the $GABA_{B(1)}$ isoform mutant mice were derived from mating together wildtype siblings generated from $GABA_{B(1a)}^{+/-}$ and $GABA_{B(1b)}^{+/-}$ heterozygous breeding. The breeding strategy was conducted in accordance with the recommendations proposed by The Jackson Laboratory to obviate genetic drift and the formation of substrains (http://jaxmice.jax.org/geneticquality/guidelines.html). Animals were maintained on a 12 hour light dark cycle. Food pellets and tap water were available ad-libitum.

All experiments were conducted in accordance with the European Directive 86/609/EEC, the Recommendation 2007/526/65/EC and approved by the Animal Experimentation Ethics Committee of University College Cork and in accordance with methods approved by the Veterinary Authority of the City of Basel. All efforts were made to minimize animal suffering and to reduce the number of animals used.

### *7.2.2 Cocaine-induced hyperlocomotion*

Locomotor activity was assessed in transparent plexiglas boxes (dimensions: 19 x 31 x 16 cm) by quantifying infrared light beam interruptions along the *x*- and *y*-axes through the walls of the box as described previously (Cryan et al., 2004). The distance travelled was automatically calculated using a TSE Moti system (TSE, Bad Homburg, Germany). Mice were individually placed in novel locomotor activity boxes and habituated for 60 min. This duration was chosen to ensure complete habituation of all mutant strains, as $GABA_{B(1b)}^{-/-}$ mice have been previously described as showing a hyperlocomotor phenotype and slower habituation in novel environment compared to wildtype and $GABA_{B(1a)}^{-/-}$ mice (Jacobson et al., 2006a). All mice were then injected with cocaine (10 mg/kg) and immediately returned to the locomotor activity box and activity recorded for a further 60 min. The procedure and dose of cocaine was selected based on previous experiments in this laboratory (Lhuillier et al., 2007).

### *7.2.3 Conditioned place preference*

Mice (n=7-11 per group) were tested in terms of cocaine induced place preference. A conditioned place preference procedure was modified from a protocol in the literature (Medvedev et al., 2005). Place preference was assessed using a 3-compartment place preference apparatus for mouse (Med Associates, VA, USA). The apparatus consisted of a central compartment (7.2cm x 12.7cm x 12.7cm) flanked on either side by two "choice" compartments (16.8cm x 12.7cm x 12.7cm). Compartments were connected via manual guillotine doors. The two choice compartments were differentiated by visual, tactile and olfactory cues. One chamber had white walls, a fine stainless steel mesh floor (6.35 mm x

6.35 mm grid) and a lemon and ginger teabag (Lipton) placed in a stainless steel pan beneath the floor out of physical contact. The other chamber had black walls, a floor consisting of 3.2mm diameter bars at intervals of 7.8mm and no specific olfactory stimulus. Preliminary experiments were performed on normal BALB/c mice to determine conditions (light intensity/ olfactory stimuli) which did not elicit any baseline preference (Appendix 2). All chambers were wiped down with 70% ethanol and dried with paper towels between each mouse.

Animals were group housed throughout the experiment and returned to their home cages subsequent to each testing session. All sessions were carried out at the same time each day for each animal during the light part of the light dark cycle. Behaviour was monitored using beam-break detection (13 beams) and analysed using Med-PC® IV (Med Associates, USA) software.

The conditioned place preference procedure consisted of three phases; an initial habituation/ pre-test phase, a conditioning phase and a test phase. The conditioning and test phases were then repeated.

*Habituation and pretest*

In the initial habituation phase mice received a single IP injection of 0.9% saline prior to being introduced to the central compartment the apparatus and being allowed to explore the apparatus freely for 30 minutes. 24 hours later this was repeated and the time spent in each chamber was recorded to determine baseline preference.

*Conditioning phase*

Mice were randomly allocated a cocaine-context pairing. Conditioning consisted of 4 alternating pairings of drug with the CS+ chamber and saline with the CS- chamber. Conditioning sessions consisted of intraperitoneal injections of either vehicle or cocaine and then placed in the appropriate chamber for 30 minutes. Locomotor activity was recorded for each conditioning session.

*Tests phase*

Animals were allowed to explore the apparatus freely for 30 minutes, with the amount of time spent in each compartment recorded.

Animals were killed by cervical dislocation followed by decapitation 24 hours subsequent to the second testing session. Brains were removed, briefly immersed in ice cold phosphate-buffered saline and 1mm slices were taken using a mouse brain matrix (RBM 2000C, Asi Instruments). Dorsal striata and nuclei accumbens were then dissected out over ice.

### *7.2.4 Tissue collection*

Wildtype mice were given either vehicle or cocaine (10 mg/kg, *n*=5) via IP injection. $GABA_{B(1a)}^{-/-}$ (n=7) and $GABA_{B(1b)}^{-/-}$ (n=12) mice received only cocaine. Two hours after cocaine or vehicle injection, mice were sacrificed by cervical dislocation, brains were removed, briefly immersed in ice cold phosphate-buffered saline and 1mm slices were taken using a mouse brain matrix (RBM 2000C, Asi Instruments). Dorsal striata and nuclei accumbens were then dissected out over ice.

### *7.2.5 Western blot*

Western blotting for cFos and ΔFosB were performed using a tehchnique modified from one previously described by our lab (Lhuillier et al., 2007). Nuclear fractions were extracted from the dorsal striatum and nucleus accumbens of mice from both the locomotor monitoring and CPP experiments using a commercially available extraction kit (Active Motif, 40010). Extracts were then separated in via SDS-PAGE, with proteins then transferred to PVDF membranes. In order to confirm that our extraction had generated a pure nuclear extract, expression levels of histone deacetylase (HDAC-1) was also measured with samples not expressing single band expression of HDAC were excluded from analyses (Galbán et al., 2003). Briefly 16-18mg of protein from each sample was separated via SDS-PAGE, before proteins were to transfered to a polyvinylidene fluoride membrane. Membranes were blocked in 5% milk in 1% TBS-Tween 20, prior to overnight incubation with primary antibody. Primary antibodies used were sc-253 rabbit anti-cfos (sc-253, Santa Cruz, CA, USA, 1:1000), rabbit anti ΔFosB (sc-48, Santa Cruz, CA, USA, 1:1000) and rabbit anti-HDAC-1 (#2062, Cell Signalling, MA, USA, 1:1000) . Membranes were subsequently incubated with horseradish peroxidase–conjugated goat anti-rabbit (111-035-003, Jackson Immunoresearch, Suffolk, UK 1:5000) prior to visualisation using Westernbright quantum HRP substrate (K-12042, Advansta, CA, USA). (Densiometric analysis of blot images was performed using MultiGauge (v.2.2) software (Fujifilm). Expression levels of cFos were measured in extracts from animals treated acutely with cocaine, whereas ΔFosB levels were examined in extracts from animals in the CPP study.

### *7.2.6 Statistical analysis*

*Locomotor response to acute cocaine*

Distance travelled during the 60 min habituation period prior to cocaine administration, the subsequent distance travelled in the 60 min after cocaine administration, were summed into 5 min intervals and analyzed using 2-way repeated measures analysis of variance (ANOVA) with factors "genotype" and "time". Summed (within animal) pre- and post-cocaine distance travelled were analyzed using one-way ANOVA. The Fisher Least Significant Difference (LSD) method was used for post hoc comparisons where indicated by significant ANOVA factors.

*Conditioned place preference*

Preference for the CS+ chamber i.e. Time in CS+ chamber minus Time in CS- chamber) at the pretest session and the two preference testing sessions, was analysed via two-way repeated measures ANOVA with the factors genotype and time. The Fisher Least Significant Difference (LSD) method was used for post hoc comparisons where indicated by significant ANOVA factors.

The number of beam breaks made by animals during each conditioning session was recorded across the conditioned place preference experiment i.e. 8 cocaine paired and 8 saline paired sessions. A two-way repeated measures ANOVA was performed to examine the effects of genotype and time. Data from cocaine paired and saline paired sessions was analysed separately. The Fisher Least Significant Difference (LSD) method was used for post hoc comparisons where indicated by significant ANOVA factors. The total number of

beam breaks for cocaine and saline paired sessions across the experiment were recorded. These were analysed via one-way ANOVA followed by a Neumann-keuls post-hoc test where appropriate.

*Molecular analyses*

Relative levels of ΔFosB expression were analysed via one-way ANOVA followed by a Neumann-keuls post-hoc test where appropriate. When analysing relative levels of cFos expression subsequent to acute cocaine treatment, initially we compared levels of cFos expression between WT animals treated with 0 and 10mg/kg of cocaine via Student's t-test. Subsequently levels of cFos expression were compared between WT, $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice treated with 10 mg/kg cocaine using one-way ANOVA followed by a Neumann-keuls post-hoc test where appropriate.

## *7.3 Results*

### *7.3.1 Cocaine-induced hyperlocomotion*

The locomotor responses of WT, $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice to acute cocaine injection is shown in Figure 1. Distance travelled in the 1 hour preceding cocaine administration was significantly affected by genotype and time (genotype: $F_{2, 371}$= 8.075, p<0.01; time: $F_{11,371}$ = 19.539, p<0.001; interaction: $F_{22,371}$=1.398, p=0.112). Post hoc analysis revealed that, overall, $GABA_{B(1b)}^{-/-}$ mice travelled a greater mean distance per 5 min block than wildtype (P < 0.001) and $GABA_{B(1a)}^{-/-}$ mice (P = 0.01), although wildtype and $GABA_{B(1a)}^{-/-}$ mice did not differ in this regard (Least square mean m per 5 min block ± sem: wildtype: 5.4 ± 0.5; $GABA_{B(1a)}^{-/-}$ 6.1 ± 0.5, $GABA_{B(1b)}^{-/-}$ 8.1 ± 0.5 ). Post hoc analysis to address the main effect of "time" demonstrated that on average, mice had habituated by approximately 30-35 min after entry to the novel locomotor box (p > 0.05 for pair-wise comparisons between 30 min and each subsequent time point, with the exception of time point 40 min versus 60 min (p < 0.01). LSD analysis to specifically address the duration of hyperlocomotor activity of $GABA_{B(1b)}^{-/-}$ indicated that this strain showed higher locomotor activity than wildtype mice at each of the 5 min timepoints from 5 to 55 min (inclusive), but had habituated to a point that was not different from wildtype mice at time-point 60 min (p > 0.05; Fig.1.). In comparison, $GABA_{B(1a)}^{-/-}$ mice differed from wildtype mice only at the first 5 min time-point (p < 0.05; Figure 1).

As expected, cocaine increased the distance travelled in all mice. Genotype and time significantly affected distance travelled after cocaine (genotype: $F_{2, 371}$ = 5.312, P < 0.05;

time: $F_{11,371} = 23.147$, $P < 0.001$; interaction: $F_{22,371} = 1.217$, $P = 0.231$). However, in contrast to the pre-cocaine habituation period, the $GABA_{B(1a)}^{-/-}$ mice travelled a greater distance than wildtype mice after cocaine ($P < 0.001$), whereas $GABA_{B(1b)}^{-/-}$ mice did not differ from $GABA_{B(1a)}^{-/-}$ or wildtype mice ($P > 0.05$; Least square mean m per 5 min block ± sem: wildtype: 4.8 ± 0.7; $GABA_{B(1a)}^{-/-}$ 8.3 ± 0.8, $GABA_{B(1b)}^{-/-}$ 6.2 ± 0.7). LSD analysis to specifically determine the duration of the hypersensitive responses to cocaine indicated $GABA_{B(1a)}^{-/-}$ mice were more active that cocaine-treated wildtype mice for the 30 min after cocaine administration, but did not different from wildtype mice subsequent to this time-point (Fig. 1).

Analysis of the summed distance travelled before (0-60 min) and after (60-95 min) cocaine mirrored that of the time course analysis (Fig. 1A). Genotype significantly affected distance travelled during pre-cocaine habituation ($F_{2, 30} = 8.075$, $P < 0.01$), with $GABA_{B(1b)}^{-/-}$ mice travelling more distance than wildtype ($P < 0.001$) and $GABA_{B(1a)}^{-/-}$ ($P = 0.01$), although wildtype and $GABA_{B(1a)}^{-/-}$ mice did not differ in this regard ($P > 0.05$). Following cocaine administration, summed distance travelled from 60-95 min was significantly greater in the $GABA_{B(1a)}^{-/-}$ mice relative to both wildtype ($P < 0.01$) and $GABA_{B(1b)}^{-/-}$ mice ($P < 0.05$), although again, wildtype and $GABA_{B(1b)}^{-/-}$ mice did not differ in this regard ($P > 0.05$; Fig. 1B.).

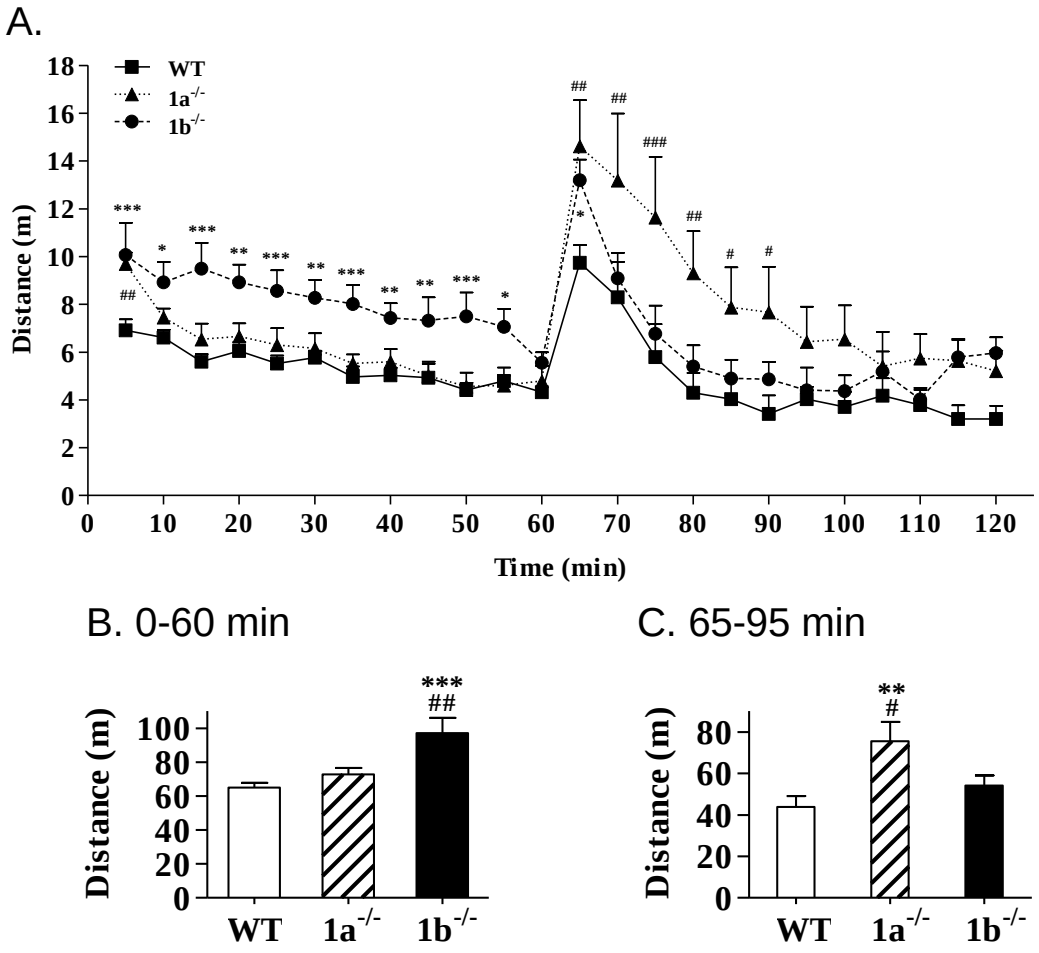


*Figure 1 - A) Time course showing locomotor activity of wildtype (WT), $GABA_{B1A}^{-/-}$ ($1a^{-/-}$) and $GABA_{B1A)}^{-/-}$ ($1b^{-/-}$) mice in a novel environment, and 60 min later in the same environment, their responses to acute cocaine administration (10 mg/kg, i.p.; Wildtype (WT) n = 11; $1a^{-/-}$ n = 9; $1b^{-/-}$ n = 11.; $^{\#}$, $^{\#\#}$, $^{\#\#\#}$ P < 0.05, P < 0.01, P < 0.001 $1a^{-/-}$ vs WT; $^{*}$, $^{**}$, $^{***}$ P < 0.05, P < 0.01, P < 0.001 $1b^{-/-}$ vs WT). B). Summed distance travelled from 0-60 min in the novel environment in the same mice ($^{***}$ P < 0.001 $1b^{-/-}$ vs WT; $^{\#\#}$ P < 0.01 $1b^{-/-}$ vs $1a^{-/-}$). C). Summed distance travelled during the 30 min following cocaine administration in the same mice ($^{**}$ P < 0.01 $1a^{-/-}$ vs WT; $^{\#}$ P < 0.05 $1a^{-/-}$ vs $1b^{-/-}$).*

### *7.3.2 Conditioned place preference*

Levels of preference of WT, $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice for the CS+ chamber are displayed in Table 1. WT, $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice displayed no preference for

either the CS+ or CS- chambers of the CPP apparatus at baseline, however none of the groups mice developed a clear preference for the drug paired chamber at either trials one or two. Two-way ANOVA showed no significant effect of time on levels of preference ($F_{(2,42)}$ = 0.52, p=0.60). Further Two-way ANOVA revealed no effect genotype on preference ($F_{(2,42)}$ = 1.0, p = 0.38) nor a time vs genotype interaction ($F_{(4,42)}$ = 0.65, p = 0.63).

| | *Preference for CS+ chamber (seconds ± SEM)* | | |
|---|---|---|---|
| *Trial Session* | *WT* | *$GABA_{B(1a)}^{-/-}$* | *$GABA_{B(1b)}^{-/-}$* |
| *Pretest* | -130.8 ± 348.5 | -115.5 ± 3941.4 | -152.9 ± 315.5 |
| *Test 1* | -410.4 ± 370.4 | 394.5 ± 307.6 | 93.30 ± 282.4 |
| *Test 2* | -177.3 ± 325.8 | 157.1 ± 168.1 | 289.9 ± 184.0 |

*Table 1 – Preference of Wildtype,* $GABA_{B(1a)}^{-/-}$ *and* $GABA_{B(1b)}^{-/-}$ *mice across the CPP paradigm. Preference data is described as the time in seconds spent in the drug paired chamber minus time spent in the saline paired chamber. Data is expressed as mean +/- SEM.*

### *7.3.3 Locomotor responses to cocaine in the CPP paradigm*

The locomotor response of WT, $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice in response to cocaine is shown in Figure 2. Two-way ANOVA of the total number of beam breaks revealed a significant effect of genotype ($F_{2,168}$=4.15, p<0.05). It is of note that this effect is time dependant, diminishing as the experiment progresses with two way ANOVA also revealing a significant effect of time ($F_{7,168}$=2.46, p<0.05). When total cumulative beam breaks were analysed using one way ANOVA, a significant effect of genotype was detected ($F_{2,26}$= 4.15, p<0.05). Post hoc analysis using the Newman-Keuls multiple comparison test revealed that $GABA_{B(1b)}^{-/-}$ mice generated more cumulative beam breaks compared to WT (p>0.05) mice and $GABA_{B(1a)}^{-/-}$ mice (p>0.05). WT mice and $GABA_{B(1a)}^{-/-}$ mice did not display significantly different numbers of beam breaks (p=0.70) (Fig 2A). Interestingly

when the locomotor activity of the animals in the CPP paradigm was analysed it was revealed that cocaine administration did not cause any locomotor activity following the first doses. Analyses of cocaine paired training sessions revealed a significant genotype-time interaction effect on beam break frequency ($F_{14,168}$=3.26, $p<0.0001$) indicating a locomotor sensitisation effect between genotypes. Furthermore, significant effects of genotype ($F_{2,168}$=8.54, $p<0.0001$) and time ($F_{7,168}$=6.66, $p<0.01$) were observed. When total cumulative beam breaks were analysed using one way ANOVA, a significant effect of genotype was detected ($F_{2,26}$= 8.55, $p<0.001$). Post hoc analysis using the Newman-Keuls multiple comparison test revealed that $GABA_{B(1b)}^{-/-}$ mice generated less cumulative beam breaks across the cocaine paired sessions than WT mice ($p<0.05$) and $GABA_{B(1a)}^{-/-}$ mice ($p>0.01$). $GABA_{B(1a)}^{-/-}$ mice trended towards an increased number of cumulative beam breaks relative to WT mice ($p=0.08$) (Fig 2B).

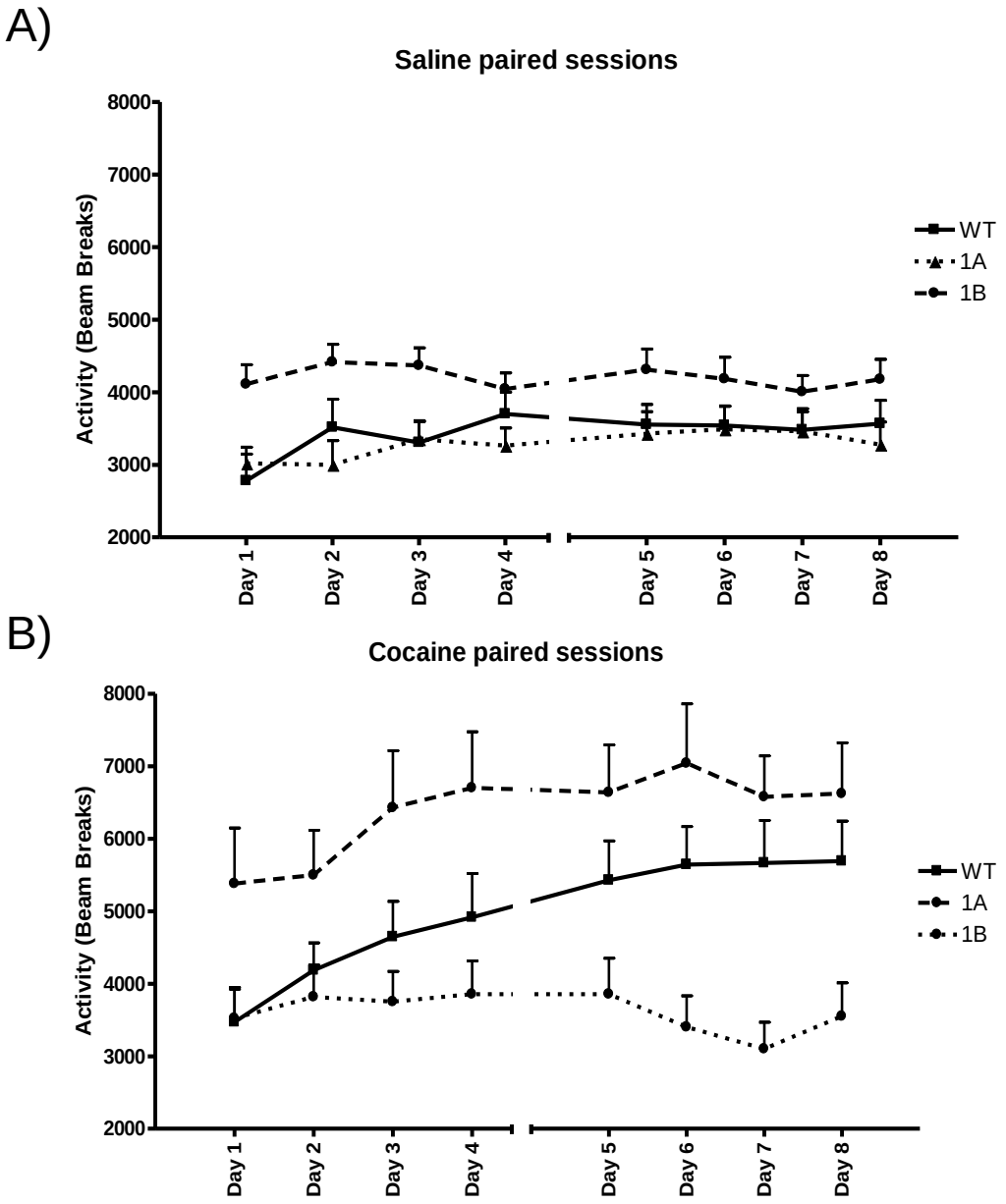


*Figure 2 – Cocaine induced hyperlocomotor response of Wildtype, $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice across the conditioning days of the CPP experiment. A) Locomotor response to saline across the conditioning period. B) Locomotor response to cocaine across the conditioning period. Data is measured as no. of beam breaks per 30 minute conditioning session and expressed as mean +/- SEM n=7-11*

### *7.3.4 Molecular responses to acute and repeated cocaine administration*

The levels of nucleus accumbens ΔFosB accumulation observed in WT, $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice is shown in Figure 3. One-way ANOVA of western blot analysis revealed no significant difference was observed between WT, $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ in terms of nucleus accumbens ΔFosB accumulation ($F_{2,23}$=2.5, p=0.10, n=8-9) (Fig 3) . An inadequate number of samples from the dorsal striatum were shown to contain pure HDAC protein via western blot for any meaningful statistical analyses. This may be due to difficulties in obtaining a pure nuclear fraction due to difficulties in extracting protein from a small sample of tissue such as the tissue collected for our studies.

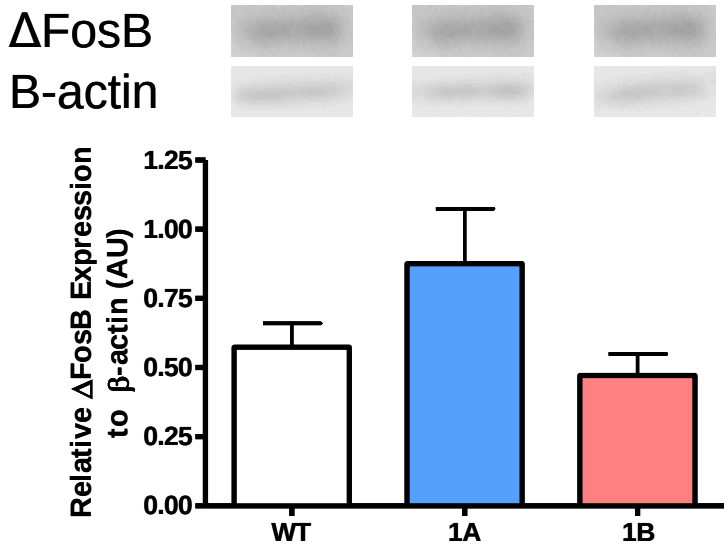


*Figure 3 – Levels of ΔFosB protein in the nucleus accumbens subsequent to the CPP experiment as measured by western blot. Protein levels are expressed in terms of densitometry of band relative to β-actin expression. Data is expressed in terms of mean +/- SEM.*

Levels of cFos expression in the dorsal striatum and nucleus accumbens of WT, $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice subsequent to acute cocaine injection is shown in Figure 4. In samples from the dorsal striatum of animals treated acutely with cocaine, students t-test revealed no significant effect of cocaine on cFos expression between wildtype saline and wildtype cocaine treated mice in either the dorsal striatum (p=0.32) or the nucleus accumbens (p=0.32). When cFos levels in the dorsal striatum and nucleus accumbens were compared between WT, $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice, one way ANOVA revealed no effect of genotype on cFos expression in either the dorsal striatum ($F_{2,17}$=0.03, p=0.97) or the nucleus accumbens ($F_{2,18}$=0.73, p=0.46).

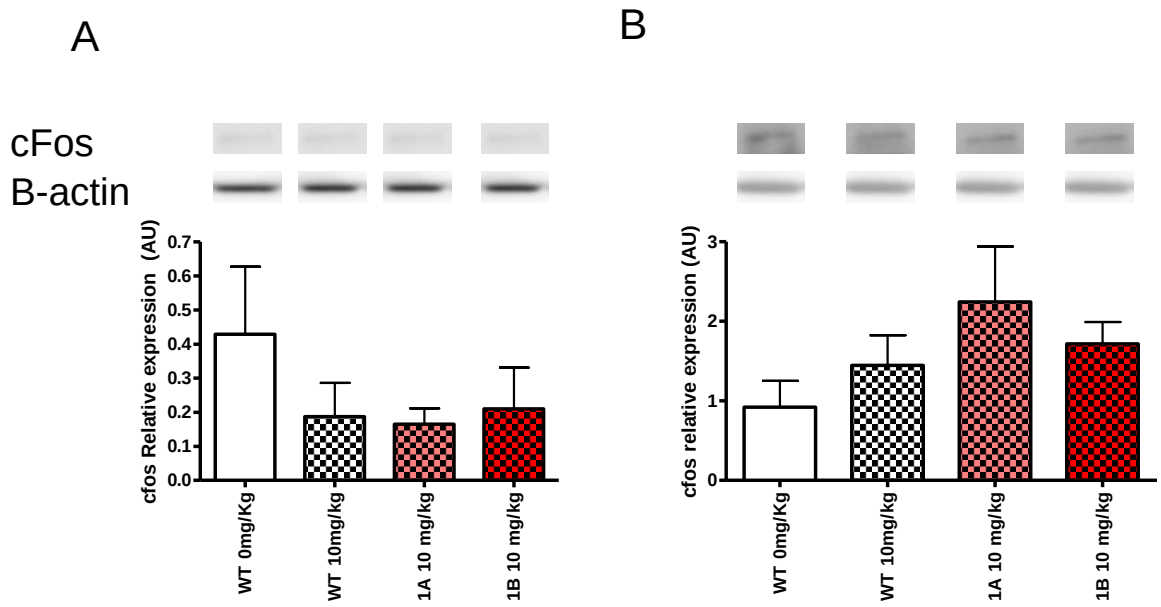


*Figure 4 – Levels of cFos protein in the A) the dorsal striatum and B) the nucleus accumbens 2 hours subsequent to acute cocaine injection (0/10 mg/kg) as measured by western blot. Protein levels are expressed in terms of densitometry of band relative to β-actin expression. Data is expressed in terms of mean +/- SEM.*

## *7.4 Discussion*

Although differing roles in regulating a variety of behaviours have been attributed to the $GABA_B$ receptor subunit isoforms, in this study we have, to our knowledge, for the first time demonstrated differing roles for the receptor subtypes in reward- and drug-related behaviour. In our study we clearly observed differing locomotor response patterns to cocaine in $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice. $GABA_{B(1a)}^{-/-}$ mice showed hypersensitive locomotor responses to acute cocaine, whereas $GABA_{B(1b)}^{-/-}$ mice responded normally relative to wildtype controls. Additionally, different patterns of sensitisation to the locomotor effects of cocaine were also observed in the $GABA_{B1}$ isoform null mice, with $GABA_{B(1a)}^{-/-}$ mice displaying increased levels of locomotor sensitisation relative to wildtype while $GABA_{B(1b)}^{-/-}$ mice did not exhibit any obvious sensitisation after repeated dosing. $GABA_{B(1b)}^{-/-}$ mice also displayed increased levels of basal locomotor activity than $GABA_{B(1a)}^{-/-}$ and wildtype mice at baseline, a replication of results previously seen in our lab (Jacobson et al., 2006a). Taken together, our results show that $GABA_{B(1a)}$ and $GABA_{B(1b)}$ receptor subunit isoforms differentially mediate at least some of the behavioural responses to cocaine administration and thus suggest that the two receptor isoforms differentially modulate dopaminergic neurotransmission *in vivo*.

Specifically, our data suggests that $GABA_{B(1a)}$ containing receptors play a crucial role in inhibiting the behavioural effects of cocaine, with $GABA_{B(1a)}^{-/-}$ mice displaying enhanced cocaine-induced hyperlocomotor activity as well as displaying a trend towards enhanced levels of cocaine-induced psychomotor stimulant sensitisation. On the other hand, mice lacking the $GABA_{B(1b)}$ subunit fail to develop sensitisation to the locomotor effects of

cocaine. Locomotor sensitisation is regarded as an animal model specifically of the clinical phenomenon of incentive sensitisation (Sanchis-Segura & Spanagel, 2006). Incentive sensitisation is the process wherein enhanced salience is attached to previously neutral drug associated cues. This is a widely accepted element of drug abuse in the clinic which forms an essential part of the transition from habitual drug consumption to drug abuse (Vanderschuren & Pierce, 2010). Our findings suggest that drug treatments aimed specifically at the $GABA_{B(1b)}$ receptor isoform may be of use in blunting or reversing this process in drug users. It must however be said that our data is taken from a CPP paradigm and that further confirmation of these findings with other chronic cocaine dosing regimens is required.

The opposing effects of $GABA_{B(1a)}$ and $GABA_{B(1b)}$ subunit ablation may be due to the differing patterns of anatomical localisation observed between the receptors . The $GABA_{B(1a)}$ isoform acts as both a heteroreceptor that inhibits glutamate release in response to spillover of GABA from GABAergic synapses as well as a postsynaptic GABA receptor, whereas the $GABA_{B(1b)}$ isoform appears to exclusively mediate the post-synaptic inhibitory effects of GABA (Vigot et al., 2006; Waldmeier et al., 2008). Recent evidence suggests that the sushi domain of the $GABA_{B(1a)}$ isoform may act as an axonal targeting signal (Biermann et al., 2010) and contribute to increased stability at the cell surface in comparison to $GABA_{B(1b)}$ containing receptors (Hannan et al., 2012). Enhanced cell surface stability of the $GABA_{B(1a)}$ subunit may underlie the non-sensitising phenotype of the $GABA_{B(1b)}^{-/-}$ mouse, in that the $GABA_{B(1b)}^{-/-}$ mouse is left a population of more stable

$GABA_{B(1a)}$ containing receptors and indeed that converse may be true in the $GABA_{B(1a)}^{-/-}$ mouse.

To our knowledge, studies designed to determine the precise anatomical location of the $GABA_B$ receptor isoforms in the dopaminergic system have yet to be performed. That said, it has been demonstrated that postsynaptic $GABA_B$ receptors couple to $K_{ir}3$ channels (Luscher et al. 1997), and that both $GABA_B$ receptors and $K_{ir}3$ channels are co-expressed on dopaminergic cell bodies in the VTA (Kalivas, 1993; Murer et al., 1997). We can posit based on our findings a model wherein the $GABA_{B(1b)}$ subunit inhibits tonic but not phasic dopaminergic tone, whereas $GABA_{B(1a)}$ containing receptors play a crucial role in inhibiting the enhanced phasic dopaminergic tone associated with acute cocaine administration. Indeed the failure of $GABA_{B(1b)}^{-/-}$ mice to sensitise to the locomotor effects of cocaine may be due to compensatory increases in $GABA_{B(1a)}$-containing receptor heterodimers. Detailed electron microscopy and electrophysiological studies will be necessary to determine the manner in which the receptor subtypes differentially modulate dopaminergic neurotransmission.

In contrast to our behavioural studies, our molecular studies demonstrated no significant differences between WT, $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice in terms of ΔFosB accumulation subsequent to the CPP paradigm, and cFos expression after acute cocaine treatment. This is surprising especially given the crucial role played by ΔFosB in mediating the neuronal and behavioural effects of drug of abuse exposure (Hiroi et al., 1997; Nestler, 2008) and in particular given the crucial role of ΔFosB in the nucleus accumbens in the

development of locomotor sensitisation to cocaine (Robison et al., 2013). Thus, we would have expected to observe reduced levels of ΔFosB expression in the $GABA_{B(1b)}^{-/-}$ mice and enhanced expression in the $GABA_{B(1a)}^{-/-}$ mice. It must however be said that in using western blot experiments, we have looked at the levels of ΔFosB throughout the brain regions in question, where as it has been previously demonstrated that ΔFosB accumulation occurs in specific neuronal subpopulations (Brenhouse & Stellar, 2006; Nestler, 2008). A more anatomically precise approach such as immunohistochemistry may have been a more accurate method for this purpose. Future studies into the role of the $GABA_{B(1)}$ isoforms in reward should examine the effects of cocaine on other molecular markers of drug exposure such as DARRP-32 and pCREB/CREB ratio (Lhuillier et al., 2007). The broad overall failure of acute cocaine injection to induce increases in cFos expression may be a result of context in which the drugs are administered, which has been shown to exert a significant influence over cfos responses to drugs of abuse in reward-related areas (Hope et al., 2006) or as a result of a behavioural anomaly in the BALB/c mouse strain, which has been characterised as having unusually weak behavioural responses to cocaine (Thomsen & Caine, 2011; Eisener-Dorman et al., 2011).

This data stands in contrast to our behavioural data. It must be stated however, that the small tissue samples used in this experiment resulted in difficulties in generating a pure nuclear extract from our samples. As such our molecular data must be interpreted with caution. The potential does exist that different neuronal subpopulations within the regions studies may underlie the behavioural differences between our genotypes. Furthermore, more precise molecular techniques such as immunohistochemistry for cFos (Singewald,

2007; O'Mahony et al., 2010) and ΔFosB (Nikulina et al., 2012) may reveal differences in neuronal activation patterns in a more precise manner. It must also be held in mind that several other molecular markers of chronic cocaine exposure such as DARRP-32 and pCREB/CREB ratio remain to be studied in these mice (Lhuillier et al., 2007).

We must point out that we have characterised the $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice in only a small subset of the available preclinical models of addiction. Further characterisation of these mice in terms of behaviour relating to other aspects of addition such as self-administration behaviour and behaviour in intracranial self-stimulation based paradigms remains an important future research direction. Likewise, further characterisation of the molecular responses to cocaine seen in these mice is crucial to our understanding of the role played by the $GABA_B$ receptor subunit isoforms in the biology underlying addiction.

An important caveat in the presented data is the failure of the $GABA_{B(1a)}^{-/-}$ mice, the $GABA_{B(1b)}^{-/-}$ mice and their wildtype controls to develop a place preference to cocaine. Similar results were also seen when animals generated in an identical manner were tested with 15 minute long testing and conditioning periods, using the same timeline and apparatus (see Appendix 3). This stands in sharp contrast to the preference developed in the same experimental procedure by $GABA_{B(2)}$-S892A mice and their wildtype controls (Chapter 6). Subtle effects of early life environment or epigenetic effects may have played a role in the difference between the animals. Indeed these mice differed from the mice used in the $GABA_{B(2)}$-S892A experiment in that they were produced via homozygous, as opposed to heterozygous breeding protocols. Handling issues have been highlighted as a

potential cause of failure in the development of conditioned place preference (Cunningham et al., 2006) and indeed a major difference was the levels of handling that mice were subjected to prior to the experiment, with the mice from the $GABA_{B(2)}$-S892A being exclusively handled from 3 weeks old by the same experimenter who performed the CPP experiment, whereas the $GABA_{B(1)}$ receptor isoform null mice were from a colony maintained by different individuals.

An important consideration regarding the use of conditioned place preference to examine cocaine reward sensitivity in the $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice is the lack of robust place preference to cocaine seen in the BALB/c mouse strain (Belzung & Barreau, 2000). The BALB/c mouse also displays lower levels of behavioural sensitivity to cocaine than other inbred mouse strains which may explain why our cfos western blots failed to shown an effect of cocaine (Thomsen & Caine, 2011; Eisener-Dorman et al., 2011). The background strain of a transgenic mouse can play a significant influence on behaviour (Jacobson & Cryan, 2007). However, it must be said that $GABA_B$ receptor knockout has thus far been shown to be lethal to mouse strains other than the BALB/c (Schuler et al., 2001) and ethical and financial constraints understandably have made researchers cautious to further experiment with other background strains. Given the vastly different levels of sensitivity to $GABA_B$ agonist treatment displayed by different mouse strains (Jacobson & Cryan, 2005) it would be unsurprising if differing effects of $GABA_B$ receptor mutation were seen across the spectrum of background strains.

In conclusion, the present data suggests that the two prevalent $GABA_B$ receptor subtypes play contrasting roles in regulating behaviours relevant to reward circuitry in the brain. Indeed, the current data suggest that inhibition of $GABA_{B(1b)}$ containing receptors or enhancement of $GABA_{B(1a)}$ containing receptors may be of potential therapeutic use in the treatment of drug addiction. However given the impossibility in pharmacologically distinguishing the receptor subtypes (Bräuner-Osborne & Krogsgaard-Larsen, 1999), it may require genetic silencing techniques such as RNA interference to exploit this knowledge in the clinic (Boudreau et al., 2011). In contrast, loss of the $GABA_{B(1a)}$ receptor isoform enhanced the hyperlocomotor responses to cocaine. Thus, both $GABA_{B(1a)}$ and $GABA_{B(1b)}$ isoforms contribute to control of dopaminergic neurotransmission, although the nature of the contribution is different for each isoform. These findings show that both $GABA_{B(1a)}$ and $GABA_{B(1b)}$ isoforms differentially regulate neurochemical processes that underlie the behavioural responses to drugs of abuse.

# *Chapter 8*

# *General Discussion and Conclusion*

## *8.1 General Discussion*

In this thesis the overarching goal was to further explore the role played by the $GABA_B$ receptor in modulating anxiety-like behaviour and cocaine sensitivity in mice with a translational perspective of anxiety and drug abuse disorders in the clinic. To this end, we used novel and established genetic and pharmacological techniques in combination with a host of preclinical behavioural assays.

Our first aim in this regard was to explore to role played by the $GABA_B$ receptor in the developmental priming of anxiety behaviour in the mouse, using pharmacological agents. Mice were treated in early life (P14-28) with the $GABA_B$ receptor agonist baclofen and the antagonist CGP52432 and the anxiety behaviour of these mice was tested in a battery of behavioural paradigms in adult life. We demonstrated that treatment in early life with the $GABA_B$ receptor agonist baclofen but not with CGP52432 results in altered anxiety behaviour in adult life (Chapter 2). To further examine the role of the $GABA_B$ receptor in the developmental processes underlying the adult anxiety behaviour, we treated mice in early life with the selective serotonin reuptake inhibitor fluoxetine, a known anxiogenic intervention (Ansorge et al., 2004, 2008; Karpova et al., 2009). We then examined the sensitivity of mice in adulthood to the hypothermic and ataxic effects of the $GABA_B$ receptor agonist baclofen. We found that fluoxetine treatment in early life does not result in alterations to $GABA_B$ receptor function during the same period (Chapter 3).

A second aim of this thesis was to examine the effects of $GABA_B$ receptor ligands on behaviour in experimental paradigms of conditioned fear. These effects had until this point

been poorly characterised in the literature. Using an established fear conditioning technique (Brinks et al., 2009; Bravo et al., 2011),we found that the $GABA_B$ receptor positive modulator GS39783 and the $GABA_B$ receptor antagonist CGP52432 do not exert behavioural effects on the acquisition, recall or extinction of cued or contextual conditioned fear (Chapter 4).

The next aim of the thesis was to characterise the anxiety behaviour and the cocaine sensitivity of a transgenic mouse line lacking the S892 phosphorylation site on the $GABA_{B2}$ subunit. $GABA_B$ receptors have been shown in vitro to display differing sensitisation and agonist potency properties to normal $GABA_B$ receptors (Bettler et al., unpublished observations). To this end, we examined the sensitivity of the $GABA_{B(2)}$-S892A mouse to acute and chronic baclofen treatment and also tested the behaviour of these mice in a battery of behavioural tests. Based on preliminary observations regarding the effects of S892 ablation on the function of $GABA_B$ receptors in the ventral tegemental area, a key region in mediating the behavioural effects of drugs of abuse, we also examined the sensitivity of these mice to the rewarding and locomotor effects of cocaine in a conditioned place preference paradigm. The $GABA_{B(2)}$-S892A transgenic mouse displays baclofen sensitivity, anxiety behaviour and cocaine sensitivity identical to those of WT littermates (Chapter 5, Chapter 6).

Further to these experiments, we characterised the cocaine sensitivity of mice wherein one isoform of the $GABA_{B1}$ subunit had been selectively ablated i.e. $GABA_{B(1a)}$ and $GABA_{B(1b)}$ receptor knockout mice. We demonstrated clearly that the $GABA_{B(1)}$ receptor

isoform knockout mice display markedly different locomotor sensitisation profiles in response to repeated cocaine treatment, with $GABA_{B(1a)}$ subunit knockout mice displaying enhanced levels of locomotor sensitisation to repeated cocaine administration, and $GABA_{B(1b)}$ subunit knockout mice displaying diminished locomotor sensitisation (Chapter 7).

## *8.2 The Role of the $GABA_B$ Receptor in the Development of Anxiety-Related Behaviour*

Prior to our studies significant amounts of preclinical data had demonstrated that the $GABA_B$ receptor played a crucial role in determining levels of anxiety-like behaviour in rodents (Cryan & Kaupmann, 2005). Furthermore some data had suggested that treatment with benzodiazepines in early life can result in altered anxiety behaviour in adulthood (Depino et al., 2008; Shen et al., 2012). It was unknown however, what role, if any, the $GABA_B$ receptor played in modulating the developmental processes that underlie the development of normal anxiety behaviour.

The major finding in this thesis is the demonstration that the $GABA_B$ receptor activation using exogenous agonists can result in enhanced anxiety behaviour in adult life (Chapter 2). This data agrees with current data demonstrating that $GABA_{B(1)}$ subunit isoforms play a crucial role in mediating the long term behavioural effects of maternal care disruption (Felice et al., 2012a). It also agrees with other studies that have examined the behavioural effects of the ionotropic $GABA_A$ receptor manipulation in early life. These studies have shown that treating mice in early life with benzodiazepines, positive modulators of the

$GABA_A$ receptor, results in enhanced anxiety behaviour in adult life (Depino et al., 2008; Shen et al., 2012).

Further research is required to elucidate the mechanism by which $GABA_B$ receptor activation alters adult anxiety behaviour. Indeed there are several possible mechanisms which may underlie this. One such mechanism by which the $GABA_B$ receptor may contribute to the developmental priming of anxiety behaviour is via interactions with the serotonergic system. A link between the $GABA_B$ receptor and the serotonergic system has been established by studies of anatomical co-localisation, electrophysiological and dialysis studies (Abellán et al., 2000). This link has also been shown by behavioural experiments demonstrating that $GABA_B$ receptor antagonists lose their behavioural effects in the absence of serotonergic function (Slattery et al., 2005a). The serotonergic system has been repeatedly demonstrated to play a crucial role in the neurological events in early life that programme adult anxiety behaviour. This has been repeatedly shown in mice using both pharmacological agents such as SSRI antidepressants, as well as by using time and brain region selective 5-$HT_{1A}$ receptor knockout mice. It has also repeatedly been shown in humans that polymorphisms in genes associated with the serotonergic system interact with early life environment to predict risk for psychiatric disorders in adult life (Nugent et al., 2011). Although the $GABA_B$ receptor has been shown to interact strongly with the serotonergic system, it must be noted that in this thesis, early life treatment with the SSRI fluoxetine has been shown to exert no effect on adult life $GABA_B$ receptor function (Chapter 3), suggesting that other mechanisms, such as interactions with other neurotransmitter systems or with the neuroendocrine system must be explored.

The $GABA_B$ receptor also displays robust interactions with the glutamatergic system, and these interactions may also underlie the developmental effects of baclofen seen in this study. These include interactions with the ionotropic NMDA receptors and the metabotropic glutamatergic receptors (mGluRs) (Tabata & Kano, 2010; Chalifoux & Carter, 2011). The relevance of these interactions in the neurodevelopmental processes underlying anxiety disorders remains unclear.

Another mechanism which may underlie the behavioural changes seen in our study is via the influence of the $GABA_B$ receptor on the neuroendocrine system. $GABA_B$ agonism has indeed been shown to induce a marked increase in growth hormone (GH) levels in healthy humans (Koulu et al., 1979). More interesting are studies that have shown that baclofen-induced increases in GH levels are attenuated in depressed patients, particularly in those with a blunted dexamethasone suppression test response (O'Flynn & Dinan, 1993). These data suggest a link between $GABA_B$ receptor function and hypothalamic-pituitary axis function. The potential thus exists for neuroendocrine alterations to underlie the anxiogenic effects of baclofen treatment. Examination of HPA-related hormone levels in baclofen-treated mouse pups and a study of long-term alterations in HPA axis function as a result of early life $GABA_B$ receptor agonism may prove interesting directions for further research.

Further studies into the developmental role of the $GABA_B$ receptor in anxiety may make use of genetic models where $GABA_B$ receptor function can be modulated in regionally and temporally selective manner. The studies would be similar in nature to studies carried out

on mice with a conditional knockdown of the 5-$HT_{1A}$ receptor (Gross et al., 2002). Based on preliminary findings from other groups, however, conditional knockdown of the $GABA_{B2}$ receptor appears to be a significant technical challenge (Bettler et al., unpublished observations). Another possible strategy for conditional knockdown of $GABA_B$ receptor function would be use of short interfering RNA (siRNA). Likewise other strategies such as engineering mice that can transiently over express $GABA_B$ receptor subunits may prove useful in the future. Another important extension of the data presented is the conduct of studies similar to those that examine the interactions between genetic variation in genes that govern serotonergic function and early life environment (Nugent et al., 2011), except examining the effects of GABAergic gene variance.

## *8.3 The Role of the $GABA_B$ Receptor in Learned Fear Responses*

Abnormal fear-learning is a clinical hallmark of anxiety disorders and although $GABA_B$ receptor-active ligands have been widely characterised in terms of their effects on unconditioned anxiety (Cryan & Kaupmann, 2005), to our knowledge, only two studies in the literature have examined the effects of $GABA_B$ receptor-active ligands in conditioned fear paradigms (Heaney et al., 2012; Li et al., 2013). Data presented in this thesis (Chapter 4) are an advance from these papers in that we use a different positive modulator (GS39783) to these papers as well as being the first study to characterise a newer generation highly-selective and potent $GABA_B$ receptor antagonist (CGP52432) in terms of its effects on fear conditioning.

Our finding that the highly selective and potent $GABA_B$ receptor antagonist CGP52432, is similar to what has been seen with the less potent $GABA_B$ receptor antagonist phaclofen

(Heaney et al., 2012). Likewise our finding that the $GABA_B$ receptor positive allosteric modulator GS39783 has no effect on conditioned fear behaviour is in agreement with findings regarding the similar modulator BHF117 (Li et al., 2013). Thus the collated finding so far relating to the utility of $GABA_B$ receptor antagonists and positive allosteric modulators in conditioned fear suggests that these drugs may not be efficacious in treating anxiety disorders characterised by pathological fear-learning.

Our data, taken with that of Li, Heaney and their colleagues (Heaney et al., 2012; Li et al., 2013), raises an interesting issue regarding the role played by GABA in the neurobiology of learned fear. *In vitro* studies have shown that GABA plays a central role in regulating neuronal activity and LTP development in the amygdala (Johansen et al., 2011; Wilensky et al., 1999). $GABA_B$ receptors do not appear to play a crucial role in these processes. Additionally, given the crucial role the $GABA_B$ receptor plays in cognition (Mondadori et al., 1993; Helm et al., 2005; Arai et al., 2009) and the fact that the neurocircuitry of fear conditioning overlaps with the circuitry that underlies other elements of cognition (Fendt & Fanselow, 1999), the absence of effect of $GABA_B$ receptor-active agents on conditioned fear in our studies raises the question of how the neural circuitries that underpin fear-learning and cognition more broadly interact.

## *8.4 Is There a Functional Role for the S892 Residue of $GABA_{B(2)}$ Receptor Subunit?*

Data presented in this thesis suggests that the $GABA_{B(2)}$-S892A mouse line displays a baclofen sensitivity, anxiety behaviour and cocaine sensitivity indistinct from its wildtype littermates (Chapters 5 and 6). This is somewhat surprising given the role played by

phosphorylation at the S892 in determining levels of receptor agonist potency and maintenance of cell surface stability. Indeed, the S892 residue has been shown to undergo significant basal PKA dependent phosphorylation in vivo, and phosphorylation of the residue has been shown to play a crucial role in inhibiting agonist induced receptor desensitisation in vitro (Couve et al., 2002). Furthermore, rates of $GABA_B$ receptor degradation have been correlated to levels of S892 residue phosphorylation (Fairfax et al., 2004). Data obtained from Xenopus oocytes conversely suggests that PKA mediated phosphorylation can effectively desensitise the receptor (Yoshimura et al., 1995).

A reason as to why no differences in behaviour were observed throughout our studies between the $GABA_{B(2)}$-S892A mouse and its wildtype littermates may be the presence of compensatory changes in the function of alternative regulatory pathways for $GABA_B$ receptor function. These other mechanisms may include protein-kinase C mediated regulation of receptor function, AMPK mediated phosphorylation or indeed CamKII mediated phosphorylation (Pontier et al., 2006; Kuramoto et al., 2007; Guetg et al., 2010; Terunuma et al., 2010a).

An even more intriguing mechanism by which compensatory modulation of the $GABA_B$ receptor could occur is via interactions with potassium channel tetramerisation domain (KCTD) proteins. These proteins are a relatively novel discovery and may indeed prove to be one of the crucial elements in determining the basis of $GABA_B$ receptor heterogeneity across the brain. These proteins have been shown to directly interact with the $GABA_B$ receptor heterodimer and have been shown to strongly influence both the processes that

underlie fast receptor sensitisation and play a crucial role in determining agonist potency for the $GABA_B$ receptor (Schwenk et al., 2010). The fact that theses proteins are differentially distributed in terms of both brain region and neurodevelopmental period (Metz et al., 2011) strongly suggests that the KCTDs play an influential role in determining $GABA_B$ receptor heterogeneity. It must however be stated that, as our data from the $GABA_{B(2)}$-S892A mouse suggests, significant levels of redundancy may exist in the processes that govern $GABA_B$ receptor function. One regulatory pathway is unlikely to be the sole determinant of heterogeneity. Indeed in light of these advances it becomes apparent that receptor phosphorylation only forms one part of a highly complex system that underlies the functional regulation of the $GABA_B$ receptor. As such characterisation of the $GABA_{B(2)}$-S892A mouse is in some ways confounded by the existence of possible compensatory changes in other molecular pathways.

Although the $GABA_{B(2)}$-S892A mouse has been extensively characterised in terms of its basic pharmacological sensitivity to baclofen as well as its anxiety behaviour, antidepressant-like behaviour and sensitivity to the rewarding effects of cocaine in this thesis, the potential for further characterisation of this animal remains. Indeed, as regards its basic pharmacology, examination via electrophysiological techniques in vivo may provide a more thorough picture than seen with the basic physiological responses to baclofen used in this thesis. Given the well-established behavioural effects of $GABA_B$ receptor positive modulators and antagonists in behavioural tests of anxiety, antidepressant activity and cocaine addiction (Cryan & Kaupmann, 2005; Lhuillier et al., 2006), it would be of interest to determine the behavioural response of the $GABA_{B(2)}$-S892A mouse in relevant

behavioural assays. The $GABA_B$ receptor has additionally been shown to modulate animal behaviour in terms of cognition (Jacobson et al., 2007b) as well as nociception and seizures (Ong & Kerr, 2000). Characterisation of the $GABA_{B2}$-S892A as regards these fields of preclinical research remains to be accomplished. The recent discovery that the $GABA_{B(1a)}$ and $GABA_{B(1b)}$ null mice are differentially sensitive to the effects of early life stress (Felice et al., 2012a) raises the further question as to whether the $GABA_{B(2)}$-S892A mouse would differ from wildtype animals in their own sensitivity to early life deprivation and represents another future research direction.

## *8.5 Addiction*

### *8.5.1 The $GABA_B$ receptor in cocaine addiction*

Prior to the studies presented in this thesis, a wealth of preclinical and clinical data had implicated the $GABA_B$ receptor as a putative target for novel anti-addictive therapies (Tyacke et al., 2010), as well as being a crucial substrate in the reward circuitry of the brain (Vlachou & Markou, 2010). The main finding as regards the role of the $GABA_B$ receptor in cocaine addiction is our data demonstrating altered locomotor sensitisation patterns for the $GABA_{B(1a)}$ and $GABA_{B(1b)}$ mice compared to WT mice. Our data suggests that the $GABA_{B(1b)}$ subunit is a crucial mediator in the development of the locomotor sensitisation to cocaine. Locomotor sensitisation is regarded as an animal model specifically of the clinical phenomenon of incentive sensitisation (Sanchis-Segura & Spanagel, 2006). Incentive sensitisation is the process wherein enhanced salience is attached to previously neutral drug associated cues. This is a widely accepted element of drug abuse in the clinic which forms an essential part of the transition from habitual drug consumption to dug abuse (Vanderschuren & Pierce, 2010). Our findings suggest that drug treatments aimed specifically at the $GABA_{B(1b)}$ receptor isoform may be of use in blunting or reversing this process in drug users. It must however be said that our data is taken from a CPP paradigm and that further confirmation of these findings with other chronic cocaine dosing regimens is required.

It must be acknowledged that in our studies we have only investigated the behavioural effects of our three transgenic mice lines in but a few of the many behavioural paradigms of drug addiction available (see Introduction, Table 7). The most obvious continuation from

the studies in this thesis is an examination of the $GABA_{B(2)}$-S892A and the $GABA_{B(1)}$ isoform specific knockout mice in other commonly used animal models of addiction such as the self-administration and intracranial self stimulation based procedures. Microdialysis studies assessing the release of dopamine in reward-related forebrain structures such as the nucleus accumbens and dorsal striatum in response to drugs of abuse in these animals may also be of interest.

It must also be borne in mind that this thesis has focussed exclusively on cocaine, which is but one of a plethora of commonly abused drugs. The $GABA_B$ receptor has also been particularly of interest as a putative drug target in the treatment of alcohol use disorders (Agabio et al., 2012). Indeed significant preclinical evidence has also suggested that the $GABA_B$ may represent a potential therapeutic avenue in smoking cessation therapy, with $GABA_B$ receptor antagonist displaying anti-addictive effects in several animal models (Paterson et al., 2008; Mombereau et al., 2007; Vlachou et al., 2011a). Assessment of the sensitivity of the transgenic mouse lines characterised in this thesis in terms of their sensitivity to the behavioural effects of alcohol represents another important future research direction.

## *8.6 Genetic Models of Altered $GABA_B$ Receptor Function – General Caveats*

### *8.6.1 Transgenic mouse models*

Certain qualifications are always associated with the use of transgenic mice in $GABA_{B2}$-S892A, $GABA_{B(1a)}$ and $GABA_{B(1b)}$ null mice, can have developmental effects not readily distinguishable from the effects of the genetic alteration in adult life. This is all the more an

important reservation in the interpretation of the findings of this thesis given the now-known developmental role of the $GABA_B$ receptor in determining adult behaviour (Felice et al., 2012a). A possible solution to this caveat is the development of siRNA and viral over expression based techniques as well as Tet-on/off conditional knockdown models allowing for a temporally control of $GABA_B$ receptor modulation.

A recurring issue in the use of genetic models of altered $GABA_B$ receptor function is that of background strain. The simple fact that $GABA_B$ receptor knockout is only achievable using the BALB/c mouse strain as a background (Schuler et al., 2001), presents significant caveats to the interpretation of data from this strain. Inbred mice strains vary significantly in their responses to common behavioural tests and the effects of an intervention in one mouse strain do not necessarily occur in others. Indeed the BALB/c mouse strain displays in some ways an unusual behavioural repertoire in tests of anxiety and depression, with some researchers going as far as to utilise them as an animal model of anxiety (O'Mahony et al., 2010). The behavioural effects of genetic and pharmacological interventions must thus be interpreted with caution. A further caveat to the use of inbred mouse strains in studying the role of the $GABA_B$ receptor in behavioural tests is the presence of significant variance as regards $GABA_B$ receptor function between mouse strains (Jacobson & Cryan, 2005).

### *8.6.2 Optogenetic mouse models*

Another crucial technology in unravelling the role played by the $GABA_B$ receptor in psychiatric illness form a preclinical perspective is the advent of optogenetic technologies.

This technology is based on the use of transgenic mice expressing light activated channel-rhodopsins in which can be targeted to neuronal populations using cell type specific promoters allowing for their activation or inhibition by direct light exposure (Deisseroth, 2011). Optogenetics provides an opportunity for unprecedented levels of temporal and spatial specificity for intervention in the mammalian brain and is already providing important insights into the neural circuitry of sleep and arousal, affective states and addiction (Touriño et al., 2013). Most recently optogenetic technology has been used to demonstrate a crucial role for GABAergic neurons in the VTA in signalling expected reward (Cohen et al., 2012), as well as a crucial role for GABAergic neurons in the raphe in mediating social avoidance behaviour (Challis et al., 2013). Further characterisation of the GABAergic neuronal populations in the brain via optogenetics, in particular $GABA_B$ receptor expressing populations will no doubt lead to further unravelling of the role played by the $GABA_B$ receptor in anxiety and substance abuse disorders.

### *8.6.3 Behavioural models of psychiatric illness*

In these studies we have made extensive use of animal models of psychiatric disease. It must be remembered that the use of these paradigms is not without their own limitations. These include questions as to the pharmacological sensitivity of these tests i.e. whether they are merely assays of benzodiazepine-like pharmacological effect as opposed to actual models of human disease (Rodgers, 2010; Cryan & Sweeney, 2011). The battery style approach employed in the studies presented provides several advantages in terms of reducing total numbers of animals used in our studies and increasing the likelihood of detecting subtle behavioural differences between treatment groups or genotypes, but does

come with the caveat that prior test experience can profoundly alter animal behaviour in subsequent tests (Cryan & Holmes, 2005). Like animal models of anxiety, animal models of cocaine addiction such as the CPP paradigm and locomotor sensitisation come with their own caveats (Sanchis-Segura & Spanagel, 2006).

### *8.6.4 Baclofen challenge as an output of $GABA_B$ receptor function*

Another major caveat to our study is the relative crudeness of the *in vivo* baclofen challenge as an output for measuring $GABA_B$ receptor function. Although treatment with baclofen produces robust and easily measurable physiological effects such as hypothermia and ataxia (Chapter 3, Chapter 5), considerably more sophisticated techniques exist to elucidate $GABA_B$ receptor function. This is especially important given that the $GABA_B$ receptors present in different brain regions, and even in different neuronal subpopulations, can differ in their pharmacological properties (Pinard et al., 2010). It must however be said that measuring the hypothermic effects of baclofen has proved an important clinical output for assessing levels of $GABA_B$ sensitivity. More sophisticated and sensitive techniques may include electrophysiology (Abellán et al., 2000), iGTP activity assays (Germany, 1993), and baclofen binding studies across the whole brain (Chu et al., 1990).

## *8.7 Perspectives and Conclusion*

The data presented in this thesis advances our understanding of the $GABA_B$ receptor as a moderator of the disease processes associated with anxiety disorders and cocaine addiction. In particular, our data suggests a role for the $GABA_B$ receptor in the developmental

processes that underlie adult anxiety behaviour and that the $GABA_B$ receptor isoforms may indeed play differential roles in the development of incentive salience, an important aspect of drug addiction. Our data furthermore suggests a limited role for $GABA_B$ receptor pS892 levels in regulating behaviour and a limited effectiveness for $GABA_B$ receptor-active ligands in modulating conditioned fear behaviour.

Recent years have seen a paradigm shift in our understanding of $GABA_B$ receptor pharmacology. The discovery of the KCTDs has transformed our understanding of the nature of $GABA_B$ receptor function in vivo and revealed that the classical heterodimer structure of the $GABA_B$ receptor is better understood as a multimeric complex of several associated proteins which exert significant influence over $GABA_B$ receptor function and may indeed underlie to a significant degree the heterogeneity of the $GABA_B$ receptor across the brain (Schwenk et al., 2010; Metz et al., 2011). Further probing of this and similar phenomena may represent the direction for future behavioural pharmacology regarding the $GABA_B$ receptor with characterisation of KCTD family knockout mice representing an obviously tempting avenue.

It must be remembered, however, that advances in preclinical behavioural pharmacology must be paralleled by advances in clinical understanding of psychiatric illness (Cryan & Sweeney, 2011). This must not only include more accurate, biomarker-based diagnostic systems, but also based development of our knowledge of $GABA_B$ receptor and other GABAergic gene polymorphisms in the population and how these contribute to the risk of anxiety disorders and drug dependence. It is only with a multidisciplinary approach involving both *in vitro* and behavioural pharmacologists, pharmaceutical chemists and

clinicians that will allow our current understanding of the $GABA_B$ receptor to bring benefit to patients.

# *Appendices*

## *Appendix 1 - Genotyping of $GABA_{B(2)}$-S892A Mice*

Genotyping was performed on offspring on tail clip samples taken from animal at ages 6-8 weeks. Tail clips were digested in 200µl non-ionic detergent (NID) buffer (50mM KCl; 10mM Tris-HCl, pH 8.3; 2mM MgCl2; 0.1 mg/ml gelatin; 0.45% NP40; 0.45% Tween-20)overnight at 56°C with addition of 3µl Proteinase K (sigma) solution 10 mg/ml. The digestion mix was then heated to 96° for 10 minutes, prior to centrifugation at 14800 rpm at room temperature for 5 minutes. The resulting supernatant was stored at -20°C prior to Polymerase Chain Reaction targeted at Exon 19 of the $GABA_B2$ gene. Primer sequences used were:

Sense - 5'-CAC TCG GAG GCT TGT GTA AC-3'

Antisense - 5'-TCC AAT GGA CGG GAG GTA GG-3'

PCR on the samples was carried out using the reaction mixture:

| | |
|---|---|
| Jumpstart buffer | 2.0µl |
| Forward primers (10mM) | 1.0µl |
| Reverse primers (10mM) | 1.0µl |
| 10x dNTPs (each dNTP at 2.5 mM) | 0.5µl |
| Jumpstart polymerase (Dq307 sigma) | 0.5µl |
| $dH_2O$ | 14µl |

Cycling parameters:

| | | |
|---|---|---|
| Step 1 | 94 | 2 min |
| Step 2 | 94 | 1 min |
| Step 3 | 55 | 1 min |
| Step 4 | 72 | 1 min |
| Step 5 | Go to step 2 for 34 cycles | |
| Step 6 | 72 | 5 min |
| Step 7 | 8 | pause |

The products from the PCR reaction were stored at -20°C prior to gel electophoresis.

Gel electrophoresis was carried out using a 2% agarose gel, for 60 minutes at 150V. Visualisation of bands was achieved by incubation of the gel in GelRed solution for 20-30 minutes and visualised under UV light using Gel Capture software. The wildtype allele formed a 514bp band with the S892A allele forming a band at 670 bp (Fig 1b).

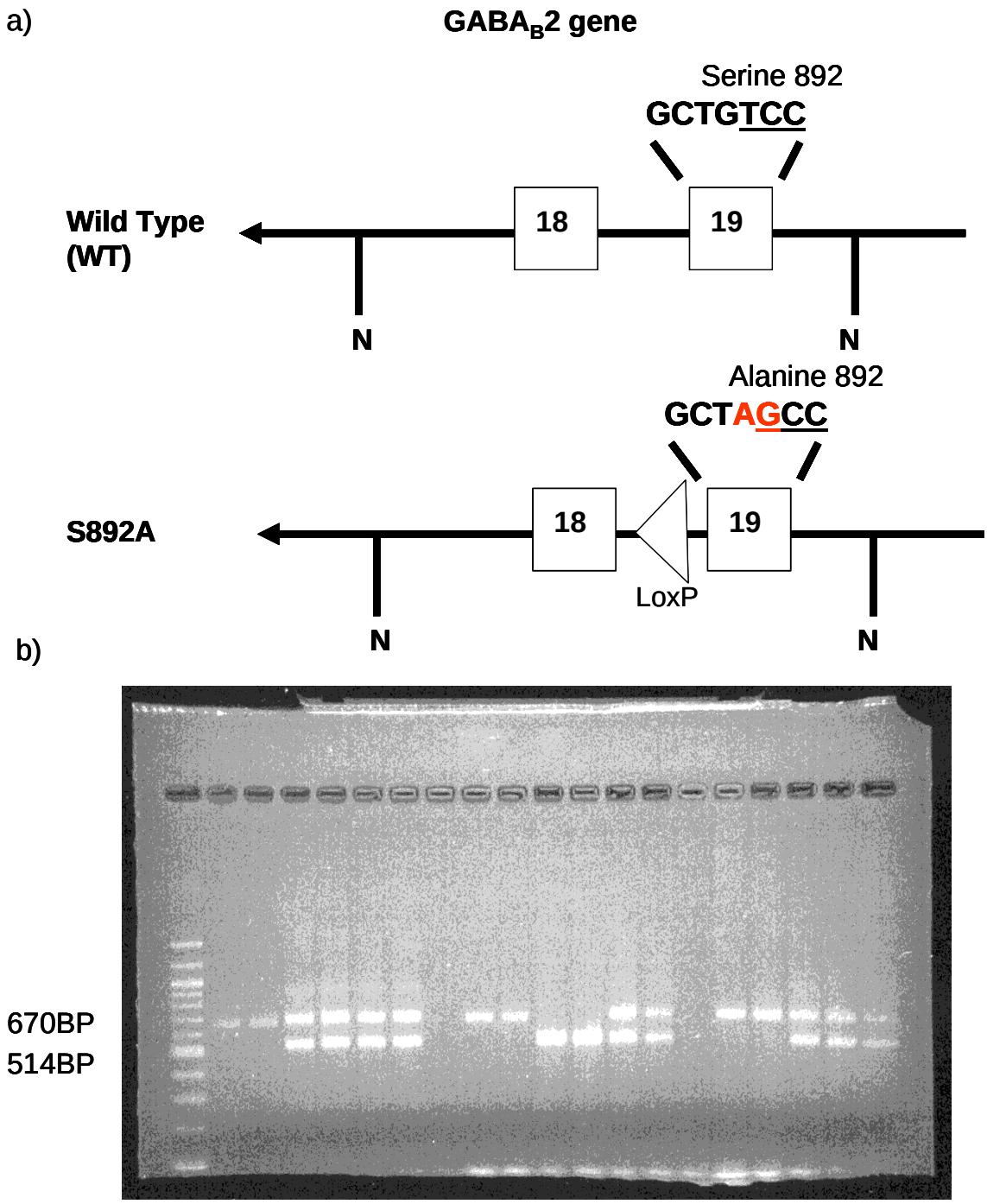


**Figure 1**

a) The GABA$_{B(2)}$-S892A mutant mouse contains a serine to alanine mutation at the serine 892 site in exon 19 of the GABA$_B$R2 gene, rendering the generated protein

unphosphorylatable at this site. The S892A mutant allele also contains an inserted LoxP site, a relic of the early stages of the mutation insertion process, which allows the S892A gene to be distinguished from the wildtype through molecular weight via electrophoresis. b) When PCR products of the $GABA_BR2$ gene are separated by gel electrophoresis, WT bands appear at 514bp and the S892A allele appears at 670bp. Heterozygous mice are identified by the presence of both bands.

## *Appendix 2 – Validation of Conditoned Place Preference Protocol*

In order to validate our CPP protocol as described in chapters 6 and 7, we initially tested 8 BALB/c mice from Harlan, UK (age6-8 weeks, with one week acclimatization to the lab). The CCP apparatus was as described in chapters 6 and 7, and 10 mg/kg of cocaine (IP injection) as our conditioned stimulus. Animals were randomly assigned to receive cocaine in the white or black chamber randomly with 4 animals being paired to black and 4 to white. Animals were allowed one 30 minute acclimatization session, followed by a pretrial session. Animals were allowed to freely explore the entire apparatus for 30 minutes. We analysed the behaviour of these animals to determine if any bias existed for either choice chamber. Behaviours analysed were the total time spent in the black and white "choice" chambers, total entries  the black and white "choice" chambers, exploration (breaks of the beam closest to the entrance of the chamber i.e. nose pokes) frequency of each chamber, activity (beam breaks of adjacent beams to the not moving away from the curent location) in each chamkber and movement (breaking a new beam and moving away from the current location). The results of our analyses are shown in Figure 1.

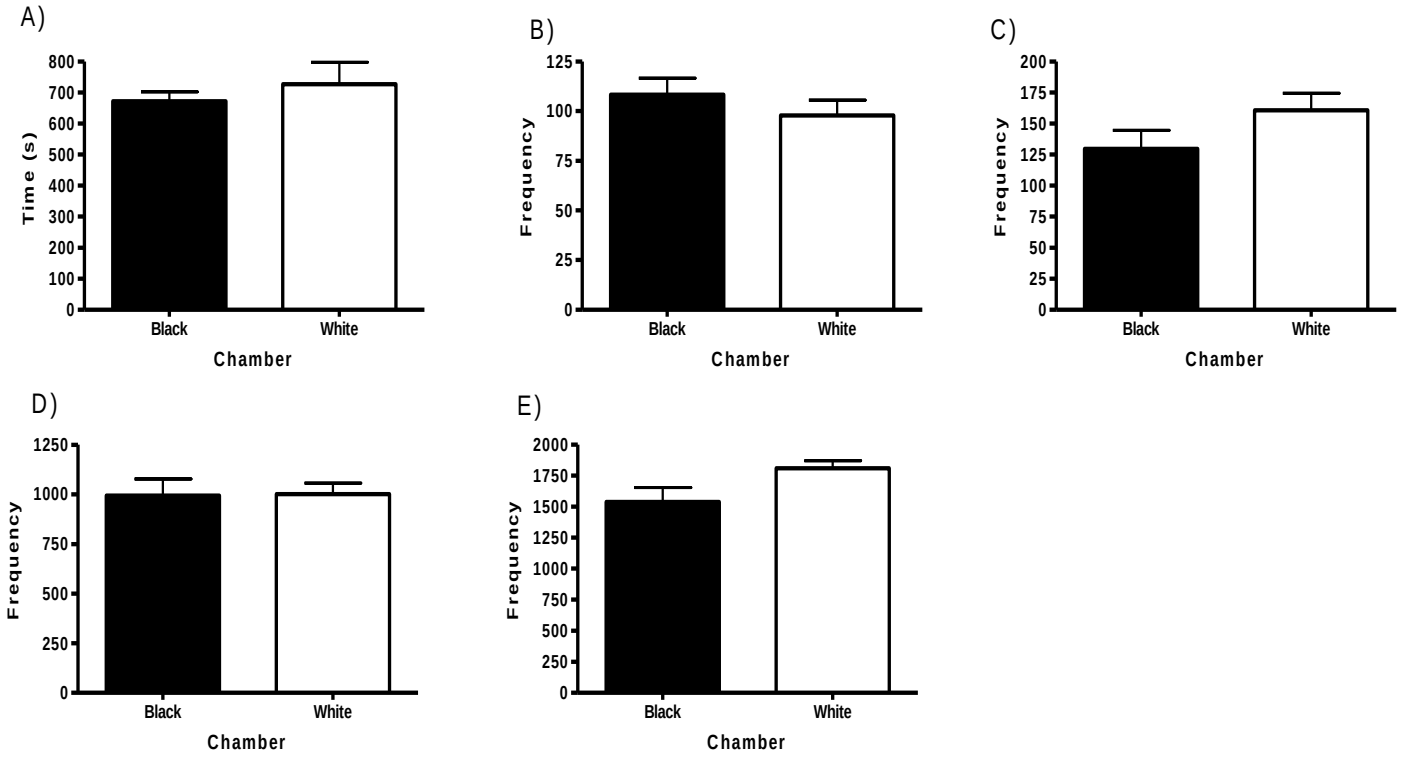


*Figure 1 – Baseline preference of BALB/c mice for the two "choice" compartments of the CPP apparatus. A) Total time spent in the white and black "choice" compartments, B) Total frequency of entries to the white and black "choice" compartment, C) Total exploration frequency in the white and black "choice" compartments, D) Total activity frequency in the white and black "choice" compartment and E) Total exploration frequency in the white and black "choice" compartments s. Data is presented as mean +/- SEM, n=8*

Students t-test revealed that although a trend towards more movement counts were observed in the white chamber ($p=0.06$) (possibly due to the novel odour), time spent in the chambers ($p=0.50$), entries to to the chambers ($p=0.38$), explorations of the chambers ($p=0.15$) and activity in the chambers ($p=0.95$) did not differ between compartments. We therefore accepted our CPP apparatus as unbiased.

Subsequent to pretesting mice place preference conditioning was performed as per chapters 6 and 7, with the test ending on the first assessment of place preference behaviour. Behviours analysed were identical to those in the pretest although with the difference that we examined preference for CS+ over CS- chambers. The results of our analyses are shown in Figure 2.

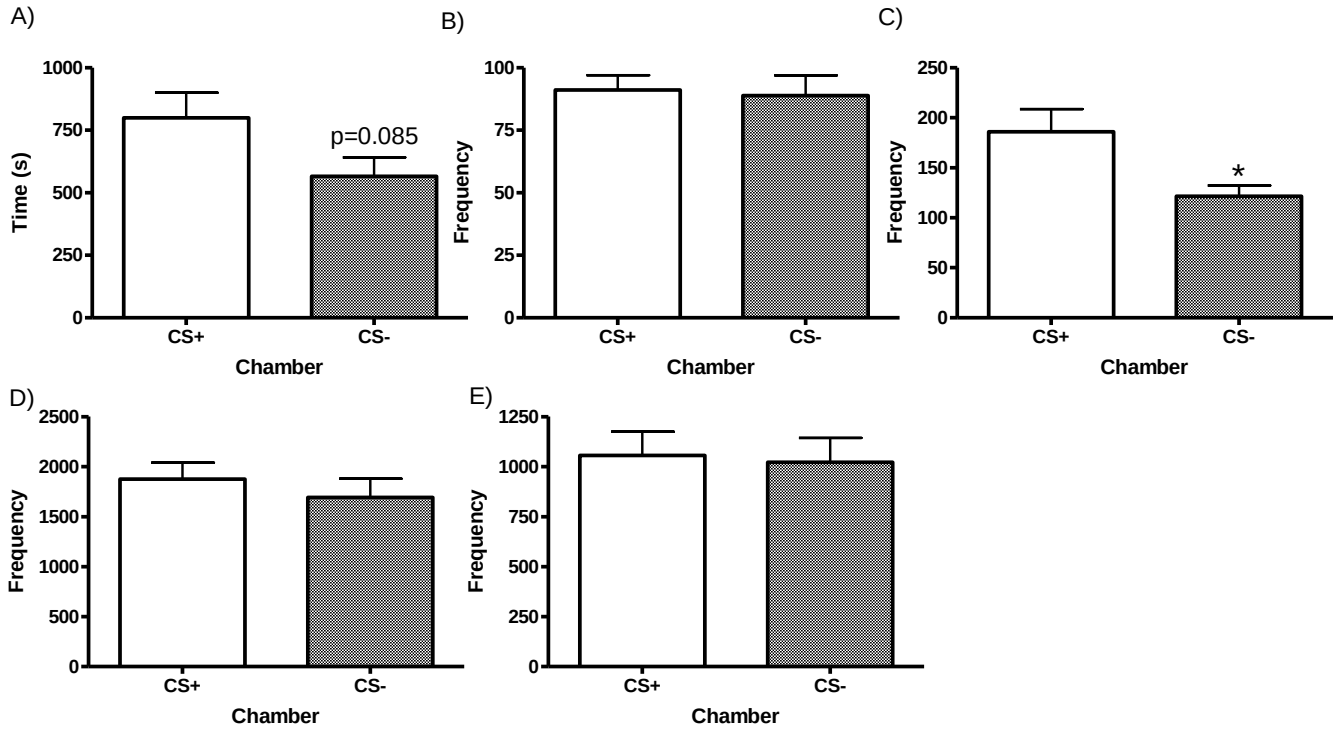


*Figure 2 – Conditioned place preference behaviour of BALB/c mice for the CS+ and CS- compartments. A) Total time spent in the CS+ and CS- compartments, B) Total frequency of entries to the CS+ and CS- compartments, C) Total exploration frequency in the CS+ and CS- compartments, D) Total activity frequency in the CS+ and CS- compartments and E) Total exploration frequency in the CS+ and CS- compartments. Data is presented as mean +/- SEM, n=8, p>0.05 is represented as *.*

Students t-test revealed that mice in our prelimninary study displayed significantly more explorations of the CS+ chamber ($p<0.05$) and trended towards spending more time in the CS+ chamber ($p=0.085$). Total chamber entries ($p=0.83$), total activity frequency ($p=0.47$) and total movement frequency ($p=0.84$) did not differ between the CS+ and CS- chambers after place preference condition

## *Appendix 3 – Preliminary CPP Characterisation of $GABA_{B(1)}$ Receptor Isoform Null Mice*

WT, $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$, as described in chapter 7, were examined using the same CPP protocol as utilised in Chapter 6 and 7, with the difference that all sessions were of 15 minutes as opposed to 30 minutes. Statistical analysis was performed as per chapter 7. Preference data is presented in Figure 1.

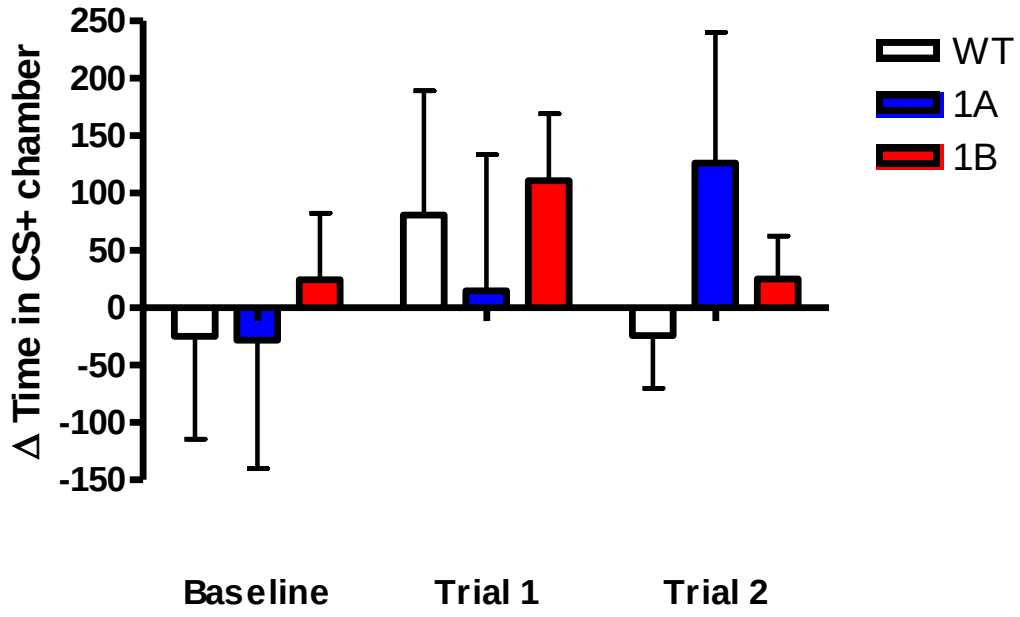


*Figure 1 - Preference of Wildtype,* $GABA_{B(1a)}^{-/-}$ *and* $GABA_{B(1b)}^{-/-}$ *mice across the CPP paradigm. Preference data is described as the time in seconds spent in the drug paired chamber minus time spent in the saline paired chamber. Data is expressed as mean +/- SEM. N=8-9*

Levels of preference of WT, $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice for the CS+ chamber are displayed in Table 1. WT, $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice displayed no preference for

either the CS+ or CS- chambers of the CPP apparatus at baseline, however none of the groups mice developed a clear preference for the drug paired chamber at either trials one or two. Two-way ANOVA showed no significant effect of time on levels of preference ($F_{(2,42)}$ = 1.3, p=0.28). Further Two-way ANOVA revealed no effect genotype on preference ($F_{(2,42)}$ = 0.11, p = 0.90) nor a time vs genotype interaction ($F_{(4,42)}$ = 0.99, p = 0.42). The use of a 15 minute protocol may have introduced a pharmacokinetic issue, in that mice reached peak CNS cocaine levels after the conditioning phases were over. In light of the failure of this method to generate robust place preference we extended conditioning and test sessions to 30 minutes for the experiments described in chapters 6 and 7.

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