

**The Role of GABA_{B(1)} Receptor Isoforms in Anxiety and Depression:
Genetic and Pharmacological Studies in the Mouse**

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Summary

Anxiety and depression disorders represent common, serious and growing health problems world-wide. The neurobiological basis of anxiety and depression, however, remains poorly understood. Further, there is a clear need for the development of better treatments for these disorders. Emerging data with genetic and pharmacological tools supports a role for GABA_B receptors in both anxiety and depression. GABA_B receptors are metabotropic GABA receptors that are comprised of two subunits, GABA_{B1} and GABA_{B2}, which form heterodimers. The GABA_{B(1)} gene is transcribed into two predominate isoforms, GABA_{B(1a)} and GABA_{B(1b)} which differ in sequence primarily by the inclusion of a pair of sushi domains (or short consensus repeats) in the GABA_{B(1a)} N-terminus. Both isoforms heterodimerize with GABA_{B2} subunits to form functional receptors. The two GABA_{B(1)} isoforms and the GABA_{B(2)} subunit constitute the majority of the molecular diversity of the GABA_B receptor. However, in the absence of any isoform-selective ligands for research, the behavioural function of mammalian GABA_{B1} receptor isoforms has been inscrutable.

Recently mice deficient in GABA_{B(1a)} and GABA_{B(1b)} isoforms were generated. Aspects of anxiety- and depression-related behaviour may be modelled in mice, by using traditional animal models, and by examining specific biological and behavioural components of the human symptomatology, or 'endophenotypes'. A preliminary aim of this thesis was to determine the utility of GABA_{B(1)} isoform-deficient mice for the dissection of GABA_{B(1a)} and GABA_{B(1b)} isoform-mediated behaviour. The main aim of this thesis was to test the hypothesis, using a combination of traditional and endophenotype murine models, that GABA_{B(1)} receptor isoforms play an important role in the mediation and anxiety and depression-related behaviour.

GABA_{B(1)} Isoforms in GABA_B Receptor Function

Preparatory work in this thesis examined the influence of genetic background on GABA_B receptor-mediated responses. Genetic background, in the form of different mouse strains, had a strong, differential effect on the classic responses to GABA_B receptor activation; hypothermia and ataxia. This underlined the necessity of including multiple experimental endpoints in the examination of GABA_B receptor function in subsequent work with GABA_{B(1)} isoform-deficient mice. Importantly, this study also demonstrated that the BALB/c mouse strain was an appropriate genetic background for carrying the GABA_{B(1)} isoform mutations.

Initial studies with GABA_{B(1)} isoform-deficient mice demonstrated that they were free of gross sensory-motor deficits that may preclude their application in behavioural tasks. Furthermore, GABA_{B(1a)} and GABA_{B(1b)} diverged in their influences on locomotor responses to novelty and circadian activity, although the GABA_B receptor agonists baclofen or γ -hydroxybutyrate (GHB) were not specific for either isoform and were unable to discriminate these differences. These findings demonstrated that the GABA_{B(1)} isoforms had differential influences on behaviour. Together these studies demonstrated that the GABA_{B(1a)}^{-/-} and GABA_{B(1b)}^{-/-} mice were applicable for testing the hypothesis that the GABA_{B(1)} isoforms were differentially implicated in anxiety and depression related behaviour.

GABA_{B(1)} Isoforms in Endophenotypes of Anxiety and Depression

Deletion of GABA_{B(1a)} and GABA_{B(1b)} isoforms had profound, differential impacts on the acquisition (GABA_{B(1a)}) and extinction (GABA_{B(1b)}) of aversive memories, as determined in a conditioned taste aversion paradigm. These effects, however, were not accompanied by differences in innate anxiety, as assessed in a comprehensive test battery of unconditioned anxiety tests, including autonomic (stress-induced hyperthermia), active (marble burying) and passive exploratory avoidance (staircase, light-dark box, elevated plus maze, elevated zero maze) behavioural readouts. There was no evidence for a specific influence of either isoform in these tests. This indicated that the GABA_{B(1)} isoforms themselves did not have a defining role in innate anxiety.

GABA_{B(1a)}^{-/-} and GABA_{B(1b)}^{-/-} mice diverged in their cognitive phenotypes. GABA_{B(1a)}^{-/-} mice were impaired in tasks of working spatial and recognition memory, but not in passive avoidance. GABA_{B(1b)}^{-/-} mice were also impaired, to a lesser degree, in a working spatial memory task, but showed preservation of working recognition memory and passive avoidance. Long term recognition memory, however, was also impaired in these mice.

The GABA_{B(1a)} isoform was specifically implicated in depression-related behaviour, as indicated by reduced immobility in a classic test of antidepressant-like behaviour – the forced swim test. This was most probably mediated *via* the striking interactions of the GABA_{B(1a)} isoform with the serotonergic system, as illustrated in particular by the profound desensitisation of presynaptic 5-HT_{1A} receptors in GABA_{B(1a)}^{-/-} mice. A lack of effect on 5-HT_{1A} receptor expression in GABA_{B(1a)}^{-/-} mice, as indicated by normal 5-HT_{1A} autoradiography densities, suggested an intracellular mechanism for this desensitisation.

Together these studies demonstrated that the GABA_{B(1)} isoforms are functionally important variants of the GABA_B receptor, with specific relevance in depression and to aversive learning and memory processes that underlie cognitive symptoms in anxiety disorders.

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Chapter 1

General Introduction

μελαγχολία

Hippocrates, c. 460 BC–c. 370 BC

“I was walking along a path with two friends—the sun was setting—suddenly the sky turned blood red—I paused, feeling exhausted, and leaned on the fence—there was blood and tongues of fire above the blue-black fjord and the city—my friends walked on, and I stood there trembling with anxiety—and I sensed an infinite scream passing through nature.”

Edvard Munch, 1863 - 1944

Anxiety disorders and depression have been synonymous with human civilization for centuries, and now constitute a fast-growing world-wide pandemic, and yet the causative factors and underlying mechanisms of these disorders still remain poorly understood. Psychiatric disorders are thus amongst the most challenging and impenetrable diseases to treat, with most current pharmacotherapies being the far from ideal benzodiazepine and monoaminergic-based drugs first discovered nearly 50 year ago. GABA_B receptors were the last of the major neurotransmitter receptors to be cloned. Pharmacological and genetic deletion studies have indicated that the GABA_B receptor is a promising target in anxiety and depression, although tools with which to probe the intricate and complex functions of this ubiquitously-expressed receptor have only relatively recently begun to be developed. The GABA_B receptor is a heterodimer of two subunits, GABA_{B(1)} and GABA_{B(2)}. Two isoforms of the GABA_{B(1)} subunit are predominant in the brain, GABA_{B(1a)} and GABA_{B(1b)}, and as such comprise the majority of the molecular diversity of the GABA_B receptor. However, with no ligands with which to probe the physiological roles of these isoforms, the impact of these isoforms on GABA_B receptor functions or in GABA_B receptor mediated influences on anxiety and depression has been a mystery.

The aim of this thesis was to use newly generated genetic tools – mice deficient in the GABA_{B(1a)} and GABA_{B(1b)} isoforms, in combination with behavioural analysis and pharmacological techniques, to evaluate the impact of the GABA_{B(1)} receptor isoforms in models of anxiety and depression.

1.1 The GABA_B Receptor

Discovery and Structure of the GABA_B Receptor

γ -aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the brain. There are two distinct classes of GABA receptor through which GABA produces its effects: the GABA_A receptor pentamer, which mediates fast inhibitory neurotransmission *via* a gated chloride ion channel, and the metabotropic GABA_B receptor which modulates slower inhibitory responses (Cooper et al. 2003). Unlike the ionotropic GABA_A receptors, GABA_B receptors predominantly mediate their effects through activation of guanine nucleotide-binding proteins (G proteins). Although aberrations in GABA neurotransmission have long been implicated in the pathophysiology of psychiatric disorders, the role of GABA_B receptors in these disorders has, until recently, been largely ignored (Cryan and Kaupmann 2005).

The first specific GABA_B receptor ligand was synthesised in 1962: β -chlorophenyl-GABA, or baclofen, a derivative of GABA; although it was some time later that the receptor itself was identified. Two stereoisomers of baclofen exist, although only one is active: L-baclofen. Baclofen was introduced into the market in 1972 as Lioresal© for the control of spasticity and muscle rigidity associated with spinal cord injury, multiple sclerosis, amyotrophic lateral sclerosis, and cerebral palsy (Bowery 1993), an indication for which it is still in clinical use, more than 30 years later (Brogden et al. 1974). Indeed baclofen remains the only GABA_B receptor-mediated therapeutic in the clinic. The existence of the GABA_B receptor itself was first proposed by Norman Bowery and colleagues in the early 1980s, following from their biochemical description of a GABA-mediated response insensitive to the GABA_A antagonist bicuculline, but sensitive to baclofen (Bowery et al. 1980). This became known as the GABA_B receptor (Bowery et al. 1981).

Cloning of the GABA_B receptor, however, proved elusive and was not completed until > 15 years after Bowery's discovery. As such, it was the last of the major neurotransmitter receptors to be cloned. The necessity for selective, high affinity ligands and the complex architecture of the receptor itself certainly contributed to this delay, and indeed made the cloning of this receptor a formidable challenge (Bettler et al. 2004; Froestl et al. 2003). Once soluble, selective, high-affinity ligands were derived, GABA_B receptor cDNAs were isolated by expression cloning using the high-affinity radiolabelled GABA_B receptor antagonist [¹²⁵I]CGP64231 (Froestl et al. 2003; Kaupmann et al. 1997). Two isoforms (then proposed as splice variants) were discovered, and designated GABA_BR1a and GABA_BR1b (Kaupmann et al. 1997). The two isoforms differed in sequence only by an extended sequence in the

extracellular NH₂-terminus (N-terminus) of the GABA_{B(1a)} isoform, which harboured two domains known as ‘Sushi motifs’ or ‘short consensus repeats’ (see section 1.4). Both GABA_{B(1a)} and GABA_{B(1b)} were comprised of the extracellular N-terminus, a seven transmembrane region and an intracellular COOH-terminus (C-terminus) region. They each share structural similarities with metabotropic glutamate receptors (mGluRs), Ca⁺²-sensing receptors (CaS), vomeronasal and taste receptors, and as such have been categorized with these receptors as members of the Family 3 (or Family C) G-protein coupled receptors (GPCRs) (Bettler et al. 2004; Foord et al. 2005; Kaupmann et al. 1997). When expressed in a recombinant system however, agonist binding affinities and effector coupling of the GABA_{B(1)} proteins were substantially below that of native receptors (Kaupmann et al. 1997).

In the year after the cloning of the GABA_{B(1)} gene, a second, structurally similar, member of the GABA_B receptor family: the GABA_{B(2)} subunit was described concomitantly by a number of research groups (Jones et al. 1998; Kaupmann et al. 1998a; Kuner et al. 1999; Martin et al. 1999; Ng et al. 1999; White et al. 1998). Furthermore, the reduced agonist affinity seen with recombinant expression of GABA_{B(1)} proteins were explained by the discovery that when expressed alone, the GABA_{B(1)} subunits were retained in the endoplasmic reticulum (ER), (Couve et al. 1998). Co-expression of the GABA_{B(1)} and GABA_{B(2)} subunits recovered both the surface expression, agonist binding affinity and for the most part, effector coupling in recombinant systems (see (Bettler et al. 2004)). The mechanism by which the subunits interacted to enable cell surface expression was subsequently shown to be *via* the masking of an ER retention motif (RSRR) in the C-terminus of the GABA_{B(1)} subunit by the C-terminus of the GABA_{B(2)} subunit, through the formation of a coiled-coil heteromer association between the two C-termini (Margeta-Mitrovic et al. 2000; Pagano et al. 2001) (although other regions in the transmembrane and extracellular domains also contribute to dimerisation - see (Bettler et al. 2004; Cryan and Kaupmann 2005)). Together these studies indicated that GABA_B receptors were functional heterodimers, the first such described for G protein-coupled receptors (Fig. 1). This previously unreported phenomenon almost certainly contributed to the difficulties encountered in the initial attempts to clone the GABA_B receptor.

The ligand binding domain for GABA_B receptor agonists and competitive antagonists has been mapped to the GABA_{B(1)} subunit. This domain conforms as a hinged, double-lobed structure that closes with ligand binding, and are thus termed the Venus flytrap modules (Bettler et al. 2004; Kubo and Tateyama 2005). The GABA_{B(2)} subunit contains the same type of ligand binding domain, but to date no GABA_B receptor ligands have been found which interact with this site (Bettler et al. 2004). The main function of the GABA_{B(2)} subunit (in

addition to masking the RSRR domain of GABA_{B(1)} appears to be the mediation of GABA_B receptor G-protein coupling (Thuault et al. 2004), although GABA_{B(1)} is clearly necessary to optimize the coupling efficiency (see (Bettler et al. 2004)).

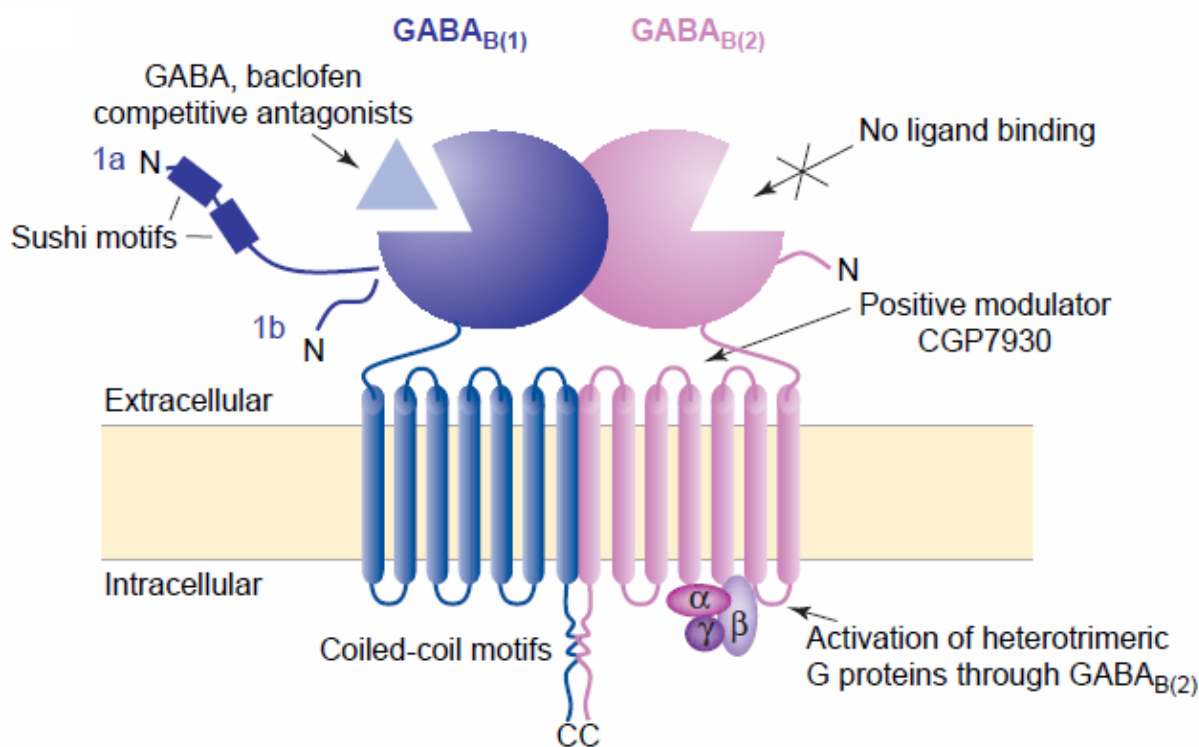


Fig. 1. The GABA_B receptor is a heterodimer comprised of GABA_{B(1)} (blue) and GABA_{B(2)} (pink) subunits. Heterodimerisation is facilitated by coiled-coil interactions between the C-termini of the two subunits. The agonist and competitive antagonist ligand binding domain is present only on the GABA_{B(1)} subunit, while G protein coupling and positive modulator binding is mediated by the GABA_{B(2)} subunit. Two isoforms of the GABA_{B(1)} subunit exist: GABA_{B(1a)} (1a) and GABA_{B(1b)} (1b) which differ in their N-termini by the inclusion of two Sushi motifs in the GABA_{B(1a)} isoform. *From Cryan and Kaupmann (2005) Trends Pharmacol Sci: 26 (1): 36-43.*

The relatively limited molecular diversity of GABA_B receptors, being comprised predominantly of the GABA_{B(1a)} or GABA_{B(1b)} isoforms dimerised with the GABA_{B(2)} subunit, came as a surprise to many researcher in the field, and is still considered today to be at odds with the reported variability and range of responses to GABA_B receptor ligands (Bettler et al. 2004; Huang 2006; Marshall et al. 1999). However, the nature of GABA_B receptor effector interactions, and the anatomical expression profile of this receptor almost certainly contribute greatly to this diversity.

GABA_B Receptor Expression and Effector Systems

GABA_B receptors predominantly mediate their effects via G protein coupling, *via* the G_{iα} and G_{oα} G protein subtypes (Asano and Ogasawara 1986; Campbell et al. 1993; Greif et al. 2000; Menon-Johansson et al. 1993; Morishita et al. 1990). However, evidence also exists for direct, non-G protein mediated actions of GABA_B receptors, for example via interactions with the transcription factor ‘activating transcription factor 4’ (ATF4, also known as cAMP response element binding protein 2 (CREB2)) (Nehring et al. 2000; Vernon et al. 2001; White et al. 2000) or with mGluR1 receptors (Tabata et al. 2004). The expression profile and thus anatomically determined effector coupling of GABA_B receptors certainly contributes to the diversity of GABA_B receptor-mediated responses. GABA_B receptors are expressed as presynaptic heteroreceptors, at post- and extra-synaptic sites and as interneuron autoreceptors (see (Bettler et al. 2004; Bowery et al. 2002; Cryan and Kaupmann 2005); Fig. 2).

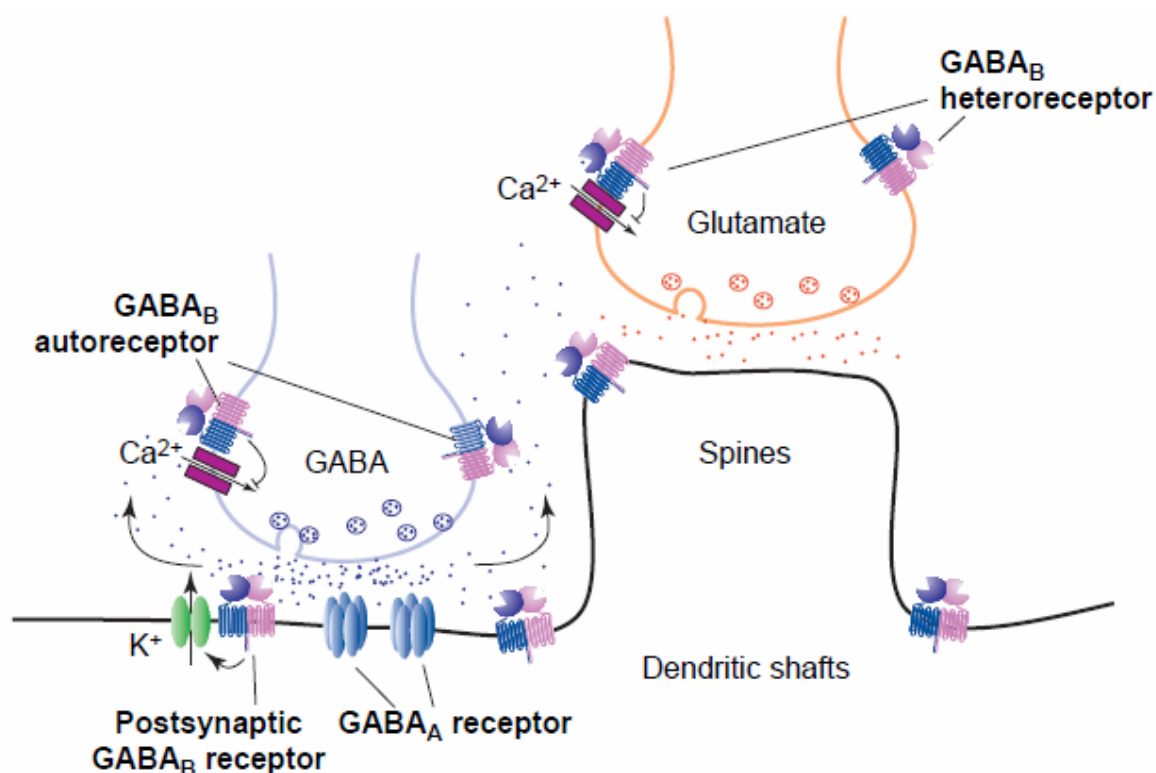


Fig. 2. GABA_B receptors are expressed as presynaptic heteroreceptors, coupling via G proteins to voltage-gated Ca²⁺ channels and modulating neurotransmitter release, and postsynaptically where they couple to inwardly-rectifying K⁺ channels to modulate slow inhibitory postsynaptic potentials. They are also expressed as autoreceptors, mediating GABA release, and at extrasynaptic sites. *From Cryan and Kaupmann (2005) Trends Pharmacol Sci: 26 (1): 36-43.*

Presynaptic receptors

Presynaptic GABA_B receptors inhibit the release of a number of different neurotransmitters (see (Bowery 1993) and (Bonanno and Raiteri 1993) for reviews), mainly by reducing calcium influx through high-voltage P/Q and N-type Ca⁺² channels (for examples see (Amico et al. 1995; Cardozo and Bean 1995; Menon-Johansson et al. 1993; Mintz and Bean 1993; Moldavan et al. 2006; Pfrieger et al. 1994; Poncer et al. 1997)), both of which are strongly implicated in neurotransmitter release (Wu and Saggau 1997). Presynaptic GABA_B receptors may also influence L- and T-type Ca⁺² channels, although both inhibition and activation of these channels by GABA_B receptors has been reported (see (Bettler et al. 2004)). In addition, direct influences of GABA_B receptors on exocytotic release machinery have also been proposed (Capogna et al. 1996; Kolaj et al. 2004; Scanziani et al. 1992). There are also indications that at the presynaptic terminal, GABA_B receptors interact with inwardly-rectifying K⁺ channels (K_{ir}3 or GIRK channels), although the channel subunit composition likely differs from the postsynaptically expressed GIRKs (Bettler et al. 2004).

Postsynaptic Receptors

Postsynaptic GABA_B receptors couple to K_{ir}3 channels, which when activated induce K⁺ efflux, hyperpolarisation and mediate slow inhibitory postsynaptic currents (IPSC) (Kolaj et al. 2004; Luscher et al. 1997; Schuler et al. 2001). Evidence for this comes, in particular, from experiments with mice deficient in either K_{ir}3.2 or the GABA_{B(1)} subunit, both of which do not show the normal baclofen-induced outward currents in hippocampal neurons (Luscher et al. 1997; Schuler et al. 2001). Other postsynaptic K⁺ currents may also be activated by GABA_B receptors, such as fast-inactivating voltage-gated K⁺ channels and small-conductance Ca⁺²-activated K⁺ channels (SK channels) (see (Bettler et al. 2004)). There is also some evidence to suggest that postsynaptic GABA_B receptors may suppress N-type high-voltage-activated calcium conductances (Kolaj et al. 2004).

In addition to coupling with ion channels *via* G proteins, GABA_B receptors negatively couple to adenylyl cyclase *via* G_{ai} and G_{ao} proteins, as evidenced, most often, by GABA_B receptor-activated inhibition of forskolin-stimulated cAMP release (for examples see (Gjoni et al. 2006; Hashimoto and Kuriyama 1997; Nishikawa et al. 1997; Wojcik and Neff 1984), and see (Bettler et al. 2004; Bowery 1993; Bowery et al. 2002) for reviews). GABA_B receptor-activated stimulation of adenylyl cyclase has also been reported (Hashimoto and Kuriyama 1997; Ren and Mody 2006). Mechanisms by which this latter effect has been proposed to occur include a reliance on crosstalk between the βγ subunits of co-expressed

stimulatory (G_s) G proteins (Bettler et al. 2004), or alternatively *via* $GABA_B$ receptor-dependent Ca^{+2} activation of Ca^{2+} /calmodulin, which in turn directly stimulates adenylyl cyclases I and/or VIII (Ren and Mody 2006). $GABA_B$ receptor inhibition of presynaptic cAMP has also been implicated in presynaptic plasticity *via* a retardation of vesicle recruitment, most probably mediated by a failure in a Ca^{2+} / cAMP-dependent vesicle priming step (Sakaba and Neher 2003). Finally, $GABA_B$ receptors have also been implicated in other second messenger pathways including the mitogen-activated protein kinase (MAPK) and protein kinase C (PKC) pathways (Kubota et al. 2003; Ren and Mody 2003; Taniyama et al. 1992).

Anatomical Expression Profile

$GABA_B$ receptors in general are widely expressed throughout the central nervous system. It has been postulated that nearly every neuron in the brain expresses $GABA_B$ receptors to some degree (Bischoff et al. 1999). The cerebral cortex (especially Layer 6b), thalamus, Purkinje processes in the molecular layer of the cerebellum, pyramidal cell layer of CA1-CA3 of the hippocampus, granular cell layer of the dentate gyrus, medial habenular nucleus, superficial layers of the dorsal horn of the spinal cord and motor neurons in the ventral horn, show particularly strong expression (Bischoff et al. 1999; Bowery et al. 1987; Charles et al. 2001; Fritschy et al. 1999; Kaupmann et al. 1997; Liang et al. 2000). The anatomical expression profile of the two $GABA_{B(1)}$ isoforms overlaps to a large degree with the $GABA_{B(2)}$ subunit, (Bettler et al. 2004) supporting the requisite heterodimerisation necessary for the expression of functional receptors.

Functions of $GABA_B$ Receptors

Well known physiological and pathophysiological roles of $GABA_B$ receptors, largely discerned from research with baclofen, include addiction, spasticity, epilepsy, gastroesophageal reflux disease, pain, cognition, anxiety and depression (for reviews see (Bettler et al. 2004; Bowery et al. 2002; Cryan and Kaupmann 2005), and see section 1.4 $GABA_B$ Receptors in Anxiety and depression for further discussions on anxiety, depression and cognition,).

Hypothermia and Ataxia: In vivo Probes of GABA_B Receptor Function

Other well known effects of GABA_B receptor activation include marked hypothermia (Cryan et al. 2004; Frosini et al. 2004; Gray et al. 1987; Humeniuk et al. 1995; Perry et al. 1998; Queva et al. 2003; Schuler et al. 2001; Serrano et al. 1985; Zarrindast and Oveissi 1988) and deficits in motor coordination (Brogden et al. 1974; Cryan et al. 2004; Frosini et al. 2004; Gassmann et al. 2004; Gray et al. 1987; Kasture et al. 1996; Schuler et al. 2001; Smith and Vestergaard 1979). These effects are well preserved in a wide range of species including mice, rats, rabbits and man. GABA_B receptor activation also induces increases in plasma growth hormone, prolactin, adrenocorticotropin hormone (ACTH) and cortisol (or corticosterone in rodents) in a range of species. This indicates roles for the GABA_B receptor in motor control, thermoregulation and endocrine modulation (Cavagnini et al. 1977; Davis et al. 1996; Hausler et al. 1993; Kimura et al. 1993; Koulu et al. 1979; Orio et al. 2001). GABA_B receptor agonist-induced hypothermia, locomotor incoordination and endocrine measures are all relatively non-invasive, cross-species translatable and simple experimental measures to obtain that can provide information about the functional *in vivo* state of GABA_B receptor. In combination with GABA_B receptor agonist challenges, they show great utility as *in vivo* probes of GABA_B receptor function in various experimental and clinical conditions.

Criteria for the valid application of pharmacological probes of central neurotransmitter *in vivo* function have been proposed (Checkley 1980), and include:

1. The response should result from the stimulation of a receptor, and be inhibited by drugs that block that receptor. The same response should occur with administration of all drugs that stimulate that receptor, and should not be inhibited by drugs that block other receptors;
2. The receptor should be centrally located;
3. Factors that can influence the response must be held constant (for example environmental, circadian, stress, hormonal rhythms);
4. Time-course studies are advocated over single time-point studies in case peak responses are missed;
5. Conscious animals should be used.

In this way, baclofen indeed has been used to probe GABA_B receptor function in animals and in clinical studies. For example, baclofen-induced locomotor impairment, antinociception and hypothermia have been used to probe GABA_B receptor function in rodents following chronic antidepressant treatments (Borsini et al. 1986; 1988; Gray et al. 1987; McManus and Greenshaw 1991). In clinical trials, blunted growth hormone to baclofen

has been seen in major depression (Marchesi et al. 1991; O'Flynn and Dinan 1993) (but see (Davis et al. 1997; Monteleone et al. 1990), alcoholism; heroin addiction (Vescovi et al. 1998; Volpi et al. 1992), and social phobia (Condren et al. 2003).

This approach for probing GABA_B receptor function is also applicable for use in mutant mice, as illustrated by studies with GABA_{B(1)}^{-/-} and GABA_{B(2)}^{-/-} mice. In these mice, the absence of hypothermia and ataxic responses to baclofen confirmed a total loss of *in vivo* GABA_B receptor-mediated responses (Gassmann et al. 2004; Queva et al. 2003; Schuler et al. 2001). Furthermore, with mutant mice this approach can be gainfully utilised in the evaluation of proposed agonists. For example, GABA_{B(1)}^{-/-} mice did not show hypolocomotor or hypothermic responses to the weak GABA_B receptor agonist and drug of abuse, γ -hydroxybutyrate (GHB), indicating GHB normally exerts its *in vivo* effects through the GABA_B receptor and not *via* a postulated GHB receptor (Kaupmann et al. 2003).

1.2 Anxiety and Depression

Anxiety and depression disorders represent common, serious and growing health problems world-wide (Kessler et al. 2005a; Miller 2006; Murray and Lopez 1997; Wong and Licinio 2001). The disorders share high levels of comorbidity (Kessler et al. 2005b; Merikangas et al. 2003), and those suffering from these disorders not only face debilitating disruptions to their psychological well-being, but are at high risk for suicide (Licinio and Wong 2005) and somatic conditions such as heart disease, gastrointestinal disorders and obesity (Harter et al. 2003; Rumsfeld and Ho 2005; Sheps and Sheffield 2001). The causative factors underlying anxiety and depression, however, remain poorly understood (Cryan and Holmes 2005; Wong and Licinio 2001; Wong and Licinio 2004), and it is clear that improvements in understanding these factors and the development of better treatments are needed (Cryan and Holmes 2005; Holmes and Cryan 2006; Wang et al. 2005).

Anxiety and depression disorders are characterised by a broad range of diverse, overlapping symptom clusters (Merikangas et al. 2003) and are classified into numerous categories and subcategories, based mainly on the subjective descriptions of symptoms (Lam et al. 2006; Wong and Licinio 2001) (Table 1 & 2). For example, two symptom criteria for major depression include: 1) depressed mood as indicated by self report or by observation (e.g. appears tearful); 2) feelings of worthlessness or excessive or inappropriate guilt (which may be delusional), not merely self-reproach or guilt about being sick (DSM-IV 1994; Lam et al. 2006; Wong and Licinio 2001) (see Table 1 for other DSM-IV symptom criteria). Anxiety

disorders are subdivided into a number of categories, distinguished from one another mainly by the nature of the anxiety or of the stimulus producing the anxiety (Cryan and Holmes 2005; Lam et al. 2006). They include generalized anxiety disorder, panic disorder (with or without agoraphobia), specific phobia, social phobia, obsessive–compulsive disorder and post-traumatic stress disorder, each with both distinct and overlapping symptom criteria (DSM-IV 1994; Lam et al. 2006) (See Table 2 for a summary).

The subjective nature of these definitions are considered by many in the field to provide challenges in clinical diagnosis (Hasler et al. 2004; Lam et al. 2006; Schulze et al. 2005; Wong and Licinio 2001), and although it is clear that some of the features of psychiatric disorders in humans cannot be modelled in mice (Tables 1 & 2), these definitions certainly contribute to challenges in modelling aspects of depression and anxiety disorders in mice and other experimental animals (Cryan and Holmes 2005; Cryan and Mombereau 2004; Holmes and Cryan 2006; Phillips et al. 2002; Tarantino and Bucan 2000).

1.3 Mutant Mice and Murine Modelling of Anxiety and Depression

The Endophenotypes Approach

Recently, there has been a move to describe psychiatric disorders by dissecting the symptomatology into objectively measurable components. That is, into individual behavioural, physiological or neurochemical endpoints - termed ‘endophenotypes’ (Gottesman and Gould 2003; Hasler et al. 2004). The promotion of endophenotypes has arisen primarily with the aim of strategising an approach to discover the genetic and neurobiological architecture of psychiatric diseases. This comes from the conceptual basis that the number of genes required to produce less complex, definable traits may be fewer (and therefore more easily discovered) than those involved in producing a more complex trait such as those seen in psychiatric diagnostic criteria (Gottesman and Gould 2003; Hasler et al. 2004). In addition, the endophenotypes approach is clearly more applicable for use in animal models (Cryan and Holmes 2005; Gottesman and Gould 2003; Hasler et al. 2004; Holmes and Cryan 2006).

Mice as experimental animals hold many practical and economic advantages over other laboratory species for use in animal modeling of human disorders. They are easy to breed, have a short generation turnover, and low maintenance costs in terms of housing. It is their unique amenability to genetic manipulations, however, that has seen the dramatic increase in the popularity of mice in psychiatric and other research, including anxiety and

depression (see (Cryan and Holmes 2005; Holmes and Cryan 2006; Jacobson and Cryan 2007; Joyner and Sedivy 2000; Phillips et al. 2002; Tarantino and Bucan 2000)). Furthermore, the availability of different inbred mouse strains, many of which are essentially isogenetic, are a particular advantage as they allow assessment of a manipulation-induced phenotypes against a reduced background variability (Festing 2004; Jacobson and Cryan 2007). As such, there have been numerous attempts to model psychiatric disease symptoms and endophenotypes in mice.

Proposed essential criteria for a valid animal model of a psychiatric disease include that it is: ‘reasonably analogous’ to the human disorder in its manifestations or symptomatology; causes a behavioural change that can be monitored objectively; produces behavioural changes that are reversed by the same treatment modalities that are effective in humans; and is reproducible between investigators (McKinney and Bunney 1969). These criteria overlap well with the endophenotype approach in terms of setting experimental endpoints that are objectively measurable, repeatable and show analogy to human symptomatology. Examples of some of the psychological and behavioural endophenotypes of depression and anxiety, and how they may be modelled in mice are shown in Tables 1 and 2 (see (Cryan and Holmes 2005; Cryan and Mombereau 2004; Cryan and Slattery 2007) for reviews).

Table 1. Modelling symptoms of major depression* in mice

Symptom	How might symptom be modelled in mice?
Depressed mood	Cannot be modelled
Markedly diminished interest or pleasure in everyday activities (anhedonia)	Reduced intracranial self-stimulation, progressive ratio responding for positive reward (for example, sucrose) and social withdrawal
Large changes in appetite or weight	Abnormal loss in body weight after exposure to chronic stressors
Insomnia or excessive sleeping	Abnormal sleep architecture (measured using electroencephalography)
Psychomotor agitation or slowness of movement	Difficulty in handling and alterations in various measures of locomotor activity and motor function
Fatigue or loss of energy	Reduced activity in home cage, treadmill/running wheel activity, nest building and active waking electroencephalogram
Indecisiveness or diminished ability to think or concentrate	Deficits in working and spatial memory and impaired sustained attention
Difficulty performing even minor tasks, leading to poor personal hygiene	Poor coat condition during chronic mild stress
Recurrent thoughts of death or suicide	Cannot be modelled
Feelings of worthlessness or excessive or inappropriate guilt	Cannot be modelled

*Symptoms used in the *Diagnostic and Statistical Manual-IV* diagnosis of major depression

From Cryan and Holmes, (2005) *Nat Rev Drug Discov* 4 (9): 775-90

Table 2. How symptoms of anxiety disorders* might be modelled in mice

Symptom	How might symptom be modelled in mice?
Avoidance of places from which escape could be difficult (agoraphobia)	Increased avoidance of exposed, well-lit areas
Sudden onset of intense fearfulness, often with respiratory distress and fear of 'going crazy' (panic attack)	Increased flight from a predator
Anxiety provoked by social situations, leading to avoidance behaviour (social phobia)	Low social interaction with unfamiliar conspecific
Anxiety provoked by a specific feared object, leading to avoidance behaviour (specific phobia)	Conditioned taste avoidance
Re-experiencing a traumatic event, leading to increased arousal and avoidance of stimuli associated with the event (post-traumatic stress disorder)	Increased freezing response to fear-conditioned cue or context
Anxiety-provoking obsessions and anxiety-reducing compulsions (obsessive-compulsive disorder)	Increased marble burying and excessive grooming
Difficulty concentrating or mind going blank (generalized anxiety disorder)	Impaired sustained attention
Sleep disturbance/insomnia	Abnormal sleep architecture (measured using electroencephalography)
Autonomic hyperarousal (tachycardia, blushing, sweating and frequent urination)	Radiotelemetric measurement of heart rate dynamics during anxiety-provocation, such as increased stress-induced hyperthermia
Flashbacks of traumatic events	Impairment in extinction of fear memory
Cognitive bias towards ambiguous or weak threat cues	Increased fear conditioning to partial threat cue
Heightened startle response, particularly in threatening contexts	Increased acoustic startle response and fear-potentiated startle response
Separation anxiety	Increased ultrasonic vocalizations in pups separated from their mother
Feelings of losing control or going crazy during a panic attack	Cannot be modelled

*Symptoms used in the *Diagnostic and Statistical Manual-IV* diagnosis of anxiety disorders.

From Cryan and Holmes, (2005) *Nat Rev Drug Discov* 4 (9): 775-90

Traditional Mouse Models and Tests in Anxiety Research

With regard to traditional animal models of anxiety, it should be noted that fear and anxiety are normal, adaptive responses to danger. Pathological anxiety has therefore been considered by some as an extreme state of the same continuum, and many animal models have been designed with this in mind (Cryan and Holmes 2005). Animal models of anxiety disorders can be broadly categorized into two categories: unconditioned and conditioned (see following section: "Modelling Cognitive Symptoms of Depression and Anxiety in Mice", page 23, for discussions on the latter).

The most common anxiety tests in the unconditioned category have capitalized on the conflict between natural avoidance behaviours and the exploratory drive of rodents, to

develop ethologically based behavioural tasks (Rodgers et al. 1997a). Examples of these tasks include the aversion to the open central area of novel open fields (see (Prut and Belzung 2003)), avoidance of brightly lit spaces in the light-dark box test (Crawley 2000), and avoidance of elevated and/or open spaces in the elevated plus maze (Crawley 2000; Holmes 2001; Rodgers 1997), elevated zero maze (Lee and Rodgers 1990; Shepherd et al. 1994), the staircase test (Simiand et al. 1984), and the mirrored arena (mirrored chamber test) (see (Belzung and Griebel 2001; Crawley 2000; Cryan and Holmes 2005; Rodgers 1997; Rodgers et al. 1997a) for reviews). Another test in this category is the four-plate test, which is based on the conflict between exploratory drive and avoidance of punishment (as shocks are delivered through the floor plates when mice move on to a new plate). All of these tests rely on a passive avoidance strategy of the animal to provide an indication of anxiety. The marble-burying and defensive burying (for example, of a shock-probe) tests, in contrast, requires the engagement of active behaviours for the expression of anxious behaviours and thus are interesting inclusions in test batteries (Broekkamp et al. 1986; Sluyter et al. 1996; Sluyter et al. 1999; Spooren et al. 2000).

Exploratory based tests are sensitive to interference by locomotor activity - for example a genetic mutation which alters baseline locomotor activity in mice may produce false negatives, or false positives, in exploratory paradigms (Cryan and Holmes 2005). The so-called 'ethological parameters', that is, species-specific behaviours and postures adapted during exploration and risk assessment, can be included as measures in many of the aforementioned tests: reductions in the number of stretch-attend postures, head-dipping over the edges of elevated apparatuses and rearing have been interpreted as heightened anxious responses in various apparatuses (Belzung 1999; Homanics et al. 1999; Rodgers 1997; Rodgers and Johnson 1995; Shepherd et al. 1994). Ethological parameters are thought to be less influenced by locomotor activity, although their particular advantage in animal models, however, originates from their basis in the evaluation of risk assessment behaviours, and thus are thought to model endophenotypes of apprehension and excessive vigilance seen in patients with anxiety disorders (Blanchard et al. 2003; Cryan and Holmes 2005; Rodgers et al. 1997a). The ethological approach has been taken further in the Mouse Defense Test Battery (MDTB), where mice are exposed to a predator threat, and panic, defensive threat/attack and risk assessment behaviours assessed, which are sensitive to panicolytic and anxiolytic pharmacotherapies, respectively (Blanchard et al. 2003).

Other examples of tests used in mice that also relatively independent from bias introduced by alterations in locomotor activity include the Vogel punished drinking test,

novelty suppressed feeding, separation-induced ultrasonic vocalisations and stress-induced hyperthermia (SIH) (Holmes and Cryan 2006). The SIH test in particular is an ideal inclusion in an anxiety test battery as it provides an indication of autonomic responses to stress, and is a translational model across strains and species (including mice and humans; see (Bouwknicht et al. 2006)).

Traditional Mouse Models and Tests in Depression Research

Currently the lack of understanding about the causative factors and the pathophysiology of depression in humans has prevented the development of an animal model from a pure aetiological basis (Cryan and Slattery 2007). Descriptions of currently used tests are summarised in Table 3. In depression research, the most widely utilized mouse models and tests are based on alterations in stress-induced coping strategies. This derives from the observation that stress and trauma, or the uncontrollability of stress ('lack of coping'), often pre-disposes human depression (Cryan and Holmes 2005; Kessler 1997). Examples of tests based on this construct include the learned helplessness, forced swim test (FST) and tail suspension tests (Table 3). These tests all show responsiveness to a range of clinical antidepressant treatments and are therefore often referred to as tests of antidepressant-like activity (Cryan and Slattery 2007). The FST is currently the most widely used test in murine antidepressant research, and is also often used in the phenotypic analysis of mutant mice (see (Cryan and Holmes 2005; Cryan and Mombereau 2004; Jacobson and Cryan 2007; Slattery and Cryan 2006) for overall reviews). Care must be taken to assess the locomotor phenotype of mutant mice used in all of the above tests, as abnormal locomotor activity produced by a genetic mutation may bias results (Cryan and Holmes 2005; Holmes and Cryan 2006; Jacobson and Cryan 2007).

The chronic mild stress (CMS) paradigm, which also relies on repeated exposure to stressful stimuli (Monleon et al. 1995; Willner 2005; Willner et al. 1987), has shown transient popularity of recent. An advantage of this model is that the experimental outcomes are usually based on hedonic measures, such as intake of a preferred sweet solution or intracranial self-stimulation (ICSS), and thus may model aspects of anhedonia seen in human depression (Harkin et al. 2002; Moreau 1997; Papp et al. 1996). In mice, deterioration of coat condition also appears to be a sensitive measure (Griebel et al. 2002; Santarelli et al. 2003). The test is also sensitive to chronic antidepressant treatment (Cryan and Holmes 2005; Cryan and Mombereau 2004; Willner 2005). The greatest disadvantage of the CMS model, however, lies

in the lack of reproducibility between different laboratories (Cryan and Holmes 2005; Jacobson and Cryan 2007) and see *Psychopharmacology* 134(4)).

Table 1 Traditional mouse models used in depression research

Animal model	Description	Reviewed in:
Learned helplessness	Animals exposed to inescapable shocks subsequently fail to escape when able to. Antidepressant treatment increases the number of escapes, not all animals develop this helpless behaviour	(Maier and Watkins 2005; Weiss et al. 1981)
Forced swim test	Rodents, placed in an inescapable container of water swim more following antidepressant administration	(Borsini 1995; Petit-Demouliere et al. 2005)
Tail suspension test	Rodents, chiefly mice, when hung from the tail will adopt an immobile posture. Antidepressant treatment increases the time animals spend in active behaviours	(Cryan et al. 2005)
Olfactory bulbectomy	Removal of the olfactory bulbs causes a constellation of behavioural and neurochemical alterations, which are only reversed by chronic antidepressant treatment	(Harkin et al. 2003; Song and Leonard 2005)
Chronic mild stress	Animals are subjected to a variety of unpredictable stressors, which leads to a constellation of symptoms, that are reversed by antidepressant treatment	(Willner 2005)
Neonatal clomipramine administration	When exposed to neonatal clomipramine, adult animals display a number of symptoms analogous to depression, including decreased reward seeking, aggressiveness and sexual behaviour. Antidepressant treatment can reverse behaviours	(Vogel et al. 1990)

Adapted from Cryan and Slattery (2007) Curr Opin Psychiatry 20 (1): 1-7.

Modelling Cognitive Symptoms of Anxiety and Depression in Mice

Human anxiety and depression and are accompanied by specific cognitive deficits. Anxiety disorders are characterised by specific cognitive deficits such as misappraisal and over-attention to threatening stimuli in panic disorder, generalized anxiety disorder and phobias, and persistence of traumatic memories in post-traumatic stress disorder (DSM-IV 1994; Lang et al. 2000). Approaches to modelling these aspects in animals have mainly focused on conditioned tests of anxiety, such as Pavlovian fear conditioning (Cryan and Holmes 2005; Kim and Jung 2006). In this paradigm, fear-related behaviours are induced by exposure to a previously innocuous stimulus (the conditioned stimulus (CS), for example, an auditory tone) that has been associated, through repeated pairings, with an innately aversive stimulus (the unconditioned stimulus (US), for example, footshocks) (Cryan and Holmes 2005; Davis 1990; Maren 2001). The tasks may be further delineated into testing contextual (fear associated with the place of conditioning, which is hippocampally-dependent) and cued (fear associated with a tone cue, and is amygdala-dependent) fear responses (Barad 2005; Bouton and Moody

2004). Freezing, startle, tachycardia, defensive burying and ultrasonic vocalizations have mainly been used as experimental outputs in these tests (see (Cryan and Holmes 2005) for a review). Interestingly, disturbances in sleep have also recently been shown to be a sensitive readout of fear conditioning in mice (Sanford et al. 2003a; Sanford et al. 2003b), which is highly relevant to the endophenotype of sleep dysfunction that is a diagnostic criterion in both depression and certain anxiety disorders (DSM-IV 1994).

Both the acquisition and extinction of fear memories can be investigated with fear conditioning. Extinction is the decline in fear observed with repeated, unreinforced exposure to the CS, and is considered a new form of learning in which new associations with the CS, in the absence of reinforcement by the US, dominate the original association (Berman and Dudai 2001; Myers and Davis 2002; Reilly and Bornovalova 2005). Investigations into the neurobiology of extinction in particular, have been applied to the modelling of anxiety disorders with persistent aversive memory and associations, such as post-traumatic stress disorder and panic disorder (Barad 2005; Cryan and Holmes 2005; Delgado et al. 2006; Ledgerwood et al. 2005; Ressler et al. 2004).

Conditioned taste aversion (CTA) is well known as an aversive, associative learning and memory paradigm (Akirav 2006; Akirav et al. 2006; Berman and Dudai 2001; Bermudez-Rattoni 2004; Lamprecht et al. 1997) and has recently been applied to the study of anxiety disorders associated with altered emotional learning and memory (Cryan and Holmes 2005; Guitton and Dudai 2004; Yasoshima and Yamamoto 2005). In CTA, an otherwise innocuous (or even preferred) taste is paired, most commonly, with experimentally induced malaise such as that induced by intraperitoneal injection of Lithium chloride. Similarly to fear conditioning, both the acquisition and extinction of the aversive association can be assessed. The test is also largely independent of influences on locomotor output (which may be problematic in tests relying on outputs such as freezing or flight). In addition to the amygdala, a well known hub in anxiety circuitry, CTA is also reliant on the insular cortex (Bermudez-Rattoni 2004; Bures 1998b), which has recently been highlighted as a highly important structure in many (if not all) human anxiety disorders (Paulus and Stein 2006). This suggests that CTA may be well suited to the investigation of the neurobiological basis of anxiety disorders with a strong cognitive component.

In depression, cognitive deficits include impaired planning, executive function and increased attention towards negative stimuli (Elliott et al. 2002). In theory, classic mouse cognitive tests of attention, working and reference, spatial and non-spatial memory could be used to study cognitive endophenotypes of depression (Cryan and Holmes 2005; Holmes and

Cryan 2006). Examples of tests could include the 5-choice serial reaction time task for attention (Patel et al. 2006; Wrenn et al. 2006), spatial alternation in T or Y mazes for working memory (Reisel et al. 2002) or Morris water maze or multi-armed food-rewarded dry mazes for spatial reference memory (Crawley 2000; Crawley and Paylor 1997). With regard to alterations in executive and prefrontal cortical function, an intra-dimensional and extra-dimensional set shifting test based on the Wisconsin card-sorting test that was developed for the assessment of prefrontal cortex cognitive functioning in rats (Birrell and Brown 2000), has recently been adapted for mice, and may show promise for application in depression-related research in the future (Brigman et al. 2005; Garner et al. 2006). In practice, there are very few such studies investigating alterations in cognitive processes in murine depression-related research. In one recent example, chronic mild stress and learned helplessness paradigms impaired the rate of acquisition and the memory probe of the hidden platform position in the Morris water maze of spatial memory (Song et al. 2006). Furthermore, these deficits were ameliorated by chronic treatment with imipramine or fluoxetine. Stress-induced increases in corticosterone and decreases in hippocampal brain-derived neurotrophic factor (BDNF) and CREB, which were also reverse by chronic antidepressant treatment, were thought to contribute to the mechanisms underlying these deficits (Song et al. 2006).

Modelling Depression and Anxiety: Conclusions

Overall, it is clear that there are many animal models and tests which may be applied to the study of the neurobiology of anxiety and depression. There are certain caveats and cautions that must be taken into account in the application of each of these tests. Genetic background, in the form of a carefully selected mouse strain, is of high importance (Crawley 2000; Jacobson and Cryan 2007). Possible abnormal locomotor phenotypes produced by a genetic mutation have been discussed, but potential alterations in other sensory-motor modalities must also be taken into account (for example, pain sensitivity in tests where foot shocks are delivered, or impairment in taste or olfactory senses in food-motivated or reward-based test). Previous test history, interactions with early-life environment and compensatory changes for constitutive genetic mutations may also influence results in anxiety and depression models (Holmes and Cryan 2006; Holmes et al. 2005). As such, a test-battery approach has been advocated for the detecting genuine phenotypes in anxiety and depression research with mutant mice, as reliance on fewer tests may give rise to erroneous interpretations depending on specific idiosyncrasies of individual tests or mutations (Cryan and Holmes 2005). Despite

these caveats, mutant mice have been greatly important for the evaluation of the function of specific genes and their down-stream molecular, circuit-related and behavioural influences (Cryan and Holmes 2005; Phillips et al. 2002; Tarantino and Bucan 2000). Mutant mice in particular are invaluable for the dissection of the functional role of a molecule that cannot be approached using more traditional strategies – perhaps the best example being that when pharmacological tools are unavailable or have poor selectivity for a particular receptor subtype (Cryan and Holmes 2005).

1.4 GABA_B Receptors in Anxiety and Depression

GABA_B Receptors and Anxiety

There are a number of indications from preclinical and clinical studies with baclofen that implicate the GABA_B receptor in anxiety (Couve et al. 2000; Cryan and Kaupmann 2005; Pile and Nowak 2005). Baclofen showed anxiolytic effects in several preclinical studies including ultrasonic vocalisation in rat pups (Nastiti et al. 1991), punished drinking (Ketelaars et al. 1988; Shephard et al. 1992), Geller-Seifter conflict test (Ketelaars et al. 1988), elevated plus maze (Andrews and File 1993) and in rats dependent on either diazepam or alcohol in the social interaction and elevated plus maze tests after drug withdrawal (File et al. 1991a; File et al. 1991b; File et al. 1992). Baclofen has also shown anxiolytic activity in some clinical settings, for example during alcohol withdrawal in alcoholics (Addolorato et al. 2002a; Addolorato et al. 2002b; Addolorato et al. 2006; Ameisen 2005; Flannery et al. 2004), and in panic disorder (Breslow et al. 1989), post-traumatic stress disorder (Drake et al. 2003) and in patients suffering from acute spinal trauma (Hinderer 1990).

Not all studies, however, have reported anxiolytic actions for baclofen (for a preclinical example see (Dalvi and Rodgers 1996), for reviews see (Couve et al. 2000; Millan 2003)). One postulated mechanism for this variability relates to the actions of GABA_B receptor agonists at GABA_B autoreceptors. Activation at this site would be expected to suppress the release of GABA, and thus could influence anxiety *via* subsequent actions at the GABA_A receptor (Dalvi and Rodgers 1996; Millan 2003). It is also clear that the dose-window for baclofen that is free of motor-impairing (and hypothermic) effects is very narrow, and as such, limit its application in both research and in the clinical treatment of affective disorders (Cryan and Kaupmann 2005).

The strongest evidence for the role of GABA_B receptors in anxiety, however, probably comes from recent genetic deletion studies in mice and the development of GABA_B receptor positive modulators (Cryan and Kaupmann 2005). Constitutive deletion of either the GABA_{B(1)} or GABA_{B(2)} receptor subunits in mice results in a complete loss of typical GABA_B receptor function, spontaneous seizures, hyperalgesia, hyperlocomotion and memory impairment (Gassmann et al. 2004; Prosser et al. 2001; Schuler et al. 2001). Furthermore, these mice show a highly anxious phenotype in exploratory-based tests of anxiety (Mombereau et al. 2004a; Mombereau et al. 2005; Mombereau et al. 2004b). Specifically, GABA_{B(1)}^{-/-} mice show profound anxiety relative to wild-type controls in the light-dark box and staircase tests (Mombereau et al. 2004a; Mombereau et al. 2004b), while GABA_{B(2)}^{-/-} mice are also anxious in the light dark box (Mombereau et al. 2005).

Positive modulators of the GABA_B receptor, such as CGP7930 and the more potent GS39783, enhance both the potency and the maximal efficacy of GABA at GABA_B receptors in native and recombinant receptor preparations, but have little or no intrinsic action (Dupuis et al. 2006; Urwyler et al. 2005; Urwyler et al. 2001; Urwyler et al. 2003). These compounds have recently been shown to interact with the 7-transmembrane domain of the GABA_{B(2)} subunit, rather than at the ligand binding domain residing on the GABA_{B(1)} subunit (Binet et al. 2004; Dupuis et al. 2006). When applied *in vivo*, GABA_B receptor positive modulators potentiate the effects of GABA_B receptor agonists, supporting their *in vitro* profile (Carai et al. 2004). GABA_B receptor positive modulators by themselves do not show the motor impairing and hypothermic profile characteristic of GABA_B receptor agonists (Cryan et al. 2004). Importantly with regard to anxiety, GS39783 by itself has an anxiolytic profile in rodents, thus providing further support for a role of the GABA_B receptors in anxiety (Cryan et al. 2004).

GABA_B Receptors and Depression

In addition to the role in anxiety, there is strong evidence from animal studies that GABA_B receptors are implicated in depression and the action of antidepressants, and that GABA_B receptor antagonists may be an attractive target for the development of novel antidepressants (see (Couve et al. 2000; Cryan and Kaupmann 2005; Pilc and Nowak 2005; Slattery and Cryan 2006)). It is now over 20 years ago since Pilc and Lloyd (1984) observed that in rats, GABA_B receptor binding in the frontal cortex was up-regulated after chronic (but not acute) antidepressant treatment. From these findings they hypothesised that GABA_B receptors may be involved in the mechanisms underlying depression and the action of antidepressants (Pilc

and Lloyd 1984). Other animal studies have since demonstrated similar findings with GABA_B receptor function or expression following antidepressant treatments, including models of electroconvulsive shock therapy (Gray et al. 1987; Gray and Green 1987; Lloyd et al. 1985; Sands et al. 2004b). Indeed, chronically administered GABA_B receptor antagonists, such as CGP36742 and SCH50,911, also up-regulate GABA_B receptor binding sites (Malcangio et al. 1993; Pibiri et al. 2005), and to a similar degree as chronically administered desipramine, further suggesting a potential application for GABA_B receptor antagonists in antidepressant therapy (Pratt and Bowery 1993).

In animal models of depression or antidepressant-like action, GABA_B receptor antagonists show an antidepressant-like profile in the rat and mouse FST (Mombereau et al. 2004a; Nowak et al. 2006), in addition to the learned helplessness (Nakagawa et al. 1999; Nowak et al. 2006), olfactory bulbectomy and chronic mild stress paradigms (Nowak et al. 2006). Furthermore, genetic deletion of either the GABA_{B(1)} or GABA_{B(2)} receptor subunits induced an antidepressant-like phenotype in the forced swim test (FST) in mice (Mombereau et al. 2004a; Mombereau et al. 2005).

There are very few clinical studies examining GABA_B receptor function in depressed patients. Mixed results are reported from studies examining GABA_B receptor function in depressed patients, using probes such as baclofen-induced growth hormone secretion or the dexamethasone suppression test (see (Slattery and Cryan 2006) for a review). However, in one, small clinical trial with depressed patients, baclofen was reported to exacerbate depressive symptoms (Post et al. 1991). Clearly more clinical studies are required to examine GABA_B receptors in depression (and preferably using GABA_B receptor antagonists).

GABA_B Receptor-Serotonin Interactions Mediate Aspects of Antidepressant Activity

The mechanisms underlying the antidepressant-like behavioural effects of GABA_B receptor antagonists have been shown to depend on an interaction with the serotonin (5-HT) system, as demonstrated by the abolition of the antidepressant-like effects of the GABA_B antagonist CGP56433A in the rat forced swim test by pre-treatment with the tryptophan hydroxylase inhibitor para-chlorophenylalanine (pCPA) (Slattery et al. 2005a). Indeed, nearly all of the 5-HT cell bodies in the dorsal and medial raphe nuclei (DRN and MRN) have been shown to express GABA_B receptors (Abellan et al. 2000a; Serrats et al. 2003; Varga et al. 2002). Activation of GABA_B receptors with baclofen, either systemically, or locally at the DRN, influences 5-HT neuron firing rate and 5-HT release at the level of the raphe nuclei and in postsynaptic structures (Abellan et al. 2000a; Abellan et al. 2000b; Tao et al. 1996). The

direction of this influence, however, differed between these aforementioned studies by Abellan and Tao. In further studies by the former group, GABA_B receptors were shown to be expressed both at postsynaptic sites on serotonin containing neurons, and presynaptically at GABAergic terminals synapsing with 5-HT neurons (Serrats et al. 2003). Thus, they proposed that activation of post-synaptic GABA_B receptors located on serotonergic neurons could explain the baclofen-induced inhibition of 5-HT release described by Tao et al., (1996), while activation of presynaptic GABA_B autoreceptors by baclofen could result in disinhibition of serotonin neurons, and an increase in firing rate and serotonin release (Abellan et al. 2000b; Serrats et al. 2003).

GABA_B Receptors and Cognitive Processes Associated with Anxiety and Depression

As previously discussed, anxiety and depression are both characterised by symptomatic deficiencies in specific cognitive functions (for example from DSM-IV-TR: reduced ability to think or concentrate, or indecisiveness in major depression; and difficulty in concentration or mind going blank in generalized anxiety). It is therefore interesting to note that GABA_B receptors have been strongly implicated in cognitive function, and in the purported underlying cellular correlate of memory, long-term potentiation (LTP) (see (Bowery et al. 2002; Davies and Collingridge 1996; Davies et al. 1991; Froestl et al. 2004)). In animal studies, GABA_B receptor antagonists improved performance in hippocampally-dependent spatial learning and memory (Helm et al. 2005; Nakagawa and Takashima 1997; Staubli et al. 1999) and in passive and active avoidance tasks (Getova and Bowery 1998; Mondadori et al. 1994; Mondadori et al. 1993). Furthermore, the GABA_B receptor antagonist SGS742 was recently investigated as a cognitive enhancer in a Phase II clinical trial for Alzheimer's disease (Froestl et al. 2004). In contrast, GABA_B receptor agonists generally (but not always: (Castellano et al. 1993; Escher and Mittleman 2004; Saha et al. 1993)) impair learning and memory (for examples in animal models, see (Cryan et al. 2004; Erickson et al. 2006; Galeotti et al. 1998; McNamara and Skelton 1996; Nakagawa et al. 1996; Nakagawa and Takashima 1997; Pitsikas et al. 2003; Tang and Hasselmo 1996)). Further evidence is provided by the phenotype of GABA_{B(1)}^{-/-} and GABA_{B(2)}^{-/-} mice, which are profoundly impaired in passive avoidance performance in a gene dose-dependent manner (Gassmann et al. 2004; Pagano et al. 2001).

1.5 The Mystery of the Sushi Repeats: GABA_{B(1)} Receptor Isoforms

As stated earlier, GABA_{B(1a)} and GABA_{B(1b)} are the most abundantly expressed variants of the GABA_{B(1)} subunit (Kaupmann et al. 1997), both of which form functional heterodimers with the GABA_{B(2)} subunit (Benke et al. 1999; Kaupmann et al. 1998a). As such, the GABA_{B(1a)} and GABA_{B(1b)} isoforms comprise an enormous proportion of the molecular diversity of the GABA_B receptor. Up until very recently, however, there has been a dearth of specific tools with which to probe the functional roles of these two isoforms in physiology and behaviour.

In recombinant studies, many groups have failed to ascribe differential pharmacological attributes to the GABA_{B(1a)} and GABA_{B(1b)} isoforms, despite the use of different experimental endpoints such as production of inositol phosphates (Brauner-Osborne and Krogsgaard-Larsen 1999), competitive binding (Green et al. 2000; Kaupmann et al. 1998a; Malitschek et al. 1998) or coupling to K_{ir}3 channels (Kaupmann et al. 1998a). There have been a few reports to the contrary, for example indicating isoform-differential G protein coupling to K_{ir}3 channels (GABA_{B(1a,2)} preferentially mediated K_{ir}3 activity via G_{oα}, while GABA_{B(1b,2)} coupled to both G_{iα} and G_{oα} variants) (Leaney and Tinker 2000). Some reports have also suggested that the anticonvulsant gabapentin interacts differentially with GABA_{B(1)} isoforms, although as attempts to repeat these findings in other laboratories have been unsuccessful, these findings remain controversial (see (Bettler et al. 2004) for a review).

There are, however, other lines of evidence to support the notion that GABA_{B(1a)} and GABA_{B(1b)} are highly important variants mediating GABA_B receptor responses. For example, GABA_{B(1a)} and GABA_{B(1b)} isoforms both show high levels of evolutionary conservation, as demonstrated by their expression in a wide range of vertebrate species including the human, rat, mouse chicken, frog and zebrafish. Interestingly, no evidence was found for the presence of GABA_{B(1a)} or GABA_{B(1b)} receptor isoforms in the fruit fly *Drosophila melanogaster* or the nematode *Haemonchus concortus*. GABA_{B(1)} and GABA_{B(2)} orthologs, however, are present in *Drosophila*, but the characteristic sushi motifs of GABA_{B(1a)} are not present (see (Bettler et al. 2004)).

Although originally postulated to be splice variants, both GABA_{B(1a)} and GABA_{B(1b)} are transcribed directly from different promoter regions of the *Gabbr1* gene (Steiger et al. 2004). The human gene coding the GABA_{B(1a)} isoform is comprised of 23 exons. The first five exons are specific only to the GABA_{B(1a)} isoform and harbour the GABA_{B(1a)} 5'-untranslated region (UTR) (exon 1), a signal peptide, and the two Sushi domains (Steiger et al. 2004). The N-terminus of GABA_{B(1b)} is produced immediately upstream from the sixth

intron of GABA_{B(1a)} (Bettler et al. 2004; Steiger et al. 2004) (Fig. 3). The remaining exons are common to both isoforms (Bettler et al. 2004; Steiger et al. 2004). As such, GABA_{B(1a)} and GABA_{B(1b)} isoforms differ in amino acid sequence only at the extracellular N-terminus domain - the 147 amino acids of the GABA_{B(1a)} isoform being replaced by a truncated 18 amino acid sequence in the GABA_{B(1b)} isoform (Kaupmann et al. 1997) (Fig. 3). In particular, the two sushi domains in the GABA_{B(1a)} N-terminus are absent in the GABA_{B(1b)} isoform (Bettler et al. 1998; Blein et al. 2004; Hawrot et al. 1998). Transcription of the two isoform mRNAs are differentially influenced by ATF4/CREB2 and depolarization-sensitive upstream stimulatory factor (USF) (Steiger et al. 2004). Other (splice) variants of the GABA_{B(1)} subunit have also been reported in the literature, including GABA_{B(1c)}, GABA_{B(1c-a)}, GABA_{B(1c-b)}, GABA_{B(1d)}, GABA_{B(1e)}, GABA_{B(1f)} (see (Bettler et al. 2004; Billinton et al. 2001; Cryan and Kaupmann 2005)). They vary in structure from the GABA_{B(1a)} and GABA_{B(1b)} isoforms mainly in the number or sequence of the N-terminus or in the C-terminus RSRR domain. However, many of these GABA_{B(1)} splice variants are either not evolutionarily conserved across different species, or not expressed in native tissues, and as such the functional relevance of these variants remains controversial (Bettler et al. 2004; Cryan and Kaupmann 2005).

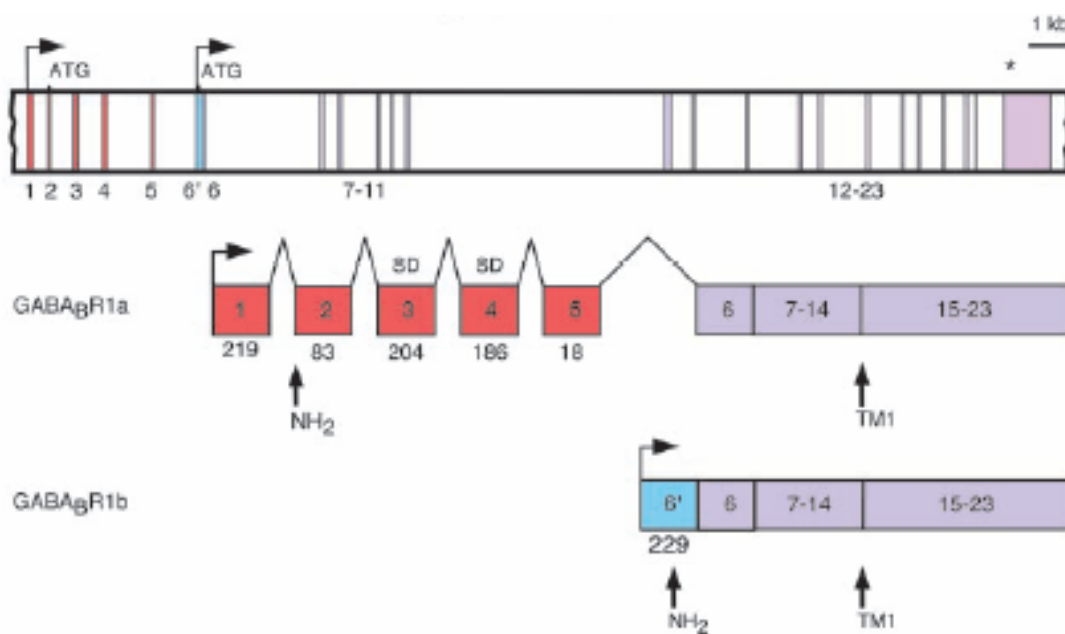


Fig. 3. *Top:* Structure of the human *Gabbr1* gene showing specific GABA_{B(1a)} (red) and GABA_{B(1b)} (blue) exons, the common exons encoding both GABA_{B(1a)} and GABA_{B(1b)} isoforms (purple), transcription start sites (arrows), ATG translation initiation codons, and translational stop codon (asterisk) (scale is indicated above right). *Bottom:* Promoter patterns for the generation of GABA_{B(1a)} (GABA_BR1a) and GABA_{B(1b)} (GABA_BR1b) mRNA, showing exons (boxes) and their size (number of base pairs, below). The GABA_{B(1a)} mRNA contains five exons at the 5'-end which are absent in the GABA_{B(1b)} transcript. Exon 1 contains the GABA_{B(1a)} 5'-UTR. Exon 6' is the alternative first exon for GABA_{B(1b)} and contains the GABA_{B(1b)} 5'-UTR and the GABA_{B(1b)}-specific coding region. The N terminus (NH₂) and first transmembrane domain (TM1) are labeled (arrows). *From Steiger et al., (2004) J Neurosci 24(27): 6115-26.*

The function of the sushi domains in the GABA_{B(1a)} isoform N-terminus have been a mystery, until very recently, and have prompted much speculation as to their function with regard to the GABA_B receptor. Sushi repeats have mostly been found in proteins that are involved in cell-cell adhesion, and prior to their identification in the GABA_{B(1a)} isoform, had not previously been described in association with a neurotransmitter receptor (Bettler et al. 2004). The function of the sushi repeats in GABA_B receptors was therefore speculated to convey protein-protein interactions at the GABA_{B(1a)} N-terminus (Couve et al. 2000), and possibly to direct pre- and postsynaptic trafficking of GABA_{B(1)} receptor isoforms (Bettler et al. 2004; Blein et al. 2004).

Many studies have demonstrated that GABA_{B(1a)} and GABA_{B(1b)} isoforms show marked differences in their pattern of anatomical distribution and relative abundance in different structures of the brain and spinal cord (Benke et al. 1999; Billinton et al. 1999; Bischoff et al. 1999; Fritschy et al. 1999; Kaupmann et al. 1998b; Liang et al. 2000; Malitschek et al. 1998; Poorkhalkali et al. 2000; Princivalle et al. 2000; Towers et al. 2000). This was particularly well demonstrated in the cerebellum, where there was good agreement between studies that the GABA_{B(1a)} isoform was predominantly expressed in the granule cell layer, while the GABA_{B(1b)} isoform was expressed mainly in the Perkinje cells (Billinton et al. 1999; Bischoff et al. 1999; Fritschy et al. 1999; Kaupmann et al. 1998b; Liang et al. 2000; Poorkhalkali et al. 2000).

Perhaps the most comprehensive neuroanatomical study conducted to investigate the expression of the two isoforms to date was that of Bischoff et al., (1999). Using isoform-specific and pan *in situ* hybridization probes, in combination with autoradiographic binding of the GABA_B receptor antagonist [³H]CGP54626, relative levels of GABA_{B(1a)} and GABA_{B(1b)} isoform expression were quantified in 87 structures and nuclei, and in four white matter regions. The greatest disparity in expression of the two isoforms were shown in the septal areas, where expression of GABA_{B(1b)} was greater than that of the GABA_{B(1a)} isoform. This profile was repeated in the majority of thalamic nuclei examined. In contrast, the midbrain and brainstem nuclei, including the substantial nigra pars reticulata, ventral tegmental area, locus ceruleus and DRN, showed a dominant expression of the GABA_{B(1a)} isoform over the GABA_{B(1b)} isoform. In the cortex, GABA_{B(1a)} tended to be somewhat dominant in most layers, and most strikingly in L4 of the parietal cortex. In L6, however, the GABA_{B(1b)} isoform was dominant (Bischoff et al. 1999). The divergent expression profile of the two isoforms lead many researchers to speculate that the two isoforms localised differentially to pre- and postsynaptic locations (Benke et al. 1999; Billinton et al. 1999; Bischoff et al. 1999;

Kaupmann et al. 1998b; Liang et al. 2000; Poorkhalkali et al. 2000; Princivalle et al. 2000; Towers et al. 2000). However, solid evidence for this was never directly demonstrated (Bettler et al. 2004), due in particular to a lack of high quality, isoform-specific antibodies suitable for electron microscopy (Vigot et al. 2006).

GABA_B receptor isoforms also demonstrate a differential expression in development. Fritschy et al., (1999) showed that GABA_{B(1a)} was expressed at higher levels than GABA_{B(1b)} in the rat neonatal brain membranes, but steadily decreased during maturation. In contrast GABA_{B(1b)} was expressed at relatively lower levels in the neonate, increased transiently around post natal day 10, then declined again toward maturity, but was still the dominant isoform in the adult (Fritschy et al. 1999). These results were similar to an earlier study by Malitschek et al., (1988) who showed that from post natal days 2 to 7, GABA_{B(1a)} in the cortex was significantly higher than GABA_{B(1b)}, while from days 14 to adulthood, the expression levels of GABA_{B(1a)} decreased and that of GABA_{B(1b)} rose.

Further indications for distinct physiological roles of the GABA_{B(1)} isoforms comes from the differential induction of GABA_{B(1a)} and GABA_{B(1b)} isoforms in neurological disease, disease models and in response to antidepressant pharmacotherapies. Specifically, GABA_{B(1a)} and GABA_{B(1b)} isoform expression was altered in different regions of the hippocampus of patients with temporal lobe epilepsy (Princivalle et al. 2003). In an animal model of Parkinson's disease, GABA_{B(1a)} was preferentially induced over GABA_{B(1b)} in the basal ganglia of rats following lesion of the nigro-striatal pathway (Johnston and Duty 2003). GABA_{B(1a)} was also enhanced over GABA_{B(1b)} in the hippocampus and spinal cord of rats following chronic treatment with the antidepressants desipramine or fluoxetine (McCarson et al. 2006; Sands et al. 2004a; Sands et al. 2004b) (although chronic amitriptyline increased both GABA_{B(1a)} and GABA_{B(1b)} equally in the spinal cord (McCarson et al. 2006)). Chronic immobilisation stress or repeated testing for withdrawal to a thermal stimulus also enhanced GABA_{B(1a)} expression in the spinal cord (McCarson et al. 2006).

Together the evolutionary conservation, differential control of transcription, and variation in anatomical, developmental and induced expression of GABA_{B(1a)} and GABA_{B(1b)} isoforms tantalizingly suggest that these isoforms are highly relevant and functionally distinct variants of the native GABA_B receptor. However, solid evidence for differential subcellular localization (e.g., pre- versus postsynaptic), or indeed for distinct physiological properties of the two isoforms, was not provided in the aforementioned studies. Furthermore, no isoform-selective ligands exist, thus precluding *in vivo* pharmacological investigations of the

contributions of GABA_{B1a} and GABA_{B1b} to physiological or behavioural functions, such as anxiety or depression.

1.6 Sushi Demystified: GABA_{B(1a)}^{-/-} and GABA_{B(1b)}^{-/-} Mice

In order to better characterise the roles of the two GABA_B receptor isoforms, mice deficient in the GABA_{B(1a)} and GABA_{B(1b)} have recently been generated (Vigot et al. 2006). Given that the two isoforms arise from differential promoter usage of the same *Gabbr1* gene, traditional knockout techniques were not applicable. Instead, the research group of Prof Bernhard Bettler at the University of Basel, in collaboration with Dr. Klemens Kaupmann and co-workers at the Novartis Institutes for BioMedical Sciences in Basel, adopted an elegant point mutation knock-in strategy to convert the start codons for GABA_{B(1a)} and GABA_{B(1b)}, respectively, into stop codons (Fig. 4). To generate the mutant mice, BALB/c gene targeting constructs with mutated initiation codons and a floxed neomycin cassette for the selection of transfected embryonic stem cells, were electroporated into BALB/c embryonic stem cells. Homologous recombination events were diagnosed, and targeted embryonic stem cells injected into C57BL/6 blastocysts, which were then implanted into foster mothers. Chimeric progeny were crossed with BALB/c mice to generate heterozygotic founding mice. The neomycin cassette was excised by crossing founder mice with BALB/c mice expressing Cre- recombinase under control of the cytomegalus virus promoter. Pups born from these matings were then scored for Cre-mediated loss of the neomycin cassette and bred to homozygosity.

In each of the GABA_{B(1a)}^{-/-} and GABA_{B(1b)}^{-/-} mutants, both GABA_{B(1a)} and GABA_{B(1b)} mRNA transcripts were expressed, as predicted. However, specific and total loss of GABA_{B(1a)} and GABA_{B(1b)} proteins was achieved in each specific mutant, respectively, thus validating that the conversion of start to stop codons prevented translation of the specific isoform proteins. Furthermore, GABA_{B(1a)}^{-/-} and GABA_{B(1b)}^{-/-} mice were viable, reproduced normally and did not show any overt physical abnormalities, indicating potential applicability for subsequent behavioural analysis.

The pharmacology of natively expressed GABA_{B(1a,2)} and GABA_{B(1b,2)} heterodimers, as assessed in cortical membranes obtained from the GABA_{B(1b)}^{-/-} and GABA_{B(1a)}^{-/-} mice respectively, was for the most part similar (Vigot et al. 2006). Inhibition of [¹²⁵I]CGP64213 antagonist binding by GABA and L-baclofen did not differ between the two mutant strains, supporting the similar results found with recombinant studies (see section 1.5). Likewise, [³H]-baclofen binding in cortical membranes was attenuated, but to a similar degree, in both

mutants. G protein coupling, as determined using GABA-stimulated GTP γ [35 S] binding in cortical membranes, was also attenuated in both mutants, but slightly more so in the GABA_{B(1b)}^{-/-}.

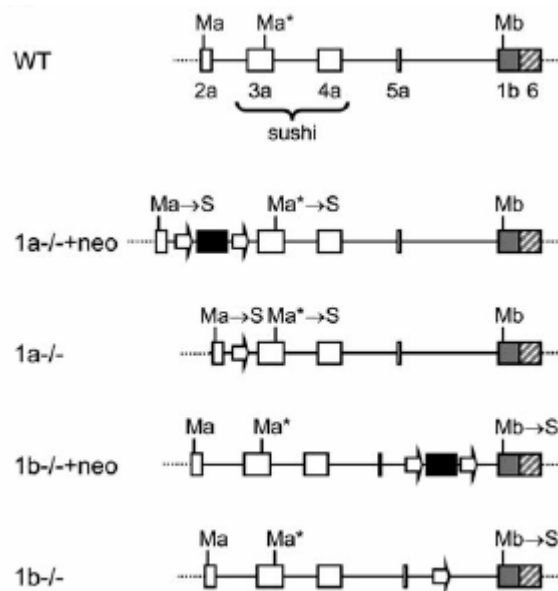


Fig. 4. Generation and genetic architecture of GABA_{B(1a)}^{-/-} (1a^{-/-}) and GABA_{B(1b)}^{-/-} (1b^{-/-}) mice. White boxes denote exons encoding the N terminus of GABA_{B(1a)}, **Ma** denotes the position of the GABA_{B(1a)} start codon. The grey box denotes the exon for the N terminus of GABA_{B(1b)}, **Mb** denotes the position of the GABA_{B(1b)} start codon. Hatched boxes denote shared downstream exons (only exon 6 is shown). **S** denotes the knockin conversion of the respective start codons, Ma and Mb, to stop codons. The black bar denotes the position of the floxed neomycin cassette inserted for selection of transfected embryonic stem cells (introduced in the introns between exons 2a/3a for GABA_{B(1a)}^{-/-} or between exons 5a/1b for the GABA_{B(1b)}^{-/-}). White arrows denote a loxP site that is left behind after Cre-mediated excision of the neomycin cassette. *From Vigot et al., (2006) Neuron 50, 589–60.*

Subsequent analysis of the mutant neuroanatomy, however, comprehensively demonstrated a pre- versus postsynaptic expression profile for GABA_{B(1a)} and GABA_{B(1b)} isoforms respectively (Perez-Garci et al. 2006; Shaban et al. 2006; Vigot et al. 2006). Ultrastructural analysis of the hippocampi and amygdalae of GABA_{B(1a)}^{-/-} and GABA_{B(1b)}^{-/-} mice, conducted with pan-GABA_{B(1)} immunogold labelling and electron microscopy, showed that in GABA_{B(1a)}^{-/-} mice the remaining GABA_{B(1b)} isoform was mostly located at postsynaptic sites opposite from glutamatergic terminals, and to a certain degree at extra synaptic sites. In contrast, in the GABA_{B(1b)}^{-/-} mice, the remaining GABA_{B(1a)} isoform was predominantly located at glutamatergic terminals (Shaban et al. 2006; Vigot et al. 2006). This expression profile was supported by studies with GFP-transfected GABA_{B(1a)} or GABA_{B(1b)} isoforms in CA1 pyramidal neurons of rat hippocampal organotypic cultures. Although both transfected isoforms were present in dendritic processes, GABA_{B(1b)}-GFP was the dominant

isoform present in dendritic spines, while in contrast, GABA_{B(1a)}-GFP targeted axons (Vigot et al. 2006).

In electrophysiological studies in the hippocampus GABA_B heteroreceptors were absent at Schaffer collateral terminals onto the CA1 in GABA_{B(1a)}^{-/-} mice, but were preserved in GABA_{B(1b)}^{-/-} mice. When examining autoreceptor function, baclofen reduced IPSC amplitudes in CA1 pyramidal neurons of both GABA_{B(1a)}^{-/-} and GABA_{B(1b)}^{-/-} mice, indicating that both isoforms fulfilled autoreceptor functions. In contrast, CA1 pyramidal slow IPSCs, as mediated by K_{ir}3.2 inwardly rectifying channels were intact in GABA_{B(1a)}^{-/-} mice, but greatly reduced in GABA_{B(1b)}^{-/-} mice, demonstrating that GABA_{B(1b)} was indeed the predominant isoform mediating GABA_B receptor mediated IPSCs (Vigot et al. 2006). Similar results were obtained in the lateral amygdala, again showing a specific presynaptic heteroreceptor function of GABA_{B(1a,2)}, a postsynaptic function of GABA_{B(1b,2)}, and both isoforms fulfilling autoreceptor functions (Shaban et al. 2006). In L5 cortical neurons, GABA_{B(1b,2)} also fulfilled a postsynaptic function, although the predominant autoreceptor was GABA_{B(1a,2)} (Perez-Garci et al. 2006). This latter finding is of particular significance as it suggests that GABA_{B(1)} autoreceptor expression can vary between the GABA_{B(1)} isoforms in a region-dependent manner.

Deletion of the respective GABA_{B(1)} isoforms had profound impacts on neurophysiological processes of GABA_{B(1a)}^{-/-} and GABA_{B(1b)}^{-/-} mice. Hippocampal LTP induction at CA3-CA1 synapses was significantly impaired in GABA_{B(1a)}^{-/-} mice (Vigot et al. 2006), which mirrored the findings with GABA_{B(1)}^{-/-} mice (Schuler et al. 2001). In contrast, LTP in GABA_{B(1b)}^{-/-} mice was normal. The deficits in LTP shown by GABA_{B(1a)}^{-/-} mice were proposed to result from adaptive changes causing a reduction in the proportion of AMPA silent synapses (Vigot et al. 2006). In the amygdala, the ultrastructural location of the GABA_{B(1)} isoform determined the nature of LTP in the lateral amygdala (LA). Specifically, in GABA_{B(1a)}^{-/-} mice, loss of GABA_{B(1a)} heterosynaptic inhibition removed the necessity for costimulation of the thalamic and cortical afferent inputs for the induction of cortico-LA presynaptic LTP (Shaban et al. 2006). In L5 cortical pyramidal neurons, GABA_{B(1b,2)} receptors on distal dendrites mediated the postsynaptic inhibition of calcium action potentials (Ca⁺²-AP), which otherwise, in the absence of inhibition, forward-propagate to generate a burst of axonal action potentials at the cell soma (Perez-Garci et al. 2006)

Overall the studies by Perez-Garci et al., (2006), Shaban et al., (2006) and Vigot et al., (2006) demonstrated that GABA_{B(1a)} and GABA_{B(1b)} isoforms showed highly specific and differential ultrastructural organisation and neurophysiological roles, most probably mediated

by differential trafficking directed by the presence of sushi repeats on the GABA_{B(1a)} isoform (Vigot et al. 2006).

1.7 Thesis Objectives and Methodological Approach

Given the role of GABA_B receptors in anxiety and depression, and the differential localisation and electrophysiological effects mediated by the GABA_{B(1a)} and GABA_{B(1b)} isoforms, it was hypothesized that these isoforms would have a differential impact on specific behaviours relevant to anxiety and depression. The aim of this thesis was therefore to determine the contribution of the GABA_{B(1)} receptor isoforms to endophenotypes of depression and anxiety-related disorders, by evaluating newly generated mice deficient in the GABA_{B(1a)} or GABA_{B(1b)} subunit isoforms in behavioural, *in vivo* pharmacology and biochemical models.

The first experiment in this series of studies (Chapter 2) aimed to evaluate GABA_B receptor function in different mouse strains using the classic GABA_B receptor agonist, L-baclofen. This provided information as to the degree of genetic contribution to GABA_B receptor-related responses, and a validation of the BALB/c mouse strain as an appropriate background strain to carry the deletion of the GABA_{B(1)} isoforms. Furthermore, it established a methodological approach for the subsequent evaluation of residual GABA_B receptor function in GABA_{B(1)} isoform-deficient mice, principally by demonstrating the importance of utilizing multiple behavioural endpoints in the assessment of GABA_B receptor function.

In initial work with the GABA_{B(1a)}^{-/-} and GABA_{B(1b)}^{-/-} mice it was important to first evaluate basic somatosensory and locomotor functions of the mutant mice (Chapter 3). This allowed determination of whether or not gross phenotypic alterations accompanied the isoform deletion which may either bias responses, or preclude application, in other tests. In this study, residual GABA_B receptor responses were also assessed in GABA_{B(1a)}^{-/-} and GABA_{B(1b)}^{-/-} mice using methods identified in Chapter 2. This allowed determination of the relative contribution of the isoforms to classic GABA_B receptor agonist-induced responses.

GABA_{B(1a)}^{-/-} and GABA_{B(1b)}^{-/-} mice were then examined in tests modelling different components of anxiety and depression-related disorders (Chapters 4-6). Mice were evaluated in a test of aversive learning and memory and, importantly, in the extinction of this memory using a CTA paradigm (Chapter 4). This methodological approach was taken as extinction of conditioned aversive learning tasks in animals are thought to model aspects of human anxiety disorders with a cognitive components, such as post-traumatic stress disorder and panic disorder (Cryan and Holmes 2005; Delgado et al. 2006; Ledgerwood et al. 2005; Ressler et al.

2004). $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice were further investigated in a battery of tests of unconditioned anxiety, and basic hypothalamic-pituitary-adrenal axis HPA characteristics measured, to determine the contribution of the $GABA_{B(1)}$ isoforms in innately anxious behaviour (Chapter 5).

Evaluation of the impact of the $GABA_{B(1)}$ isoforms in depression-related behaviour are described in Chapter 6, in which $GABA_{B(1)}$ isoform-deficient mice were examined in classic tests of antidepressant-like activity: the forced swim test. This study included an investigation of whether serotonin- $GABA_B$ receptor interactions may contribute to the antidepressant-like phenotype of $GABA_{B(1a)}^{-/-}$ mice.

Finally, to further understand the contribution of $GABA_{B(1)}$ isoforms in cognitive endophenotypes $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice were investigated in cognitive tests of recognition, spatial working and avoidance memory (Appendix 1, Chapter 7).

Chapter 2

Differential Sensitivity to the Motor and Hypothermic Effects of the GABA_B Receptor Agonist Baclofen in Various Mouse Strains

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2.1 Abstract

Rationale: Comparison of different mouse strains can provide valuable information about the genetic control of behavioural and molecular phenotypes. Recent evidence has demonstrated the importance of GABA_B receptors in anxiety and depression. Investigation of the pharmacogenetics of GABA_B receptor activation may aid in the understanding of mechanisms underlying the role of GABA_B in affect. *Objectives:* The aim of current study was to determine the relative sensitivity of different mouse strains to GABA_B receptor agonism in two models of GABA_B receptor function, namely hypothermia and motor incoordination. *Methods:* Mice each from 11 strains (BALB/cByJlco, DBA/2Jlco, OF1, FVB/Nlco, CD1, C3H/HeOulco, 129/SvPaslco, NMRI, C57BL/6Jlco, A/JOlHsd and Swiss) were trained to walk on a rotarod for 300 s. On the following day mice received 0, 3, 6 or 12 mg/kg of L-baclofen *p.o.* Rectal temperature and rotarod performance were measured at 0, 1, 2 and 4 hours after drug application. *Results:* L-baclofen produced a significant dose-dependent hypothermia and ataxia in most, but not all, mouse strains examined. The magnitude and duration of response was influenced by strain, with mice of the 129/SvPaslco strain showing largest hypothermic response to 12 mg/kg L-baclofen and C3H/HeOulco the lowest, whereas the BALB/cByJlco strain demonstrated greatest ataxic response on the rotarod, and NMRI the least. Interestingly, some strains (notably C3H/HeOulco) had marked differential hypothermic and ataxic responses, with minimal body temperature responses to L-baclofen but significant ataxia on the rotarod observed. *Conclusion:* There is differential genetic control on specific GABA_B receptor populations that mediate hypothermia and ataxia. Further, these studies demonstrate that background strain is an important determinant of GABA_B receptor mediated responses, and that hypothermic and ataxic responses may be influenced by independent genetic loci.

2.2 Introduction

Comparison of different mouse strains provides valuable information about the importance of genetic background in behavioural and pharmacodynamic phenotypes. Such information is important when considering which strains of mice are most suitable for both genetic manipulation studies and pharmacological analyses (Crawley 2000; Crawley et al. 1997). Recent studies have demonstrated widespread differences in the sensitivity of mice strains to GABA_A / benzodiazepine receptor ligands (Griebel et al. 2000), and serotonergic and noradrenergic antidepressants (Lucki et al. 2001) in animal models of anxiety and depression.

Emerging genetic and pharmacological evidence suggests a role for the metabotropic GABA_B receptor as a therapeutic target for anxiety and depression (Cryan and Kaupmann 2005; Mombereau et al. 2004a). These receptors were first pharmacologically characterized as receptors insensitive to the GABA_A antagonist bicuculline by Bowery et al., (1980), and designated as GABA_B receptors in 1981 (Bowery et al. 2002; Bowery et al. 1981). Presynaptic GABA_B receptors modulate neurotransmitter release by depressing Ca²⁺ influx *via* voltage-activated Ca²⁺ channels (Bowery et al. 2002). Postsynaptic GABA_B receptors predominantly couple to inwardly rectifying K⁺ channels (Luscher et al. 1997) and mediate slow inhibitory postsynaptic potentials (Bowery et al. 2002). GABA_B receptor proteins are abundantly expressed in the brain and are localized in many neuronal cell types including interneuron populations, as well as in certain glial cells, where they form functional heterodimers.

The prototypical GABA_B receptor agonist baclofen (β -p-chlorophenyl-GABA) was first synthesized in 1962 and shown to exert potent muscle relaxant and analgesic properties (see (Froestl et al. 2003)). Baclofen has been invaluable in elucidating the role of GABA_B receptors in various disorders including epilepsy, cognition, pain, gastroesophageal reflux disease and addiction (Bowery et al. 2002). Further, baclofen (Lioresal®) has been in clinical use for the treatment of spasticity for over 30 years (Brogden et al. 1974). Baclofen induces marked hypothermia and deficits in motor coordination, and these have been widely used as behavioural/physiological indices of GABA_B receptor activation in rodents (Brogden et al. 1974; Cryan et al. 2004; Gray et al. 1987; Lehmann et al. 2003; Schuler et al. 2001). Despite this, to our knowledge, there is no data investigating whether these responses are under the same genetic control. Indeed recent strain comparisons in mice suggest that individual behavioural responses to GABA_A receptor ligands (ethanol, diazepam and pentobarbital), including motor in-coordination and hypothermia, have differential genetic determinants (Crabbe et al. 1998; Crabbe et al. 1994; Crabbe et al. 2002).

The aim of these studies, therefore, were to investigate if genetic influences exist in the response profile of 11 different mouse strains to L-baclofen, in tests for hypothermia and motor in-coordination, by recording temperature and rotarod endurance in parallel. It was also of interest to see if the pharmacodynamics of both responses differed within each given strain.

2.3 Methods

Animals

Forty to 41 naive male mice from each of 11 strains were obtained from four different suppliers. Iffa Créo (Charles River), France, bred and supplied the inbred FVB/Nlco, BALB/cByJlco, C57BL/6Jlco, DBA/2Jlco, C3H/HeOulco, 129/SvPaslco and outbred OF1 mice strains; Janvier, France, supplied the outbred strain Swiss (IOPS Orl); Charles River, Germany supplied the outbred CD1 mice; and Harlan, Netherlands, supplied the inbred A/JOlalHsd and outbred NMRI strains. All mice weighed between 15-20 grams on arrival at the laboratory and were tested within 3 weeks. Mice were group-housed 5 per cage on sawdust in macrolon cages with one red, triangular, polycarbonate Mouse House® (Nalgene) per cage and tissue paper nesting materials. Housing was at a constant room temperature of 22-24°C in a 12 h light:dark cycle with lights on at 6 A.M. Food pellets and tap water were available *ad libitum*. All animal experiments were conducted during the light phase, with dosing beginning between 9:30 and 11 A.M. The rotarod and a radio were used to provide acclimatising and background noise prior to and throughout all experimentation. All animal experiments were conducted in accordance with the Veterinary Authority of Basel Stadt, Switzerland.

Rotarod

The rotarod apparatus consists of a 28 mm diameter rod partitioned into 5 available lanes 58 mm wide to accommodate individual mice. The rod was positioned 30 cm above a surface and rotated at a constant speed of 12 rpm. Each day the rod lanes were tightly lined with fresh paper towelling. The test was carried out as previously described (Cryan et al. 2004; Dunham and Miya 1957).

Rectal Temperature

Rectal temperature was measured with a lubricated ELLAB Instruments thermistor probe inserted 20 mm in to the rectum. Mice were hand-held by the tail base and inverted against the cage wall during temperature recording, and the probe held in place until a stable temperature measurement was achieved (about 15 seconds). Care was taken to ensure minimal disturbance to other mice in the cage until all 5 mice in each cage had been measured.

Procedure

Mice strains were tested in two cohorts of about 20 animals on separate days, with the exception of the FVB/Nlco and CD1 strains, where all mice were tested on a single day. Two days prior to testing, the Mouse House® and tissue paper nesting materials were removed from each cage to reduce possible inter- and intra-cage variations in mouse body temperatures on the day of testing. On the day prior to testing, mice were acclimatized to the rectal thermistor probe by performing a single body temperature measurement and were then trained to walk on the rotarod for 300 s. Rotarod training was performed in two to four sessions, depending on the innate ability demonstrated by each strain to walk on the rotarod. Mice were returned to the home cage for an interval of about 30 minutes between training sessions. The number of falls during training was recorded for each mouse.

On the day of testing, mice were moved to the experimental lab at least 2 hours before dosing. One hour before dosing, rectal temperature was taken. At time 0 hr, rectal temperature was taken for all animals in a cage, and then the animals placed on the rotarod for 300 seconds to re-establish training and provide an experimental baseline. All rotarod data were disregarded from animals falling more than once at this time point; however, animals were placed on the rod at all time-points to mimic the conditions of full participants. Drug treatments were still administered and temperature data were still collected from these animals. All animals were immediately dosed with their allocated treatment on completing 300 seconds on the rotarod. One, 2 and 4 hours thereafter rectal temperature and endurance on the rotarod were recorded.

Occasionally, mice clung to the rotarod for full revolutions without attempting to walk on top of the rod. Animals that clung to the rod in this manner for $\geq 80\%$ of the time that they spent on the rod were thus considered ataxic and were allocated a score of 0 s endurance. Animals that clung to the rod for full revolutions totalling less than 80% were correspondingly recorded for the amount of time they spent actively walking on the rod.

An index of the degree of hypothermia was calculated by totalling the differences between control (pre-dose) rectal temperature and the temperatures at 1, 2 and 4 h post treatment for each animal. An index of the degree of ataxia was calculated by determining the difference between cumulative total time on the rotarod 1, 2 and 4 h after drug or vehicle application as a proportion of the total possible (i.e. percent reduction from 900 seconds).

Drugs

L-baclofen (the active enantiomer of baclofen) was synthesised in-house (Novartis Pharma). Baclofen doses were prepared fresh daily as a suspension in 0.5% methylcellulose in water. Vehicle or baclofen were administered orally to deliver a final dose of 0, 3, 6 and 12 mg/kg in a volume of 10ml/kg. The experimenter was blinded to drug treatments during the entire experimental procedure.

Statistics

Mouse strain effects on basal rectal temperature (taken 1 h before drug administration on the day of testing) were analysed with one-way analysis of variance (ANOVA) followed by Fishers LSD post hoc comparisons. Within-strain analysis of the effect of baclofen treatment on body temperature employed a two-way repeated measures ANOVA (with dose and time as factors) followed by Fishers LSD post hoc comparisons. The cumulative effect of each baclofen dose within each strain on the degree of hypothermia was analysed with one-way ANOVA followed by Fishers LSD post hoc comparisons.

The relationship between mean strain basal body temperature and hypothermia with baclofen at 12 mg/kg, as well as the correlation between mean strain hypothermia and ataxia at 12 mg/kg, were analysed using Pearson product moment correlation. This method has been previously reported to estimate the degree of shared genetic control between traits (Crabbe et al. 2002; Hegmann and Possidente 1981).

The effect of strain on falls from the rotarod during training were analysed by Kruskal-Wallis one-way ANOVA on ranks, due to failure of normality in assumption testing, followed by Dunn's method for post hoc comparisons. Within-strain analysis of the effects of baclofen dose on endurance on the rotarod was analysed using Kruskal-Wallis one-way ANOVA on ranks within time, due to failures in normality and equal variance testing, followed by Dunn's Method for post hoc comparisons. The cumulative effect of dose within each individual strain on rotarod endurance after drug or vehicle application was analysed with Kruskal-Wallis one-way ANOVA on ranks, due to failure in normality testing, followed by Dunn's Method for post hoc comparisons.

Effect of strain on ataxia (summed endurance at 1, 2 and 4 h post-drug or vehicle application) and hypothermia (summed $\Delta T^{\circ}\text{C}$) were analysed with one-way ANOVA followed by Fishers LSD Method for post hoc comparisons. All statistical analyses were performed using SigmaStat[®] for Windows version 2.03, 1992-1997, SPSS Inc.

2.4 Results

General

Inter-animal aggression in FVB/Nlco resulted in 10 of these animals being removed from the study before experimentation began, giving a final *n* of 30 animals. Inter-male aggression has been described in this mouse strain previously (Mineur and Crusio 2002; Pugh et al. 2004).

There were 11 incidences of animals in the C57BL/6Jlco strain clinging to the rotarod for $\geq 80\%$ of their endurance. There were three such incidences in the DBA/2Jlco strain, three in the FVB/Nlco strain, one in the OF1 strain, two in the CD1 strain, five in the 129/SvPaslco strain and six in the Swiss mice. In the A/JOlalHsd mice there were five incidences of clinging for 80% or more of their endurance, and three incidences of full-revolution clinging that lasted for less than 80% of the endurance time, interspersed with attempts to walk on the rod. Only animals receiving baclofen demonstrated rod-clinging behaviour.

Effects of baclofen on hypothermia

Strains differed in basal body temperature [ANOVA: $F(10, 429) = 5.41$, $P < 0.001$], with the FVB/Nlco strain showing the highest basal body temperature ranking of the 11 strains investigated, and the BALB/cByJlco the lowest (Table 1).

Table 1. Basal rectal temperature of different mouse strains (taken 1 h before start of the experiment).

Strain	Temperature ($^{\circ}\text{C}$) †	Rank-order	n
FVB/Nlco	36.42 (± 0.12)a	1	31
CD1	36.24 (± 0.12)a	2	40
NMRI	36.23 (± 0.10)a	3	40
Swiss	36.17 (± 0.07) ab	4	40
A/JOlalHsd	35.97 (± 0.09) bc	5	48
OF1	35.93 (± 0.06) bc	6	40
C57BL/6Jlco	35.93 (± 0.08)bc	7	40
C3H/HeOulco	35.92 (± 0.08) bc	8	41
129/SvPaslco	35.85 (± 0.11)cd	9	39
DBA/2Jlco	35.82 (± 0.09)cd	10	40
BALB/cByJlco	35.65 (± 0.10)d	11	41

†Data shows means \pm SEM. Means with different superscripts are significantly different ($P < 0.05$).

Body temperature responses of the strains were differentially affected by baclofen dose and the time of measurement after dosing (Figure 1). This resulted in a drop in body temperature in most strains. When analysed as a two-way repeated measure ANOVA, with time and dose as factors, body temperatures in BALB/cByJlco [Dose: $F(3, 132) = 30.52$, $P < 0.001$] and C57BL/6Jlco [Dose: $F(3, 108) = 11.95$, $P < 0.001$] were affected only by the highest dose of baclofen used (12 mg/kg), whereas DBA/2Jlco [Dose: $F(3, 108) = 15.19$, $P < 0.001$], FVB/Nlco [Dose: $F(3, 81) = 16.67$, $P < 0.001$], 129/SvPaslco [Dose: $F(3, 105) = 44.68$, $P < 0.001$], A/JOlalHsd [Dose: $F(3, 132) = 30.52$, $P < 0.001$] and the out-bred strains NMRI [Dose: $F(3, 108) = 7.97$, $P < 0.001$], Swiss [Dose: $F(3, 108) = 12.06$, $P < 0.001$], CD1 [Dose: $F(3, 108) = 6.14$, $P < 0.01$] and OF1 [Dose: $F(3, 108) = 12.98$, $P < 0.001$] all showed hypothermic responses to both 6 and 12 mg/kg baclofen (Figure 1).

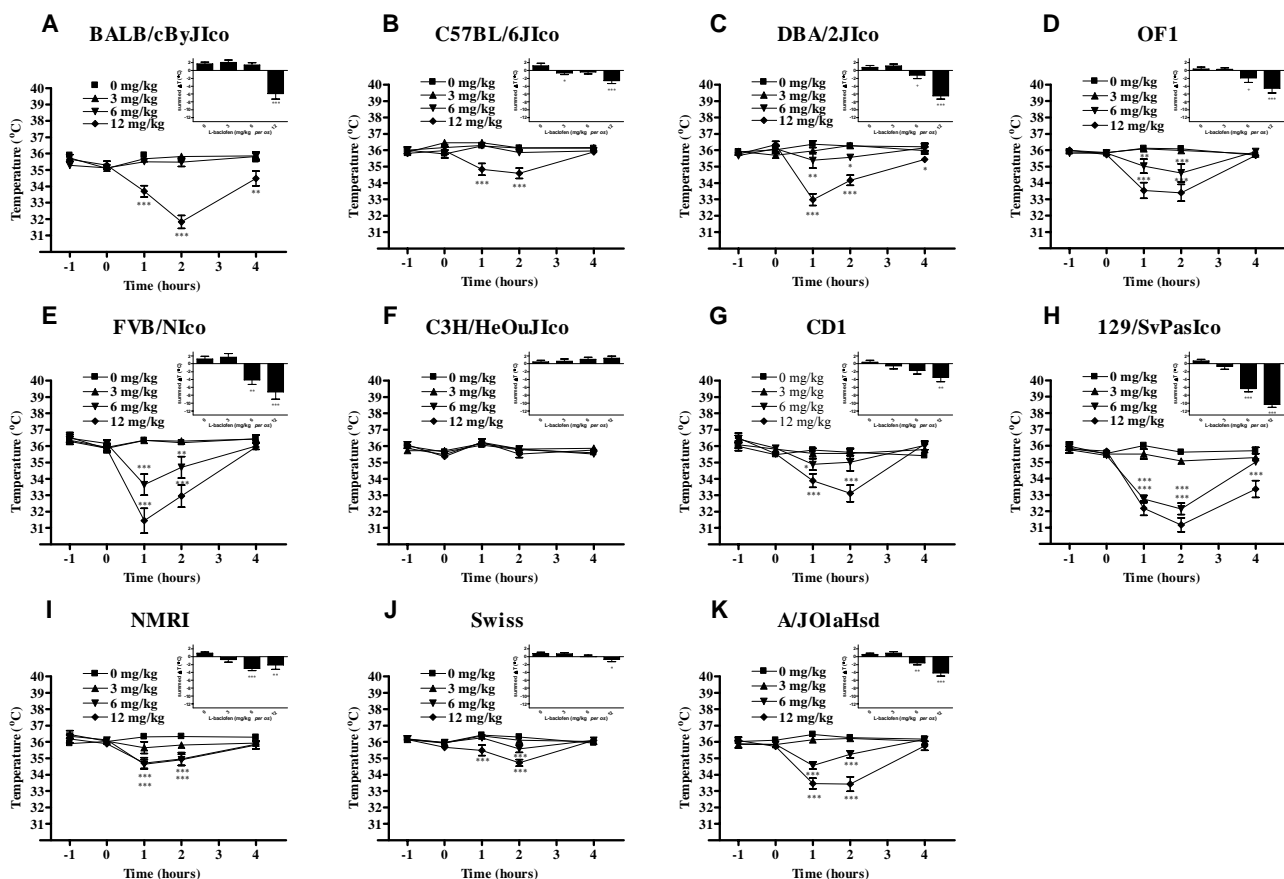


Fig. 1 (a-k). Mean (+ SEM) body temperature responses to baclofen at 0, 3, 6 and 12 mg/kg p. o. in 11 different mouse strains at -1, 0, 1, 2 and 4 h relative to the time of drug application. *Inset graphs* are mean (+ SEM) summed total body temperature change from baseline within animal, pooled over time for each different baclofen dose. *, **, *** Groups that differed significantly compared to vehicle-treated mice ($P < 0.05$, < 0.01 , and < 0.001 , respectively).

Peak hypothermic responses to baclofen were reached by 1 hour post-dosing for the strains DBA/2Jlco [Interaction: $F(9, 108) = 14.13, P < 0.001$], FVB/Nlco [Interaction: $F(9, 81) = 11.19, P < 0.001$] and NMRI [Interaction: $F(9, 108) = 2.64, P < 0.01$], whereas BALB/cByJlco [Interaction: $F(9, 111) = 12.65, P < 0.001$], C57BL/6Jlco [Interaction: $F(9, 108) = 6.29, P < 0.001$], 129/SvPaslco [Interaction: $F(9, 105) = 19.31, P < 0.001$], A/JOlacHsd [Interaction: $F(9, 132) = 18.36, P < 0.001$] and the three outbred strains CD1 [Interaction: $F(9, 108) = 8.23, P < 0.001$], OF1 [Interaction: $F(9, 108) = 8.76, P < 0.001$] and Swiss [Interaction: $F(9, 108) = 5.57, P < 0.001$] showed peak hypothermia at two hours post-dosing. The body temperature of all strains had returned to normal within 4 hours of dosing, with the exception of BALB/cByJlco, DBA/2Jlco and 129/SvPaslco when given the 12 mg/kg baclofen dose only. Interestingly, the C3H/HeOulco strain showed no body temperature responses to baclofen at any dose or time point investigated [Dose: $F(3, 111) = 0.77, P = 0.52$; Interaction: $F(9, 111) = 0.89, P = 0.54$].

Pearson's product moment correlation of strain means showed no significant relationship between basal body temperature and hypothermic responses to 12 mg/kg baclofen ($r = 0.19, P = 0.58$). When assessed as a cumulated hypothermic response to baclofen over time and within strain (Figure 1 insets), again it can be seen that BALB/cByJlco mice [$F(3, 37) = 19.41, P < 0.001$] respond significantly only to the 12 mg/kg dose of baclofen. Similar effects were observed with the out-bred CD1 [$F(3, 36) = 3.72, P < 0.05$] and Swiss [$F(3, 36) = 2.98, P < 0.05$] strains, with the latter strain showing only relatively small cumulative hypothermia to baclofen. C57BL/6Jlco [$F(3, 36) = 7.17, P < 0.001$], DBA/2Jlco [$F(3, 36) = 25.81, P < 0.001$], FVB/Nlco [$F(3, 27) = 11.90, P < 0.001$], 129/SvPaslco [$F(3, 35) = 51.74, P < 0.001$], A/JOlacHsd [$F(3, 45) = 20.83, P < 0.001$] and the outbred OF1 [$F(3, 36) = 7.45, P < 0.001$] and NMRI [$F(3, 36) = 5.51, P < 0.01$] strains show a significant incremental increase in hypothermia with increasing doses of baclofen above 3 mg/kg. However the incremental increase in response in the C57BL/6Jlco to the lower doses of baclofen were relatively small in comparison with that observed in other strains (Table 1). Again C3H/HeOulco, when assessed in this manner, had a blunted body temperature response to all of the doses of baclofen used in this experiment [$F(3, 37) = 0.72, P = 0.55$].

Effects of baclofen on rotarod endurance

Strains differed in the number of falls accumulated during rotarod training (Kruskal-Wallis: $H = 125.3, P < 0.001$), with the 129/SvPaslco falling most often, and the NMRI strain the least (Figure 2).

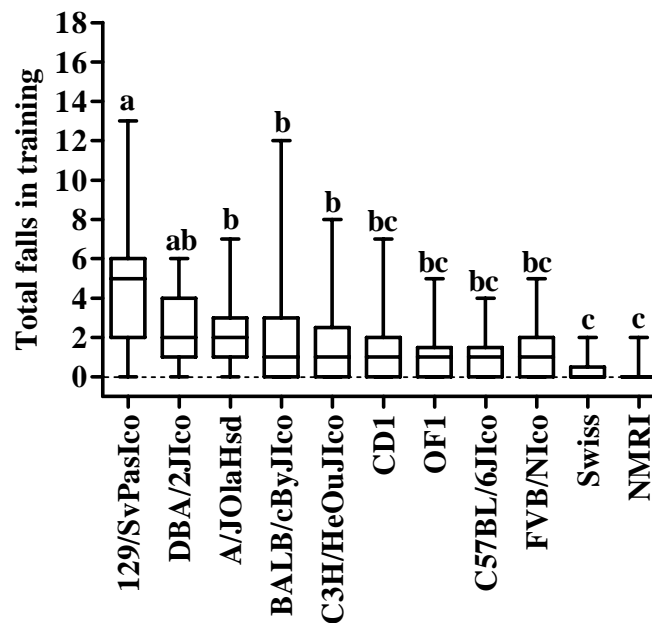


Fig. 2. Median (\pm 25% quartile, *white box* ; \pm 75% quartile, *error bar*) number of falls from the rotarod during training sessions, medians with different superscripts are significantly different ($P < 0.05$).

Endurance on the rotarod, like body temperature, was affected differently and temporally across the strains by baclofen (Figure 3). Similar to the hypothermic effects, BALB/cByJIco (1h: $H = 28.63$, $P > 0.001$, 2 h: $H = 28.58$, $P < 0.001$) and C57BL/6JIco (1 h: $H = 18.57$, $P > 0.001$, 2 h: $H = 12.68$, $P < 0.01$) were ataxically affected only by baclofen at 12 mg/kg. Although unlike their temperature responses, this was also the case for FVB/NIco (1 h: $H = 11.85$, $P < 0.01$; 2 h: $H = 14.89$, $P < 0.01$), and the out-bred strains OF1 (1 h: $H = 11.52$, $P < 0.01$), CD1 (1 h: $H = 19.78$, $P < 0.001$; 2 h: $H = 9.18$, $P < 0.05$) and Swiss (1 h: $H = 17.61$, $P < 0.001$; 2 h: $H = 18.32$, $P < 0.001$). The DBA/2JIco (1 h: $H = 22.26$, $P < 0.001$; 2 h: $H = 12.47$, $P < 0.01$), 129/SvPasIco (1 h: $H = 27.13$, $P < 0.001$; 2 h: $H = 26.79$, $P < 0.001$), and A/JOlHsd (1 h: $H = 23.18$, $P < 0.001$; 2 h: $H = 13.92$, $P < 0.01$) mice showed responses to both 6 and 12 mg/kg. NMRI was the only strain which did not display significant ataxia to any doses of baclofen or at any time point investigated in the study. Of note, the C3H/HeOuJIco strain also showed significant responses to baclofen on the rotarod (1 h: $H = 16.74$, $P < 0.001$; 2 h: $H = 12.24$, $P < 0.01$), in comparison to no effect on body temperature. All mice strains had returned to a normal level of coordination not statistically different from baseline within 4 h after dosing.

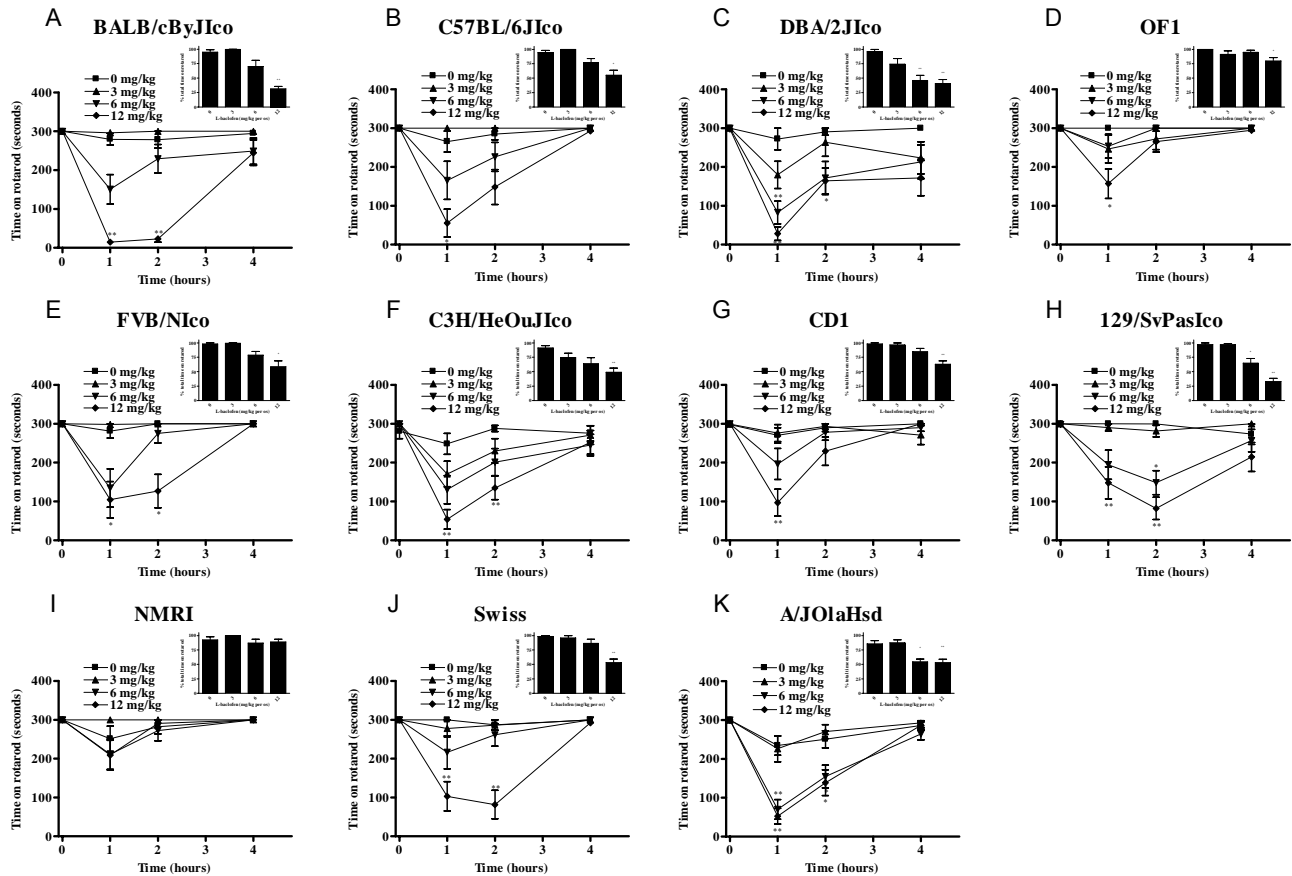


Fig. 3 (a-k). Mean (+ SEM) time spent on the rotarod (up to a maximum of 300 s per time point) following baclofen at 0, 3, 6 and 12 mg/kg *p. o.* in 11 different mouse strains 0, 1, 2 and 4 h after drug application. *Inset graphs* are mean (+SEM) percentages of summed total time spent on the rotarod 1, 2 and 4 h after drug or vehicle application relative to the maximum possible duration (900 s) for the different baclofen doses. Statistical analyses were performed on raw summed data. *, **, *** Groups that differed significantly compared to vehicle-treated mice ($P < 0.05$, < 0.01 , and < 0.001 , respectively).

Confirmation of the ataxic responses to only the highest dose of baclofen were demonstrated by analysing summed endurance (Figure 3 insets) for the inbred strains BALB/cByJlco ($H = 26.33$, $P < 0.001$), C57BL/6Jlco ($H = 19.78$, $P < 0.001$), FVB/Nlco ($H = 11.99$, $P < 0.01$), C3H/HeOulco ($H = 11.42$, $P = 0.01$), and the outbred strains OF1 ($H = 14.27$, $P < 0.01$), CD1 ($H = 19.12$, $P < 0.001$) and Swiss ($H = 24.52$, $P < 0.001$). Likewise, summed endurance for NMRI ($H = 6.41$, $P = 0.09$) failed to demonstrate significant ataxic responses to any of the doses of baclofen used when analysed in this way. Although the NMRI strain approached statistical significance for ataxia at the highest dose of baclofen used ($P = 0.06$), the reduced endurance on the rod at 12 mg/kg was small – about an 11% mean reduction from the possible cumulated time spent on the rod at the three rotarod sessions after dosing (Figure 4a). DBA/2Jlco ($H = 19.79$, $P < 0.001$), 129/SvPaslco ($H = 28.07$, $P < 0.001$),

and A/JOl_aHsd ($H = 20.35$, $P < 0.001$) mice, in comparison, show significant dose-related ataxia to both the 6 and 12 mg/kg doses of baclofen (Figure 3 insets).

Ranking of strains for sensitivity to baclofen

Comparison of the different strains for summed hypothermic sensitivity to 12 mg/kg baclofen revealed a significant effect of strain [$F(10, 100) = 10.05$, $P < 0.001$] allowing the ranking of strains from least to most sensitive ($P < 0.05$, Table 2, Figure 4a). There was also an influence of strain on the ataxic sensitivity to 12 mg/kg of baclofen [$F(10, 96) = 7.71$, $P < 0.001$], with post hoc analysis again allowing ranking of the different strains from least to most sensitive ($P < 0.05$, Table 2, Figure 4a).

The rank order of ataxia, from least to most sensitive strains, differed from the hypothermic sensitivity rank order (Figure 4b). Mean strain hypothermic sensitivity and ataxia to 12 mg/kg baclofen were not significantly correlated ($r = 0.35$, $P = 0.30$).

Table 2. Rank orders of strain sensitivity (from least to most sensitive) to baclofen (12 mg/kg) on the basis of hypothermic body temperature response (ΔT from baseline within animal ($^{\circ}\text{C}$) summed over 4 h), and ataxic responses on the rotarod (total summed endurance on the rotarod over three 5-min sessions at 1, 2 and 4 hours after drug or vehicle application)

Strain	Hypothermia		Strain	Time on Rotarod	
	$\Sigma(\Delta T)\dagger$	Rank		Σ Endurance (s) \dagger	Rank
C3H/HeO _u Jlco	1.42 (± 1.74)a	1	NMRI	801 (± 42)a	1
Swiss	-0.69 (± 0.56)ab	2	OF1	717 (± 53)ab	2
NMRI	-2.12 (± 1.10)bc	3	CD1	567 (± 53)bc	3
C57BL/6Jlco	-2.63 (± 0.70)bc	4	FVB/Nlco	531 (± 88)cd	4
CD1	-3.44 (± 1.11)cd	5	C57BL/6Jlco	497 (± 75)cd	5
A/JOl _a Hsd	-4.14 (± 0.75)cde	6	A/JOl _a Hsd	778 (± 54)cd	6
OF1	-4.56 (± 1.16)cdef	7	Swiss	478 (± 57)cd	7
BALB/cByJlco	-5.88 (± 1.41)def	8	C3H/HeO _u Jlco	442 (± 67)cde	8
DBA/2Jlco	-6.45 (± 0.80)ef	9	DBA/2Jlco	365 (± 63)de	9
FVB/Nlco	-7.16 (± 1.85)f	10	129/SvPaslco	297 (± 48)e	10
129/SvPaslco	-10.32 (± 0.78)g	11	BALB/cByJlco	283 (± 38)e	11

\dagger Data shown are means (\pm SEM). Means with different superscripts are significantly different ($P < 0.05$)

2.5 Discussion

The present study shows that baclofen produces different degrees of hypothermia and ataxia in the eleven mouse strains investigated. In addition, the strains most sensitive to the hypothermic responses were not necessarily the same as those giving the greatest ataxia and vice versa. These findings point to a differential genetic influence on various GABA_B receptor mediated responses *in vivo*, which may have marked implications regarding choice of strain for genetic and pharmacological studies influenced by GABA_B receptor function.

GABA_B receptors have long been known to influence body temperature in mammals. Previous studies have demonstrated baclofen-induced hypothermia in C57BL/6 mice (Gray et al. 1987), C57BL/6:129Sv hybrid mice (Queva et al. 2003), BALB/c mice (Humeniuk et al. 1995; Schuler et al. 2001), OF1 mice (Cryan et al. 2004), rats (Serrano et al. 1985; Zarrindast and Oveissi 1988), rabbits (Frosini et al. 2004) and in man (Perry et al. 1998). The weak GABA_B receptor agonist and GABA metabolite, γ -hydroxybutyrate (GHB), has also been shown to produce a reduction in body temperature (Kaupmann et al. 2003). The GABA_B receptor antagonist CGP35348 blocks the hypothermic response to baclofen in rats (Jackson and Nutt 1991) and in BALB/c mice (Humeniuk et al. 1995). Furthermore, GABA_{B(1)} or GABA_{B(2)} receptor subunit-deficient mice do not demonstrate any hypothermic response to GABA_B receptor agonists, although their basal body temperature is lower than that of their wild-type litter mates (Gassmann et al. 2004; Kaupmann et al. 2003; Queva et al. 2003; Schuler et al. 2001).

Results from the present study indicate that although baclofen can induce hypothermia in many mice strains, the degree of hypothermia produced is dependent on their genetic background. Further, this response may be absent altogether, as we observed in the C3H/HeOulco strain (Table 2, Figure 1f, Figure 4a). Variations in the body temperature response profiles of different mouse strain have also been previously reported for pentobarbital (Crabbe et al. 2002), ethanol (Crabbe et al. 1994), diazepam (Crabbe et al. 1998) and morphine (Belknap et al. 1998). Although it is not necessarily expected that different drugs will give similar hypothermic responses in different strains, it is interesting to note that in our studies the C3H/HeOulco strain body temperature was unaffected by baclofen, and in the study of Belknap and colleagues (1998) the C3H/HeJ strain did not show any hypothermia to morphine. Conversely, the 129/SvPaslco strain in this study responded with a high degree of both ataxia and hypothermia to baclofen, and the 129P3/J (formerly 129/J; (Festing et al. 1999) strain was shown to be highly susceptible to the hypothermic and ataxic effects of pentobarbitone in comparison with other strains (Crabbe et al. 2002). However, it should be

noted that extensive genetic variability between the 129 substrains exist (Simpson et al. 1997; Threadgill et al. 1997), which makes drawing comparisons about like-phenotypes within this strain more complicated.

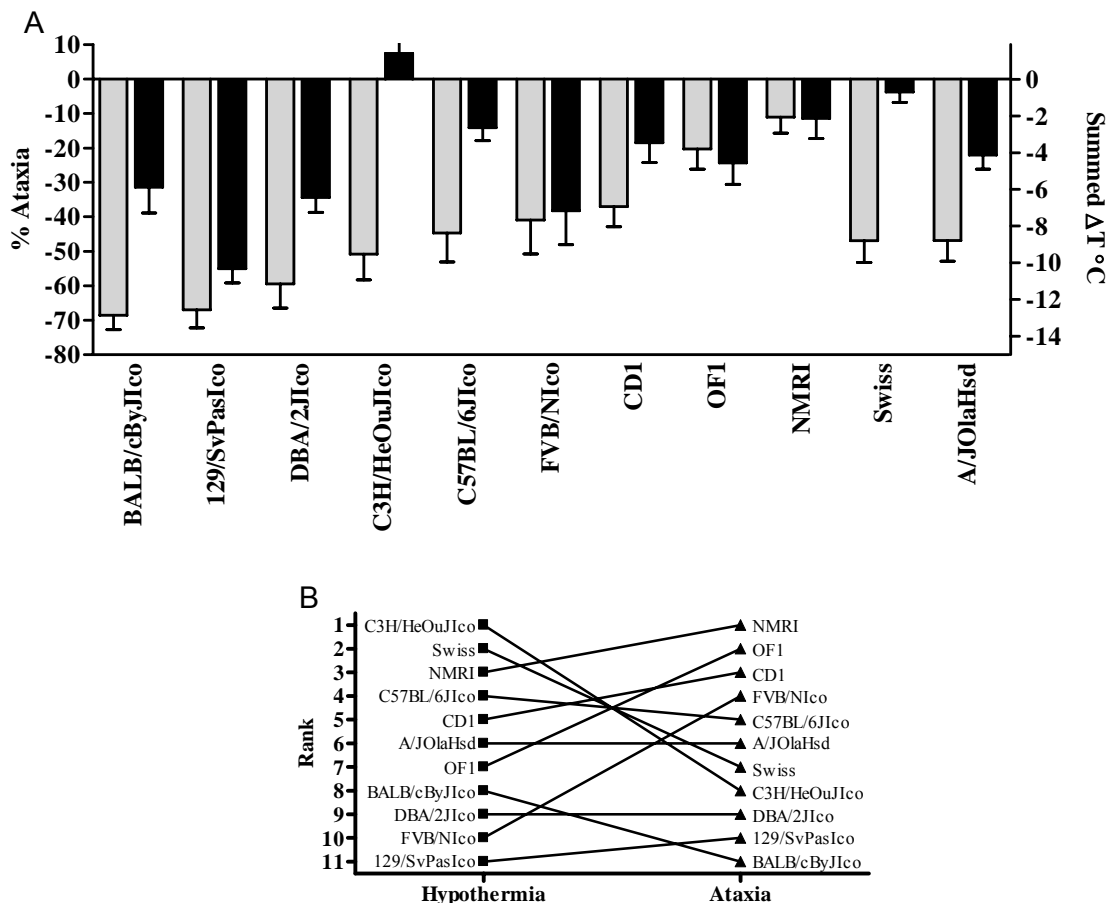


Fig 4. Relative ataxic and hypothermic sensitivity to the highest dose of baclofen (12 mg/kg) in 11 different mouse strains. A) Pale grey bars: mean (+ SEM) percent reduction in total time spent on rotarod from a possible maximum of 900 s (300 s at each of the three post-drug time points tested) following baclofen dosing. Black bars: mean (+ SEM) change in temperature (ΔT), for each mouse relative to own control, summed over the 4 time points tested, following baclofen dosing. B) Schematic showing disparity of rank order in mouse strain hypothermic and ataxic sensitivity to baclofen (least sensitive = 1, most sensitive = 11).

Baclofen has also long been known to produce motor discoordination in a range of species, for example in BALB/c (Gassmann et al. 2004; Schuler et al. 2001), OF1 (Cryan et al. 2004) and C57BL/6 mice (Gray et al. 1987), rats (Kasture et al. 1996; Smith and Vestergaard 1979) and rabbits (Frosini et al. 2004). Clearly, it is these muscle relaxant effects

that have made baclofen the drug of choice for treatment of spasticity in man (Bowery et al. 2002). The results of the present study indicate that indeed, as for hypothermia, the severity of baclofen-induced ataxia in mice is highly dependent on the background strain, and may be absent altogether, as in the case of the NMRI strain (Table 2, Figure 3i, Figure 4a). Other strains, such as C3H/HeOJlco, DBA/2Jlco, 129/SvPaslco and BALB/cByJlco, show relatively severe ataxic responses to baclofen. Interestingly, recent studies (Rustay et al. 2003a) have demonstrated that the BALB/cByJ and DBA/2 strains (and also FVB/N) failed to show ataxia with 2 g/kg of ethanol on the accelerating rotarod. However, on a fixed speed rotarod (rotating at 10 rpm), which is a similar speed to that used in our study (12 rpm), all mice strains investigated were similarly impaired by the three doses (1, 2 and 3 g/kg) of ethanol studied (Rustay et al. 2003a). Crabbe and co-workers have also investigated the ataxic effects of the GABA_A allosteric modulators ethanol (Crabbe et al. 1994), pentobarbital (Crabbe et al. 2002) and diazepam (Crabbe et al. 1998) in different mouse strains. Overall, when comparing the strains used in these aforementioned studies with the strains most similar to those used in our studies, there appears to be little consistent agreement on ataxic ranking. This is to be expected to a certain degree as the primary sites of drug action differ between these studies. Additionally, we acknowledge that caution must be taken when comparing rotarod data amongst different laboratories, as parameters such as rotarod speed, or accelerating versus fixed speed protocols can influence animal performance and hence the interpretation of result (Rustay et al. 2003b).

Multiple mechanisms may underlie the different ataxic and hypothermic responses to baclofen in the mouse strains. Firstly, it is possible that genetic influence on pharmacokinetics and metabolism of baclofen may influence the different sensitivity between strains. Certainly, variations in mouse strain responses to pentobarbital and diazepam appear to be at least somewhat influenced by differences in pharmacokinetics, although pharmacodynamic contributions were much greater (Crabbe et al. 1998; Crabbe et al. 2002). To our knowledge, there is no inter-strain comparison of the pharmacokinetics of GABA_B receptor agonists. However, when making within-strain comparison of GABA_B receptor-mediated responses, there is unlikely to be a major pharmacokinetic influence. We purposely investigated simultaneously, two distinct responses in the same animals in an effort to minimize any potential interpretation of a pharmacokinetic basis for the effects. Thus the differential responses of baclofen on motor in-coordination and temperature in certain strains, such as the C3H/HeOJlco and NMRI, are largely due to pharmacodynamic influences.

It may be postulated that strains with different basal temperature set points could respond to hypothermia-induction in a congruent fashion. However, poor correlations between the strain mean hypothermic responses and basal body temperature illustrate that these two parameters are not significantly related at least with regard to baclofen. Just as basal body temperature varies with strain, so to does innate ability on the rotarod, in terms of both baseline performance and ability to learn the task over time (Bothe et al. 2004; Brooks et al. 2004; McFadyen et al. 2003; Tarantino et al. 2000). Additionally, widespread strain differences in cognitive ability have been reported (Crawley 2000; Crawley et al. 1997). Hence it is plausible that strain learning ability on rotarod could influence performance in the present study. However, the baselines at time zero, the day after training, with the exception of animals of the A/JOlHsd strain, were stable showing that the mice had learned the task adequately. The generally poor performance of the A/J strain on the fixed speed rotarod has also been demonstrated in other studies (Rustay et al. 2003a). Nevertheless, given the fact that GABA_B receptors modulate cognitive performance (although the specific mechanisms are still not fully understood; Bowery et al. 2002; Schuler et al. 2001), one cannot totally rule out potential cognition-altering effects of baclofen on rotarod performance.

Potential regional differences in GABA_B receptor expression may also play a role in the altered hypothermic or ataxic responses to baclofen in the different mouse strains. Body temperature is coordinated primarily by the anterior hypothalamus and preoptic area (Boulant 2000; Frosini et al. 2004), and is mediated at least partially through GABA_B receptors in these regions (Jha et al. 2001; Pierau et al. 1997; Yakimova et al. 1996). The posterior hypothalamus and brainstem nuclei are also implicated in body temperature regulation. In the rat, the GABA_{B(1a)} receptor isoform is relatively heavily expressed in the supraoptic nucleus of the hypothalamus (Liang et al. 2000), and the GABA_{B(1)} receptor subunit is predominantly expressed (over that of the GABA_{B(2)} receptor subunit) in most serotonergic and catecholaminergic neurons of the brainstem nuclei that are involved in regulation of autonomic functions (Burman et al. 2003).

GABA_B receptors are also widely expressed in many neuroanatomical structures involved in motor control and coordination such as the cerebellum, thalamus, striatum, sensory motor cortex and spinal cord (see (Benke et al. 1999; Bowery et al. 2002; Chen et al. 2004; Fritschy et al. 2004; Liang et al. 2000; Waldvogel et al. 2004). Given such an expression profile it would be expected that baclofen could induce motor impairment at numerous different anatomical levels. Indeed, baclofen has been proposed to have direct actions on various regions known to influence motor in-coordination including the motor

cortex (Frosini et al. 2004), substantia nigra (Chan et al. 1998; Turski et al. 1990), cerebellum (Dar 1996) and at the spinal level (Bettler et al. 2004). Detailed information on specific cross strain differences in GABA_B receptor expression patterns have not been reported to date. However, it is conceivable that variations in GABA_B receptor expression and abundance in motor- and temperature-related anatomical regions may underlie strain differences in sensitivity to baclofen.

Finally, other neurotransmitter systems can influence body temperature and ataxia. The neurochemistry of temperature control is complex, and certainly involves many neurotransmitter receptor systems including 5-HT_{1A} (Cryan et al. 1999; Hedlund et al. 2004), 5-HT_{2C}, dopamine D₂ receptors (Cryan et al. 2000), 5-HT₇ (Hedlund et al. 2004), GABA_A receptor ethanol, benzodiazepine, and barbiturate binding sites (Crabbe et al. 1998; Crabbe et al. 1994; Crabbe et al. 2002); cholinergic (Unal et al. 1998); noradrenergic (Myers et al. 1987) and the opioid receptor system (Adler et al. 1988; Belknap et al. 1998). On the other hand, the glutamate, dopamine, adrenergic and cholinergic systems are the main neurotransmitters associated with motor control in mice (Svensson et al. 1995). GABA_B has long been known to interact with monoaminergic systems, as demonstrated by baclofen-induced increases in striatal dopamine (Carlsson et al. 1977), and attenuation of baclofen-induced increases in noradrenaline turnover by the α 2-adrenergic receptor agonist clonidine (Sawynok and Reid 1986). The interactions between GABA_B and 5-HT have been shown particularly at the level of the dorsal raphe nucleus (Judge et al. 2004; Mannoury la Cour et al. 2004), and may occur through presynaptic GABA_B heteroreceptor inhibition of 5-HT release, or possibly through G-protein coupling interactions between 5-HT_{1A} and GABA_B receptor complexes (Mannoury la Cour et al. 2004). It is noteworthy that although GABA_B may influence serotonergic-mediated effects, the reverse, at least as determined via hypothermic responses to GABA_B and 5-HT_{1A} agonists, may not necessarily hold true. Gray et al., (1987) demonstrated that serotonin depletion via ICV 5,7-dihydroxytryptamine administration abolished the hypothermic effect of the 5-HT_{1A} receptor agonist 8-OH DPAT, but did not alter baclofen-induced hypothermia. Of particular relevance to motor control, GABA_B receptor agonism has also been shown to influence neostriatal glutamatergic excitability, most likely through inhibition of presynaptic glutamate release via GABA_B inhibition of Ca⁺² channels (Barral et al. 2000). Finally, large strain differences in monoamine systems also exist, for example in brain 5-HT, noradrenaline and dopamine levels in C57BL/6 and BALB/c mice (Daszuta and Barrit 1982; Daszuta et al. 1982a; Daszuta et al. 1982b) and these divergences may indirectly influence strain responses to baclofen.

The present study indicates that baclofen can have distinct, independent effects (including no effect at all) on ataxia and hypothermia within a given strain. This is demonstrated in particular by mice in the NMRI strain, which showed hypothermia without significant ataxia, and by mice of the C3H/HeOulco strain, which exhibited ataxia without simultaneous hypothermia, in response to the same dose of baclofen (Table 2, Figure 4a). This is of particular interest for two reasons: 1) it demonstrates the importance of genetic background in responses to GABA_B receptor activation, and; 2) it suggests that hypothermia and ataxia may be under independent genetic control (despite activation of a common receptor). The study of inbred mouse strains assists in identification of specific genetic influences on given behaviours, as inbred mice from the same strain are essentially genetically identical i.e. homozygous for each gene. Thus, fluctuations in the mean responses of diverse strains are a reflection of differences in genetic makeup (Crabbe et al. 2002). Genetic background has been shown to influence the sensitivity of mice to many drugs (Crawley 2000; Crawley et al. 1997) and the present study shows that this is true also for the response of mice to baclofen. It should be cautioned, however, that epigenetic factors have also been shown to have a strong influence on behaviour (Francis et al. 2003) and these also may alter the responses to baclofen. Clearly our data also suggests that it is prudent to investigate multiple responses (both behavioural and physiological) when investigating pharmacological effects following genetic manipulations such as targeted deletions, as using a single parameter may not indicate whether or not the responses to a given ligand are truly changed, as demonstrated here with hypothermia and ataxia.

Recently, GABA_B receptor positive modulators have been identified (Urwyler et al. 2003) which lack the hypothermic and ataxic properties of baclofen (Cryan et al. 2004). Allosteric positive modulation of metabotropic receptors provide a novel means for the pharmacological manipulation of G-protein-coupled receptors acting at a distinct site apart from the orthosteric binding region of the receptor protein. The prototypical GABA_B positive modulator GS39783 has shown potential as anti-addictive and anti-anxiety agent (Cryan et al. 2004; Smith et al. 2004). Future studies must determine whether similar strain differences are manifested in behaviours related to these disorders as occurs with baclofen-induced responses.

Overall, our data clearly show a strong genetic influence on GABA_B receptor-mediated responses. Such genetically determined effects may directly influence the therapeutic efficacy of GABA_B agonists and suggest that perhaps pharmacogenetic factors should be considered in patients prescribed baclofen. These findings also have implications

for the selection of mouse strains for research investigating the role of GABA_B in physiology and behaviour. Further, these studies demonstrate that hypothermic and ataxic responses may be influenced by independent genetic loci.

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Chapter 3

GABA_{B(1)} Receptor Subunit Isoforms Exert a Differential Influence on Baseline but not GABA_B Receptor Agonist - Induced Changes in Mice

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3.1 Abstract

GABA_B receptors agonists produce hypothermia and motor in-coordination. Two GABA_{B(1)} receptor subunit isoforms exist, but because of lack of specific molecular or pharmacological tools, the relevance of these isoforms in controlling basal body temperature, locomotor activity or *in vivo* responses to GABA_B receptor agonists has been unknown. Here we use mice deficient in the GABA_{B(1a)} and GABA_{B(1b)} subunit isoforms to examine the influence of these isoforms on both baseline motor behaviour and body temperature and on the motor-incoordinating and hypothermic responses to the GABA_B receptor agonists L-baclofen and γ -hydroxybutyrate (GHB). GABA_{B(1b)}^{-/-} mice were hyperactive in a novel environment and showed slower habituation than either GABA_{B(1a)}^{-/-} or wild-type mice. GABA_{B(1b)}^{-/-} mice were hyperactive throughout the circadian dark phase. Hypothermia in response to L-baclofen (6 and 12 mg kg⁻¹) or GHB (1 g kg⁻¹), baclofen-induced ataxia as determined on the fixed-speed rotarod, and GHB-induced hypolocomotion were significantly, but for the most part similarly, attenuated in both GABA_{B(1a)}^{-/-} and GABA_{B(1b)}^{-/-} mice. We conclude that L-baclofen and GHB are non-selective for either GABA_{B(1)} receptor isoforms in terms of *in vivo* responses. However, GABA_{B1} receptor isoforms have distinct and different roles in mediating locomotor behavioural responses to a novel environment. Therefore GABA_{B(1a)} and GABA_{B(1b)} isoforms are functionally relevant molecular variants of the GABA_{B(1)} receptor subunit, which are differentially involved in specific neurophysiological processes and behaviors.

3.2 Introduction

GABA_B receptors are heterodimeric G protein-coupled receptors composed of GABA_{B(1)} and GABA_{B(2)} subunits. They are located pre- and postsynaptically, where they modulate neurotransmitter release and slow inhibitory postsynaptic potentials, mainly via actions on presynaptic Ca⁺² channels and postsynaptic inwardly rectifying K⁺ channels, respectively, and are also expressed as interneuron autoreceptors (Couve et al., 2000; Bettler et al., 2004). GABA_B receptors are implicated in epilepsy, addiction, pain and gastrointestinal disease (Bettler et al., 2004). Emerging data also supports a role for GABA_B receptors in anxiety, depression (Mombereau et al., 2004a, 2005; Cryan and Kaupmann, 2005) and cognition (Bowery, 2006). These findings promote further investigations into the mechanisms of action of the GABA_B receptors because of their potential importance as a therapeutic target (Bettler et al., 2004; Cryan and Kaupmann, 2005).

The *Gabbr1* gene is transcribed from two different promoter sites to generate two predominant isoforms in the brain: GABA_{B(1a)} and GABA_{B(1b)} (Steiger et al., 2004), both of which can heterodimerise with the GABA_{B(2)} subunit to form functional receptors (Bettler et al., 2004). The two isoforms differ in sequence only by the inclusion of a pair of sushi domains (also called short consensus repeats) at the N-terminus on the GABA_{B(1a)} isoform, which are absent in the GABA_{B(1b)} isoform (Blein et al., 2004). Other GABA_{B(1)} receptor splice variants have been reported in recombinant systems, although many of these variants are either not expressed in native tissues, or are not evolutionarily conserved across different species, and as such the functional relevance of these variants remains controversial (Bettler et al., 2004; Cryan and Kaupmann, 2005). Therefore, the molecular diversity of native GABA_B receptors has therefore been regarded as being relatively limited, which is at odds with the reported variability in the nature of responses to GABA_B receptor ligands (Marshall et al., 1999; Bettler et al., 2004; Huang, 2006).

In recombinant systems, many studies attest to a lack of pharmacological differences between the GABA_{B(1a,2)} and GABA_{B(1b,2)} isoforms (Kaupmann et al., 1998a; Malitschek et al., 1998; Brauner-Osborne and Krogsgaard-Larsen, 1999; Green et al., 2000), although there are also a few findings to the contrary, that differences exist in the pharmacology of the receptor isoforms (see (Bettler et al., 2004). However, to date, research tools with which to probe the *in vivo* pharmacology of GABA_{B(1a)} and GABA_{B(1b)} isoforms have not been available. The anatomical expression profile of GABA_{B(1a)} and GABA_{B(1b)} receptor isoforms diverges in many structures (Benke et al., 1999; Bischoff et al., 1999; Liang et al., 2000; Fritschy et al., 2004), which has given rise to speculation that the isoforms may have functional heterogeneity, possibly mediated by differential synaptic localisation (Bettler et al., 2004). Furthermore, many studies have shown pharmacological differences between hetero- and autoreceptors, and some GABA_B receptor ligands have been postulated to act preferentially at either presynaptic or postsynaptic locations (for reviews see Bowery et al., 2002 and Bettler et al., 2004). However, the possible contribution of these different isoforms to characteristic *in vivo* responses to GABA_B receptor activation, such as hypothermia and motor performance in response to baclofen (Gray et al., 1987; Jacobson and Cryan, 2005), are currently unknown.

Recently, mice deficient in the GABA_{B(1a)} and GABA_{B(1b)} subunit isoforms have been generated using a knock-in genetic approach (Vigot et al., 2006). Electron microscopy and electrophysiological characterisation of the mice revealed that, at least in the hippocampus (Vigot et al., 2006) and in the lateral amygdala (Shaban et al., 2006), the GABA_{B(1a)} isoform

was predominantly a presynaptic heteroreceptor, while the GABA_{B(1b)} isoform was mainly located postsynaptically, and both were autoreceptors (Shaban et al., 2006; Vigot et al., 2006). In addition, this distribution has been shown to generalise to layer 5 cortical neurons, where the GABA_{B(1b)} isoform was also shown to be predominantly postsynaptically located, while GABA_{B(1a)} was the main isoform represented at presynaptic terminals of local interneurons synapsing on the dendritic tuft (Perez-Garci et al., 2006). As such, GABA_{B(1a)}^{-/-} and GABA_{B(1b)}^{-/-} mice provide an ideal tool with which to investigate the roles of the two GABA_{B(1)} isoforms in the *in vivo* pharmacology of GABA_B receptor agonist-induced responses. Therefore, the aim of the present study was to determine the influence of the two GABA_{B(1)} isoforms on both on motor-incoordinating and hypothermic responses to the GABA_B receptor agonists baclofen and γ -hydroxybutyrate (GHB), and on baseline locomotor behaviour and body temperature.

3.3 Materials and Methods

Animals and Housing

The generation (Vigot et al., 2006) and breeding strategy (Jacobson et al., 2006b) of wild-type (WT), GABA_{B(1a)}^{-/-} and GABA_{B(1b)}^{-/-} mice as used in the present studies has been described previously. In brief, mice were generated using a knock-in point mutation strategy, whereby GABA_{B(1a)} and GABA_{B(1b)} initiation codons were converted to stop codons by targeted insertion of a floxed neo-cassette. All mutant and WT mice were maintained on a pure inbred BALB/c genetic background. GABA_{B(1a)}^{-/-} and GABA_{B(1b)}^{-/-} mice used in the present experiments were derived from homozygous breeding (F3-4) of siblings originating from the founding heterozygotic mice. Homozygous WT controls for the GABA_{B(1)} isoform mutant mice were derived from mating together WT siblings generated from GABA_{B(1a)}^{+/-} and GABA_{B(1b)}^{+/-} heterozygous breedings (F3-4). The breeding strategy was applied in accordance with the recommendations proposed by The Jackson Laboratory (Bar Harbor, ME) to obviate genetic drift and the formation of substrains (<http://jaxmice.jax.org/geneticquality/guidelines.html>).

Mice were singly housed in macrolon cages with sawdust bedding, tissue paper nesting materials and one red, triangular, polycarbonate Mouse House® (Nalgene, Nalge and Nunc International, Rochester, NY) per cage. Housing was at a constant room temperature of 22-24°C in a 12-h light dark cycle with lights on at either 6 A.M. to 6.30 A.M. Food pellets and tap water were available *ad libitum* (except during experimentation, unless stated). Separate cohorts of mice were used for each experiment. All mice were drug-naïve before

experimentation. Male mice were used in all experiments with the exception of GHB-induced hypolocomotion, for which only females were available. In certain experiments as indicated, experimental replication was also carried out in a cohort of female mice. All animal experiments were conducted during the light phase with the exception of continuous locomotor activity assessments which were made during both the light and dark phases. All animal experiments were conducted in accordance with Swiss guidelines, and approved by the Veterinary Authority of Basel-Stadt, Switzerland.

Drugs

All drug solutions were prepared freshly prior to use. L-baclofen (Novartis, Basel, Switzerland) and GHB (Novartis) were dissolved in 0.5% methyl cellulose (vehicle) and applied p.o. in a volume of 10 ml/kg.

Primary Observation Test

A battery of behavioural and physiological observations were made as previously described (Cryan et al., 2003) to investigate if $GABA_{B(1a)}^{-/-}$ or $GABA_{B(1b)}^{-/-}$ mice had any gross differences compared with WT mice. This was important to investigate, as both $GABA_{B(1)}$ - and $GABA_{B(2)}$ -deficient mice have been shown previously to develop an enhanced susceptibility to seizures (Prosser et al., 2001; Schuler et al., 2001). Mice used in this experiment were singly-housed WT, $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice. The experiment was replicated separately in both male and female mice. The mean age (\pm S.E.M.) of the mice was 20.0 ± 1.0 weeks for males and 23.1 ± 0.8 weeks for females ($n = 11-12$ for each genotype and gender). The observations quantified were the presence of twitches, tremor, convulsions, piloerection, stereotyped behaviour, lacrimation, salivation, ptosis, catalepsy, passivity, falling convulsion and ataxia. In addition the frequency and quality of breathing was observed. Alterations in skin colour, tail position, pelvic position, limb tonus, abdominal tonus and pupil width were observed. The nature of locomotion, motility and rearing in the home cage were quantified, as was overall flight and startle reactions. In addition, novelty behaviour was observed and a series of reflexes checked, including pinna reflex, toe-pinch, tail-pinch and provoked biting. Body temperature was also quantified. This battery of tests has been validated in our laboratories to detect stimulant and sedative effects in mice in addition to other effects of pharmacological agents.

Locomotor Activity of $GABA_{B(1a)}^{-/-}$, $GABA_{B(1b)}^{-/-}$ and WT Mice in a Novel Environment

Locomotor activity of mutant and WT mice when placed in a novel environment was investigated in two separate experiments with male and female mice, respectively. Horizontal locomotor activity of the mice [$n = 10-11$ for each genotype and sex, mean age (\pm S.E.M.) was $17.5 (\pm 0.5)$ and $14.5 (\pm 0.4)$ weeks for males and females, respectively] was recorded for 1 h after individuals were placed into novel enclosure (transparent Plexiglas boxes, 19 x 31 x 16 cm), with motion detection determined by infrared light beam interruptions along the x - and y -axes. Distance travelled was automatically calculated using a TSE Moti system (TSE, Bad Homburg, Germany).

Continuous 3-Day Locomotor Activity of Male $GABA_{B(1a)}^{-/-}$, $GABA_{B(1b)}^{-/-}$ and WT Mice

Horizontal locomotor activity of age-matched, singly housed male mice (WT, $n = 9$; $GABA_{B(1a)}^{-/-}$, $n = 9$; $GABA_{B(1b)}^{-/-}$, $n = 6$; mean age \pm S.E.M., 15.6 ± 0.6 weeks) was continuously recorded over 67 h (TSE Moti system). Mice were transferred to new home cages at 1 P.M. in the afternoon. Food and water was provided *ad libitum*, as usual, although tissue paper nesting material and the Mouse House were removed. Motion detection was determined in the new home cages from the time immediately after re-housing over the following 3 dark and 2.5 light cycles, by infrared light beam interruptions along the x - and y -axes. Distance travelled was automatically calculated using a TSE Moti system (TSE, Bad Homburg, Germany). The testing room in which the experiment took place was undisturbed during the course of the experiment.

Influence of L-Baclofen on Rotarod Endurance and Body Temperature of $GABA_{B(1a)}^{-/-}$, $GABA_{B(1b)}^{-/-}$ and WT Mice

The effect of the $GABA_B$ receptor agonist, L-baclofen, on motor coordination and body temperature of $GABA_{B(1a)}^{-/-}$, $GABA_{B(1b)}^{-/-}$ and WT mice were investigated in two experiments: one with male and one with female mice, respectively. Each experiment was conducted with two cohorts of mice. In the first cohort, 0 and 12 mg/kg of L-baclofen was examined in age-matched mutant and WT mice (male mice: $n = 19 - 20$ per genotype; mean age \pm S.E.M., 15.2 ± 0.3 weeks; female mice: $n = 18 - 21$ per genotype; mean age \pm S.E.M., 18 ± 0.3 weeks). In the second cohort, 0 and 6 mg/kg of L-baclofen was examined (male mice: $n = 9 - 10$ per genotype; mean age \pm S.E.M., 15.2 ± 0.3 weeks; female mice: $n = 8 - 10$ per genotype; mean age \pm S.E.M., 25.2 ± 0.5 weeks).

In the experimental protocol, performance on the fixed-speed rotarod (Dunham and Miya, 1957) was combined with the evaluation of rectal temperature within each animal, as

described previously (Cryan et al., 2004; Jacobson and Cryan, 2005). The rotarod apparatus consisted of a 28 mm diameter rod of approximately 300mm length, which was partitioned into 5 lanes of 58 mm wide to accommodate individual mice. The rod was positioned 30 cm above a surface and rotated at a constant speed of 12 rpm. Each day the rod lanes were tightly lined with fresh paper towelling. Rectal temperature was recorded (ELLAB Instruments thermistor probe, Copenhagen, Denmark) from individual mice while hand held near the base of the tail against the wall of the home cage. The probe was left in place until steady readings were obtained (approximately 15 s).

Two days prior to testing, the Mouse House and tissue paper nesting materials were removed from each cage to reduce possible inter-cage variations in mouse body temperatures on the day of testing. On the day before testing mice were acclimatized to the rectal thermistor probe by performing a single body temperature measurement and were then trained to walk on the rotarod for 300 s. Rotarod training was performed in two to four sessions, depending on the innate ability of each animal. The number of falls during training was recorded. Mice were returned to the home cage for an interval of approximately 30 min between training sessions.

On the day of testing, mice were moved to the experimental lab at least 2 hours before dosing. One hour before dosing, rectal temperature was taken. At time 0 h, rectal temperature was taken, then the mice were placed on the rotarod for 300 s to re-establish training and to provide an experimental baseline. Each mouse was then immediately dosed with its allocated treatment on completing 300 s on the rotarod. Rectal temperature and endurance on the rotarod were recorded 1, 2 and 4 h thereafter.

An index of the degree of hypothermia (summed ΔT) was calculated by totalling the differences between control (predose) rectal temperature and the temperatures at 1, 2 and 4 h post treatment, respectively, within each animal. An index of the degree of ataxia was calculated by determining the difference between cumulative total time on the rotarod 1, 2 and 4 hours after drug or vehicle application as a proportion of the total possible (i.e. percent reduction from 1200 s).

Effect of GHB on Body Temperature of Male $GABA_{B(1a)}^{-/-}$, $GABA_{B(1b)}^{-/-}$ and WT Mice

The effect of GHB (1 g/kg) on body temperature has been shown to be $GABA_{B(1)}$ receptor dependent (Kaupmann et al., 2003). Therefore, we assessed whether either receptor isoforms conferred susceptibility to GHB-induced hypothermia. This dose was selected based on our dose-response data obtained in BALB/c mice (Kaupmann et al., 2003), the background strain

for the GABA_{B(1)} isoform mutant and WT mice. Rectal temperature (ELLAB Instruments thermistor probe, as described above) of singly housed, age-matched, male mice (WT, $n = 7$; GABA_{B(1a)}^{-/-}, $n = 9$; GABA_{B(1b)}^{-/-}, $n = 13$; mean age \pm S.E.M., 27.0 ± 1.7 weeks) was recorded 1 h before, immediately before, and 30, 60, 120 and 240 min after, p.o. administration of GHB [1 g/kg in methyl cellulose (0.5%)] in a volume of 10 ml/kg.

Effect of GHB on Locomotor Activity of Female GABA_{B(1a)}^{-/-}, GABA_{B(1b)}^{-/-} and WT Mice

Previous studies have shown that the motor impairing effects of GHB (1g/kg) are GABA_{B(1)} receptor mediated (Kaupmann et al., 2003). Therefore, it was important to assess whether either of the GABA_{B(1)} receptor subunit isoforms conferred susceptibility to GHB-induced hypoactivity. Singly housed, age-matched, female mice (WT, $n = 23$; GABA_{B(1a)}^{-/-}, $n = 16$; GABA_{B(1b)}^{-/-}, $n = 34$; mean age \pm S.E.M., 26.7 ± 0.6 weeks) were given GHB (1 g/kg) or vehicle (0.5% methyl cellulose) p.o. in a volume of 10 ml/kg. The doses of GHB was selected based on previous dose-response studies showing maximal effects at this dose in this background mouse strain (Kaupmann et al., 2003). One hour after dosing, mice were placed in a transparent Plexiglas boxes (19 x 31 x 16 cm), and motion detection was determined over the following hour by infrared light beam interruptions along the x - and y -axes. Distance travelled was automatically calculated using a TSE Moti system (TSE, Bad Homburg, Germany).

Statistical Analyses

Because studies that were carried out in both male and female mice were conducted in independent experiments, data for each sex were analysed separately. Live-weight and rectal temperature as determined in the primary observation test were analysed with one-way analysis of variance (ANOVA). Locomotor activity in a novel environment and continuous 3-day locomotor activity were analysed for the effects of genotype and time using two-way repeated measures ANOVA. Summed mean distance travelled during the first 2 and the full 12 h of the three complete dark cycles and during the 12 h of the two complete light cycles, was analysed for the effect of genotype using one-way ANOVA. Basal body temperature (at time points -1 and 0 h in the combined rotarod - temperature experiment) were analysed for the effect of replicate, genotype and time using three-way ANOVA. Fisher's least significant difference post hoc comparisons were made where indicated by significant ANOVA factors. Falls during rotarod training, and body temperature responses to GHB were analysed for the effect of genotype in a pair-wise fashion using the Mann-Whitney rank sum method. All

variables from the POT, with the exception of live-weight and rectal temperature (see above), summed rotarod data (within each baclofen dose) and distance travelled after vehicle or GHB treatment (within treatment and time point) were analysed for the effect of genotype using Kruskal Wallis one-way ANOVA on ranks. Dunn's Method post hoc comparisons were made where indicated by significant Kruskal Wallis ANOVA factors, with the exception of the influence of GHB on locomotor activity, where pair-wise Mann-Whitney Rank Sum comparison were used as a post hoc comparison method when appropriate.

3.4 Results

POT

Unlike $GABA_{B(1)}$ - and $GABA_{B(2)}$ -deficient mice, in which spontaneous seizures were observed (Schuler *et al.*, 2001; Gassmann *et al.*, 2004), this phenotype was not behaviourally apparent in $GABA_{B(1a)}$ ^{-/-} or $GABA_{B(1b)}$ ^{-/-} mice. To assess whether ablation of $GABA_{B(1a)}$ or $GABA_{B(1b)}$ receptor isoforms had any other effects on gross behaviour and physiology, male and female $GABA_{B(1a)}$ ^{-/-} or $GABA_{B(1b)}$ ^{-/-} mice were subjected to an extensive POT battery.

Within the male mice, $GABA_{B(1b)}$ ^{-/-} mice were significantly heavier than either WT ($P < 0.01$) or $GABA_{B(1a)}$ ^{-/-} ($P < 0.001$) mice, and WT mice were heavier than $GABA_{B(1a)}$ ^{-/-} mice ($P < 0.001$). Mean (\pm SEM) weights of male mice were WT, 32.5 (\pm 0.6) g; $GABA_{B(1a)}$ ^{-/-}, 29.2 (\pm 0.5) g; $GABA_{B(1b)}$ ^{-/-}, 34.8 (\pm 0.6) g (genotype $F_{2,34} = 23.73$, $P < 0.001$).

Female $GABA_{B(1b)}$ ^{-/-} mice were slightly, but significantly heavier than female WT mice ($P < 0.01$, mean \pm S.E.M. weights were 27.6 \pm 0.7 g and 24.5 \pm 0.7 g, respectively). However, there were no differences between the weight of female $GABA_{B(1a)}$ ^{-/-} mice (25.9 \pm 0.8 g) and either WT or $GABA_{B(1b)}$ ^{-/-} female mice (genotype $F_{2,35} = 4.20$, $P < 0.05$).

Otherwise the POT battery revealed no significant differences between $GABA_{B(1a)}$ ^{-/-}, $GABA_{B(1b)}$ ^{-/-} and WT mice in any of the other 31 parameters assessed (see Materials and Methods). This included body temperature as assessed in the home cage, where mean rectal temperature for both the male and female mice was not significantly influenced by genotype, respectively (males: mean rectal temperature \pm SEM: 35.9 \pm 0.13 °C; genotype $F_{2,34} = 0.36$, $P = 0.70$; females: mean rectal temperature 36.2 \pm 0.15 °C; genotype $F_{2,35} = 1.20$, $P = 0.32$).

Locomotor Activity of $GABA_{B(1a)}$ ^{-/-}, $GABA_{B(1b)}$ ^{-/-} and WT Mice in a Novel Environment

Data from one male and one female $GABA_{B(1a)}$ ^{-/-} mouse were excluded from analysis as statistical outliers. The male mouse travelled 158 m in 1 h, compared with the mean for the remaining nine male $GABA_{B(1a)}$ ^{-/-} mice of 79.6 \pm 5.5. The female mouse travelled 208 m in 1

h, compared with the mean for the remaining 10 female mice of 54.1 ± 3.6 m. Both of these animals showed intermittent periods of repetitive, stereotypic circling behaviour in the home cage, which might have increased locomotor activity during the test. No other signs of stereotyped behaviour were observed in any genotype across all other experiments.

Genotype influenced the pattern of locomotor activity in male mice (time $F_{11, 347} = 15.39$, $P < 0.001$; genotype $F_{2, 347} = 1.67$, $P > 0.05$; interaction $F_{22, 347} = 1.708$, $P < 0.05$) (Fig. 1a). Post hoc analysis revealed the male $GABA_{B(1b)}^{-/-}$ mice were more active than WT and $GABA_{B(1a)}^{-/-}$ male mice within the first 5 min, and at other time-points within the first 20 min of the experiment (Fig. 1a).

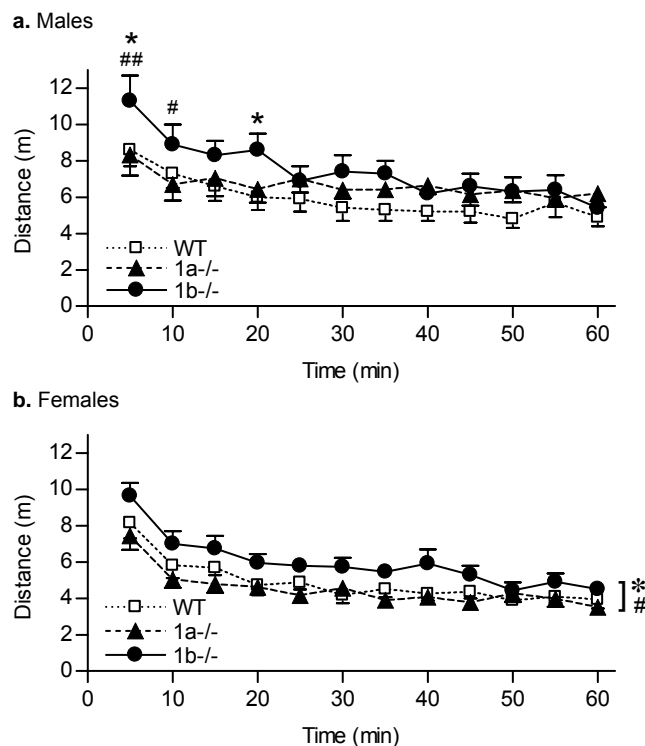


Fig. 1. $GABA_{B(1b)}^{-/-}$ mice were hyperactive in a novel environment. Distance travelled during spontaneous locomotor activity in a novel enclosure by male (a) and female (b) WT, $GABA_{B(1a)}^{-/-}$ ($1a^{-/-}$) and $GABA_{B(1b)}^{-/-}$ ($1b^{-/-}$) mice. * $P < 0.05$ vs WT, # $P < 0.05$ vs $1a^{-/-}$, ### $P < 0.01$ vs $1a^{-/-}$.]' denotes ANOVA main effects for genotype and time.

Genotype and time influenced the locomotor activity of female mice, with the $GABA_{B(1b)}^{-/-}$ mice showing consistently greater distance travelled over the duration of the experiment than WT or $GABA_{B(1a)}^{-/-}$ mice, as revealed by a main effect of genotype (time $F_{11, 347} = 28.27$, $P < 0.001$; genotype $F_{2, 347} = 4.13$, $P < 0.05$; interaction $F_{22, 347} = 0.744$, $P > 0.05$) (Fig. 1b). Post hoc analysis revealed that female $GABA_{B(1b)}^{-/-}$ mice were more active over the hour than both WT and $GABA_{B(1a)}^{-/-}$ female mice ($P < 0.05$, respectively).

Continuous 3-Day Locomotor Activity of $GABA_{B(1a)}^{-/-}$, $GABA_{B(1b)}^{-/-}$ and WT Mice

Male $GABA_{B(1b)}^{-/-}$ mice travelled significantly greater distances than either WT or $GABA_{B(1a)}^{-/-}$ mice during the 1st h of habituation to a new enclosure, and during the following three dark phases, whereas $GABA_{B(1a)}^{-/-}$ mice travelled similar distances to that of WT mice during the dark phases (genotype $F_{2, 1607} = 2.62$, $P = 0.096$; time $F_{66, 1607} = 34.08$, $P < 0.001$; interaction $F_{132, 1607} = 1.75$, $P < 0.001$) (Fig. 2, a and b). The hyperactivity of the $GABA_{B(1b)}$ mice was particularly prevalent during the first 2 h of the dark phase, as the distance travelled (averaged over the 3 dark phases and expressed as a proportion the WT mean) during this time was 154% that of the WT controls (genotype: $F_{2,23} = 6.697$, $P < 0.01$) (Fig. 2c). This pattern of behavior was confirmed when assessing data as mean summed distance (within animal) in the dark, as $GABA_{B(1b)}^{-/-}$ mice travelled a greater mean distance during the dark phase than either WT or $GABA_{B(1a)}^{-/-}$ mice ($P < 0.05$, respectively; genotype $F_{2, 23} = 3.81$, $P < 0.05$) (Fig. 2d). In comparison, during the two complete light phases, the $GABA_{B(1a)}^{-/-}$ mice travelled a greater mean distance per light phase than the WT mice (genotype $F_{2, 23} = 4.15$, $P < 0.05$) (Fig 2e).

Influence of L-Baclofen on Rotarod Endurance and Body Temperature of $GABA_{B(1a)}^{-/-}$, $GABA_{B(1b)}^{-/-}$ and WT Mice

Temperature data from one female $GABA_{B(1a)}^{-/-}$ mouse in the baclofen 12 mg/kg dataset was eliminated as a statistical outlier (the two pre-drug body temperature measurements on the day of the experiment were less than 35.0 °C). Rotarod data from one male $GABA_{B(1a)}^{-/-}$ mouse in the vehicle-treated dataset were eliminated due to repeated voluntary jumping from the rotarod. Data from one female $GABA_{B(1a)}^{-/-}$ mouse at a time-point 4 hours after vehicle dosing were lost due to repeated voluntary jumping from the rotarod.

Body temperature at times -1 and 0 h, as well as rotarod training performance and duration on the rotarod at time 0 h of the experiment, did not differ between the two experimental cohorts for either of the experiments involving male or female mice (temperature: males, cohort $F_{1,175} = 1.399$, $P = 0.24$; females, cohort $F_{1,171} = 0.47$, $P = 0.50$; all mice walked for 300 s on the rotarod at time point 0), therefore temperature and rotarod data for the two cohorts were pooled (within experiments).

Mean body temperature before drug treatment was slightly, but significantly, lower in female $GABA_{B(1a)}^{-/-}$ mice than either the WT ($P < 0.05$) or $GABA_{B(1b)}^{-/-}$ mice ($P < 0.05$), although the $GABA_{B(1a)}^{-/-}$ and WT mice were not different from each other [$P > 0.05$; genotype $F_{2,171} = 3.37$, $P < 0.05$; mean temperature \pm S.E.M. (°C): WT, 35.87 ± 0.08 ; $GABA_{B(1a)}^{-/-}$, 35.60 ± 0.08 ; $GABA_{B(1b)}^{-/-}$, 35.85 ± 0.06].

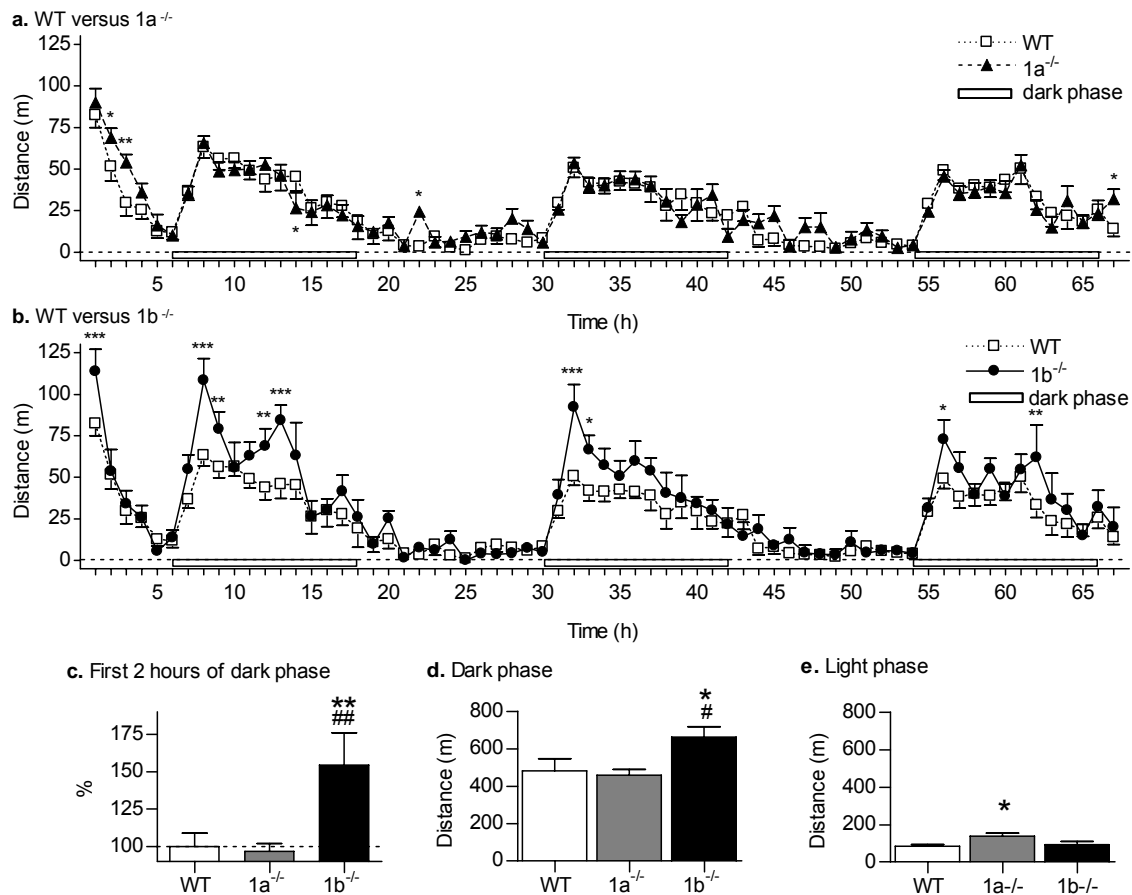


Fig. 2. $GABA_{B(1b)}^{-/-}$ mice are hyperactive during the circadian dark phase. Distance travelled during 67 hours of continuously monitored locomotor activity by male wild-type (WT) and $GABA_{B(1a)}^{-/-}$ ($1a^{-/-}$) mice (a.), and of the WT versus $GABA_{B(1b)}^{-/-}$ ($1b^{-/-}$) mice (b.). Mean (+ SEM) summed distance travelled by WT, $1a^{-/-}$ and $1b^{-/-}$ mice in the 2 h immediately after the start of the dark phase, averaged over the three dark phases and expressed as a proportion of the WT mean (c.). Mean (+ SEM) summed distance travelled per 12 h for male WT, $1a^{-/-}$ and $1b^{-/-}$ mice during the three complete dark phases (d.) and two complete light phases (e.). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs WT; # $P < 0.05$, ## $P < 0.01$ vs $1a^{-/-}$.

In contrast, in the experiment with male mice, $GABA_{B(1b)}^{-/-}$ mice had a slightly higher mean basal body temperature than either the WT ($P < 0.05$) or $GABA_{B(1a)}^{-/-}$ mice ($P < 0.05$), although the $GABA_{B(1a)}^{-/-}$ and WT mice were not significantly different from each other [$P > 0.05$; genotype $F_{2,175} = 5.11$, $P < 0.01$; mean temperature \pm S.E.M. ($^{\circ}$ C): WT, 35.94 ± 0.10 ; $GABA_{B(1a)}^{-/-}$, 36.09 ± 0.11 ; $GABA_{B(1b)}^{-/-}$, 36.40 ± 0.10].

Baclofen produced profound, long lasting hypothermia in male WT mice (male WT: baclofen dose $F_{2,149} = 13.74$, $P < 0.001$; time $F_{4,149} = 13.99$, $P < 0.001$; interaction $F_{8,149} = 16.36$, $P < 0.001$) (Fig. 3a). Baclofen at 12 mg/kg, but not 6 mg/kg, also induced hypothermia

in male $GABA_{B(1b)}^{-/-}$ mice (male $GABA_{B(1b)}^{-/-}$: baclofen dose $F_{2,149} = 5.95$, $P < 0.01$; time $F_{4,149} = 9.03$, $P < 0.001$; interaction $F_{8,149} = 2.95$, $P < 0.01$) (Fig. 3c). In contrast, neither the 6 nor 12 mg/kg doses of baclofen induced hypothermia in the male $GABA_{B(1a)}^{-/-}$ mice, relative to vehicle-treated $GABA_{B(1a)}^{-/-}$ males, at the three postdrug time points measured (male $GABA_{B(1a)}^{-/-}$: baclofen dose $F_{2,139} = 2.78$, $P = 0.08$; time $F_{4,139} = 1.73$, $P = 0.149$; interaction $F_{8,139} = 0.61$, $P = 0.76$) (Fig. 3b). When examining the total hypothermic response to baclofen over the duration of the experiment, both $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ male mice showed an attenuated summed ΔT in response to 12 mg/kg baclofen in comparison to the WT mice, although neither of the mutant mice strains differed from each other in this regard (males summed ΔT : genotype $F_{2,87} = 3.26$, $P < 0.05$; baclofen dose $F_{2,87} = 10.64$, $P < 0.001$, interaction $F_{4,87} = 4.66$, $P < 0.01$) (Fig. 3d).

Baclofen also induced time-dependent hypothermia in female WT mice (female WTs: baclofen dose $F_{2,129} = 52.85$, $P < 0.001$; time $F_{4,129} = 18.17$, $P < 0.001$; interaction $F_{8,129} = 18.11$, $P < 0.001$) (Fig. 3e). Baclofen at 12 mg/kg, but not 6 mg/kg, also induced hypothermia in both $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ female mice (female $GABA_{B(1a)}^{-/-}$: baclofen dose $F_{2,154} = 6.27$, $P < 0.01$; time $F_{4,154} = 4.04$, $P < 0.01$; interaction $F_{8,154} = 5.11$, $P < 0.001$; female $GABA_{B(1b)}^{-/-}$: baclofen dose $F_{2,149} = 4.58$, $P < 0.05$; time $F_{4,149} = 1.14$, $P = 0.34$; interaction $F_{8,149} = 3.77$, $P < 0.001$) (Fig. 3, f and g). However, the degree of hypothermia was greatly attenuated in both mutant strains of mice in comparison to the WT mice. As for the experiment using the male mice, this attenuation was particularly apparent when examining the summed hypothermic responses to baclofen. The degree of summed hypothermia in response to 12 mg/kg of baclofen was greatly attenuated in both mutant strains of female mice in comparison to the WT mice, but did not differ significantly between the two mutant lines (females: genotype $F_{2,86} = 26.09$, $P < 0.001$; baclofen dose $F_{2,86} = 50.78$, $P < 0.001$; interaction $F_{4,86} = 15.19$, $P < 0.01$) (Fig. 3h).

It is interesting to note that in the experiments with both male and female $GABA_{B(1b)}^{-/-}$ mice, the lower dose of baclofen appeared to induce hyperthermia 1 hour after baclofen administration (Fig. 3, c and 3g). In the female mice, post hoc analysis within the 6 mg/kg group showed significant increase in body temperature from the -1 to the 0 h time point ($P < 0.01$), and a tendency to further increase from the 0 to the 1 h time point ($P = 0.077$). In male mice, post hoc analysis within the 6 mg/kg baclofen treatment, similar to the females, showed a significant increase in body temperature from the -1 to the 0 h time point ($P < 0.05$). However, mean body temperature at time points 0 and 1 h were not significantly different from each other ($P = 0.144$).

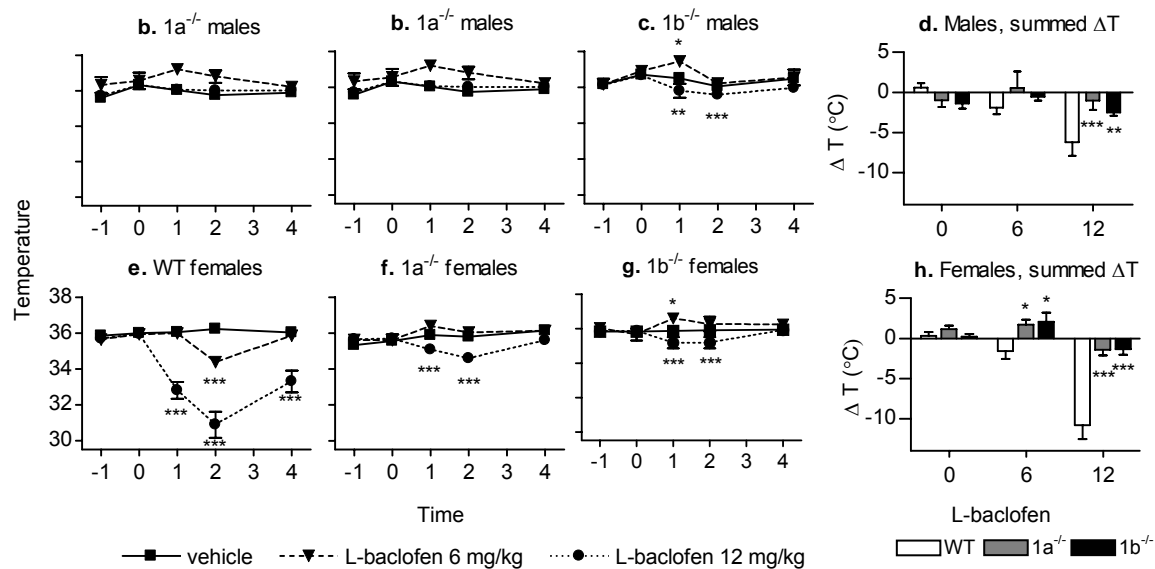


Fig. 3. Hypothermic responses to baclofen are attenuated in $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice. Mean (\pm SEM) body temperature of male (a - c.) and female (e - g.) wild-type (WT), $GABA_{B(1a)}^{-/-}$ ($1a^{-/-}$) and $GABA_{B(1b)}^{-/-}$ ($1b^{-/-}$) mice following vehicle or L-baclofen (6 and 12 mg/kg) administration. Data are also presented as mean (\pm SEM) summed area under the curve (ΔT , calculated within animal) for male (d.) and female (h.) WT, $1a^{-/-}$ and $1b^{-/-}$ mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs WT.

During training for the rotarod experiment, the mean number of falls from the rotarod during training for male mice was not significantly affected by genotype ($P > 0.05$ for all the pairings; mean \pm S.E.M.: WT, 0.73 ± 0.24 ; $GABA_{B(1a)}^{-/-}$, 0.43 ± 0.17 ; $GABA_{B(1b)}^{-/-}$, 0.67 ± 0.19). In comparison, female $GABA_{B(1a)}^{-/-}$ mice fell from the rotarod more often during training than either WT or $GABA_{B(1b)}^{-/-}$ mice ($P < 0.05$, for each respective pairing; mean \pm S.E.M.: WT, 0.15 ± 0.07 ; $GABA_{B(1a)}^{-/-}$, 0.94 ± 0.25 ; $GABA_{B(1b)}^{-/-}$, 0.13 ± 0.06). On the day of testing, however, all mice walked on the rotarod for the allocated 300 s on the first experimental time point (immediately prior to dosing), thus indicating they had learned the task adequately.

Baclofen significantly impaired rotarod endurance in male WT mice in a time- and dose-dependant manner (male WT: baclofen dose $F_{2, 119} = 6.99$, $P < 0.01$; time $F_{3, 119} = 17.06$, $P < 0.001$; interaction $F_{6, 119} = 7.64$, $P < 0.001$) (Fig. 4a). Baclofen similarly reduced rotarod endurance in male $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice by 1 h after dosing, although the duration of impairment was shorter than that of the WT mice in both mutant strains of mice ($GABA_{B(1a)}^{-/-}$: baclofen dose $F_{2, 111} = 2.44$, $P = 0.107$; time $F_{3, 111} = 9.63$, $P < 0.001$; interaction $F_{6, 111} = 3.72$, $P < 0.01$; $GABA_{B(1b)}^{-/-}$: baclofen dose $F_{2, 119} = 5.82$, $P < 0.01$; time $F_{3, 119} = 5.60$, $P < 0.01$; interaction $F_{6, 119} = 5.63$, $P < 0.001$) (Fig. 4, b and c). Overall, when examining total

summed endurance on the rotarod, male $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice performed similarly to WT at 0-, 6- and 12-mg/kg doses of baclofen (vehicle, $H = 3.30$, $P = 0.19$; baclofen 6 mg/kg, $H = 4.51$, $P = 0.162$; baclofen 12 mg/kg $H = 1.20$, $P = 0.55$) (Fig. 4d).

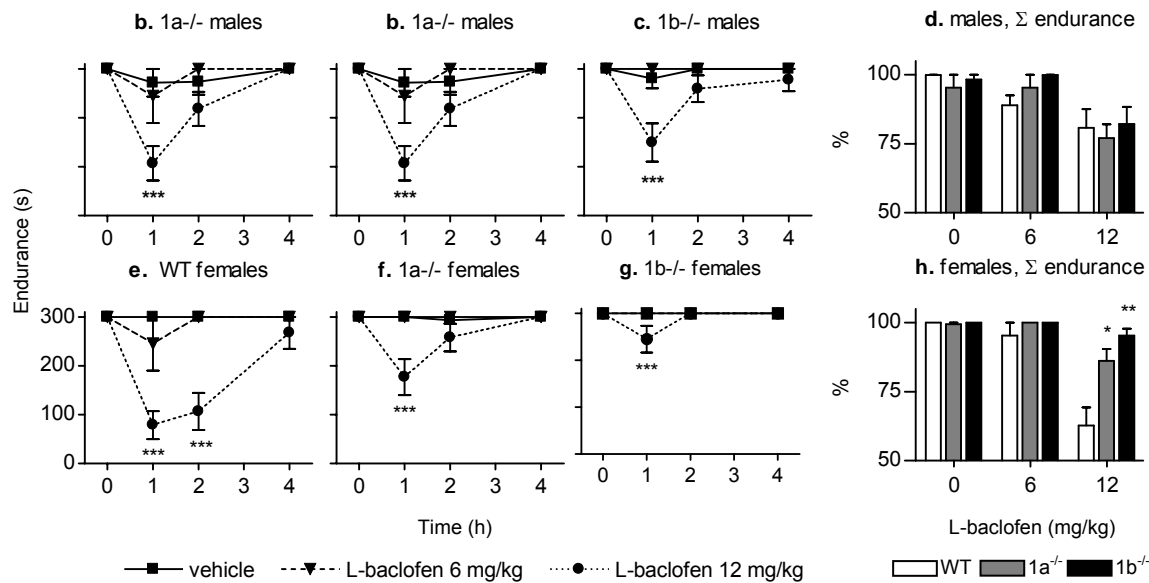


Fig. 4. Ataxic responses to baclofen are attenuated in $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice. Mean (\pm SEM) endurance on a fixed-speed rotarod (12 rpm, maximum allowed was 300 s per time point) for male (a - c.) and female (e - g.) wild-type (WT), $GABA_{B(1a)}^{-/-}$ (1a^{-/-}) and $GABA_{B(1b)}^{-/-}$ (1b^{-/-}) mice following vehicle or L-baclofen (6 and 12 mg/kg) administration. Data are also presented as a proportion of the total time available (1200 s) which was spent walking on the rotarod by male (d.) and female (h.) WT, 1a^{-/-} and 1b^{-/-} mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs WT.

Baclofen also impaired rotarod endurance in female WT mice in a dose and time dependent manner (female WT: baclofen dose $F_{2, 103} = 26.96$, $P < 0.001$; time $F_{3, 103} = 17.34$, $P < 0.001$; interaction $F_{6, 103} = 15.51$, $P < 0.001$) (Fig. 4e.). Baclofen likewise impaired rotarod endurance in female $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice at 1 h after dosing, although to a lesser duration and degree to that of WT in both mutant strains ($GABA_{B(1a)}^{-/-}$: baclofen dose $F_{2, 122} = 8.66$, $P = 0.001$; time $F_{3, 122} = 4.55$, $P < 0.01$; interaction $F_{6, 122} = 5.82$, $P < 0.001$; $GABA_{B(1b)}^{-/-}$: baclofen dose $F_{2, 119} = 3.71$, $P < 0.05$; time $F_{3, 119} = 3.03$, $P < 0.05$; interaction $F_{6, 119} = 3.71$, $P < 0.01$) (Fig. 4, f and g). When examining summed endurance, mice of the three genotypes treated with either vehicle or 6 mg/kg baclofen showed similar rotarod performances (vehicle, $H = 4.10$, $P = 0.129$; baclofen 6 mg/kg, $H = 2.50$, $P = 0.81$) (Fig. 4h). However, post hoc analyses for data at 12 mg/kg of baclofen demonstrated both $GABA_{B(1a)}^{-/-}$

and $GABA_{B(1b)}^{-/-}$ mice had similarly attenuated ataxic responses in comparison with the WT mice ($P < 0.05$; baclofen 12 mg/kg, $H = 12.26$, $P = 0.002$) (Fig. 4h).

Effect of GHB on Body Temperature of $GABA_{B(1a)}^{-/-}$, $GABA_{B(1b)}^{-/-}$ and WT Mice

All mice responded to GHB with hypothermia, relative to pre-drug application body temperature. However, the degree of hypothermia was influenced by genotype. Thirty minutes after GHB application, $GABA_{B(1a)}^{-/-}$ mice had a significantly lower mean reduction in body temperature than that of the WT mice ($P < 0.05$) (Fig. 5). One hour after p.o. application, both $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice had attenuated hypothermic responses to the GHB compared with WT mice ($P < 0.05$ for $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice vs WT, respectively) (Fig. 5). Body temperature of the mice before drug administration was not influenced by genotype ($P > 0.05$).

Effect of GHB on Locomotor Activity of $GABA_{B(1a)}^{-/-}$, $GABA_{B(1b)}^{-/-}$ and WT Mice

Interestingly, the genotype did not affect the distance travelled for vehicle-treated mice at any of the post-drug administration time intervals investigated ($P > 0.05$). In contrast, in the GHB-treated mice, genotype influenced the median distance travelled in the 15 to 20 min ($H = 6.02$, $P < 0.05$), 20 to 25 min ($H = 6.25$, $P < 0.05$), 25 to 30 min ($H = 9.01$, $P < 0.05$), 30 to 35 min ($H = 6.93$, $P < 0.05$) and 40 to 45 min intervals ($H = 6.61$, $P < 0.05$). Post hoc comparison revealed that the $GABA_{B(1b)}^{-/-}$ mice tended to be more active than WT mice during the 15 to 20 min interval ($P = 0.056$). However, at the other time intervals, $GABA_{B(1a)}^{-/-}$ mice travelled a greater distance than the WT mice (20 – 25 min, $P < 0.05$; 25 – 30 min, $P < 0.01$; 30 – 35 minutes, $P < 0.05$; 40 – 45 min, $P < 0.01$) (Fig. 6). This indicated that both $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice were less sensitive to the locomotor suppressing effects of GHB.

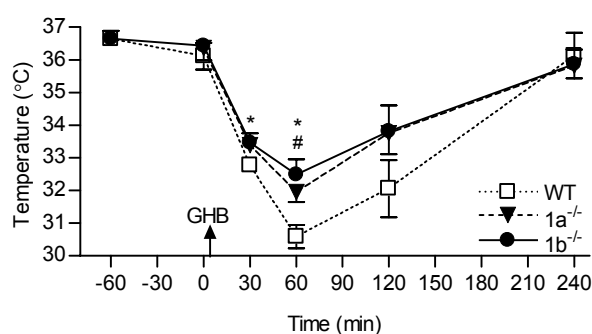


Fig. 5. Hypothermic responses to GHB were attenuated in $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice. Mean (\pm SEM) body temperature of male wild-type (WT), $GABA_{B(1a)}^{-/-}$ ($1a^{-/-}$) and $GABA_{B(1b)}^{-/-}$ ($1b^{-/-}$) mice following administration of GHB (1 g/kg). * $P < 0.05$ $1a^{-/-}$ vs WT. # $P < 0.05$ $1b^{-/-}$ vs WT.

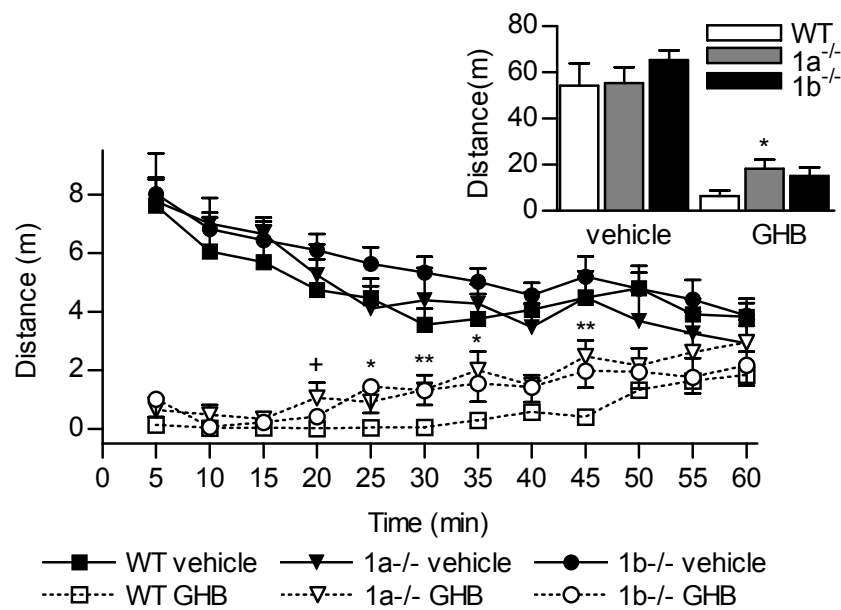


Fig. 6. Hypolocomotor effects of GHB are attenuated in $GABA_{B(1)}$ isoform-deficient mice. Mean (\pm SEM) distance travelled by female wild-type (WT), $GABA_{B(1a)}^{-/-}$ ($1a^{-/-}$) and $GABA_{B(1b)}^{-/-}$ ($1b^{-/-}$) mice following administration of vehicle or GHB (1 g/kg). Note: analysis performed with non-parametric testing. + $P < 0.10$ GHB-treated $1b^{-/-}$ vs WT; * $P < 0.05$, GHB-treated $1a^{-/-}$ vs WT.

3.5 Discussion

The *Gabbr1* gene is predominantly transcribed into two differentially expressed isoforms, $GABA_{B(1a)}$ and $GABA_{B(1b)}$, which differ in sequence primarily by the inclusion of a pair of evolutionary-conserved sushi repeats (also known as short consensus repeats) in the $GABA_{B(1a)}$ N terminus (Bettler et al., 2004). The recent generation of $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice (Vigot et al., 2006) has opened up new possibilities for understanding the functions of these sushi domains. Here we show that $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice possessed normal overt behavioural responses as observed in the primary observation test, as well as in basic righting and motor function, as indicated by unimpaired baseline performance on the rotarod. In addition, unlike the full $GABA_{B(1)}^{-/-}$ mice (Prosser et al., 2001; Schuler et al., 2001), basic observations of the mice suggested that neither $GABA_{B(1a)}^{-/-}$ nor $GABA_{B(1b)}^{-/-}$ mice had spontaneous seizures. However, the characteristic hypothermia and motor impairment in response to the $GABA_B$ receptor agonists L-baclofen or GHB were markedly attenuated, and interestingly, to a relatively similarly degree in both $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice compared to WT controls. Moreover, the two mutant lines of mice diverged significantly in their baseline behaviour. The $GABA_{B(1b)}^{-/-}$ mice were hyperactive in

a novel environment and habituated more slowly than either the WT or $GABA_{B(1a)}^{-/-}$ mice. Furthermore, $GABA_{B(1b)}^{-/-}$ mice were more active throughout the dark phase than either the $GABA_{B(1a)}^{-/-}$ or WT mice. Finally, the findings of the lack of overt phenotype in the POT, similar attenuation of baclofen-induced hypothermia and ataxia in both mutants, and hyperlocomotor responses to a novel environment by $GABA_{B(1b)}^{-/-}$ mice were replicated in separate experiments with both male and female mice, attesting to the reproducibility of these phenotypes.

$GABA_B$ receptors play a crucial role in mediating normal motor responses (Jacobson and Cryan, 2005). Further, deletion of the $GABA_{B1}$ receptor subunit results in a complex locomotor response (Mombereau et al., 2004a; Vacher et al., 2006). This includes marked hyperlocomotion when exposed to a novel environment whereas in a familiar environment, $GABA_{B(1)}^{-/-}$ mice display an altered pattern of circadian activity but no hyperlocomotion (Vacher et al., 2006). Therefore, the baseline motor hyperactivity of $GABA_{B(1b)}^{-/-}$ mice in a novel environment was not entirely surprising. Although the effect of $GABA_{B(1b)}$ isoform deletion on locomotor activity seemed somewhat modest, the magnitude of the effect is more apparent when examined as a relative proportion of the WT controls. For example, male $GABA_{B(1b)}^{-/-}$ mice travelled approximately 140% of the distance of WT controls in the first five minutes of exposure to a novel environment, and 154% of that of wild type mice in the first 2 h of the dark cycle. Furthermore, hyperlocomotor responses were demonstrated on four occasions with three different cohorts of mice: novelty-induced hyperlocomotion was replicated in a separate cohort of female mice; in another cohort of male mice in the 3 day locomotor experiment during the 1st h in a new home cage; and hyperlocomotion of $GABA_{B(1b)}^{-/-}$ in the dark phase was subsequently demonstrated during the following three dark cycles. Together these data suggest that the loss of the $GABA_{B(1b)}$ isoform may contribute to a significant degree to the aforementioned hyperlocomotion of $GABA_{B(1)}^{-/-}$ mice.

Although the locomotor behaviour of $GABA_{B(1a)}^{-/-}$ mice was similar to WT controls in the present investigation, in other test systems differences to WT and $GABA_{B(1b)}^{-/-}$ mice have been identified. We have previously shown that $GABA_{B(1a)}^{-/-}$ mice were impaired in an object recognition task (Vigot et al., 2006), whereas the $GABA_{B(1b)}^{-/-}$ mice were not. Furthermore, $GABA_{B(1a)}^{-/-}$ mice had deficits in the acquisition of conditioned taste aversion (Jacobson et al., 2006b) and in the generalisation of fear conditioning-induced freezing (Shaban et al., 2006). Correspondingly, the $GABA_{B(1a)}^{-/-}$, but not the $GABA_{B(1b)}^{-/-}$, mice were also deficient in hippocampal (Vigot et al., 2006) and amygdala long-term potentiation (Shaban et al., 2006). In addition, Perez-Garci et al., (2006) have also shown specific and differential roles for the

GABA_{B(1a)} and GABA_{B(1b)} isoforms in mediating different components of GABA_B receptor-induced inhibition of layer 5 cortical neurons. Together with the hyperactive phenotype in locomotor activity of the GABA_{B(1b)}^{-/-} mice shown in the present study, these data show that GABA_{B(1)} receptor isoforms have divergent and functionally relevant influences on behavioural output and the underlying neurophysiology.

Given the phenotypic differences between these two isoform-deficient lines of mice, it was interesting to note that although both GABA_{B(1a)}^{-/-} and GABA_{B(1b)}^{-/-} mice both showed attenuated responses to the GABA_B receptor agonists baclofen, the degree of attenuation was largely similar in both mutant strains of mice. In GABA_{B(1)}^{-/-} mice, baclofen does not induce the hypothermic or ataxic effects normally seen in WT mice (Schuler et al., 2001; Queva et al., 2003). This indicated that the full GABA_B receptor heterodimer is necessary for the actions of these agonists and, likewise, that the actions of these agonists are specific to the GABA_B receptor. Results of the present study, however, suggest that neither the GABA_{B(1a)} nor GABA_{B(1b)} isoforms are solely responsible for the hypothermic or ataxic actions of baclofen. The data also show that neither isoform can fully compensate for the loss of the other with regard to agonist-induced responses. To further confirm this we investigated the effects of another GABA_B receptor agonist, GHB. We have previously shown that the hypothermic and motor-impairing effects of GHB (1g/kg) are completely absent in mice lacking the GABA_{B(1)} receptor (Kaupmann et al., 2003). Here we show that the effects of GHB were modestly but significantly attenuated in mice lacking either of the two GABA_{B(1)} isoforms. Thus, we can conclude, as in the case of baclofen, that neither the GABA_{B(1a)} nor GABA_{B(1b)} isoforms are solely responsible for the hypothermic or ataxic actions of GHB. These *in vivo* data are in support of a number of recombinant studies demonstrating that neither baclofen nor GHB appear to show specificity for either one of the two predominant GABA_{B(1)} subunit isoforms (see Bettler et al., 2004).

Paradoxically, at the lower dose of 6 mg/kg, baclofen seemed to increase body temperature in GABA_{B(1b)}^{-/-} mice. Examination of post-hoc statistical comparisons indicated that the bulk of the temperature increase of mice in this treatment group occurred between time -1 and 0 hours, both of which preceded baclofen injection. Therefore, it seems likely that handling stress may have induced the apparent hypothermic responses in these mice. However, it should be noted that the GABA_B agonist GHB at low doses has been previously reported to induced hyperthermia as opposed to hypothermia (Kaufman et al., 1990). Therefore, it remains possible that some influence of residual GABA_{B(1)} activity in the GABA_{B(1b)}^{-/-} mice may have contributed to a hyperthermic response to baclofen in these mice.

Control of body temperature and motor activity is anatomically and neurochemically heterogeneous (Cryan et al., 1999; Cryan et al., 2000; Jacobson and Cryan, 2005). Both GABA_{B(1a)} and GABA_{B(1b)} isoforms are abundantly expressed throughout the brain, although their expression level relative to each other and distribution profile within structures diverges in many regions, including in many of those involved in the control of body temperature or motor activity and coordination (Benke et al., 1999; Bischoff et al., 1999; Liang et al., 2000; Fritschy et al., 2004). Therefore, it may seem reasonable to have expected that, irrespective of a lack of specificity of the isoforms for baclofen or GHB, responses to the agonists may yet have varied between the two mutant mouse lines. In the present study, however, this was not the case. Previously we have demonstrated that genetic background (in the form of different mouse strains) can greatly influence hypothermic and ataxic responses to baclofen (Jacobson and Cryan, 2005). The present study may indicate that this is not necessarily because of overall variations in the relative expression of the GABA_{B(1)} subunit isoforms. Indeed, given that baseline body temperatures of the GABA_{B(1a)}^{-/-} and GABA_{B(1b)}^{-/-} mice showed negligible differences, it may be that normal homeostasis of body temperature is not specifically controlled by one or other of the GABA_{B(1)} subunit isoforms either. This is in contrast to the full GABA_{B(1)}^{-/-} mice, where basal body temperature was shown to be approximately 1°C less than that of the WT controls (Kaupmann et al., 2003; Queva et al., 2003).

The lack of differential responses to baclofen and GHB between the GABA_{B(1)} isoform-deficient mice may also be in part the result of the complex neural control of body temperature and motor activity. For example, when strongly activated by pharmacological means, loss of either presynaptic or postsynaptic inhibition in a complex multisynaptic system may ultimately appear similar downstream (i.e., appearing as an increase in excitability in a convergent output). With regard to the contribution of different cellular components in these systems, the prospective roles of interneurons in GABA_B receptor agonist-induced hypothermia and ataxia are unknown at the present time. Hippocampal and lateral amygdala autoreceptor function was preserved in both GABA_{B(1a)}^{-/-} and GABA_{B(1b)}^{-/-} mice (Shaban et al., 2006; Vigot et al., 2006), but was completely absent in the GABA_{B(1)}^{-/-} mice (Prosser et al., 2001; Schuler et al., 2001). This indicated that in these structures, both isoforms can act as autoreceptors. However, GABAergic interneurons synapsing on distal dendrites of layer 5 cortical neurons appeared to preferentially express the GABA_{B(1a)} isoform, but not the GABA_{B(1b)} isoform (Perez-Garci et al., 2006). This shows that different interneuron populations may variably express the two GABA_{B(1)} subunit isoforms. Clearly, further studies

are needed to evaluate the expression profile and roles of GABA_{B(1)} isoforms autoreceptors in GABA_B receptor-mediated hypothermia and ataxia.

In conclusion, GABA_{B(1a)} and GABA_{B(1b)} isoforms are functionally relevant molecular variants of the GABA_{B(1)} receptor subunit, which are differentially involved in specific neurophysiological processes and behaviors. It is evident from the present study, however, that the GABA_B receptors agonists baclofen and GHB were unable to pharmacologically discriminate these differences, at least with regard to body temperature and motor coordination. As the sequence of the GABA_{B(1a)} and GABA_{B(1b)} isoforms differ primarily in the N-terminus, and not in the region coding the ligand binding domain (Kaupmann et al., 1997; Bettler et al., 2004), future studies should focus on strategies to uncover novel interaction sites at either receptor isoforms in order to enable specific pharmaceutical intervention.

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Chapter 4

GABA_{B(1)} Receptor Isoforms Differentially Mediate the Acquisition and Extinction of Aversive Taste Memories

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4.1 Abstract

Conditioned taste aversion (CTA), is a form of aversive memory where an association is made between a consumed substance and a subsequent malaise. CTA is a critical mechanism for the successful survival, and hence evolution, of most animal species. The role of excitatory neurotransmitters in the neurochemical mechanisms of CTA is well recognized, however, less is known about the involvement of inhibitory receptor systems. In particular the potential functions of metabotropic GABA_B receptors in CTA have not yet been fully explored. GABA_B receptors are metabotropic GABA receptors that are comprised of two subunits, GABA_{B(1)} and GABA_{B(2)}, which form heterodimers. The *Gabbr1* gene is transcribed into two predominate isoforms, GABA_{B(1a)} and GABA_{B(1b)} which differ in sequence primarily by the inclusion of a pair of sushi domains (or short consensus repeats) in the GABA_{B(1a)} N-terminus. The behavioural function of mammalian GABA_{B1} receptor isoforms is currently unknown. Here, using a point mutation strategy in mice we demonstrate that these two GABA_{B(1)} receptor isoforms are differentially involved in critical components of CTA. In contrast to GABA_{B(1b)}^{-/-} and wild-type mice, GABA_{B(1a)}^{-/-} mice failed to acquire CTA. On the other hand, GABA_{B(1b)}^{-/-} mice, robustly acquired CTA but failed to show any extinction of this aversion. The data demonstrate that GABA_B receptors are involved in both the acquisition and extinction of CTA; however, receptors containing the GABA_{B(1a)} or the GABA_{B(1b)} isoform differentially contribute to the mechanisms used to learn and remember the salience of aversive stimuli.

4.2 Introduction

Conditioned taste aversion (CTA) is an associative learning phenomenon whereby the characteristics of a consumed substance are paired with the memory of a subsequent malaise (Bermudez-Rattoni, 2004). CTAs are long-lasting and specific memories which can be induced with a single pairing of the conditioned stimulus (CS, being the consumed substance) and the unconditioned stimulus (US, the malaise). As such, CTA is a critical mechanism for the successful survival, and hence evolution, of most animal species (Bures, 1998a). Moreover, because CTA declines reliably with repeated non-reinforced exposure to the CS, the use of CTA in the laboratory allows the investigation of the processes involved in both the acquisition and extinction of aversive memories (Bahar et al., 2003; Bermudez-Rattoni, 2004).

The importance of excitatory neurotransmitters in the acquisition and extinction of CTA is well known (Berman and Dudai, 2001; Bermudez-Rattoni, 2004). In contrast, there is a paucity of studies investigating the role of inhibitory neurotransmitters in CTA. Some studies have demonstrated that ionotropic GABA_A-modulating drugs such as benzodiazepines alter the acquisition and certain aspects of extinction of CTA (Roache and Zabik, 1986; Delamater and Treit, 1988; Yasoshima and Yamamoto, 2005) whereas the role of GABA_B receptors is largely uninvestigated.

GABA_B receptors are comprised of two subunits, GABA_{B(1)} and GABA_{B(2)}, which form heterodimers. The *Gabbr1* gene is predominantly transcribed into two differentially expressed isoforms, GABA_{B(1a)} and GABA_{B(1b)}, which differ in sequence primarily by the inclusion of a pair of evolutionary conserved sushi repeats (a.k.a. short consensus repeats) in the GABA_{B(1a)} N-terminus (Bettler et al., 2004). The function of the sushi repeats, and hence of the different receptor isoforms, has been a mystery until recently. Vigot et al. (2006) demonstrated that the sushi repeats define the morphological localization of GABA_B receptors: the GABA_{B(1a)} isoform was mainly a presynaptic heteroreceptor at glutamatergic terminals, whereas the GABA_{B(1b)} isoform was predominantly located postsynaptically. Given the importance of GABAergic mechanisms in emotional learning (Akirav, 2006; Davis et al., 2006) we used GABA_{B(1a)}^{-/-} and GABA_{B(1b)}^{-/-} mice to address the hypothesis that GABA_B receptor isoforms could play distinctive roles in the acquisition and extinction of aversive memories.

4.3 Materials and Methods

Establishment of a Conditioned Taste Aversion Protocol in BALB/c Mice

Because there are marked strain differences in emotional behaviour in mice (Cryan and Holmes, 2005), it was important first to establish an appropriate CTA protocol in the background strain of our genetically modified mice. A two-bottle choice CTA protocol was validated using singly-housed male mice [BALB/cByJlco (Charles River Laboratories, L'Abresse Cedex, France; ~12 weeks of age, n=30)]. Mice were trained to drink water from a 15 ml plastic drinking tube in two 30 - minute sessions (morning and afternoon) per day for 5 d. Mice were then presented with a saccharin solution (0.5% in tap water) in their drinking tube. Thirty minutes after the end of the 30 minute saccharin - drinking period, they were injected (i.p., 10 ml/kg) with either vehicle (saline, unconditioned mice) or the malaise-inducing agent lithium chloride (LiCl; Sigma-Aldrich Chemie, Steinheim, Germany) at a dose

of either 3 or 6 mEq/kg (0.3 or 0.6 M LiCl) (conditioned mice). Over the following 7 days, mice were presented with both water and the saccharin solution in the morning drinking sessions. Drinking tubes containing the saccharin solution were always presented in the same spatial order relative to the water tube (e.g. always on the right). Afternoon drinking sessions remained water-only throughout the experiment.

Conditioned Taste Aversion in Wild-Type, $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ Mice

The generation of $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ has been described previously (Vigot et al., 2006). Briefly, a knock-in point mutation strategy was adopted, whereby $GABA_{B(1a)}$ and $GABA_{B(1b)}$ initiation codons were converted to stop codons by targeted insertion of a floxed neo-cassette. Gene targeting constructs and embryonic stem cells were of BALB/c origin. Embryonic stem cells were injected into C57BL/6 blastocysts and chimerics crossed with BALB/c mice to generate heterozygotic founding mice. The neo-cassette was excised by crossing to BALB/c mice expressing Cre recombinase and breeding to homozygosity. Consequently, all mutant and wild-type mice were maintained on a pure inbred BALB/c genetic background. $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice used for the evaluation of CTA were derived from subsequent homozygous breeding (F5-6) of siblings originating from the founding heterozygotic mice. Homozygous wild-type controls for the $GABA_{B(1)}$ isoform mutant mice were derived from mating together wild-type siblings generated from $GABA_{B(1a)}^{+/-}$ and $GABA_{B(1b)}^{+/-}$ heterozygous breedings (F5-6). The breeding strategy was applied in accordance with the recommendations proposed by The Jackson Laboratory (Bar Harbor, ME) to obviate genetic drift and the formation of substrains (<http://jaxmice.jax.org/geneticquality/guidelines.html>).

A similar protocol to that validated in-house (see above) was used to evaluate CTA in singly housed male wild-type ($n = 19$, 29.3 ± 0.6 weeks of age), $GABA_{B(1a)}^{-/-}$ ($n = 15$, 26.8 ± 0.6 weeks of age) and $GABA_{B(1b)}^{-/-}$ ($n = 18$, 26.4 ± 0.6 weeks of age) (Fig. 1). Mice from each genotype were allocated to either an unconditioned (saline injection after saccharin presentation) or conditioned (6 mEq / kg LiCl after saccharin presentation) treatment. The dose of lithium was selected based on the validation experiment. Furthermore, mice were subjectively scored in a blind fashion for the presence or absence of malaise behaviour after LiCl or saline injections (Hayley et al., 1999; Anisman et al., 2001). Malaise was defined as prolonged periods of non-sleeping immobility, piloerection, contraction of the flanks, prostrate elongated body posture and/or excessive defecation or diarrhoea. Mice displaying malaise behaviour were given a score of 1. Animals not showing malaise behaviour were

given a score of 0. Sleeping animals were not scored. For 2 weeks after conditioning, mice experienced a once – daily preference test in which they were presented with both saccharin and water for 30 minutes. In the afternoons they were given water only for 30 minutes. They were then returned to an *ad libitum* water regime for a further week. Thereafter, animals were water-deprived overnight and again presented with the choice of saccharin or water in the morning. This allowed us to assess whether the aversion was altered over 1 week in the absence of saccharin exposure. Animals were then returned to an *ad libitum* water regime for an additional week. Thereafter, animals were water deprived overnight and again presented with the choice of saccharin or water in the morning with the difference that the spatial order of tube presentation was reversed, which allowed us to determine whether or not perseverative behaviour was contributing to the choice of drinking fluid. The following day, the saccharin or water option was presented again, but in their usual order.

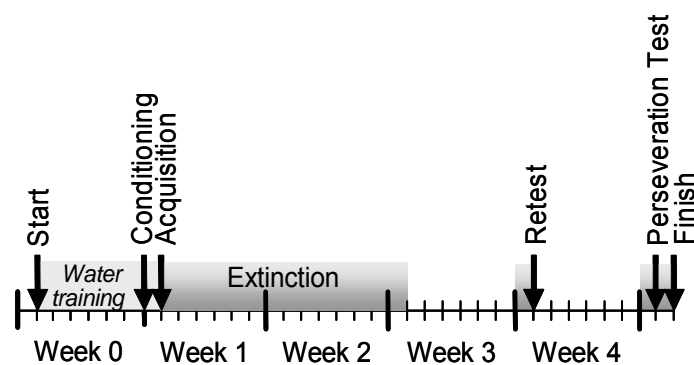


Fig. 1. Schematic of a CTA protocol used in $GABA_{B(1)}$ isoform mutant and wild-type mice.

Calculations and Statistical Analyses.

All drinking tubes were weighed before and after presentation to the mice to obtain the weight of fluid consumed. An aversion index (AI) for the saccharin solution was calculated as follows: $AI (\%) = [\text{water intake (g)} / [\text{saccharin intake (g)} + \text{water intake (g)}]] \times 100$. Data were analysed with one-way, two-way or two-way repeated measure ANOVA, followed by Fisher's LSD *post hoc* comparisons, where appropriate.

4.4 Results

Conditioned Taste Aversion in BALB/c Mice

BALB/c mice acquired a robust aversion to both 3 and 6 mEq/kg doses of LiCl in comparison to the unconditioned (saline-treated) animals (Fig. 2A. LiCl dose $F_{2,29} = 9.87$, $P < 0.001$).

Mice treated with LiCl at 3 mEq/kg had extinguished the aversion by 5 d, whereas the mice treated with LiCl at 6 mEq/kg took 7 d to extinguish (Fig. 3A, $P > 0.05$, respectively). Therefore, this protocol was chosen as appropriate for detecting alterations in CTA acquisition or extinction in genetically modified mice bred on a BALB/c genetic background.

Conditioned Taste Aversion in $GABA_{B(1a)}^{-/-}$, $GABA_{B(1b)}^{-/-}$ and Wild-Type Mice

Mice of all three genotypes readily consumed the saccharin solution on the day of conditioning (mean \pm sem saccharin solution intake: WT, 1.91 ± 0.08 ml; $GABA_{B(1a)}^{-/-}$, 2.12 ± 0.13 ml; $GABA_{B(1b)}^{-/-}$, 1.95 ± 0.09 ml. Genotype; $F_{2,51} = 11.11$, $P = 0.34$).

$GABA_{B(1a)}^{-/-}$ that received LiCl after saccharin exposure (conditioned) failed to acquire an aversion to the saccharin solution, relative to conditioned wildtype and $GABA_{B(1b)}^{-/-}$ mice ($P < 0.01$), and showed a preference for the saccharin solution to a level not different from that of unconditioned $GABA_{B(1a)}^{-/-}$, $GABA_{B(1b)}^{-/-}$ or wild-type mice. In comparison, both conditioned wild-type and $GABA_{B(1b)}^{-/-}$ mice developed similar, robust levels of aversion to the saccharin solution, relative to unconditioned controls ($P < 0.001$), (Fig. 2B. LiCl $F_{1,51} = 39.77$, $P < 0.001$; genotype $F_{2,51} = 6.57$, $P < 0.01$; interaction $F_{2,51} = 4.60$, $P < 0.05$). The failure of the conditioned $GABA_{B(1a)}^{-/-}$ mice to acquire an aversion to the saccharin solution was not attributable to insensitivity to LiCl-induced malaise, as indicated by the demonstration of malaise behaviour in 100% of the $GABA_{B(1a)}^{-/-}$ mice 1 hour after LiCl injections (Table 1).

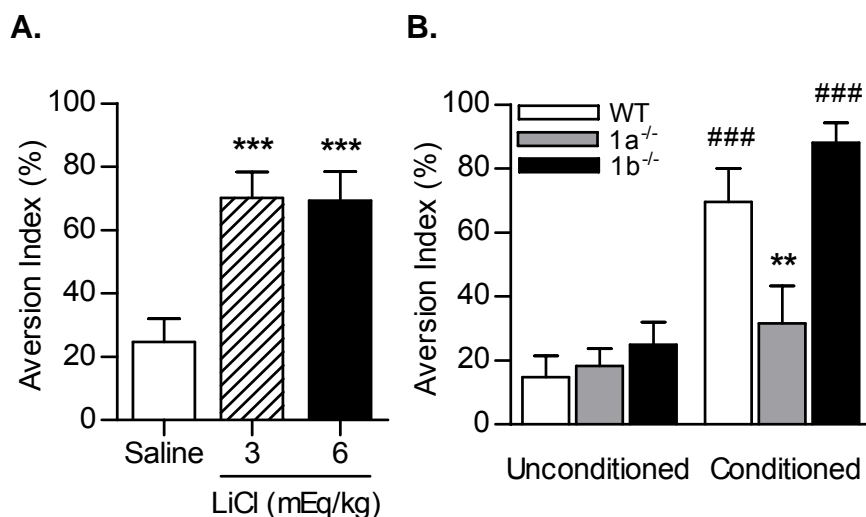


Fig. 2. Acquisition of CTA. **A.** BALB/c mice acquire CTA to a saccharin solution when paired with malaise induced by LiCl at 3 and 6 mEq/kg (***) $P < 0.001$ vs saline). **B.** The $GABA_{B(1a)}$ receptor isoform is essential for acquisition of a CTA (WT, wild-type; $1a^{-/-}$, $GABA_{B(1a)}^{-/-}$; $1b^{-/-}$, $GABA_{B(1b)}^{-/-}$; ** $P < 0.01$ vs conditioned wild-type; *** $P < 0.001$ vs unconditioned within genotype).

Table 1. LiCl (6 mEq/kg, i.p.) induced malaise to an equivalent degree in wildtype, $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice.

Treatment (N)	Wildtype			$GABA_{B(1a)}^{-/-}$			$GABA_{B(1b)}^{-/-}$											
	Saline (9)			LiCl (10)			Saline (7)			LiCl (8)			Saline (9)			LiCl (9)		
Time (h)	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
% sick (# sick)	0	0	0	90	90	67	0	0	0	100	83	57	11	0	0	89	78	100
				(9)	(9)	(6)				(8)	(6)	(4)	(1)			(8)	(7)	(7)
% sleep (# sleep)	11	87	44	0	0	10	0	29	71	0	25	13	0	44	38	0	0	22
	(1)	(8)	(4)			(1)	(2)	(2)	(5)	(2)	(2)	(1)	(4)	(4)	(3)			(2)

Only mice that were awake were scored for the presence or absence of malaise (% sick).

In striking contrast, although $GABA_{B(1b)}^{-/-}$ mice readily acquired the aversion, they failed to show any reduction of this aversion over the following 30 days of the experiment, relative to both unconditioned $GABA_{B(1b)}^{-/-}$ mice and to conditioned wild-type and $GABA_{B(1a)}^{-/-}$ mice (Fig. 3B. Conditioning within $GABA_{B(1b)}$: LiCl $F_{1,323} = 27.39$, $P < 0.001$; time $F_{17,323} = 2.71$, $P < 0.001$; interaction $F_{17,323} = 0.72$, $P = 0.78$. Genotype within conditioned treatment: genotype $F_{2,480} = 7.32$, $P < 0.01$; time $F_{17,480} = 5.06$, $P < 0.001$; interaction $F_{34,480} = 1.90$, $P < 0.01$).

Post hoc comparisons revealed significant differences between the conditioned $GABA_{B(1b)}^{-/-}$ and conditioned wild-type mice from day 5 onward (Fig. 3B). This was because of extinction in the conditioned wild-type mice, which reached a low level of aversion not different to that of unconditioned wild-type mice by day 5 after conditioning (Fig.3B. Conditioning within wild-type: LiCl $F_{1,326} = 8.22$, $P = 0.01$; day $F_{17,326} = 5.2$, $P < 0.001$; interaction $F_{17,326} = 2.85$, $P < 0.001$). The reversal of drinking tube presentation on day 29 demonstrated that the $GABA_{B(1b)}^{-/-}$ mice were not simply drinking from the same tube position each day, but were actively avoiding the saccharin solution.

In the unconditioned mice, there was no effect of genotype on the AI although the overall AI decreased over the duration of the experiment until the perseveration test on day 29, when it was transiently elevated (Fig. 3B .Genotype within unconditioned: $F_{2,434} = 1.49$, $P = 0.25$; day $F_{17,434} = 5.04$, $P < 0.001$; interaction $F_{34,434} = 1.08$, $P = 0.36$).

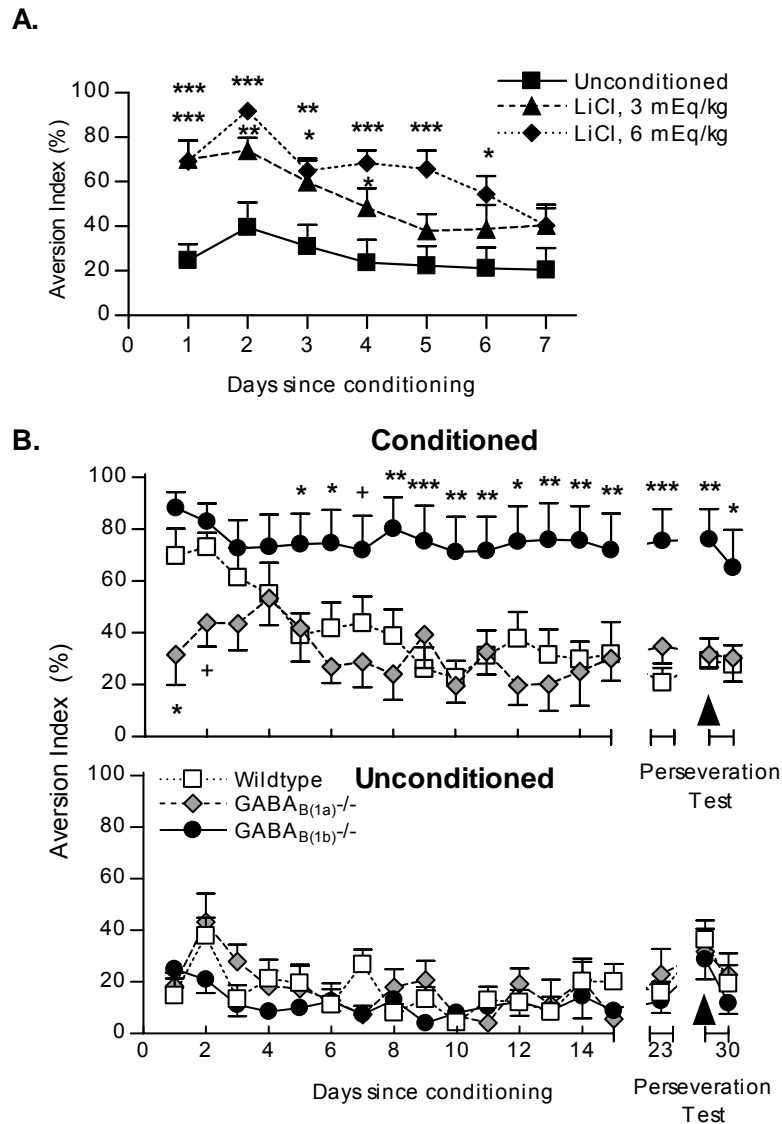


Fig. 3. Extinction of CTA. **A.** Time to extinguish a CTA in BALB/c mice was determined by dose of the malaise-inducing agent, LiCl ($***P < 0.001$, $**P < 0.01$, $*P < 0.05$ vs saline, within day). **B.** **Conditioned** Deletion of the $GABA_{B(1b)}$ receptor isoform profoundly impairs extinction of CTA ($***P < 0.001$, $**P < 0.01$, $*P < 0.05$, $+P < 0.10$ vs wildtype). **Unconditioned** $GABA_{B(1)}$ isoforms do not influence the development of preference for a saccharin solution in unconditioned mice.

4.5 Discussion

Our data demonstrate a critical role for $GABA_B$ receptors in CTA. Specifically, the two $GABA_{B(1)}$ subunit isoforms are differentially involved in the acquisition and extinction of CTA. Acquisition of CTA requires the $GABA_{B(1a)}$ isoform, whereas extinction requires the $GABA_{B(1b)}$ receptor isoform.

It has recently been shown that the presence of specific sushi domains directs GABA_{B(1a)} isoforms to a presynaptic localisation and that this localisation is critical for cognitive performance as assessed using an object recognition task (Vigot et al., 2006). Further, GABA_{B(1a)}^{-/-} mice have impaired hippocampal LTP and lack presynaptic GABA_B-ergic inhibition of glutamatergic excitability (Vigot et al., 2006). Given that glutamate signalling is essential for the acquisition of CTA (Yasoshima et al., 2000; Bermudez-Rattoni, 2004; Akirav, 2006), it therefore seems plausible that GABA_{B(1a)} isoform modulation of presynaptic glutamate release may underlie the mechanisms CTA acquisition. The brain regions involved in such modulation are unknown presently, because the GABA_{B(1a)} receptor isoform is widely expressed throughout the brain (Benke et al., 1999; Bischoff et al., 1999; Fritschy et al., 1999). However, lesion or inactivation of the pontine parabrachial nucleus, amygdala or insular cortex disrupts the acquisition of CTA (Bermudez-Rattoni and Yamamoto, 1998; Bures, 1998b; Bermudez-Rattoni, 2004) which points to GABA_{B(1a)} receptors in these structures as being crucial for CTA acquisition.

Given the differential localization of GABA_{B(1)} isoforms (Perez-Garci et al., 2006; Vigot et al., 2006) the very dissimilar phenotype of GABA_{B(1b)}^{-/-} compared to GABA_{B(1a)}^{-/-} mice was not entirely unexpected. Indeed, unlike GABA_{B(1a)}^{-/-} mice, GABA_{B(1b)}^{-/-} mice readily acquired CTA but failed to extinguish the aversion despite repeated unreinforced exposures to the CS. Similar to the acquisition of associative learning, its extinction is also believed to be a learning process that results from the formation of new memories as opposed to simple forgetting (Myers and Davis, 2002; Davis et al., 2006). It has been suggested that the study of CTA may have direct implications for the study of anxiety disorders associated with altered emotional learning (Bahar et al., 2003; Bermudez-Rattoni, 2004; Guitton and Dudai, 2004; Cryan and Holmes, 2005). Therefore, the understanding of the molecular mechanisms that underlie the extinction of established aversive memories would be a considerable breakthrough in the treatment and management of anxiety disorders (Ressler et al., 2004; Barad, 2005; Davis et al., 2006).

Until recently, no unique pharmacological or functional properties could be assigned to GABA_{B(1a)} or GABA_{B(1b)} (Perez-Garci et al., 2006; Vigot et al., 2006). However, it has been proposed that there exists auxiliary proteins that modify receptor activity, pharmacology, and localization (Marshall et al., 1999). Our data clearly show differential functions of GABA_{B(1)} receptor isoforms in the acquisition (GABA_{B(1a)}) and extinction (GABA_{B(1b)}) of CTA. Thus future studies must focus on uncovering potential novel protein interacting sites at either receptor isoforms to enable pharmaceutical intervention. Together, our data

demonstrate that isoforms of the GABA_{B(1)} receptor, which differ only in the presence or absence of a pair of sushi repeats at their N-terminal ectodomain, play differential, yet critical roles in the evolutionary conserved mechanisms used to learn and remember the salience of aversive stimuli.

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Chapter 5

Behavioural Evaluation of Mice Deficient in GABA_{B(1)} Receptor Isoforms in Tests of Unconditioned Anxiety

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5.1 Abstract

Rationale: Emerging data support a role for GABA_B receptors in anxiety. GABA_B receptors are comprised of a heterodimeric complex of GABA_{B1} and GABA_{B2} receptor subunits. The predominant neuronal GABA_{B1} receptor isoforms are GABA_{B(1a)} and GABA_{B(1b)}. Recent findings indicate specific roles for these isoforms in conditioned fear responses, although their influence on behaviour in tests of unconditioned anxiety is unknown. *Objective:* To examine the role of the GABA_{B(1)} isoforms in unconditioned anxiety. *Methods:* Mice deficient in the GABA_{B(1a)} or GABA_{B(1b)} receptor isoforms were examined in a battery of anxiety tests. *Results:* In most tests, genotype did not significantly affect anxious behaviour, including the elevated plus maze, marble burying, or stress-induced hyperthermia tests. Corticosterone and ACTH levels were similarly unaffected by genotype. Female, but not male, GABA_{B(1a)}^{-/-} and GABA_{B(1b)}^{-/-} mice showed increased anxiety relative to wild-type controls in the elevated zero maze. In the staircase test, male GABA_{B(1b)}^{-/-} mice defecated more than male GABA_{B(1a)}^{-/-} mice, although no other test parameter was influenced by genotype. In the light-dark box, female GABA_{B(1a)}^{-/-} mice spent less time in the light compartment compared to the GABA_{B(1b)}^{-/-} females whereas male GABA_{B(1b)}^{-/-} mice made fewer light-dark transitions than GABA_{B(1a)}^{-/-} males. *Conclusions:* Specific roles for either GABA_{B(1)} isoform in unconditioned anxiety were not explicit. This differs from their contribution in conditioned anxiety, and from the anxious phenotype of GABA_{B1} and GABA_{B2} subunit knockout mice. The findings suggest that the GABA_{B(1)} isoforms have specific relevance for anxiety with a cognitive component, rather than for innate anxiety *per se*.

5.2 Introduction

G-protein coupled γ -aminobutyric acid (GABA) receptors, GABA_B receptors, are heterodimers comprised of GABA_{B(1)} and GABA_{B(2)} subunits. They are expressed both as presynaptic heteroreceptors and also postsynaptically, where they respectively modulate neuronal excitability. Heteroreceptors modulate the release of (excitatory) neurotransmitters, mainly *via* actions on presynaptic Ca⁺² channels, and postsynaptic GABA_B receptors activate slow inhibitory postsynaptic potentials via activation of inwardly-rectifying K⁺ channels. GABA_B receptors also function as autoreceptors on interneurons. Additionally, GABA_B receptors are negatively coupled to adenylyl cyclase, through which they influence downstream molecular pathways (Bettler et al. 2004; Couve et al. 2000).

There is a growing body of evidence indicating that GABA_B receptors play a critical role in anxiety (Cryan and Kaupmann 2005; Pilc and Nowak 2005). The classic GABA_B receptor agonist, baclofen, has shown anxiolytic activity in some clinical settings. Baclofen reduced anxiety in post-traumatic stress disorder (PTSD) patients (Drake et al. 2003), in alcoholics following alcohol withdrawal (Addolorato et al. 2002a; Addolorato et al. 2006; Ameisen 2005; Flannery et al. 2004), in panic disorder (Breslow et al. 1989) and in patients suffering from acute spinal trauma (Hinderer 1990). Baclofen has also demonstrated anxiolytic effects in several preclinical studies including ultrasonic vocalisation in rat pups (Nastiti et al. 1991), increased punished drinking (Shephard et al. 1992), elevated plus maze (Andrews and File 1993) (but see (Dalvi and Rodgers 1996)) and in the social interaction and elevated plus maze tests following withdrawal of dependent rats from either diazepam or alcohol (File et al. 1991a; File et al. 1991b; File et al. 1992). GABA_B receptor positive modulators such as GS39783 also demonstrate an anxiolytic profile in both rats and mice in a wide range of tests (Cryan et al. 2004).

Perhaps the strongest preclinical evidence to date for a role of GABA_B receptors in anxiety was demonstrated by the phenotype of GABA_B receptor-deficient mice. Deletion of either the GABA_{B(1)} or GABA_{B(2)} receptor subunits results in a complete loss of typical GABA_B functions and induces a highly anxious phenotype in mice in exploratory-based tests of anxiety (Mombereau et al. 2004a; Mombereau et al. 2004b). For example, GABA_{B(1)}^{-/-} mice show profound anxiety relative to wild-type controls in the light-dark box and staircase tests (Mombereau et al. 2004a; Mombereau et al. 2004b). Similarly, GABA_{B(2)}^{-/-} mice are also anxious in the light dark box (Mombereau et al. 2005).

The GABA_{B(1)} subunit is predominantly expressed as one of two isoforms: GABA_{B(1a)} or GABA_{B(1b)}, both of which are transcribed from different promoters of the GABA_{B(1)} gene (Steiger et al. 2004) and heterodimerize with the GABA_{B(2)} subunit to form functional receptors (Bettler et al. 2004). The primary sequence of the GABA_{B(1a)} isoform differs from that of the GABA_{B(1b)} isoform by the inclusion of short-consensus repeats (or “sushi domains”) at the N-terminus (Blein et al. 2004). Currently there are no pharmacological tools available to dissect the physiological roles of these isoforms. Recently, however, the generation of mice deficient in either the GABA_{B(1a)} or GABA_{B(1b)} isoforms has facilitated the demonstration of specific and differential roles for the GABA_{B(1)} isoforms. The two isoforms localise differentially: the GABA_{B(1a)} isoform was predominantly expressed as a presynaptic heteroreceptor in the hippocampus (Vigot et al. 2006) and lateral amygdala (Shaban et al. 2006). In contrast the GABA_{B(1b)} isoform was predominantly located postsynaptically in these

structures (Shaban et al. 2006; Vigot et al. 2006). Both the GABA_{B(1a)} and GABA_{B(1b)} isoforms also fulfilled autoreceptor functions in the hippocampus and amygdala (Vigot et al. 2006)), although in the apical dendrites of layer 5 cortical neurons, the GABA_{B(1a)} isoform was shown to be the predominant autoreceptor (Perez-Garci et al. 2006). The GABA_{B(1)} isoforms also have differential impacts on neurophysiological processes, such as LTP in the hippocampus (Vigot et al. 2006) and amygdala (Shaban et al. 2006). Finally, deletion of either isoform demonstrates a divergence of behavioural phenotypes in conditioned aversive learning and memory paradigms (Jacobson et al. 2006b; Shaban et al. 2006; Vigot et al. 2006). Specifically, in a conditioned fear paradigm GABA_{B(1a)}^{-/-} mice showed generalized freezing to both paired and unpaired tones (Shaban et al. 2006). In a conditioned taste aversion paradigm (CTA), GABA_{B(1a)}^{-/-} mice failed to acquire an aversion to a saccharin solution when paired with a lithium chloride-induced malaise, while GABA_{B(1b)}^{-/-} mice acquired CTA, but demonstrated a profound failure in the extinction of this aversion (Jacobson et al. 2006b).

It is therefore well-established that GABA_B receptors play a role in anxiety, and that the GABA_{B(1)} subunit isoforms are functionally relevant molecular variants of the GABA_B receptor which convey differential behavioural responses in tests of aversive learning and memory. However, the influence of the two GABA_{B(1)} isoforms on behaviour in tests of unconditioned anxiety is currently unknown. The aim of the present study therefore, was to determine the influence of the different GABA_{B(1)} subunit isoforms on innate anxiety-related behaviours, using mice deficient in either the GABA_{B(1a)} or GABA_{B(1b)} isoforms.

5.3 Materials and Methods

Animals

The generation of wild-type, GABA_{B(1a)}^{-/-} and GABA_{B(1b)}^{-/-} mice as used in the present studies has been described previously (Vigot et al. 2006). Briefly, a knock-in point mutation strategy was adopted, whereby GABA_{B(1a)} and GABA_{B(1b)} initiation codons were converted to stop codons by targeted insertion of a floxed neo-cassette. All mutant and wild-type mice were maintained on a pure inbred BALB/c genetic background. GABA_{B(1a)}^{-/-} and GABA_{B(1b)}^{-/-} mice used in the present experiments were derived from homozygous breeding of siblings originating from the founding heterozygotic mice (F3-4). Homozygous wild-type controls for the GABA_{B(1)} isoform mutant mice were derived from mating together wild-type siblings generated from GABA_{B(1a)}^{+/-} and GABA_{B(1b)}^{+/-} heterozygous breedings (F3-4). The breeding strategy was applied 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>).

Male and female mice were investigated in separate experiments for all tests performed with the exception of corticosterone and ACTH levels, in which only male mice were used. All mice were singly-housed after achieving at least 8 weeks of age, with the exception of those used in the marble burying test that were group-housed 2 – 4 mice per cage. Mice were housed in macrolon cages with sawdust bedding, tissue paper nesting materials and one red, triangular, polycarbonate Mouse House® (Nalgene) per cage. Housing was at a constant room temperature of 22-24°C in a 12 h light : dark cycle with lights on at 6 – 6.30 am. Food pellets and tap water were available *ad libitum* (except during experimentation). Mice were allowed to settle for a minimum of 1 week after single-housing before testing began. All mice were 8 weeks of age or older at testing. All mice were drug-naïve, and mice used in the stress-induced hyperthermia (SIH), light-dark box (LDB), marble burying tests and in the corticosterone and ACTH assessment were experimentally-naïve. Mice used in the staircase test were the same as those from the SIH test, with an interval of approximately 3 weeks and 5 weeks between tests for the male and female mice, respectively. Mice used in the marble-burying test were singly-house immediately following the test and subsequently tested in the elevated plus maze after an interval of approximately 5 weeks and 1 week for male and female mice, respectively. Mice tested with the elevated zero maze had been previously exposed to a 5 minute handling period approximately 1 week prior to testing, during which basic sensory and sensory-motor characteristics of the mutant mice and wild-type controls were examined (Jacobson et al. 2006a). All animal experiments were conducted during the light phase. All animal experiments were conducted in accordance with Swiss ethics guidelines, and approved by the Veterinary Authority of Basel Stadt, Switzerland.

Stress-Induced Hyperthermia (SIH)

The SIH test is an ideal inclusion in an anxiety test battery as it is not unduly influenced by alterations in locomotor activity, and is a translational model across strains and species (including mice and humans; see (Bouwknicht et al. 2006). The test procedure for SIH was adapted from that reported by Van der Heyden and colleagues (Van der Heyden et al. 1997) and was conducted essentially as described by Cryan *et al.*, (2003), with the exception that mice in the present study were already singly housed prior to the time of SIH testing. Two SIH experiments were conducted, one with male and one with female mice. Age-matched (within gender), experimentally-naïve male and female wild-type, $GABA_{B(1a)}^{-/-}$ and

GABA_{B(1b)}^{-/-} mice (mean age: 10 ± 0.4 weeks for males and 13 ± 0.3 weeks for females, n = 10 per sex and genotype) were re-housed in new cages overnight in the testing room. They had free access to water and food, but were without nesting materials or Mouse Houses®. The following day rectal temperature was measured in each mouse twice, i.e. at $t = 0$ min (T_1) and $t = +15$ min (T_2). The first measurement of temperature serves as the stressor and results in a rapid hyperthermic response. The difference in temperature ($T_2 - T_1$) was considered to reflect the SIH. Time-points were based on previous experiments which showed that a $T_2 - T_1$ interval of 15 min was optimal in terms of SIH (Spooren et al. 2002). Rectal temperature was measured to the nearest 0.1 °C by an ELLAB instruments thermometer (Copenhagen, Denmark Model DM 852), by inserting a lubricated thermistor probe model PRA-22002-A (ELLAB) 2.2 mm diameter 20 mm into the rectum; the mouse was hand-held at the base of the tail during this determination and the thermistor probe was left in place for 15 s.

Staircase Test

The staircase test in mice (Simiand et al. 1984) allows the distinction between locomotor versus anxiolytic responses by comparing the ratio of steps climbed to rearings performed. Anxiolysis is thus interpreted when reductions in rearing are accompanied by an unchanged, or an increased, number of steps climbed. The test shows pharmacological selectivity for anxiolytics, and indeed, locomotor stimulants tend to decrease both parameters in the test (Simiand et al. 1984). Additionally, the number of faecal boli and urination spots made by the mice are easily quantifiable in this test and provide a further indicator of stress responses (Cryan et al. 2003; Gray and Lalljee 1974).

The test was carried out essentially as previously described (Cryan et al. 2003; Simiand et al. 1984). The apparatus comprised an enclosed staircase with five steps made of grey plastic. Each step was 2.5 cm in height, 7.5 cm in length and 11 cm in width. The apparatus was 45 cm in length, with one end 12 cm and the other 25 cm in height. Mutant and wild-type mice utilized in the staircase test in the present study were the same animals as those for the SIH test, with the exception that of the wild-type female mice, only 8 rather than 10 mice were used. Experiments with male and female mice were conducted separately. Animals were moved in their home cages to the testing room at least 1 h prior to testing commencement. Mice were placed on the bottom level facing away from the stairs. The number of steps ascended and rearings made in a 3-min period were observed. The apparatus was briefly wiped with a wet paper towel and dried between animals.

Light - Dark Box

The light-dark box test (LDB) is a conflict-based anxiety test whereby the tendency to explore a novel environment contrasts against the natural aversion of mice to brightly-lit spaces (Crawley 2000). The test was included in the present study as most reliable indicators of anxiety, such as light-dark transitions and time spent in the light (in that order) (Crawley and Davis 1982; Holmes et al. 2002), are expressed as passive avoidance behaviours, thus increasing the range of behaviours used in the present study to evaluate anxiety. The test was carried out similarly to that previously described (Cryan et al. 2003; Holmes et al. 2002; Mombereau et al. 2004a). The apparatus consisted of a Plexiglas enclosure (44 x 21 x 21 cm) divided into two compartments (one light and one dark) by a partition in which there was a small opening (12 x 5 cm) at the floor level. The light compartment was open-roofed, with walls of transparent Plexiglas and was brightly illuminated by a 60W desk lamp overhead (approximately 1000 Lux). The smaller, dark compartment (14 cm width) was closed-roofed and was constructed of black Plexiglas. Male and female mice were investigated in separate experiments. Age-matched, experimentally-naive wild-type, $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice (mean age: 12 ± 0.4 weeks, $n = 10 - 11$ per sex and genotype) were individually placed in the center of the light compartment, facing away from the partition and allowed to explore the apparatus freely for 10 min. The apparatus was cleaned thoroughly between subjects. The number of light – dark transitions, time spent in the light compartment, and latency to enter the dark, were recorded by a trained observer.

Marble-Burying Test

The Marble-Burying test was similar to that previously described (Broekkamp et al. 1986; Spooen et al. 2000). This test was included as animals who are more anxious must engage in active behaviours (defensive marble burying) as opposed to passive behaviours utilized to avoid anxiogenic stimuli in the light-dark box and elevated mazes. Male and female mice were investigated in separate experiments. Mice were group-housed, age-matched (within gender), experimentally-naive male and female wild-type, $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice (Males: mean age, 15.2 ± 0.4 weeks; wild-type $n = 14$, $GABA_{B(1a)}^{-/-}$ $n = 19$, $GABA_{B(1b)}^{-/-}$ $n = 26$. Females: mean age, 20.6 ± 0.6 ; wild-type $n = 23$, $GABA_{B(1a)}^{-/-}$ $n = 16$, $GABA_{B(1b)}^{-/-}$ $n = 35$). Mice were placed individually in small cages ($26 \times 20 \times 14$ cm), in which 10 marbles had been equally distributed on top of a 5 cm – deep bed of sawdust, and a wire lid placed on top of the cage. Mice were left undisturbed for 30 min, after which the number of buried marbles (i.e., those covered by sawdust three-quarters or more) were counted.

Elevated Plus Maze

The elevated plus maze is a widely used test for assessing anxiety behaviour in mice (Crawley 2000; Holmes 2001; Rodgers 1997). Similarly to the light-dark box, the elevated plus maze is based on the conflict between exploratory drive and avoidance of the innately-aversive open arms, and in the expression of passive behaviours that are employed to avoid the more anxiogenic areas of the mazes. However, the tests differ both in the nature of the anxiogenic stimuli (heights versus a brightly lit space), and in the starting place of the mice (choice point versus maximally anxiogenic area) (Crawley 2000; Rodgers 1997). As such, results between the two tests can be expected to vary, and therefore the inclusion of both tests in the present study provides a more comprehensive test battery. Separate experiments were conducted with male and female mice. Mice were the same animals as those utilized in the Marble-Burying test, with the exception that of the female $GABA_{B(1b)}^{-/-}$ mice, only 34, rather than 35 mice were used. The elevated plus maze was carried out as described previously (Cryan et al. 2003; Rodgers et al. 1997b). The apparatus comprised two open arms (30×5 cm) and two enclosed arms ($30 \times 5 \times 15$ cm), which extended from a common central platform (5×5 cm). The configuration formed the shape of a plus sign, with like-arms arranged opposite one another, and the apparatus was elevated 60 cm above floor level on a central pedestal. The maze floor was made of black Plexiglas, while the side- and end-walls of the enclosed arms were made from clear Plexiglas. Grip on the edges of open arms was facilitated by inclusion of a small raised edge (0.25 cm) around their perimeter. Animals were transported from the holding room to the laboratory at least 1 h before testing. Mice were placed onto the central platform facing an enclosed arm. A 6-min trial was performed and, between subjects, the maze was thoroughly cleaned. Direct registrations were made by an observer sitting close to the maze using the following conventional parameters: number of open and closed arm entries (arm entry defined as all four paws entering an arm), time spent on open arms (excluding the central platform).

Elevated Zero Maze

The elevated zero maze is an ideal complementary test to the elevated plus maze. It eliminates the central area of the plus maze and provides a continuous circular track to facilitate exploration, while maintaining endpoint parameters similar in construct to that of the elevated plus maze (Lee and Rodgers 1990; Shepherd et al. 1994). Furthermore, we have previously shown that mice lacking the $GABA_{B(1)}$ subunit jumped off the maze in a panic-like response (Mombereau et al. 2004a). It was therefore of interest to assess the influence of $GABA_{B(1)}$

receptor isoform deletion on mice in this test. The elevated zero maze test was conducted as previously described (Cryan et al. 2004). Male and female mice were investigated in separate experiments. Age-matched (within gender), experimentally-naïve male and female wild-type, $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice (mean age: 21.2 ± 1.4 weeks for males and 23.8 ± 0.8 weeks for females, $n = 11 - 12$ for each sex and genotype) were used. Mice had been individually handled for about 5 minutes each one week prior to testing in the elevated zero maze. The apparatus was a 5.5 cm-wide circular track constructed of grey Plexiglas with an inside diameter of 34 cm, a midtrack circumference of approximately 121 cm, and an elevation of 40 cm. It consisted of two open quadrants with a raised, 2-mm edge and two closed quadrants with walls 11 cm high. Mice were placed in one of the closed quadrants designated as the starting quadrant and were allowed to investigate the zero maze for a period of 5 min. During this time, an observer scored mice on several anxiety-related variables as identified in previous studies (Cryan et al. 2004; Shepherd et al. 1994; Tarantino et al. 2000). These included time spent in both open and closed quadrants, number of transitions between quadrants, latency to leave the closed quadrant, stretch-attend postures (SAP, elongated body posture with at least snout over open/closed divide) into the open quadrant, rearing, and head dips.

Corticosterone and ACTH

In order to investigate the effects of deletion of the $GABA_{B(1a)}$ or $GABA_{B(1b)}$ isoforms on hypothalamic-pituitary-adrenal (HPA) axis activity, corticosterone and adrenocorticotrophic hormone (ACTH) levels at 0700 – 0800 hr were measured in trunk blood of male, experimentally-naïve wild-type ($n = 9$), $GABA_{B(1a)}^{-/-}$ ($n = 9$) and $GABA_{B(1b)}^{-/-}$ ($n = 6$) male mice (mean age (\pm SEM) 15.6 ± 0.6 weeks). Home cages for each individual mouse were removed from the housing room one at a time to another room where mice were decapitated immediately on arrival and trunk blood collected into EDTA-treated 1.5 ml tubes (Milian S.A., Meyrin, Switzerland). Blood samples were kept on ice for up to about 20 min until centrifugation at 10 000 rpm for 15 min in a refrigerated centrifuge (4 °C). The plasma fraction was collected and stored at -80 °C until subsequent analysis for corticosterone and ACTH. Plasma corticosterone and ACTH concentrations were measured using commercially available radioimmunoassay kits (ICN Biomedicals, Costa Mesa, CA, USA).

Statistical Analyses

As studies which were carried out in both male and female mice were conducted in independent experiments, data for each sex were analysed separately. Corticosterone levels and the number of steps taken in the staircase test were analyzed using one-way Analysis of Variance (ANOVA). Parameters measured within the SIH, staircase, light-dark box, elevated plus maze tests and ACTH data were analyzed using Kruskal-Wallis one-way Analysis of Variance on Ranks, followed by Dunn's Method *post hoc* comparisons where indicated by significant ANOVA factors.

5.4 Results

Stress-Induced Hyperthermia (SIH)

SIH was not influenced significantly by genotype in either of the experiments using male or female mice (Fig 1a and b; $H = 4.25$, $P = 0.12$ and $H = 1.64$, $P = 0.44$ for males and females, respectively). In the male mice the first temperature recording, T1, tended to be higher for the $GABA_{B(1a)}^{-/-}$ mice (Fig 1a; $H = 5.27$, $P = 0.072$), although in the female mice this trend was not apparent (Fig 1b; $H = 3.582$, $P = 0.17$). The second temperature recording, T2, was not significantly influenced by genotype in either the male or female mice (Fig 1a and b; $H = 2.24$, $P = 0.33$ and $H = 3.40$, $P = 0.18$ for male and female mice, respectively).

Staircase Test

The number of steps taken, incidence of rearing and the ratio of steps to rears in the staircase test were not significantly affected by genotype in either of the experiments using male or female mice (Males: steps $F_{2,29} = 0.26$, $P = 0.772$; rears $H = 0.96$, $P = 0.62$; ratio $H = 0.88$, $P = 0.64$. Females: steps $F_{2,27} = 0.996$, $P = 0.38$; rears $H = 3.61$, $P = 0.17$; ratio $H = 1.25$, $P = 0.536$; Fig 2a and b).

With regard to physiological indicators of stress (Fig 2a and b), genotype significantly influenced the number of faecal boli produced by male mice during the test ($H = 14.13$, $P < 0.001$). *Post hoc* comparisons demonstrated the $GABA_{B(1b)}^{-/-}$ mice defecated more than $GABA_{B(1a)}^{-/-}$ mice ($P < 0.01$), although neither mutant strain differed from the wild-types in this regard ($P > 0.05$). Faecal boli production was not significantly affected by genotype in the female mice, as was also the case for the number of urine spots produced by male or female mice (Females faecal boli $H = 2.37$, $P = 0.31$; males urination score $H = 3.73$, $P = 0.16$; females urination score $H = 3.74$, $P = 0.154$).

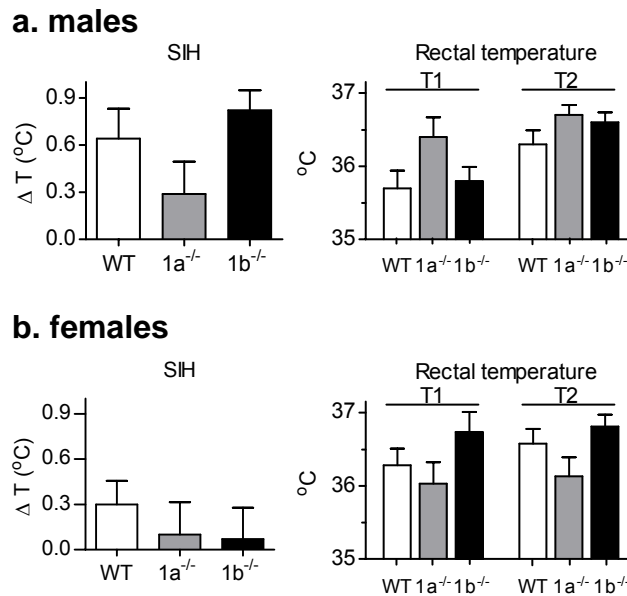


Fig. 1. Stress-Induced Hyperthermia (SIH). Genotype did not significantly influence SIH or rectal temperature at the two time points measured in experiments with (a) male ($n = 10$ per genotype) or (b) female ($n = 10$ per genotype) wild-type (WT), $GABA_{B(1a)}^{-/-}$ ($1a^{-/-}$) or $GABA_{B(1b)}^{-/-}$ ($1b^{-/-}$) mice. Bars represent means + SEM. First rectal temperature recording (T1), second rectal temperature recording taken 15 min later (T2), temperature difference between T1 and T2 (ΔT).

Light - Dark Box

In the experiment with male mice, three wild-type mice, one $GABA_{B(1a)}^{-/-}$ and one $GABA_{B(1b)}^{-/-}$ mouse made no transitions during the test – all of their time in the test was spent in the light compartment in which they were initially placed, and the latency to enter the dark side was therefore greater than the 600 sec duration of the test. As a consequence, absolute data values for latency for these mice were not determined. Furthermore, data from these mice may have introduced bias in other test measures such as time in the light compartment and light-dark transitions, therefore these mice were excluded from statistical analysis.

Genotype influenced the number of light-dark transitions in male mice ($H = 6.48$, $P = 0.039$, Fig. 3a). Post hoc comparisons revealed that male $GABA_{B(1b)}^{-/-}$ mice conducted significantly less light-dark transitions than $GABA_{B(1a)}^{-/-}$ mice ($P < 0.05$), although neither mutant differed from the wild-type controls in this regard. The latency to enter the dark compartment and the total time spent in the light compartment were not significantly influenced by genotype in the male mice (Latency: $H = 1.55$, $P = 0.46$; Time in light: $H = 2.26$, $P = 0.32$; Fig. 3a).

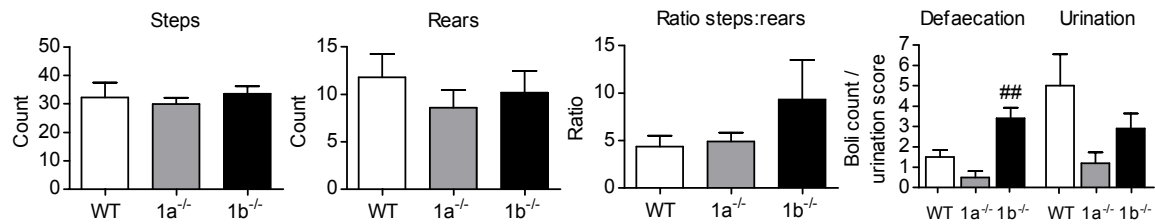
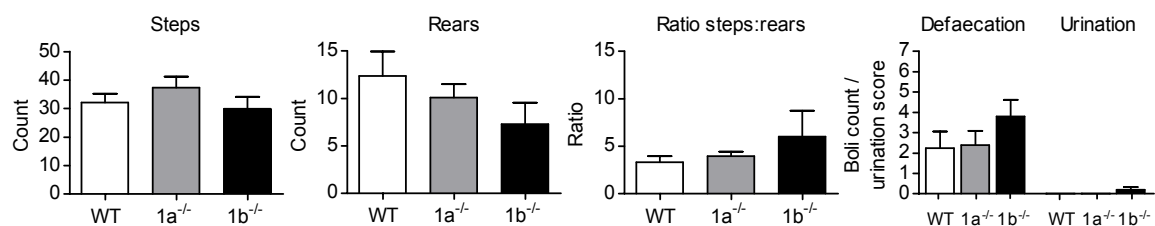
a. males**b. females**

Fig. 2. Staircase. Deletion of GABA_{B(1a)} or GABA_{B(1b)} receptor subunit isoforms did not influence the number of ascending steps taken (Steps), incidence of rearing (Rears), the ratio between these two parameters (Ratio) or the amount of urination in the staircase test in experiments with (a) male (n = 10 per genotype) and (b) female (n = 10 per genotype) mice, although male GABA_{B(1b)}^{-/-} mice defecated more than GABA_{B(1a)}^{-/-} male mice. Bars represent means ± SEM. # P < 0.05 for GABA_{B(1a)}^{-/-} vs GABA_{B(1b)}^{-/-}. Wild-type (WT), GABA_{B(1b)}^{-/-} (1a^{-/-}), GABA_{B(1b)}^{-/-} (1b^{-/-}).

In the experiment with female mice, some mice also failed to enter the dark compartment (two wild-type, one GABA_{B(1a)}^{-/-} and two GABA_{B(1b)}^{-/-} mice) and were therefore excluded from statistical analysis. The number of light-dark transitions made by female mice was not significantly affected by genotype (H = 1.99, P = 0.37, Fig. 3b). However, GABA_{B(1b)}^{-/-} female mice spent more time in the light compartment than GABA_{B(1a)}^{-/-} female mice (P < 0.05, Fig. 3b), although neither mutant genotype differed significantly from the wild-types (H = 6.58, P = 0.037, Fig. 3b). As for the experiment conducted with the male mice, latency to enter the dark compartment was not significantly affected by genotype in the female mice (H = 0.71, P = 0.70).

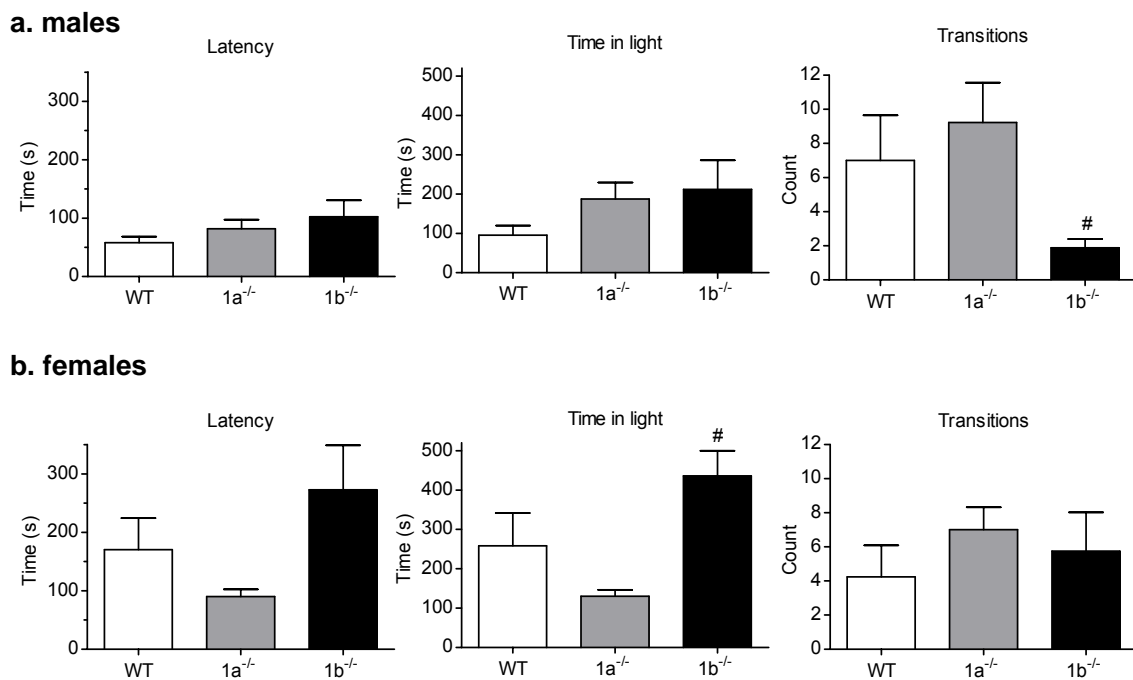


Fig. 3. Light-Dark Box. In male mice (a), $GABA_{B(1b)}^{-/-}$ ($1b^{-/-}$) mice made fewer transitions between the light and dark compartments (Transitions) relative to the $GABA_{B(1a)}^{-/-}$ ($1a^{-/-}$) mice ($n = 10$ per genotype). In a separate experiment with female mice (b), $GABA_{B(1b)}^{-/-}$ mice spent more time in the light compartment (Time in light) than $GABA_{B(1a)}^{-/-}$ mice ($n = 10 - 11$ per genotype). Genotype did not influence the time take for mice of either sex to initially enter the dark compartment (Latency). Bars represent means + SEM. # $P < 0.05$ for $GABA_{B(1a)}^{-/-}$ vs $GABA_{B(1b)}^{-/-}$. Wild-type (WT).

Marble Burying Test

The number of marbles buried in the experiments with either male or female mice were not significantly affected by genotype ($H = 0.33$, $P = 0.85$ and $H = 2.49$, $P = 0.29$ for males and females, respectively; Table 1).

Table 1. Mean (\pm SEM) number of marbles buried by male (wild-type $n = 14$, $GABA_{B(1a)}^{-/-}$ $n = 19$, $GABA_{B(1b)}^{-/-}$ $n = 26$) and female (wild-type $n = 23$, $GABA_{B(1a)}^{-/-}$ $n = 16$, $GABA_{B(1b)}^{-/-}$ $n = 35$) wild-type and $GABA_{B(1)}$ isoform-deficient mice.

	Wild-type	$GABA_{B(1)}^{-/-}$	$GABA_{B(1b)}^{-/-}$
Males	7.6 (\pm 0.6)	7.4 (\pm 0.6)	7.3 (\pm 0.4)
Females	6.7 (\pm 0.5)	6.6 (\pm 0.6)	7.5 (\pm 0.3)

Elevated Plus Maze

Genotype did not significantly influence any of the measured parameters in the elevated plus maze in the experiments involving the male or the female mice. The number of open arm entries for male ($H = 0.71$, $P = 0.70$) or female mice ($H = 2.62$, $P = 0.27$) was not affected by genotype, nor were closed arm entries for male or female mice ($H = 0.91$, $P = 0.63$; $H = 0.71$, $P = 0.70$ for males and females respectively), nor the total number of arm entries ($H = 0.87$, $P = 0.65$; $H = 1.67$, $P = 0.44$ for males and females, respectively). With regard to total arm entries, some mice made only one or no arm entries during the experiment. In female mice, this was the case for 4 of the 23 wild-type, 1 of the 16 $GABA_{B(1a)}^{-/-}$ and 9 of the 35 $GABA_{B(1b)}^{-/-}$ mice. In the experiment with male mice, all of the wild-type and $GABA_{B(1a)}^{-/-}$ mice made more than one arm entry, while 2 of the 26 $GABA_{B(1b)}^{-/-}$ males made one or no arm entries. The ratio of the number of open to closed arm entries was not affected by genotype in the experiments with either sex ($H = 2.16$, $P = 0.34$ and $H = 0.87$, $P = 0.65$ for males and females, respectively). Finally, the amount of time spent on the open arms by the mice in each respective experiment was not significantly affected by genotype ($H = 2.35$, $P = 0.31$ and $H = 2.05$, $P = 0.36$ for males and females, respectively; Table 2)

Table 2. Parameters measured in the elevated plus maze were not significantly influenced by deletion of the $GABA_{B(1a)}$ or $GABA_{B(1b)}$ isoforms. Data are means (\pm SEM)

(n)	Males			Females		
	Wild-type (14)	$GABA_{B(1a)}^{-/-}$ (19)	$GABA_{B(1b)}^{-/-}$ (26)	Wild-type (23)	$GABA_{B(1a)}^{-/-}$ (16)	$GABA_{B(1b)}^{-/-}$ (34)
Open arm entries	5.0 (± 1.0)	4.8 (± 0.6)	4.3 (± 0.6)	3.3 (± 0.5)	3.3 (± 0.7)	2.3 (± 0.3)
Closed arm entries	7.9 (± 1.4)	6.4 (± 1.0)	5.8 (± 0.8)	3.6 (± 0.7)	4.0 (± 0.9)	3.4 (± 0.6)
Ratio open : closed entries	0.39 (± 0.03)	0.48 (± 0.05)	0.44 (± 0.04)	0.49 (± 0.06)	0.52 (± 0.06)	0.49 (± 0.1)
Total arm entries	12.9 (± 2.3)	11.2 (± 1.4)	10.1 (± 1.3)	6.9 (± 1.1)	7.3 (± 1.4)	5.7 (± 0.8)
Time open (s)	109.1 (± 13.3)	153.3 (± 20.3)	135.8 (± 16.8)	103.0 (± 18.2)	137.9 (± 25.8)	110.1 (± 20.7)

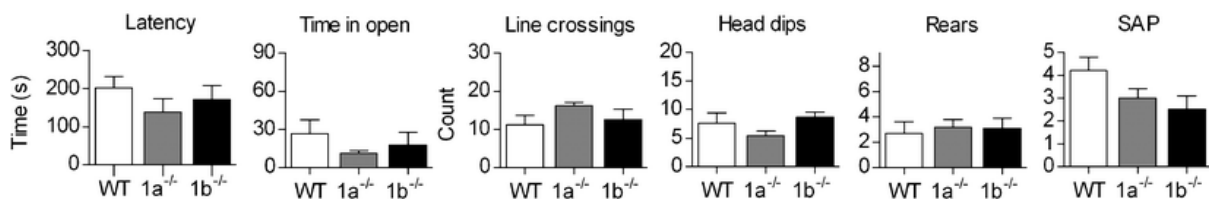
Elevated Zero Maze

In the experiment with male mice, the latency to enter the open side, time spent on the open side, the number of head dips over the side of the open areas and number of rears were not significantly affected by genotype ($H = 1.98$, $P = 0.371$; $H = 0.62$, $P = 0.733$; $F_{2,34} = 1.75$, $P = 0.19$ and $H = 1.18$, $P = 0.55$, respectively). There was a tendency for genotype to influence the

distance travelled in the maze (line crossings: $H = 5.50$, $P = 0.064$), and the number of SAPs performed by the mice ($F_{2,34} = 2.577$, $P = 0.092$; Figure 4a).

In the experiment with female mice, genotype significantly influenced the majority of parameters measured in the elevated zero maze. The latency to enter the open sided area of the maze ($H = 11.39$, $P < 0.01$) was significantly longer in both $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice than in the wild-type mice. Both mutant strains also performed significantly less head dips over the maze open sides ($F_{2,35} = 21.07$, $P < 0.001$) and rearings ($H = 13.52$, $P = 0.001$) than the wild type mice. Genotype also influenced the time spent on the open sided areas ($H = 11.60$, $P < 0.01$) and the number of line crossings ($H = 9.09$, $P < 0.05$), and *post-hoc* comparisons revealed a significant difference between $GABA_{B(1b)}^{-/-}$ mice and wild-types in these parameters. The mutants did not differ significantly from each other in *post hoc* comparisons of the aforementioned parameters. The only parameter measured in the elevated zero maze that was not significantly influence by genotype in the female mice was the number of SAPs ($F_{2,35} = 0.80$, $P = 0.46$; Figure 4b).

a males



b females

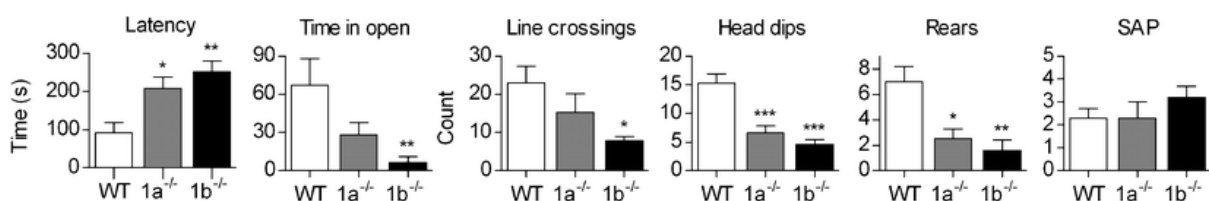


Fig. 4. Elevated Zero Maze. In male mice (a) deletion of either the $GABA_{B(1a)}$ or $GABA_{B(1b)}$ isoforms did not significantly influence the time taken to enter an open-sided quadrant (Latency), time spent in the open-sided quadrants (Time in open), ambulation in the maze (Line crossings), or the ethological parameters: incidence of dipping the head over the open sides (Head dip), rearing (Rears) and the number of stretch-attend postures (SAP) performed into the open quadrants (n: WT = 12, $1a^{-/-}$ = 11, $1b^{-/-}$ = 12). In a separate experiment with female mice (b) genotype influenced all elevated zero maze parameters measured with the exception of SAPs (n: WT = 12, $1a^{-/-}$ = 12, $1b^{-/-}$ = 12). Bars represent means + SEM. * $P < 0.05$, ** $P < 0.01$ vs wild-type (WT). $GABA_{B(1a)}^{-/-}$ ($1a^{-/-}$), $GABA_{B(1b)}^{-/-}$ ($1b^{-/-}$).

Basal Corticosterone and ACTH

Mean plasma corticosterone appeared to be slightly elevated in the $GABA_{B(1a)}^{-/-}$ mice compared to the $GABA_{B(1b)}^{-/-}$ mice (Fig. 6a), however, ‘genotype’ did not achieve statistical significance ($F_{2,12} = 1.91$, $P = 0.17$). Two data points were removed from the ACTH dataset as statistical outliers: one from the $GABA_{B(1a)}^{-/-}$ mice (922 pg/ml) and one from the $GABA_{B(1b)}^{-/-}$ mice (1176 pg/ml). The genotype did not significantly influence basal ACTH levels (Fig 6b; $H = 1.93$, $P = 0.38$).

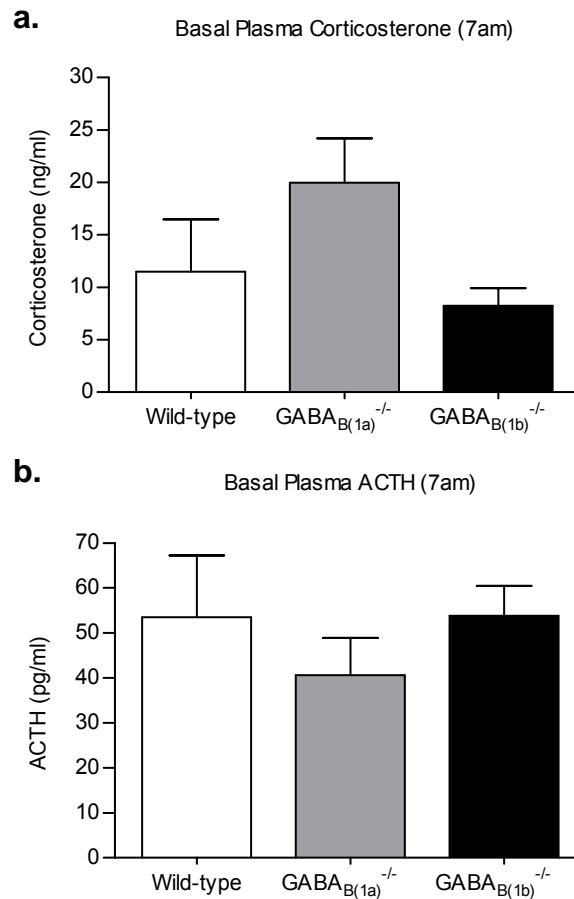


Fig. 5. Hypothalamic-Pituitary-Adrenal Axis Characteristics. Deletion of $GABA_{B(1a)}$ or $GABA_{B(1b)}$ isoforms did not significantly influence plasma corticosterone (a) or ACTH (b) of male mice as measured within 1 hour of the start of the circadian light phase (Basal) (n : wild-type = 9, $GABA_{B(1a)}^{-/-}$ = 9, $GABA_{B(1b)}^{-/-}$ = 6). Bars represent means + SEM.

5.5 Discussion

The present study aimed to determine the influence of $GABA_{B(1)}$ subunit isoform deletion on innate, unconditioned anxiety-related behaviours in mice. To this end, $GABA_{B(1a)}^{-/-}$, $GABA_{B(1b)}^{-/-}$ and wild-type control mice were investigated in a comprehensive battery of

anxiety tests that employed a number of different types of experimental endpoints including autonomic, passive and active avoidance and ethological parameters. The test-battery approach has been advocated for the detecting of genuine differences in anxiety phenotypes in mutant mice, as reliance on fewer tests may give rise to erroneous interpretations depending on specific idiosyncrasies of individual tests or mutations (Cryan and Holmes 2005). Overall, the present study demonstrated that the constitutive genetic deficiency of either the $GABA_{B(1a)}$ or $GABA_{B(1b)}$ isoform did not, for the most part, result in an alteration of innate, unconditioned anxiety (Table 3).

Table 3. Summary of results for the behavioural assessment of innate anxiety in $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice. X = no alteration in phenotype, ↓ = anxiolytic-like phenotype, ↑ = anxiogenic-like phenotype, relative to wild-type controls.

Paradigm	Parameter	Endpoint Category	Males		Females	
			$GABA_{B(1a)}^{-/-}$	$GABA_{B(1b)}^{-/-}$	$GABA_{B(1a)}^{-/-}$	$GABA_{B(1b)}^{-/-}$
Stress-induced hyperthermia						
	SIH	Autonomic	x	x	x	x
Staircase						
	Steps	Passive avoidance	x	x	x	x
	Rears	Ethological	x	x	x	x
	Ratio	Combined	x	x	x	x
	Boli and urine	Autonomic	x	x	x	x
Light dark box						
	Latency	Active avoidance	x	x	x	x
	Time in light	Passive avoidance	x	x	x	x
	Transitions	Passive avoidance	x	x	x	x
Marble burying						
	Marbles buried	Active avoidance	x	x	x	x
Elevated plus maze						
	Open arm	Passive avoidance	x	x	x	x
	Closed arm	Passive avoidance	x	x	x	x
	Ratio	Passive avoidance	x	x	x	x
	Total arm	Passive avoidance	x	x	x	x
	Time open	Passive avoidance	x	x	x	x
Elevated zero maze						
	Latency	Passive avoidance	x	x	↑	↑
	Time open	Passive avoidance	x	x	x	↑
	Line X	Passive avoidance	x	x	x	↑
	Head dips	Ethological	x	x	↑	↑
	Rears	Ethological	x	x	↑	↑
	SAP	Ethological	x	x	x	x
Basal corticosterone						
		Autonomic	x	x	-	-
Basal ACTH						
		Autonomic	x	x	-	-

No anxiety-related phenotype was detected for either the $GABA_{B(1a)}^{-/-}$ or $GABA_{B(1b)}^{-/-}$ mice, in experiments with either gender, in the SIH, marble burying and elevated plus maze paradigms. Furthermore, in male mice at least, genotype did not influence the levels of HPA axis hormones (Table 3). In the staircase, light-dark box and elevated zero maze tests, $GABA_{B(1b)}^{-/-}$ mice showed some minor differences in behaviour when compared with $GABA_{B(1a)}^{-/-}$ mice. However, given that neither mutant differed from the control wild-type mice in these measures, or for that matter in the majority of the response parameters measured in these tests, one cannot place too much credence on such subtle effects with relation to anxiety-related behaviours

Deletion of either $GABA_{B(1)}$ subunit isoforms significantly influenced a number of parameters in the elevated zero maze in female mice. Locomotor activity can influence aspects of performance in exploratory-based tests of anxiety (Cryan and Holmes 2005); for example, a hyperactive phenotype may falsely suggest anxiolysis, and likewise, hypoactivity anxiogenesis. $GABA_{B(1b)}^{-/-}$ mice (both females and males) have previously been demonstrated to be hyperactive in a novel environment, while the distance traveled by $GABA_{B(1a)}^{-/-}$ mice was not different to that of the wild-type controls (Jacobson et al. 2006a). However, these observations are at odds with the reductions in exploratory activity by both mutant strains relative to the wild-types in the elevated zero maze in the present study, as indicated by an increased latency to enter the open sided area in both female mutant genotypes and by the reduced line crossings made by the $GABA_{B(1b)}^{-/-}$ females. Furthermore, the ethological measures of head-dipping and rearing were also reduced in both $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ female mice in this test. Reductions in the parameters of latency to enter an innately aversive area, head-dipping over the edges of elevated apparatuses and rearing, have been interpreted as heightened anxious responses in various apparatuses, including the elevated zero maze (Belzung 1999; Homanics et al. 1999; Rodgers 1997; Rodgers and Johnson 1995; Shepherd et al. 1994). The elevated zero maze is considered a more sensitive test than the elevated plus maze, mainly due to the elimination of the central square of the elevated plus maze, which can produce difficulties in data interpretation (Lee and Rodgers 1990; Shepherd et al. 1994), and to the facilitation of locomotor exploration given by the circular track of the zero maze, thus eliminating corners in which the mice may barricade themselves (Rodgers 1997; Shepherd et al. 1994). The present findings therefore suggest a subtle, sex-specific role for the $GABA_{B(1)}$ isoforms in innate anxiety. It should be noted, however, that in the light-dark box test, $GABA_{B(1b)}^{-/-}$ female mice spent an increased amount of time in the light side relative to the $GABA_{B(1a)}^{-/-}$ female mice, an anxiolytic-like response. Together with the data

from other anxiety paradigms where no robust anxiety phenotype was observed, these results indicate that the influence of GABA_{B(1)} isoforms on anxiety in female mice is most likely highly dependent on the environment.

Failure to enter the dark compartment of the light-dark box was shown by a small number of both male and female mice, and occurred to a reasonably similar level in wild-type and mutant mice. The proportion of mice of each genotype making 0 or 1 total arm entries in the elevated plus maze was also similar to that of the light dark box, although only in female mice. These behaviours may reflect ‘freezing’, neophobic avoidance of newly-discovered compartments, or failure to explore extensively enough to find additional compartments (Rodgers 1997), and thus may indicate anxiety in these animals. As genotype did not significantly influence these behaviours, they may be a consequence of the background strain. The BALB/c substrains have previously been reported as anxious in comparison to other mouse strains (Belzung 1999; Belzung and Griebel 2001; Cryan and Holmes 2005; Griebel et al. 2000). It could therefore be posited that innate anxiety may have been near-maximal in the background strain for the GABA_{B(1)} isoform mutant mice, and thus further increases in anxiety induced by the genetic manipulations may be difficult to detect (Crawley et al. 1997). However, this was certainly not the case with either of the GABA_{B(1)}^{-/-} or GABA_{B(2)}^{-/-} mice, both of which were also maintained on a BALB/c background (Gassmann et al. 2004; Schuler et al. 2001), and both of which show profoundly increased anxiety relative to wild-type controls in exploratory anxiety tests (Mombereau et al. 2004a; Mombereau et al. 2005; Mombereau et al. 2004b). GABA_{B(1)}^{-/-} mice were substantially more anxious than wild-types in the light-dark box (Mombereau et al. 2004a; Mombereau et al. 2004b) and staircase tests (Mombereau et al. 2004a), while in the elevated zero maze, all GABA_{B(1)}^{-/-} mice jumped from the maze, a response indicating heightened flight or panic behaviour (Mombereau et al. 2004a). Clearly this indicates that the tests used are suitable to detect increases in anxious behaviours in the BALB/c strain.

The anxious phenotype of the aforementioned GABA_{B(1)}^{-/-} mice differs considerably from that of the specific GABA_{B(1)} isoform-deficient mice in the present study. Both GABA_{B(1)}^{-/-} and GABA_{B(2)}^{-/-} mice show spontaneous seizures, hyperalgesia, hyperlocomotion, memory impairments and significantly elevated exploratory anxiety (Gassmann et al. 2004; Mombereau et al. 2004a; Mombereau et al. 2005; Mombereau et al. 2004b; Schuler et al. 2001). In these mutant mice, classical GABA_B receptor agonist responses are abolished (Gassmann et al. 2004; Kaupmann et al. 2003; Schuler et al. 2001). In contrast, there is only partial GABA_B receptor loss in the GABA_{B(1a)} and GABA_{B(1b)}^{-/-} mice (Vigot et al. 2006), and

some residual agonist-induced function remains, albeit blunted, in both the $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice (Jacobson et al. 2006a; Perez-Garci et al. 2006; Shaban et al. 2006; Vigot et al. 2006). In combination with the lack of overt anxious phenotype of the $GABA_{B(1)}$ isoform mutant mice in the present study, these findings together demonstrate that at least some heterodimeric $GABA_B$ receptor function is essential for the prevention of an increase in anxiety behaviour, and furthermore, that the presence of either the $GABA_{B(1a)}$ or $GABA_{B(1b)}$ isoforms can fulfil this task.

The indistinct innate anxiety phenotype of $GABA_{B(1b)}^{-/-}$ and $GABA_{B(1a)}^{-/-}$ mice demonstrated in the present study contrasts with their reported phenotypes in aversive taste memory (Jacobson et al. 2006b) and conditioned freezing paradigms (Shaban et al. 2006). $GABA_{B(1a)}^{-/-}$ mice did not acquire a CTA to a saccharin solution paired with LiCl-induced malaise, while $GABA_{B(1b)}^{-/-}$ mice acquire CTA well, but were profoundly impaired in the extinction of this aversion (Jacobson et al. 2006b). In conditioned freezing, $GABA_{B(1a)}^{-/-}$ mice show subsequent generalized freezing to sound cues irrespective of whether or not they were paired with high-intensity (0.9mA) foot shocks during conditioning (Shaban et al. 2006). In theory, it should not necessarily be expected that conditioned and unconditioned tests of fear and anxiety should demonstrate similar phenotypes, or indeed, even to produce similar phenotypes within each of these categories (Rodgers 1997). Certainly, dissociations between aversive conditioning and exploratory anxiety has also been reported for other mutant mice, such as forebrain-selective glycine transport 1 (GlyT1)-deficient mice (Yee et al. 2006) and 5-HT_{1A} receptor knockout mice (Klemenhagen et al. 2006), although it should be noted that in each of these studies only one unconditioned anxiety paradigm was utilized. However, the differentiation between conditioned and unconditioned responses appears particularly clearly delineated with $GABA_{B(1)}$ isoform deficient mice. CTA is well known as an aversive, associative learning and memory paradigm (Akirav 2006; Akirav et al. 2006; Berman and Dudai 2001; Bermudez-Rattoni 2004; Lamprecht et al. 1997; Welzl et al. 2001) and has recently been applied to the study of anxiety disorders associated with altered emotional learning (Cryan and Holmes 2005; Guitton and Dudai 2004; Yasoshima and Yamamoto 2005). Conditioned freezing in rodents is thought to model emotive cognition aspects of human anxiety disorders such as post-traumatic stress disorder and panic disorder (Barad 2005; Cryan and Holmes 2005; Delgado et al. 2006; Ledgerwood et al. 2005; Ressler et al. 2004). It should also be noted that the $GABA_{B(1a)}$ isoform has been demonstrated as necessary for both hippocampal (Vigot et al. 2006) and amygdala (Shaban et al. 2006) long-term potentiation, and the ability to discriminate successfully between novel and familiar objects in

a mouse object recognition paradigm – a task which requires the presence of an intact hippocampus (Broadbent et al. 2004; Clark et al. 2000). Together these findings suggest that the GABA_{B(1a)} and GABA_{B(1b)} isoforms of the GABA_{B(1)} subunit have specific relevance for anxiety with a cognitive component, rather than for innate anxiety *per se*. Indeed, it remains possible that the specific and differential deficiencies in emotive learning and memory in these mutant mice could be a product predominantly of cognitive impairments, rather than one of emotive processing in itself.

In conclusion, previous studies have demonstrated that genetic ablation of all functional GABA_B receptors results in increases in unconditioned anxiety behaviour (Cryan and Kaupmann 2005). Results of the present study suggest that this not due to the specific loss of either one of the predominant GABA_{B(1)} subunit isoforms, and that the role for the isoforms in innate anxiety is relatively indistinct. In contrast, these isoforms appear to be more explicitly involved in anxious behaviours that are associated with a cognitive component.

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Chapter 6

Antidepressant-Like Effects and Blunted 5-HT_{1A} Receptor Responses in Mice Lacking GABA_{B(1a)} but not GABA_{B(1b)}

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6.1 Abstract

GABA_B receptors are heterodimers of GABA_{B1} and GABA_{B2} receptor subunits. The predominant GABA_{B1} receptors isoforms are GABA_{B1a} and GABA_{B1b}. There is accumulating evidence for a role of GABA_B receptors in depression. In preclinical studies, GABA_B receptor antagonists induce antidepressant-like responses, and GABA_{B1} and GABA_{B2} deficient mice show an antidepressant-like profile in the forced swim test (FST). However, the contribution of GABA_{B1} isoforms to the antidepressant-like behavior is unknown. We therefore studied mutant mice deficient in either the GABA_{B1a} or GABA_{B1b} receptor isoforms in the FST. As there is strong evidence for interactions between GABA_B receptor and the serotonergic system, and 5-HT_{1A} receptors are implicated in depression and antidepressant actions, we also evaluated 5-HT_{1A} receptor function in these mice. Both male and female GABA_{B1a}^{-/-} mice showed an antidepressant-like profile in the FST, relative to wild-types. GABA_{B1b}^{-/-} deficient mice were without a phenotype in this test. Further, GABA_{B1a}^{-/-} mice demonstrated profoundly blunted hypothermic and motoric responses to the 5-HT_{1A} receptor agonist 8-OH-DPAT. In addition, 8-OH-DPAT-induced corticosterone and ACTH release was specifically attenuated in GABA_{B1a}^{-/-} mice. Interestingly, 5-HT_{1A} receptor binding sites, as detected by autoradiography with [³H]-MPPF and [³H]-8-OH-DPAT, were largely unaffected by genotype. The data indicate that the GABA_{B1a} isoform, but not the GABA_{B1b} isoform, may underlie GABA_B receptor-related antidepressant-like effects. We conclude that GABA_B receptors containing the GABA_{B1a} isoform regulate the serotonergic system and contribute to the neurobiological mechanisms underlying depression.

6.2 Introduction

GABA_B receptors are heterodimers of GABA_{B(1)} and GABA_{B(2)} subunits. There are two predominant isoforms of the GABA_{B(1)} receptor subunit expressed in brain, GABA_{B(1a)} and GABA_{B(1b)}, both of which heterodimerize with the GABA_{B(2)} subunit to form functional receptors (Bettler et al. 2004; Cryan and Kaupmann 2005). Both isoforms are encoded by the same gene, *Gabbr1*, and are generated by different promoter usage (Steiger et al. 2004). The isoforms differ in sequence primarily by the inclusion of an extended N-terminus ('short consensus repeats' or 'sushi domains') on the GABA_{B(1a)} isoform (Kaupmann et al. 1998a).

A hypothesis for the role of GABA_B receptors in the mechanisms underlying depression and the action of antidepressants was first proposed over 20 years ago (Pile and Lloyd 1984). This followed the observation that after chronic (but not acute) treatment of rats

with antidepressants of varied mechanisms of action, GABA_B receptor binding in the frontal cortex was up-regulated (Pilc and Lloyd 1984). Other animal studies have since demonstrated similar findings on GABA_B receptor function or expression following antidepressant administration (Gray and Green 1987; Sands et al. 2004b) or models of antidepressant electroconvulsive shock therapy (Gray and Green 1987; Lloyd et al. 1985). This includes the enhancement of hypothermia induced by GABA_B receptor activation via the prototypic agonist baclofen, a classic indicator of GABA_B receptor function (Gray et al. 1987). However, further advances have been hampered by a lack of tools with which to probe the molecular diversity of the GABA_B system.

Accumulating recent evidence has demonstrated that GABA_B receptor antagonists may be an attractive target for the development of antidepressants (Cryan and Kaupmann 2005; Slattery and Cryan 2006). Specifically, genetic deletion of either the GABA_{B(1)} or GABA_{B(2)} receptor subunits induced an antidepressant-like phenotype in the forced swim test (FST) in mice (Mombereau et al. 2004a; Mombereau et al. 2005). Furthermore, GABA_B receptor antagonists show antidepressant-like actions in the rat and mouse FST (Mombereau et al. 2004a; Nowak et al. 2006; Slattery et al. 2005a) and in the learned helplessness (Nakagawa et al. 1999; Nowak et al. 2006), olfactory bulbectomy and chronic mild stress paradigms (Nowak et al. 2006). The mechanisms underlying the antidepressant-like behavioral effects of GABA_B receptor antagonists have been shown to depend on an interaction with the serotonergic system (Slattery et al. 2005a), as it was abolished by pre-treatment with the tryptophan hydroxylase inhibitor para-chlorophenylalanine.

GABA_B receptors are expressed both presynaptically and postsynaptically, where they modulate neuronal excitability through inhibition of neurotransmitter release via interactions with Ca⁺² channels, and inhibitory post synaptic potentials via interactions with inwardly-rectifying K⁺ (GIRK) channels, respectively (see (Bettler et al. 2004) for a review). Activation of GABA_B receptors with baclofen either systemically or locally has been shown to modulate 5-HT release at the level of the raphe nuclei and in postsynaptic structures (Abellan et al. 2000a; Abellan et al. 2000b; Tao et al. 1996).

Currently, no GABA_{B(1)} isoform-selective ligands exist, and so to date pharmacological analysis of the physiological roles of GABA_{B(1a)} and GABA_{B(1b)} receptor isoforms has been impossible. However, recently the generation of mice deficient in these two isoforms (Vigot et al. 2006) has enabled such investigations. Studies with these mice have facilitated the demonstration of distinct behavioral and physiological roles for the GABA_{B(1a)} and GABA_{B(1b)} isoforms such as locomotor activity (Jacobson et al. 2006a), recognition

memory (Vigot et al. 2006), conditioned taste aversion (Jacobson et al. 2006b) and conditioned fear (Shaban et al. 2006). The influence of the specific isoforms on depression related behaviors, however, has not been investigated to date.

Given the interactions between the 5-HT and GABA_B receptor systems, and the aforementioned specific phenotypes of GABA_{B(1)} isoform-deficient mice, we hypothesized that the GABA_{B(1)} isoforms may have differential influences on the 5-HT system and 5-HT-related behavior of relevance to depression. Dysregulation of the 5-HT system is accompanied by alterations in 5-HT receptor function, and in particular by 5-HT_{1A} autoreceptor desensitization – which has been proposed as an important mechanism by which antidepressants exert their effects (see Blier and Ward 2003 for a review). In the present study we therefore aimed to test our hypothesis using GABA_{B(1a)}^{-/-} and GABA_{B(1b)}^{-/-} mice in a classic test of antidepressant-like function, and in paradigms designed to probe pre- and postsynaptic 5-HT_{1A} receptor function.

6.3 Materials and Methods

Animals

The generation (Vigot et al. 2006) and breeding strategy (Jacobson et al. 2006a; Jacobson et al. 2006b) of wild-type, GABA_{B(1a)}^{-/-} and GABA_{B(1b)}^{-/-} mice used in the present studies have been described previously. Briefly, a knock-in point mutation strategy was adopted, whereby GABA_{B(1a)} and GABA_{B(1b)} initiation codons were converted to stop codons by targeted insertion of a floxed neo-cassette. All mutant and wild-type mice were maintained on a pure inbred BALB/c genetic background. Mutant mice were derived from homozygous breeding of siblings originating from the founding heterozygotic mice (F3-F6). Homozygous wild-type controls for the GABA_{B(1)} isoform mutant mice were derived from mating together wild-type siblings generated from GABA_{B(1a)}^{+/-} and GABA_{B(1b)}^{+/-} heterozygous breedings (F3-F6).

Mice were singly-housed in macrolon cages with sawdust bedding, tissue paper nesting materials and one Mouse House® (Nalgene) per cage. Housing was at a constant room temperature of 22-24°C in a 12 h light:dark cycle with lights on at 6-6.30 am. Food pellets and tap water were available *ad libitum* (except during experimentation). Testing began a minimum of about 3 weeks after single-housing. Experiments investigating body temperature responses to (±)8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) were conducted with the mice while in their home cages. In these and the forced swim test (FST) and hypothalamic-pituitary-adrenal (HPA) axis experiments, mice were moved to an

experimental room a minimum of 1 hour beforehand. In experiments necessitating euthanasia, mice were moved, individually in their home cages, to another room where they were immediately decapitated. Male mice were used in all experiments. In certain experiments as indicated, experimental replication was also carried out in a cohort of female mice. All animal experiments were conducted in accordance with the Swiss guidelines and regulations, and approved by the Veterinary Authority of Basel Stadt, Switzerland.

Drugs

The 5-HT_{1A} receptor agonist (±)8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT; Cat No. H-8520, Sigma-Aldrich) was prepared freshly each day in saline. A dose of 0.5 mg/kg was used in all experiments that involved 8-OH-DPAT. This dose was selected on the basis of previous studies showing that 0.5 mg/kg of 8-OH-DPAT was optimal for the production of hypothermia in mice (Gardier et al. 2001; Young et al. 1994), including mice of the BALB/c strain (Man et al. 2002; McAllister-Williams et al. 1999). All 8-OH-DPAT and vehicle injections were delivered subcutaneously at the back of the neck in a volume of 10 ml/kg.

Forced Swim Test (FST)

Wild-type, GABA_{B(1a)}^{-/-} and GABA_{B(1b)}^{-/-} were investigated for duration of immobility in a FST in two separate experiments conducted with male and female mice. Ten mice of each genotype for each gender were used. The mean (± SEM) age at testing of the mice was 10.6 (± 0.3) weeks of age for male mice, and 14.2 (± 0.3) for the female mice. The FST was conducted as previously described (Cryan et al. 2003; Mombereau et al. 2004a). Briefly, cylinders (21 cm diameter x 45 cm high) were filled to a depth of 15 cm with water adjusted to a temperature of 23-25°C. Mice were then placed into the cylinders where they remained for a duration of 6 minutes. The test sessions were recorded on video tapes from a camera positioned directly above the cylinders. The amount of time mice spent immobile in the last 4 minutes of the test, that is when making only those movements necessary to keep their heads above water, were scored manually from video tapes by a trained observer blind to the animals genotypes.

5-HT_{1A} Receptor Activation, Hypothermia and Behavior

Male Mice. Wild-type, GABA_{B(1a)}^{-/-} and GABA_{B(1b)}^{-/-} mice (n = 5 per genotype, mean (± SEM) age = 31.2 (± 0.8) weeks) were investigated for their body temperature responses to the 5-HT_{1A} receptor agonist, 8-OH-DPAT. All mice received 8-OH-DPAT (0.5 mg/kg, s.c.). A

baseline body temperature measurement was taken immediately prior to 8-OH-DPAT administration to serve as a within-animal pre-drug control. Body temperature was subsequently measured 30, 60, 90 and 120 minutes after 8-OH-DPAT administration. Body temperatures were measured to the nearest 0.1 °C using an ELLAB instruments thermometer (Copenhagen, Denmark Model DM 852) and lubricated thermistor probe (ELLAB model PRA-22002-A, 2.2 mm diameter) inserted 20 mm into the rectum while the mouse was gently hand-held at the base of the tail. The thermistor probe was left in place for 15 s until a stable recording was obtained.

Female Mice. Only $GABA_{B(1a)}^{-/-}$ (n = 17) and $GABA_{B(1b)}^{-/-}$ (n = 16) female mice (mean (\pm SEM) age = 28.4 (\pm 0.8) weeks) were available at the time of experimentation. Consequently, the objective of this experiment was to verify whether or not the different responses to 8-OH-DPAT of the two mutant strains, as seen with the male mice, was also present in a cohort of female mice. Due to the absence of wild-type controls, the experimental design was altered to include both vehicle (saline) and 8-OH-DPAT treatments (0.5 mg/kg, s.c) for each genotype. Pre-treatment baseline temperatures were also obtained, enabling each mouse to serve as its own control. Body temperature was recorded as described above, immediately before injection with either vehicle or 8-OH-DPAT, and then again 30, 60, 90 and 120 minutes after injection.

Influence of 5-HT_{1A} Receptor Activation on Behavior

We took advantage of the 8-OH-DPAT hypothermia experiments to evaluate the behavioral responses of the mice to the 8-OH-DPAT treatment. As such, the presence or absence of a flat body posture was recorded for male and female mice in the aforementioned experiments, 30-50 minutes after treatment.

Influence of 5-HT_{1A} Receptor Activation on Indicators of Hypothalamic-Pituitary-Adrenal (HPA) Axis Activity

Male mice only were used in this experiment (n: WT = 25, $GABA_{B(1a)}^{-/-}$ = 21, $GABA_{B(1b)}^{-/-}$ = 22). The mean (\pm SEM) age of the mice was 20.9 (\pm 0.5) weeks. Mice were allocated to 8-OH-DPAT (0.5 mg/kg) or vehicle (saline) treatments. Mice were injected 30 minutes before sacrifice by decapitation and trunk blood was collected into 1.5 ml tubes pre-prepared with EDTA (Milian S.A., Meyrin, Switzerland). The tubes were held on ice until centrifugation within 25 minutes of collection (10 000 rpm, 15 minutes, 4 ° C). The plasma supernatant was collected and stored at -80 ° C until analysis for corticosterone and adrenocorticotropin

hormone (ACTH) concentration using commercially available radioimmunoassay kits (ICN Biomedicals, Costa Mesa, CA, USA).

5-HT_{1A} Receptor Autoradiography

Brains of experimentally-naïve male wild-type, GABA_{B(1a)}^{-/-} and GABA_{B(1b)}^{-/-} (n = 6 per genotype, mean age 15.9 ± 0.7 weeks) were harvested immediately after decapitation and stored at -80°C. Brains were subsequently cut in 10 µm sections with a microtome-cryostat and thaw-mounted on poly-l-lysine coated slides. Sequential brain sections at regions of interest (see Fig. 6a and b) were analysed for 5-HT_{1A} receptor binding by autoradiographic analysis as described below, based on previously published methods with the 5-HT_{1A} receptor antagonist [³H]MPPF ((2'-methoxy)-phenyl-1-[2'-(N-2"-pyridinyl)-p-fluorobenzamido]ethyl-piperazine) (Hensler 2002; Kung et al. 1996), and with the 5-HT_{1A} receptor agonist [³H]8-OH-DPAT (Hoyer et al. 1992; Palacios et al. 1993).

[³H]MPPF autoradiography. Sections were pre-incubated for 30 min at room temperature in a 170 mM Tris-HCl buffer (pH 7.6), then incubated for a further 90 min in the same buffer supplemented with 10 nM [³H]MPPF (71.6 Ci/mmol; Perkin Elmer). Non-specific binding was determined in a set of adjacent slides by incubation in the presence of 10 µM 5-HT. Sections were then washed twice for 5 min in ice-cold incubation buffer followed by a brief dip in ice-cold water. Finally, sections were dried under a stream of cold air. Autoradiograms were generated by exposing the labeled sections to BioMax MR Films (Eastman Kodak Company, Rochester, NY) for a period of 8 weeks.

[³H]8-OH-DPAT autoradiography. Sections were pre-incubated for 30 min at room temperature in a buffer solutions containing 170 mM Tris-HCl (pH 7.6), 4 mM CaCl₂, 0.01% ascorbic acid, 1 µM pargyline and 1 µM fluoxetine. Sections were then incubated for 60 min in the same buffer supplemented with 2 nM [³H]8-OH-DPAT (230 Ci/mmol; Amersham). Non-specific binding was determined in a set of adjacent slides by incubation in the presence of 10 µM 5-HT. Sections were then washed twice for 5 min in ice-cold incubation buffer followed by a brief dip in ice-cold water. Sections were finally dried under a stream of cold air. Autoradiograms were generated by exposing the labeled tissues to BioMax MR Films (Eastman Kodak Company, Rochester, NY) for a period of 3 weeks.

Statistics

As data from male and female mice were derived from separate experiments, they were analyzed separately within each gender. Immobility in the forced swim test, [³H]MPPF and

[³H]8-OH-DPAT binding within the brain regions investigated, were analyzed using 1-way analysis of variance (ANOVA). Body temperatures of male and female mice in the 8-OH-DPAT experiments were analyzed, respectively, using a 2-way repeated measures ANOVA. Fishers LSD *post hoc* comparisons were made where indicated by significant ANOVA terms.

Corticosterone and ACTH data did not meet assumptions for parametric testing. These data were therefore analyzed within genotype for the effect of treatment using pair-wise Mann-Whitney Rank Sum Tests, and within treatment for the effect of genotype using Kruskal-Wallis 1-way ANOVA on Ranks. Dunn's *post hoc* comparisons were made where indicated by significant terms in Kruskal-Wallis 1-way ANOVAs.

6.4 Results

Forced Swim Test

Male mice. Genotype significantly affected immobility time of male mice in the forced swim test ($F_{2,27} = 9.227$, $P = 0.001$, Fig. 1a). *Post hoc* comparisons revealed that the $GABA_{B(1a)}^{-/-}$ mice had significantly reduced immobility scores relative to wild-type mice, to more than half that of the wild-type controls (58% decrease). The $GABA_{B(1a)}^{-/-}$ mice also had a significantly lower mean immobility score than the $GABA_{B(1b)}^{-/-}$ mice ($p < 0.05$). Mean immobility score of the $GABA_{B(1b)}^{-/-}$ mice, however, did not differ from that of wild-type controls ($P > 0.05$).

Female mice. FST immobility in female mice was similarly affected by genotype (Genotype: $F_{2,27} = 5.798$, $P < 0.01$), and likewise, *post hoc* tests showed the $GABA_{B(1a)}^{-/-}$ mice had a lower mean immobility than the wild-type controls (44% decrease, $P < 0.05$) and $GABA_{B(1b)}^{-/-}$ mice ($P < 0.01$; Fig 1b). Female $GABA_{B(1b)}^{-/-}$ mice did not differ from the wild-type controls in this regard.

8-OH-DPAT, Hypothermia and Behavior

Male Mice. Genotype significantly influenced body temperature responses to 8-OH-DPAT (Genotype: $F_{2,74} = 4.712$, $P < 0.05$. Time: $F_{2,74} = 31.396$, $P < 0.001$. Interaction: $F_{2,74} = 6.589$, $P < 0.001$. Fig. 2). *Post hoc* comparison within each of the wild-type and $GABA_{B(1b)}^{-/-}$ genotypes showed profound, long-lasting hypothermia in response to 8-OH-DPAT, relative to pre-drug temperatures (Wild-type: $P < 0.001$ for Time 0 versus 30 and 60 min. $GABA_{B(1b)}^{-/-}$: $P < 0.001$ for Time 0 versus 30 and 60 min, $P < 0.01$ for Time 0 versus 90 min). Body temperature had returned to a level not different from that measured before 8-OH-DPAT administration by 90 minutes in the wild-type mice, and by 120 min in the $GABA_{B(1b)}^{-/-}$ mice

($P > 0.05$). In contrast, $GABA_{B(1a)}^{-/-}$ mice failed to demonstrate any significant hypothermia in response to 8-OH-DPAT relative to their own pre-drug body temperatures ($P > 0.05$ for Time 0 versus all other time-points). Likewise, body temperature of $GABA_{B(1a)}^{-/-}$ mice was significantly higher than that of the wild-type or $GABA_{B(1b)}^{-/-}$ mice 30 and 60 minutes after 8-OH-DPAT administration ($P < 0.001$, Fig. 2).

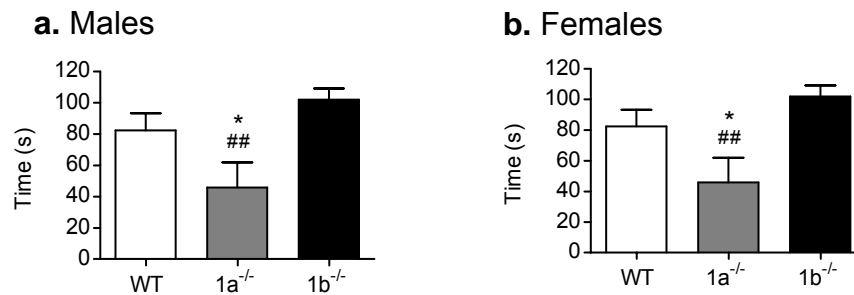


Fig. 1. Duration of immobility in the Forced Swim Test of (a.) male and (b.) female wild-type (WT), $GABA_{B(1a)}^{-/-}$ (1a^{-/-}) and $GABA_{B(1b)}^{-/-}$ (1b^{-/-}) mice (n = 10 per genotype). * $P < 0.05$ versus WT; ## $P < 0.01$ versus 1b^{-/-}.

Female mice. As for the male $GABA_{B(1a)}^{-/-}$ mice, female $GABA_{B(1a)}^{-/-}$ mice did not demonstrate hypothermia in response to 8-OH-DPAT (within $GABA_{B(1a)}^{-/-}$: Treatment $F_{1,64} = 2.978$, $P = 0.112$; Time $F_{4,64} = 30.101$, $P < 0.001$; Interaction $F_{4,64} = 4.284$, $P < 0.01$; Fig. 3). *Post hoc* analyses revealed that vehicle-treated $GABA_{B(1a)}^{-/-}$ mice showed an increase in body temperature 30 minutes after injection relative to that taken at the start of the experiment ($P < 0.001$). 8-OH-DPAT-treated $GABA_{B(1a)}^{-/-}$ mice, in comparison, demonstrated neither an increase nor decrease in body temperature at 30 minutes post injection ($P > 0.05$), and as such, their mean temperature was lower than that of the vehicle-treated $GABA_{B(1a)}^{-/-}$ mice at this time ($P < 0.01$, Fig. 3). However, 60 minutes after injection, the mean body temperature of 8-OH-DPAT-treated $GABA_{B(1a)}^{-/-}$ mice had also risen in comparison to that recorded pre-drug ($P < 0.001$), and was no longer different to that of vehicle treated mice ($P > 0.05$).

$GABA_{B(1b)}^{-/-}$ female mice demonstrated a similar pattern of response to the male $GABA_{B(1b)}^{-/-}$ and wild-type mice with regard to hypothermic responses to 8-OH-DPAT (within $GABA_{B(1b)}^{-/-}$: Treatment $F_{1,69} = 16.810$, $P = 0.001$; Time $F_{4,69} = 20.343$, $P < 0.001$; Interaction $F_{4,69} = 17.837$, $P < 0.01$; Fig. 3). That being a profound, long-lasting hypothermia with 8-OH-DPAT relative to both their own mean pre-drug body temperature (at 30 and 60 minutes, $P < 0.001$, respectively) and to vehicle-treated mice of the same genotype at the same time points ($P < 0.001$ at 30 and 60 min post-injection, respectively, Fig. 3).

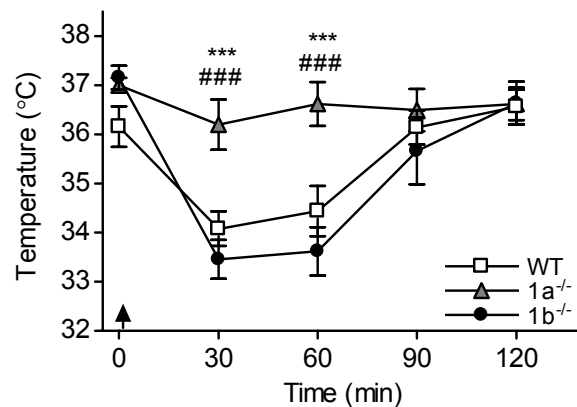


Fig. 2. Body temperature of male $GABA_{B(1a)}^{-/-}$ ($1a^{-/-}$), $GABA_{B(1b)}^{-/-}$ ($1b^{-/-}$) and wild-type (WT) mice ($n = 5$ per genotype) before (Time = 0 min) and after a single administration (arrow head) of (\pm) 8-OH-DPAT (0.5 mg/kg s.c.). *** $P < 0.001$ versus WT; #### $P < 0.001$ versus $1b^{-/-}$. All mice received 8-OH-DPAT. Note also that mean body temperatures at times 30 and 60 min differed significantly from that taken pre-drug within both WT and $1b^{-/-}$ mice ($P < 0.001$) and for $1b^{-/-}$ at time 90 min ($P < 0.01$).

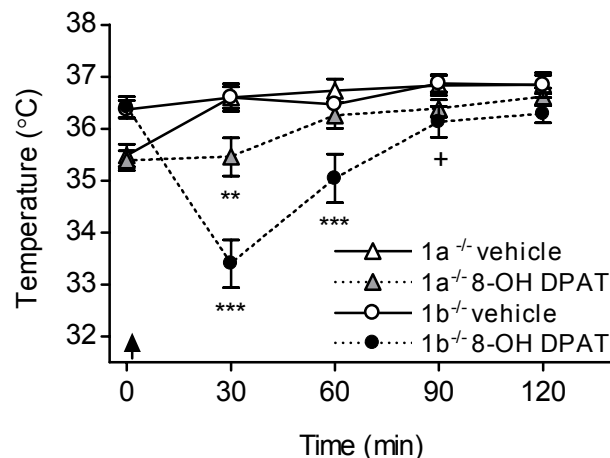


Fig. 3. Body temperature of female $GABA_{B(1a)}^{-/-}$ ($1a^{-/-}$, $n = 17$) and $GABA_{B(1b)}^{-/-}$ ($1b^{-/-}$, $n = 16$) mice before (time 0) and after administration (arrow head) of either (\pm) 8-OH-DPAT (0.5 mg/kg, s.c) or saline vehicle. *** $P < 0.001$, ** $P < 0.01$, + $P < 0.10$ versus vehicle-treated mice within genotype and time-point. Note also that mean temperatures at time 30 min increased significantly from that taken pre-drug for vehicle-treated $GABA_{B(1a)}^{-/-}$ mice, and at 60, 90 and 120 minutes post drug for vehicle and 8-OH-DPAT-treated $GABA_{B(1a)}^{-/-}$ mice ($P < 0.001$ vs Time 0 min for each time-point within treatment). 8-OH-DPAT-treated $GABA_{B(1b)}^{-/-}$ mice had significantly lower body temperature relative to that taken pre-drug at time 30 and 60 min ($P < 0.001$, respectively).

Influence of 5-HT_{1A} Receptor Activation on Behavior

The behavioral activities of mice of the three genotypes differed after 8-OH-DPAT administration. In the male mice, only one of the GABA_{B(1a)}^{-/-} mice showed a flat body posture after 8-OH-DPAT. In contrast, 3 out of 5 mice of each of the wild-type and GABA_{B(1b)}^{-/-} genotypes had flat body postures after 8-OH-DPAT.

The number of female GABA_{B(1a)}^{-/-} mice showing a flat body posture after vehicle (saline) or 8-OH-DPAT treatment was zero in both groups. In comparison, 5 of the 8 GABA_{B(1b)}^{-/-} female mice treated with 8-OH-DPAT had flat body postures, and one of these mice also showed twisting motions of the tail while in a flat posture. None of the vehicle-treated GABA_{B(1b)}^{-/-} female mice had a flat body posture.

Influence of 5-HT_{1A} Receptor Activation on Indicators of Hypothalamic-Pituitary-Adrenal (HPA) Axis Activity

Treatment with 8-OH-DPAT significantly increased plasma corticosterone in each of the three genotypes (Wild-type T = 66, P < 0.001; GABA_{B(1a)}^{-/-} T = 85, P < 0.01; GABA_{B(1b)}^{-/-} T = 187, P < 0.001; Fig. 4a). However, GABA_{B(1a)}^{-/-} mice had a significantly attenuated corticosterone response to 8-OH-DPAT in comparison with wild type mice (H = 6.638, P < 0.05, Fig. 4a). There was no significant differences in plasma corticosterone between the genotypes of vehicle-treated mice (H = 0.792, P = 0.673; Fig. 4a).

Two ACTH data points were eliminated from analysis as statistical outliers, one from the wild-type vehicle group (3680 pg/ml) and the other from the wild-type 8-OH-DPAT-treated group (1287pg/ml).

8-OH-DPAT increased plasma ACTH in the wild-type mice (T = 59.00, P < 0.001, Fig. 4b). There was a tendency for 8-OH-DPAT to increase ACTH in GABA_{B(1b)}^{-/-} mice as well (T = 155, P = 0.066). However, no significant effect of 8-OH-DPAT treatment on plasma ACTH concentration was detected for GABA_{B(1a)}^{-/-} mice (T = 122, P = 0.559).

Plasma ACTH levels in vehicle treated mice were not significantly affected by genotype (H = 1.808, P = 0.405). However, within the mice treated with 8-OH-DPAT, GABA_{B(1a)}^{-/-} mice had a lower ACTH levels than either wild type or GABA_{B(1b)}^{-/-} mice (P < 0.05, respectively). The GABA_{B(1b)}^{-/-} mice did not differ significantly from the wild-type controls in this regard (H = 7.334, P > 0.05; Fig 4b).

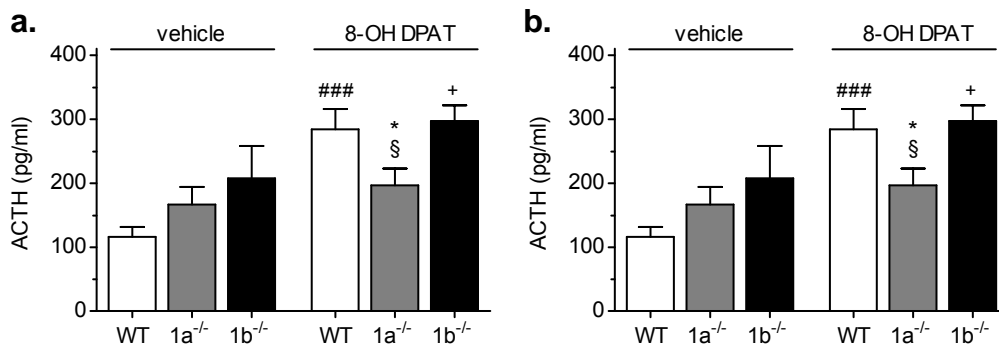


Fig. 4. Mean (+ SEM) plasma corticosterone (a.) and ACTH (b.) responses to vehicle (saline, s.c.) or (\pm) 8-OH-DPAT (0.5 mg/kg, s.c.) in male wild-type (WT, $n = 25$), $GABA_{B(1a)}^{-/-}$ (1a^{-/-}, $n = 21$) and $GABA_{B(1b)}^{-/-}$ (1b^{-/-}, $n = 22$) mice. ### $P < 0.001$, * $P < 0.10$ versus vehicle within genotype; + $P < 0.05$ versus WT within 8-OH-DPAT; § $P < 0.05$ versus 1b^{-/-} within 8-OH-DPAT-treated mice.

5-HT_{1A} Receptor Autoradiography

Few differences were apparent between wild-type, $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice in 5-HT_{1A} receptor autoradiographic binding over most of the brain regions analyzed (see Fig. 5 for representative images). 5-HT_{1A} receptor antagonist ($[^3H]$ MPPF) and agonist ($[^3H]$ 8-OH-DPAT) binding densities, respectively, were not significantly affected by genotype in the caudate putamen, nucleus accumbens, cerebral cortex, dorsal endopiriform nucleus, dorsal or ventral hippocampus (or the CA1 region of the dorsal hippocampus), amygdala nuclei, medial or posterior hypothalamus, amygdalohippocampal area, dorsal and median raphé nuclei or the entorhinal cortex ($P > 0.05$ for ANOVA factor ‘Genotype’, Fig. 6 a and b).

Small but significant differences were apparent with $[^3H]$ MPPF binding in the lateral septum (Genotype $F_{2,17} = 3.73$, $P < 0.05$) and medial septum (limb of the diagonal band) (Genotype $F_{2,17} = 3.834$, $P < 0.05$). *Post hoc* comparisons revealed a slightly higher mean binding density in the lateral septum of $GABA_{B(1a)}^{-/-}$ mice in comparison with $GABA_{B(1b)}^{-/-}$ mice ($P < 0.05$), but not versus wild-type mice ($P > 0.05$, Fig. 6a). In the medial septum, mean $[^3H]$ MPPF binding density was higher in the $GABA_{B(1a)}^{-/-}$ mice than either the wild-type or $GABA_{B(1b)}^{-/-}$ mice, which did not differ from each other in this regard (Fig. 6a). However, $[^3H]$ 8-OH-DPAT binding densities in either the medial or lateral septum were not significantly influenced by genotype ($P > 0.05$).

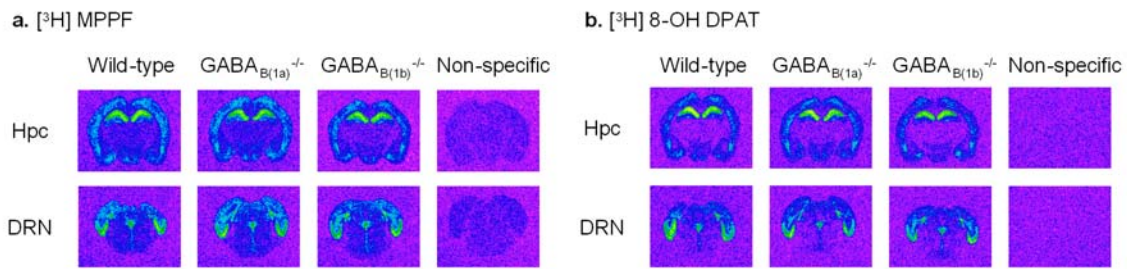


Fig. 5. Representative autoradiograms of 5-HT_{1A} receptor antagonist ($[^3\text{H}]$ MPPF; **a.**) and agonist ($[^3\text{H}]$ 8-OH-DPAT; **b.**) binding in wild-type, $\text{GABA}_{\text{B}(1\text{a})}^{-/-}$ and $\text{GABA}_{\text{B}(1\text{b})}^{-/-}$ mice ($n = 18$) in the hippocampus (Hpc) and dorsal raphe nucleus (DRN).

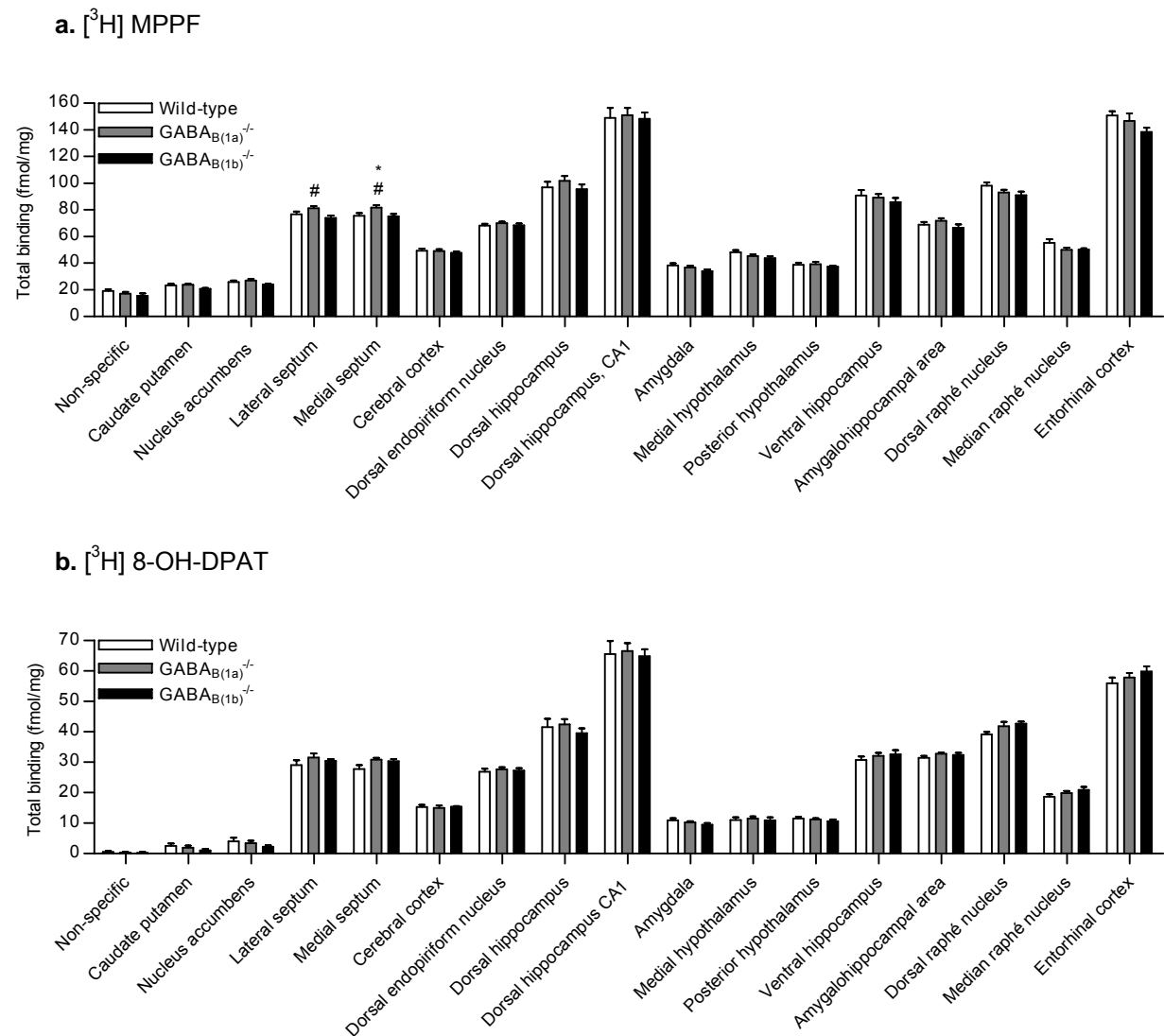


Fig. 6. Density of 5-HT_{1A} receptor binding sites in different brain regions in wild-type, $\text{GABA}_{\text{B}(1\text{a})}^{-/-}$ and $\text{GABA}_{\text{B}(1\text{b})}^{-/-}$ male mice ($n = 18$) as determined using (a.) and $[^3\text{H}]$ 8-OH-DPAT (b.) autoradiography. * $P < 0.05$ versus wild-type, # $P < 0.05$ versus $\text{GABA}_{\text{B}(1\text{b})}^{-/-}$.

6.5 Discussion

The present study demonstrated that constitutive genetic ablation of the GABA_{B(1a)} isoform, but not the GABA_{B(1b)} isoform, produced an antidepressant-like phenotype in the FST. This finding was replicated in separate cohorts of both male and female mice, attesting to the robustness of the phenotype. The present study also demonstrated that GABA_{B(1a)}^{-/-} mice, but not GABA_{B(1b)}^{-/-} mice, showed a profound desensitization to the hypothermic and behavioral effects of 5-HT_{1A} receptor activation, as assessed using the agonist 8-OH-DPAT. This finding was replicated in two different experimental protocols with male and female mice. Similarly, in GABA_{B(1a)}^{-/-} mice, but again not in GABA_{B(1b)}^{-/-} mice, HPA activation in response to 5-HT_{1A} receptor stimulation were attenuated. Furthermore, the influence of GABA_{B(1a)} isoform ablation on 5-HT_{1A} receptor desensitization appeared to be largely independent from the level of 5-HT_{1A} receptor expression. These findings indicate that the GABA_{B(1)} isoforms have a differential involvement in antidepressant-like effects in the FST, and in functional interactions with the 5-HT_{1A} receptor.

The FST in the mouse is one of the most widely used paradigms for assessing antidepressant-like behaviour (Cryan and Holmes 2005; Cryan and Mombereau 2004; Petit-Demouliere et al. 2005). We have previously shown that mice lacking functional GABA_B receptors have an antidepressant-like effect in this test (Mombereau et al. 2004a; Mombereau et al. 2005) which confirms the antidepressant-like effects of pharmacological antagonism of GABA_B receptors in the FST (Mombereau et al. 2004a; Nowak et al. 2006; Slattery et al. 2005a). The results of the present study demonstrate that the GABA_{B(1a)} isoform is important for mediating the GABA_B receptor-mediated antidepressant-like effects in the FST. It is important to note that we have previously shown that GABA_{B(1a)}^{-/-} mice did not show differences in locomotor activity from that of wild-type controls (while, in contrast, GABA_{B(1b)}^{-/-} mice were hyperactive) (Jacobson et al. 2006a). This indicates that the reduced of immobility in the FST of GABA_{B(1a)}^{-/-} mice in the present study was not a non-specific effect due to enhanced locomotor activity.

Previously we have shown that the antidepressant-like behavioral effects of GABA_B-receptor antagonists depends on an interaction with the serotonergic system (Slattery et al. 2005a). Of all the 5-HT receptors, the 5-HT_{1A} receptor is perhaps the most important for influencing the antidepressant response both clinically and in animal models (Cryan and Leonard 2000). In particular, 5-HT_{1A} autoreceptor desensitization is thought to contribute to antidepressant actions in humans and in animal models (see (Blier and Ward 2003; De Vry

1995; Hensler 2003) for reviews). This is further supported in the clinic by the enhancement of antidepressant onset of action and efficacy by combining SSRIs with a 5-HT_{1A} antagonist, the latter being proposed to act as a mimic of 5-HT_{1A} desensitization (Artigas et al. 1996; Blier et al. 1997). Therefore it seemed likely that altered 5-HT_{1A} function may contribute to the antidepressant like phenotype of GABA_{B(1a)}^{-/-} mice. Indeed, our data suggest that the phenotype of GABA_{B(1a)}^{-/-} mice in the FST was a result of 5-HT_{1A} autoreceptor desensitization, leading to dysregulated 5-HT release

5-HT_{1A} receptors are expressed both as presynaptic autoreceptors on serotonergic cell bodies in the raphé nuclei, and at postsynaptic and extrasynaptic sites in limbic structures of the forebrain (see (Hensler 2006) and (Hoyer et al. 2002) for reviews). It is well known that activation of 5-HT_{1A} receptors induces hypothermia in a range of different species including mice, rats and humans (for examples, see (Blier et al. 2002; Cryan et al. 1999; Larsson et al. 1990; Millan et al. 1993; Rausch et al. 2006)). In mice, this phenomenon is largely thought to be mediated by the presynaptic autoreceptors on serotonergic cell bodies in the dorsal raphé nucleus (DRN) (Bill et al. 1991; Goodwin et al. 1985; Martin et al. 1992). In the present study, 8-OH-DPAT completely failed to induce hypothermia in GABA_{B(1a)}^{-/-} mice, thus suggesting that these mice have altered presynaptic 5-HT_{1A} autoreceptor function.

GABA_{B(1a)} and GABA_{B(1b)} receptors have recently been shown to localize to differential synaptic sites in the hippocampus, amygdala and layer 5 cortical neurons. GABA_{B(1a)} was predominantly the heteroreceptor, while GABA_{B(1b)} was mostly postsynaptic (Perez-Garci et al. 2006; Shaban et al. 2006; Vigot et al. 2006). In addition, in the hippocampus and amygdala, both isoforms were expressed as autoreceptors (Shaban et al. 2006; Vigot et al. 2006), while in layer 5 of the cortex, the GABA_{B(1a)} isoform was expressed as an autoreceptor, but not GABA_{B(1b)} (Perez-Garci et al. 2006). In situ hybridization studies suggest that GABA_{B(1a)} is the prevalent GABA_{B(1)} isoform in the DRN (Bischoff et al. 1999). Most 5-HT cell bodies in the dorsal and medial raphé nuclei are co-positive for GABA_{B(1)} (Abellan et al. 2000a; Varga et al. 2002). This suggests that it is the GABA_{B(1a)} isoform which is predominantly expressed on serotonergic cell bodies in the DRN.

GABA_B and 5-HT_{1A} receptors are both coupled via G-proteins to GIRK2 channels in the DRN (Innis et al. 1988), and hypothermic responses to either baclofen or 8-OH-DPAT are similarly attenuated in GIRK2^{-/-} mice (Costa et al. 2005). Furthermore, in serotonin transporter-deficient (5-HTT^{-/-}) mice, the capacity of baclofen or 5-HT_{1A} receptor agonists to inhibit 5-HT cell firing was reduced, as was agonist-stimulated [³⁵S] GTPγS binding by both of these receptors (Fabre et al. 2000; Mannoury la Cour et al. 2001; Mannoury la Cour et al.

2004). These studies provide evidence that these receptors share G-proteins in the DRN, leading Mannoury la Cour et al., (2004) to speculate that in 5HTT^{-/-} mice, desensitization of 5-HT_{1A} and GABA_B receptor agonist-induced responses was due to a down-regulation of the G-protein pool shared by both of these receptors. In the present study there was a lack of, or negligible, influence of deletion of the GABA_{B(1a)} (or GABA_{B(1b)} for that matter) isoforms on 5-HT_{1A} receptor binding density as determined with either a 5-HT_{1A} receptor agonist or antagonist, despite the demonstrated complete loss of presynaptic 5-HT_{1A} receptor physiological function in the GABA_{B(1a)}^{-/-} mice. It will therefore be of interest in future studies to investigate downstream 5-HT_{1A} receptor signalling mechanisms in GABA_{B(1a)}^{-/-} mice. Interestingly, other studies using treatments that enhance serotonergic transmission have shown reductions in 5-HT_{1A} receptor-dependent G-protein coupling which were independent of changes in 5-HT_{1A} receptor ligand autoradiographic binding (Castro et al. 2003; Hensler 2002; Sim-Selley et al. 2000).

In contrast with presynaptic mediation of 5-HT_{1A} receptor-induced hyperthermia, stimulation of the postsynaptic 5-HT_{1A} receptors is thought to mediate agonist-induced stimulation of the HPA axis (Bagdy 1996; Bluet Pajot et al. 1995; Feldman et al. 2000) and behaviors characterizing serotonin-syndrome, such as a flat body posture and abnormal tail motions (Berendsen et al. 1991; Green and Backus 1990; Hamon 2000). The attenuation of 8-OH-DPAT-induced behavioral responses typical of the serotonin syndrome and attenuated HPA activation in GABA_{B(1a)}^{-/-} mice suggests that postsynaptic 5-HT_{1A} receptors, albeit to a much lesser extent than presynaptic receptors, may also be desensitized.

Overall the present study has indicated that GABA_{B(1)} isoforms show differential roles in the mediation of FST behavior and of 5-HT_{1A} receptor function at pre- and postsynaptic sites. Further work is needed to investigate the ultrastructural expression of the GABA_{B(1)} isoforms in the serotonergic system, particularly at the level of the DRN. Our data identify an interaction of GABA_B receptors containing the 1a isoform with the serotonergic system and suggest that the GABA_{B(1a)} isoform may be a potential therapeutic target for the development of novel antidepressants.

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Chapter 7

Specific Roles of GABA_{B(1)} Receptor Isoforms in Cognition

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7.1 Abstract

The GABA_B receptor is a heterodimer of GABA_{B(1)} and GABA_{B(2)} subunits. There are two isoforms of the GABA_{B(1)} subunit: GABA_{B(1a)} and GABA_{B(1b)}. Recent studies with mutant mice suggest a differential role for the two GABA_{B(1)} isoforms in behavioural processes. As pharmacological and genetic studies have implicated GABA_B receptors in cognition we investigated the behaviour of GABA_{B(1a)}^{-/-} and GABA_{B(1b)}^{-/-} mice in different types of cognitive paradigms. GABA_{B(1a)}^{-/-} and GABA_{B(1b)}^{-/-} mice were both impaired relative to wildtype controls in a continuous spontaneous alternation behavior test of working spatial memory. In contrast to the reported phenotype of GABA_{B(1)}^{-/-} mice, however, neither GABA_{B(1a)}^{-/-} nor GABA_{B(1b)}^{-/-} mice were deficient in a passive avoidance task. On the other hand, GABA_{B(1a)}^{-/-} mice were impaired in familiar and novel object recognition. We conclude that GABA_{B(1)} isoforms contribute differentially to GABA_B receptor-mediated cognitive processes.

7.2 Introduction

GABA_B receptors are metabotropic GABA receptors formed by the heterodimerization of GABA_{B(1)} and GABA_{B(2)} subunits. They modulate excitability as presynaptic hetero- and autoreceptors that inhibit the release of neurotransmitters. They are also expressed postsynaptically, where they induce inhibitory postsynaptic potentials via activation of inwardly-rectifying potassium channels (reviewed in (Bettler et al. 2004)).

Many studies with GABA_B receptor ligands have implicated the GABA_B receptor in cognitive processes (reviewed in (Bowery et al. 2002)). GABA_B receptor antagonists improved performance in a number of different cognitive tests, for example hippocampal-dependent spatial learning and memory (Helm et al. 2005; Nakagawa and Takashima 1997; Staubli et al. 1999), and passive (Mondadori et al. 1994; Mondadori et al. 1993) and active avoidance (Getova and Bowery 1998). In contrast, GABA_B receptor agonists generally (but not always (Castellano et al. 1993; Escher and Mittleman 2004)) impair learning and memory in these tasks (see (Bowery et al. 2002)). In addition, deletion of the GABA_{B(1)} receptor subunit profoundly impaired passive avoidance performance in a gene dose-dependent manner (Schuler et al. 2001).

The GABA_{B(1)} subunit is predominantly expressed in the CNS as one of two isoforms: GABA_{B(1a)} or GABA_{B(1b)} (Kaupmann et al. 1998a). Currently no isoform-specific ligands exist with which to dissect their influences on cognitive processes. Recently, however, mutant

mice deficient in either the GABA_{B(1a)} or GABA_{B(1b)} isoforms have been generated (Vigot et al. 2006). We have previously reported that mice deficient in the GABA_{B(1a)} isoform were impaired in hippocampal LTP and in an object recognition task (Vigot et al. 2006). GABA_{B(1a)}^{-/-} mice also did not discriminate between paired and unpaired tones in a conditioned fear paradigm (Shaban et al. 2006), and failed to acquire a conditioned taste aversion (CTA) to a novel saccharin solution paired with a LiCl-induced malaise (Jacobson et al. 2006b). GABA_{B(1b)}^{-/-} mice, in contrast, acquired CTA normally, but failed to extinguish their aversion up to 30 days later (Jacobson et al. 2006b). These studies indicate that the GABA_{B(1)} isoforms convey specific and differential components of cognitive processes. However, the impact of the GABA_{B(1)} isoforms on other types of cognitive processes has yet to be elaborated on.

In this study, we utilized GABA_{B(1a)}^{-/-} and GABA_{B(1b)}^{-/-} mice to examine the influence of the isoforms in three different types of cognitive paradigms. We first aimed to assess novel and familiar object recognition, thus replicating our previous findings with a larger cohort of mice (Vigot et al. 2006). We also examined the mice in a test indicative of spatial working memory, namely continuous spontaneous alternation behaviour (SAB). Finally passive avoidance was investigated for comparison with the reported impairment of GABA_{B(1)}^{-/-} mice, lacking both GABA_{B(1)} isoforms (Schuler et al. 2001).

7.3 Methods and Materials

The generation of GABA_{B(1a)}^{-/-}, GABA_{B(1b)}^{-/-} and wildtype mice has been described previously (Jacobson et al. 2006b; Vigot et al. 2006). Briefly, a point mutation knock-in strategy was adopted whereby initiation codons for the respective isoforms were replaced with stop codons. Mice were generated and maintained on a pure BALB/c genetic background. Mice were housed with sawdust bedding, tissue paper nesting materials and one Mouse House® (Nalgene, Nalge Nunc International, Rochester, NY) per cage. Housing was at a temperature of 22-24°C in a 12 h light : dark cycle with lights on at 6.30 am. Food pellets and tap water were available *ad libitum* (except during experimentation). All experiments were conducted during the light cycle, using male mice only. All animal experiments were conducted according to the Swiss recommendations and guidelines and approved by the Basel-Stadt Cantonal Veterinary Authority.

Experimentally naïve, singly-housed male wildtype (n = 19), GABA_{B(1a)}^{-/-} (n = 15) and GABA_{B(1b)}^{-/-} (n = 18) mice (mean age 24 (± 0.4) weeks) were tested in an object recognition

task as previously described (Vigot et al. 2006). The test was based on the original principles of Ennaceur and Delacour, which relies upon the natural tendency of rodents to attend to a novel object more than to a familiar one (Ennaceur and Delacour 1988). Briefly, mice were habituated overnight to a new cage (22 x 37 x 15 (h) cm, containing sawdust bedding and *ad libitum* standard food and water). The following day at time = 0 min a clean PVC disc (2 (h) x 5 (d) cm) was placed for 3 min in the centre of the cage. The disc was then removed and 10 min later replaced by a new, identical clean disc. This was repeated at time = 24 h. At time = 24 h + 10 min, a clean grey PVC cone (6 (h) x 5 (d) cm) was placed in the cage. All objects were presented for a duration of 3 min and were handled only with tissue paper to prevent contamination with odours. All sessions were recorded on video. The frequency of stretch attend postures (SAP, defined as head and shoulders extended towards the object) was scored at least twice for each mouse by a trained observer blinded to the animals' genotypes. The mean of duplicate scores for each animal were used for analysis. Data from one $GABA_{B(1a)}^{-/-}$ mouse was eliminated due to stereotypic circling behaviour during the test which was unrelated to its attention towards the objects. Data were analysed within genotype using a 1-way repeated measures analysis of variance (ANOVA), followed by Fisher LSD post hoc comparisons as appropriate.

In a separate cohort of mice, continuous SAB was assessed in a Y-maze constructed from 3 walled arms (8 cm-long, 6 cm-wide) in a radial arrangement each ending in a 10 cm square. A clean sheet of paper was placed under the maze and the walls wiped clean for each mouse, to reduce odour traces between animals. Wildtype (n = 12), $GABA_{B(1a)}^{-/-}$ (n = 9) and $GABA_{B(1b)}^{-/-}$ (n = 9) singly-housed mice (mean age 21 (\pm 0.6) weeks) were placed individually into the Y-maze facing the end wall of a randomly allocated arm, and allowed to explore for 5 minutes. The order of the arms (1, 2 or 3) visited were recorded. Overlapping triplets of 3 arm visits that included each of the three arms was counted as 1 complete spontaneous alternation. The number of alternations performed was divided by the total number of arms visited minus 2 (i.e. the number of total possible alternations) to obtain % spontaneous alternation (%SA). Data were analyzed as using 1-way ANOVA for the effect of genotype followed by Fishers LSD post hoc comparisons. Additionally, within each genotype, the difference between mean %SA was assessed against the chance score (50%) using a one-sample T-test.

A one-trial step-through passive avoidance paradigm was performed as previously described (Schuler et al. 2001; Venable and Kelly 1990). Briefly, wildtype (n = 23), $GABA_{B(1a)}^{-/-}$ (n = 17) and $GABA_{B(1b)}^{-/-}$ (n = 18) singly-housed mice (aged 23 (\pm 0.6) weeks)

were placed in the light side of a two-compartment trough-shaped apparatus. The barrier between the compartments was opened, the time taken to break a photobeam located 10.5 cm inside the dark compartment was recorded automatically (training latency) and a 0.5 mA foot shock (Campden Instruments 521 C Source Shock, rectangular current wave) immediately delivered through the floor and walls. The shock duration was a maximum of 5 s or until the animal escaped back into the light side. Animals with a training latency >150 s were not shocked and were excluded from further testing (one wildtype and two $GABA_{B(1a)}^{-/-}$ mice). The retention test was performed 24 hours later and was identical to the training trial except no shock was delivered. Maximum latency allowed in the retention test was 300 s. Data were analysed using a 2-way repeated measures ANOVA.

7.4 Results and Discussion

In the object recognition test, SAPs of wildtype mice were affected by time (the three disc presentations) and novelty (cone presentation) ($F_{3,72} = 9.45$, $P < 0.001$; Fig. 1). The number of SAPs to the disc was decreased after a 10-min interval ($P < 0.001$) indicating that the mice remembered the object. Interestingly, the number of SAPs to the disc was also reduced 24 h later ($P < 0.001$). In our previous study, wildtype mice did not demonstrate recognition of the familiar disc after a 24-h interval (Vigot et al. 2006). Using a greater number of animals in the present study ($n = 19$ in this study versus 7 previously), it was clear that a more persistent memory trace of the object occurred in the wildtype mice. When presented with the novel cone at 24 hr 10 min, the number of SAPs increased relative to the disc at maximum recognition (i.e. to the 10 min disc presentation, $P < 0.001$) and to the disc presented at time = 24 h ($P < 0.01$), indicating that the mice recognised the cone as a novel object.

In contrast, no significant alterations were seen in the number of SAPs performed by $GABA_{B(1a)}^{-/-}$ mice, irrespective of the delay between presentation of familiar objects, or of the novelty of the object ($F_{3,55} = 0.70$, $P = 0.56$; Fig. 1). This indicated that the $GABA_{B(1a)}^{-/-}$ mice failed to recognise a familiar object, or to discriminate between the novel and familiar objects. This finding replicates that of our previous study (Vigot et al. 2006).

The number of SAPs made by $GABA_{B(1b)}^{-/-}$ mice towards the objects was influenced by time and novelty ($F_{3,71} = 7.35$, $P < 0.001$; Fig. 1). Like the wild type mice, and in accordance with our previous findings, $GABA_{B(1b)}^{-/-}$ mice recognised the familiar disc after a 10-min delay, as indicated by a reduction in the number of object-oriented SAPs ($P < 0.001$). However, similar to our original findings (Vigot et al. 2006), the present study indicated that the $GABA_{B(1b)}^{-/-}$ mice did not, on average, maintain a persistent memory trace of the disc ($P =$

0.43). In accordance with our previous findings, however, the ability of the $GABA_{B(1b)}^{-/-}$ mice to discriminate between a familiar object (disc at 10 min) and a novel one (cone at 24 h 10 min) was intact ($P = 0.001$).

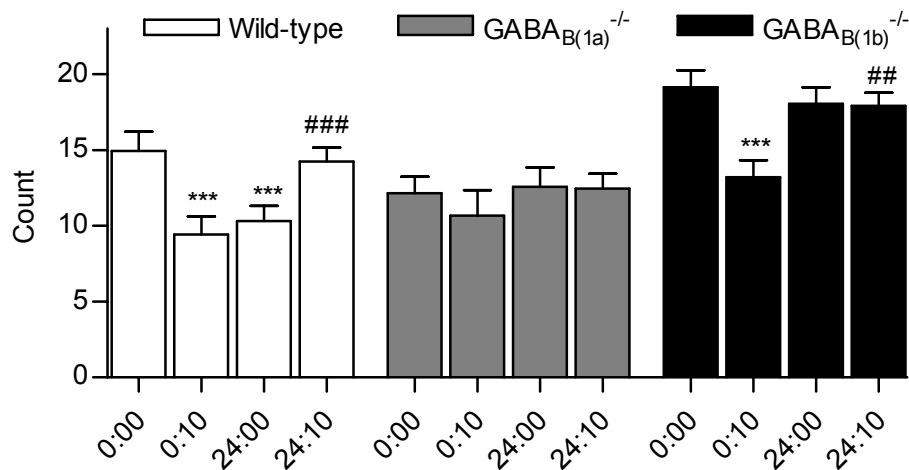


Fig. 1. Number of stretch attend posture performed in an object recognition task by wildtype ($n = 19$), $GABA_{B(1a)}^{-/-}$ ($n = 14$) and $GABA_{B(1b)}^{-/-}$ ($n = 18$) mice towards a disc at initial presentation (Time = 0:00), to the same (clean) disc 10 min and 24 hr later (Time = 0:10 and 24:00, respectively) and to a novel cone-shaped object (Time 24:10). *** $P < 0.001$ vs Time 0:00 within genotype; ### $P < 0.001$, ## $P < 0.01$ versus Time 0:10 within genotype.

In the SAB test, both $GABA_{B(1)}$ isoform mutant strains were impaired relative to wildtype controls in the proportion of completed alternations (% SAB; $F_{2,27} = 5.067$, $P < 0.05$; Fig. 2). When examined against a chance performance score of 50%, wildtype ($P < 0.001$) and $GABA_{B(1b)}^{-/-}$ mice ($P < 0.05$) performed significantly better than chance, whereas the $GABA_{B(1a)}^{-/-}$ mice did not ($P = 0.196$). The number of arms entered, a measure of locomotor activity, was higher in the $GABA_{B(1b)}^{-/-}$ mice than either the wildtype or $GABA_{B(1a)}^{-/-}$ mice ($P < 0.001$ and $P < 0.05$, respectively; Fig. 2). Increased locomotor responses to a novel environment have been previously reported for $GABA_{B(1b)}^{-/-}$ mice (Jacobson et al. 2006a). However, the greater number of arms visited by the $GABA_{B(1b)}^{-/-}$ mice in this study did not improve the proportion of correct alternations in the SAB test. Although there are criticism of continuous SAB as a cognitive task *per se* (Hughes 2004), results of the present study suggest that both the $GABA_{B(1)}$ isoforms may have a role in working spatial memory. Further delineation of the roles of the isoforms in other more comprehensive tests of spatial working and reference memory are therefore warranted.

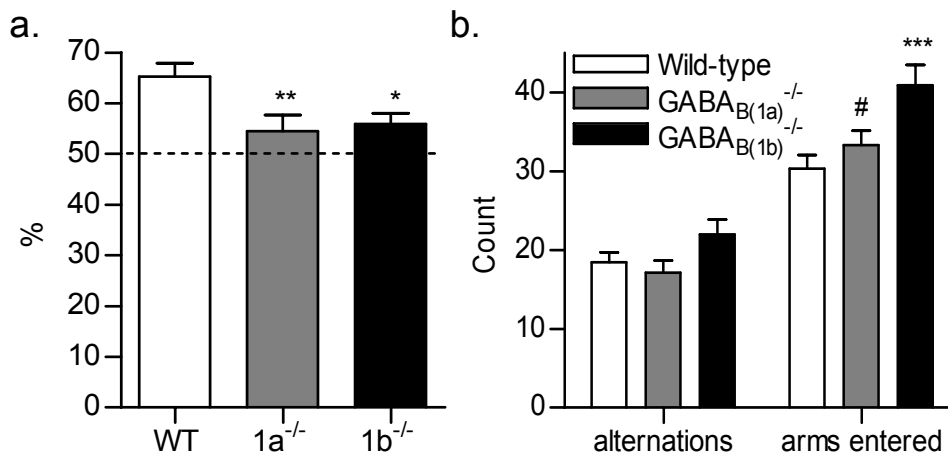


Fig. 2. Percentage of correct alternations (**a.**), number of correct alternations and total number of arms visited (**b.**) by wildtype ($n = 12$), $GABA_{B(1a)}^{-/-}$ ($n = 9$) and $GABA_{B(1b)}^{-/-}$ ($n = 9$) mice in a Y-maze continuous spontaneous alternation task. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ versus wildtype; # $P < 0.05$ versus $GABA_{B(1b)}^{-/-}$.

In passive avoidance training all mice vocalized on receiving the shock, indicating that pain sensory modalities were functional in both isoform-deficient mutant strains. Latency to enter the dark side was greater at retention testing than during training, although there was no effect of genotype on either training or retention latency (Genotype $F_{2,107} = 0.55$, $P = 0.58$; Training $F_{1,107} = 77.11$, $P < 0.001$; Interaction $F_{2,107} = 0.30$, $P = 0.74$; Fig. 3). This indicated that the memory of the shock received in the dark compartment was functional in both mutant strains. This finding contrasts with that of the aforementioned $GABA_{B(1)}^{-/-}$ mice, which lack both functional isoforms and show profound memory deficits in the retention phase of this test (Schuler et al. 2001). It should be noted however, that in $GABA_{B(1)}^{-/-}$ mice, $GABA_B$ receptor agonist-induced responses are completely abolished (Kaupmann et al. 2003; Schuler et al. 2001). In contrast, in the $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice the loss of $GABA_B$ receptor function is partial (Vigot et al. 2006), and some $GABA_B$ receptor agonist-induced functions remain in both of these mutant strains (Jacobson et al. 2006a; Perez-Garci et al. 2006; Shaban et al. 2006; Vigot et al. 2006). Together these findings indicate that heterodimeric $GABA_B$ receptor function is essential for the retention of passive shock-avoidance training, and that this may be accomplished with either one of the $GABA_{B(1)}$ isoforms.

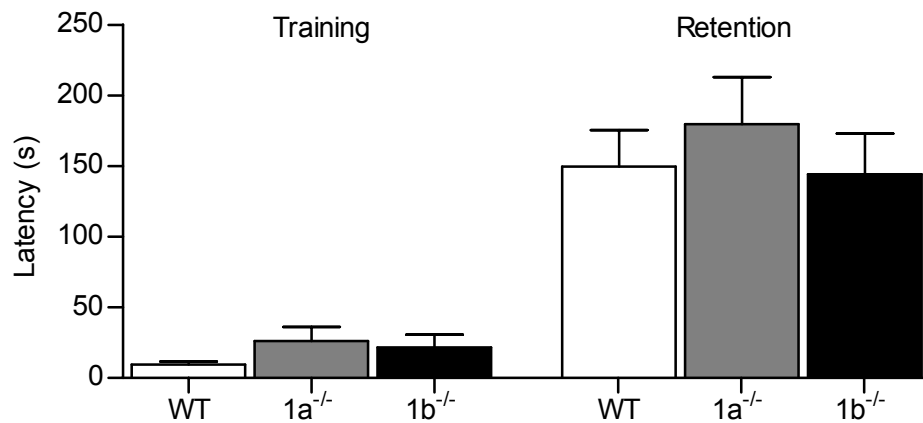


Fig. 3. Latency of wildtype ($n = 23$), $GABA_{B(1a)}^{-/-}$ ($n = 17$) and $GABA_{B(1b)}^{-/-}$ ($n = 18$) mice to enter the dark compartment during training and 24 h later during retention testing in a one-trial step-through passive avoidance paradigm.

The present study indicated that the $GABA_{B(1)}$ isoforms contribute differentially to distinct cognitive capabilities. Currently it is difficult to speculate about the neuronal and molecular mechanisms underlying these differences. The hippocampus is implicated in spontaneous alternation (Lalonde 2002), object recognition (Broadbent et al. 2004; Clark et al. 2000) and passive avoidance (Impey et al. 1998; Stubley-Weatherly et al. 1996). As the $GABA_{B(1a)}^{-/-}$ mice are deficient in hippocampal LTP (Vigot et al. 2006), an impairment in this mutant in all three of these tasks could have been expected, although this was not the case as passive avoidance capabilities were preserved in these mice. However, it is also clear that learning can occur in mice in the absence of LTP (reviewed in (Bannerman et al. 2006)). Other brain regions are of course also important in all these tasks, for example the rhinal cortices are crucial for both passive avoidance (for example, (Phillips and LeDoux 1995)) and object recognition learning (reviewed in (Steckler et al. 1998)). $GABA_{B(1)}$ isoforms, however, are widely expressed throughout the brain, and the isoforms diverge in expression profile both between and within various structures, including within the anatomical divisions of the hippocampus and neocortex (Bischoff et al. 1999). Furthermore, the ultrastructural expression of the $GABA_{B(1)}$ isoforms differs markedly. In the hippocampus and lateral amygdala the $GABA_{B(1a)}$ isoform is a presynaptic heteroreceptor at glutamatergic terminals, while $GABA_{B(1b)}$ is mostly postsynaptically located, and both are autoreceptors (Shaban et al. 2006; Vigot et al. 2006). This pattern is preserved to a certain degree in layer 5 pyramidal cortical neurons, with the exception that the autoreceptor is predominantly the $GABA_{B(1a)}$ isoform (Perez-Garci et al. 2006).

To summarise, the present study demonstrated that the GABA_{B(1a)} isoform is essential for object recognition and discrimination, while the GABA_{B(1b)} isoform may be required more for either the retrieval or long-term storage of this type of memory. Impairments of both isoform-deficient mice in continuous SAB indicate that further examination of spatial working memory are warranted, and may confirm that both isoforms contribute to this cognitive capability. In contrast, although previous research has demonstrated that complete GABA_B receptor heterodimers are required for effective passive avoidance performance, the present study shows that the presence of either of the GABA_{B(1)} isoforms is sufficient for normal performance. Future research may benefit from pursuit of development of isoform-specific ligands ultimately for the development of cognitive therapeutics. We conclude that the GABA_{B(1a)} and GABA_{B(1b)} isoforms are molecular variants of the GABA_B receptor system which contribute differentially to specific components of GABA_B receptor-mediated cognitive functioning.

Acknowledgements

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Chapter 8

General Discussion

The experiments described in this thesis demonstrated an important role for the GABA_{B(1)} isoforms, GABA_{B(1a)} and GABA_{B(1b)}, in specific aspects of aversive memory and depression-related behaviour and neuropharmacology, but not in innate anxiety. Preparatory work underlined the necessity of including multiple experimental endpoints in the study of GABA_B receptor function using *in vivo* pharmacological approaches, and established the influence of genetic background on GABA_B receptor-mediated responses. Importantly, it was demonstrated that the BALB/c mouse strain was appropriate for carrying the GABA_{B(1)} isoform genetic mutations. Initial studies with GABA_{B(1)} isoform-deficient mice showed GABA_{B(1a)} and GABA_{B(1b)} isoforms diverged in their influences on locomotor responses to novelty and circadian activity, although the GABA_B receptor agonists baclofen or GHB were not specific for either isoform and were unable to discriminate these differences.

In tests related to anxiety and depression, the two isoforms had profound, differential effects impacts on the acquisition (GABA_{B(1a)}) and extinction (GABA_{B(1b)}) of aversive memories. These effects, however, were not accompanied by differences in innate anxiety. The GABA_{B(1a)} isoform was specifically implicated in depression-related behaviour, most probably mediated *via* its striking interactions with the serotonergic system. Finally, the GABA_{B(1)} isoforms were differentially implicated in distinct cognitive tasks.

Together these studies have demonstrated that the GABA_{B(1)} isoforms are functionally important variants of the GABA_B receptor, with specific relevance in depression and to aversive learning and memory processes that may underlie cognitive symptoms in anxiety disorders.

8.1 The Utility of GABA_{B(1a)}^{-/-} and GABA_{B(1b)}^{-/-} Mice

Prior to initiation of the research outlined in this thesis, no specific functions had been assigned to the GABA_{B(1)} receptor isoforms (see General Introduction). Genetic-based manipulation of the machinery involved in the translation of the isoform proteins represented an elegant molecular strategy with great potential for determining the *in vivo* functions of the

isoforms (Huang 2006; Vigot et al. 2006). The result of this approach was the demonstration that GABA_{B(1)} isoforms localised specifically to presynaptic heteroreceptor and postsynaptic sites, as well as fulfilling autoreceptor functions. This immediately illustrated why it had not previously been possible to delineate differential functions of GABA_{B(1)} isoforms. Cellular assays lack the necessary morphological organisation, and application of GABA_B receptor ligands in electrophysiological bath preparations of tissue from normal wildtype animals (Shaban et al. 2006), or to normal wildtype animals in *in vivo* studies, will affect both the GABA_{B(1)} isoforms at all of their different sites. Thus, genetic manipulation of mice probably represented the only method available for determining the functions of the individual GABA_{B(1)} isoforms, and particularly with regard to assessment of the behavioural impact of the isoforms.

In the generation of GABA_{B(1)} isoform deficient mice, a specific concern was the potential to generate an epileptiform phenotype similar to that observed in the full GABA_{B(1)}^{-/-} and GABA_{B(2)}^{-/-} mice. Both of these mutant strains show overt, spontaneous epileptiform and audiogenic seizures (Gassmann et al. 2004; Prosser et al. 2001; Queva et al. 2003; Schuler et al. 2001). When generated on a C57BL/6 or 129 mouse strain genetic background, the life-span of GABA_{B(1)}^{-/-} mice was very short - only a few weeks - due to the seizures, and limited their application in behavioural studies (Prosser et al. 2001; Queva et al. 2003). However, on a BALB/c background GABA_{B(1)}^{-/-} mice had a much longer life-span – well into adulthood (thus enabling behavioural studies), although seizure activity was still present (Gassmann et al. 2004; Schuler et al. 2001). Before generation of GABA_{B(1)} isoform specific mice, it was unknown whether or not one of the isoforms may have been responsible for the epileptiform activity. Therefore GABA_{B(1)} isoform-deficient mice were generated on a BALB/c background in case an epileptiform phenotype accompanied either one of the mutations. Choice of a BALB/c background would further allow comparisons with other aspects of the GABA_{B(1)}^{-/-} and GABA_{B(2)}^{-/-} phenotypes. Ultimately, by observation, it appeared that neither of the GABA_{B(1)} isoform-specific mutants demonstrated overt clonic-tonic seizures, although confirmation by EEG analysis has yet to be conducted. Indeed, GABA_{B(1a)}^{-/-} and GABA_{B(1b)}^{-/-} mice were viable, healthy, bred relatively normally and, in a comprehensive test battery, demonstrated no gross morphological or sensory-motor deficiencies (Chapter 3). This indicated they were applicable to behavioural research.

BALB/c Mice as the Background Strain for Studying GABA_{B(1)} Receptor Isoform Function

The importance of the background strain in the phenotypic analysis of mutant mice is well known (Crawley 2000; Crawley et al. 1997; Tarantino and Bucan 2000). With regard to the aims of this thesis, the BALB/c genetic background is particularly well suited for anxiety- and depression-related research. They show higher levels of anxiety than other strains in several anxiety paradigms such as the light-dark box and open field test (Griebel et al. 2000; Kim et al. 2002), leading to speculations that the BALB/c strain may be a relevant strain to model trait, pathological anxiety (Belzung and Griebel 2001). In addition, BALB/cJ mice have a high baseline sensitive in many models used in depression-related research (see (Jacobson and Cryan 2007)), and are broadly responsive to selective serotonin reuptake inhibitor (SSRI) antidepressants in both acute (Crowley et al. 2005; Lucki et al. 2001) and chronic (Dulawa et al. 2004) tests, although there are a few findings to the contrary (see (Cervo et al. 2005) and see (Jacobson and Cryan 2007) for a review). The BALB/cJ background for the GABA_{B(1)} isoform deletion was therefore ideally suited for the aims of this thesis, to determine the roles of the isoforms in anxiety and depression-related behaviour.

It should be noted, however, that some BALB/c substrains may not be ideal backgrounds for cognition research. At the very least, test protocols which are effective in other strains may require significant adaptation for use with BALB/c strains (Jacobson and Cryan, unpublished observations). Performance of BALB/cJ in the Morris water maze (MWM) reference spatial memory paradigm has been reported as intermediate, but poor in the BALB/cByJ substrain (Francis et al. 1995; Zaharia et al. 1996) (reviewed in (Schimanski and Nguyen 2004)) - although interestingly, BALB/cByJ pups cross-fostered by C57BL/6 mothers were not impaired in the MWM (Zaharia et al. 1996). Similarly, reported performance of BALB/cByJ in cued and contextual conditioned fear vary from intermediate (Balogh and Wehner 2003; Bolivar et al. 2001) to poor (Schimanski and Nguyen 2005). Care must be taken also with the BALB/cJ strain in object recognition tests. In a recent study BALB/cJ mice showed intense, long-lasting interest in a novel object relative to other strains (Kim et al. 2005). The object, a Styrofoam cube wrapped in paper, gave many hours of entertainment to the BALB/cJ mice, which showed perseverative responses towards it and were highly industrious in gnawing and stripping the paper from it. In traditional object recognition tasks, a reduction of time spent 'exploring' the object is thought to reflect familiarity, and thus recognition memory of the object (Ennaceur and Delacour 1988). Clearly with BALB/cJ mice in such a task, evaluations must be made circumspectly. With this in

mind, an appropriate object recognition protocol was adopted and successfully used in BALB/c mice in this thesis.

With regard to GABA_{B(1)} isoform deletions, perhaps one of the most important things to assess in BALB/c mice as the background strain for the mutations was their native GABA_B receptor function. Classic *in vivo* probes of GABA_B receptor function, motor incoordination and hypothermic responses, varied within and between mouse strains (Chapter 2) demonstrating that genetic background has a strong effect on the responses to the GABA_B receptor agonist, baclofen. These studies also demonstrated that hypothermic and ataxic responses to GABA_B agonists are influenced by independent genetic loci, and therein highlighted the necessity of including more than one experimental endpoint in an *in vivo* assessment of GABA_B receptor function. Indeed, the BALB/c strain showed both hypothermic and ataxic response to baclofen, and was thus validated as an appropriate strain for carrying the GABA_{B(1)} isoform genetic mutations. Inappropriate strains would have included NMRI, which showed very low ataxic and hypothermic responses to baclofen, or the C3H, which showed ataxia, but no demonstrable hypothermia to the agonist.

Overall, the BALB/cJ background strain was appropriate for carrying the GABA_{B(1)} isoform deletions and for anxiety- and depression-related behavioural research. This strain provided a safeguard from a possible epileptic phenotype, levels of normal GABA_B receptor function that would allow identification of a loss of GABA_B function, and sensitivity in tests of anxiety and depression.

Clearly genetic mutations, in the appropriate background strain, represent a powerful tool in gene-to-phenotype research. However, some general caveats in the use of genetically modified mice, as mentioned in the introduction, are worth revisiting. Behavioural analysis of genetically modified mice can be influenced by developmental compensations, effects on other gene products, and/or altered endocrine and neuronal feedback loops (Cryan and Holmes 2005; Pfaff 2001; Phillips et al. 2002). Further, epigenetic and environmental factors, such as maternal care, need to be considered (Holmes et al. 2005). These alterations may result in ectopic expression of other proteins which may markedly influence behaviour (Pfaff 2001). Pleiotropy, where one gene which has many (sometimes seemingly unrelated) effects, and overlap of individual genes, are common occurrences that may add to the complexities of phenotypic analysis in genetically modified animals (Pfaff 2001).

Overcoming of compensations in constitutive modifications may be addressed by cre-lox conditional mutation techniques for controlling time and/or tissue dependent expression of the mutation (Huerta et al. 2000; Iwasato et al. 2000; Tsien et al. 1996). Indeed, cre-lox

conditional mutation techniques have been developed for the GABA_{B(1)} receptor subunit (Haller et al. 2004). Alternatively, much interest has been generated in the potential of RNA interference (RNAi), as a means to effectively knockdown genes in the adult mouse brain (Thakker et al. 2006; Thakker et al. 2004; Thakker et al. 2005). However, whether or not these techniques are applicable for conditional, selective knock-down of GABA_{B(1a)} or GABA_{B(1b)} proteins has yet to be investigated.

Overall, the generation of viable, GABA_{B(1a)}^{-/-} and GABA_{B(1b)}^{-/-} mice opened new opportunities for behavioural research into the roles of the GABA_{B(1)} isoforms. Indeed, studies in this thesis have demonstrated that GABA_{B(1a)}^{-/-} and GABA_{B(1b)}^{-/-} mice are highly useful tools for the *in vivo* assessment of GABA_{B(1)} isoform functions, and were appropriate for addressing the hypothesis that these isoforms would have a differential impact on specific behaviours relevant to anxiety and depression.

8.2 GABA_{B(1)} Isoforms and GABA_B Receptor Function

GABA_B Receptor Agonists: Baclofen and GHB

Amongst the first studies conducted with GABA_{B(1a)}^{-/-} and GABA_{B(1b)}^{-/-} mice, responses to baclofen or GHB in GABA_{B(1)} isoform-deficient mice were assessed and found to be similarly attenuated in both mutant strains (Chapter 3). This demonstrated that, in support of *in vitro* findings (Brauner-Osborne and Krogsgaard-Larsen 1999; Green et al. 2000; Kaupmann et al. 1998a; Malitschek et al. 1998), baclofen does not have preferential specificity for the isoforms. Furthermore, either GABA_{B(1a)} or GABA_{B(1b)} may compensate to a certain degree for the loss of the other to produce baclofen-induced hypothermia or ataxia. These findings also suggested that response variation in the different mouse strains with baclofen (Chapter 2) was therefore unlikely to be a product of grossly dissimilar expression of the GABA_{B(1a)} and GABA_{B(1b)} isoforms *per se*. It was interesting to note that the baclofen-induced hypothermic response was almost completely blocked in both GABA_{B(1a)}^{-/-} and GABA_{B(1b)}^{-/-} mice, whereas baclofen-induced motor-incoordination was attenuated to a lesser degree in both mutant strains. This confirmed the conclusions from Chapter 2 that both of these responses are under divergent genetic control, and that it is important to investigate more than one agonist-induced response in genetically modified animals.

GABA_{B(1a)}^{-/-} and GABA_{B(1b)}^{-/-} mice are ideally suited for the testing of possible isoform-selective ligands in the hypothermia/ataxia *in vivo* assay. Selective, isoform-specific

agonists, for example, should induce hypothermia and/or ataxia in only one of the two mutant strains. $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice will therefore be valuable tools for the assessment of putative $GABA_{B(1)}$ isoform-specific ligands in the future.

GABA_{B(1)} Isoforms and Baseline Locomotor Behaviour

$GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice diverged significantly in their locomotor behaviour, although baseline body temperature was not greatly affected by isoform deletion. In particular, $GABA_{B(1b)}^{-/-}$ mice were hyperactive in a novel environment and in the circadian dark phase (Chapter 3). In the hippocampus, amygdala and L5 neocortex, the $GABA_{B(1b)}$ isoform is predominantly expressed postsynaptically (Perez-Garci et al. 2006; Shaban et al. 2006; Vigot et al. 2006). Postsynaptic $GABA_B$ receptors are coupled to K_{ir3} channels (Luscher et al. 1997) and $K_{ir3.2}$ and $GABA_B$ receptors are co-localised at excitatory postsynaptic sites in the hippocampus (Kulik et al. 2006). Thus, it seems likely that the $GABA_{B(1b)}$ isoform may be coupled to K_{ir3} channels, at least in these regions.

K_{ir3} channels are strongly implicated in motor function, as is well illustrated by the phenotype of the weaver mutant mouse which have a mutation in the $K_{ir3.2}$ subunit (Patil et al. 1995) and show pronounced motor impairments including ataxia, trembling and locomotor hyperactivity (Rakic and Sidman 1973a; b). Genetic deletion of $K_{ir3.2}$ in mice induces hyperactivity in a novel environmental and in the (early) circadian dark phase (Blednov et al. 2001; Blednov et al. 2002). The novelty-induced and circadian hyperlocomotion phenotypes of $K_{ir3.2}^{-/-}$ mice are similar to that of the $GABA_{B(1b)}^{-/-}$ mice (Fig. 1), and together with the aforementioned colocalisation studies, suggests the same $K_{ir3.2}$ mediated-mechanisms may underlie these phenotypes in both mutants.

Dopaminergic neurons in the VTA are of prime importance in mediating the hyperlocomotor effects of dopamine and dopaminergic agents (Kalivas and Stewart 1991). Both $K_{ir3.2}$ channels (Murer et al. 1997) and $GABA_B$ receptors are expressed on dopaminergic cell bodies in the VTA (Kalivas 1993). There is evidence to suggest that VTA dopaminergic neurons are under tonic inhibitory control by $GABA_B$ receptors, as local application of $GABA_B$ receptor antagonists increased VTA extracellular dopamine levels (Giorgetti et al. 2002), and systemic application increased the firing rate of VTA dopaminergic neurons (Erhardt et al. 2002). In contrast, activation of $GABA_B$ receptors decreases the excitability and firing rate of VTA dopaminergic neurons and reduced dopamine release in the nucleus accumbens (Chen et al. 2005; Lacey 1993; Olpe et al. 1977; Westerink et al. 1996; Wirtshafter and Sheppard 2001).

Furthermore, baclofen, or the GABA_B positive modulator GS39783, dose dependently attenuated acute cocaine-induced hyperlocomotion (Lhuillier et al. 2006). Together these findings may indicate that tonic inhibition of VTA dopaminergic neurons may be compromised in GABA_{B(1b)}^{-/-} mice, mediated by a coupling failure with the K_{ir}3.2 channel, and thus may underlie their hyperlocomotor phenotype.

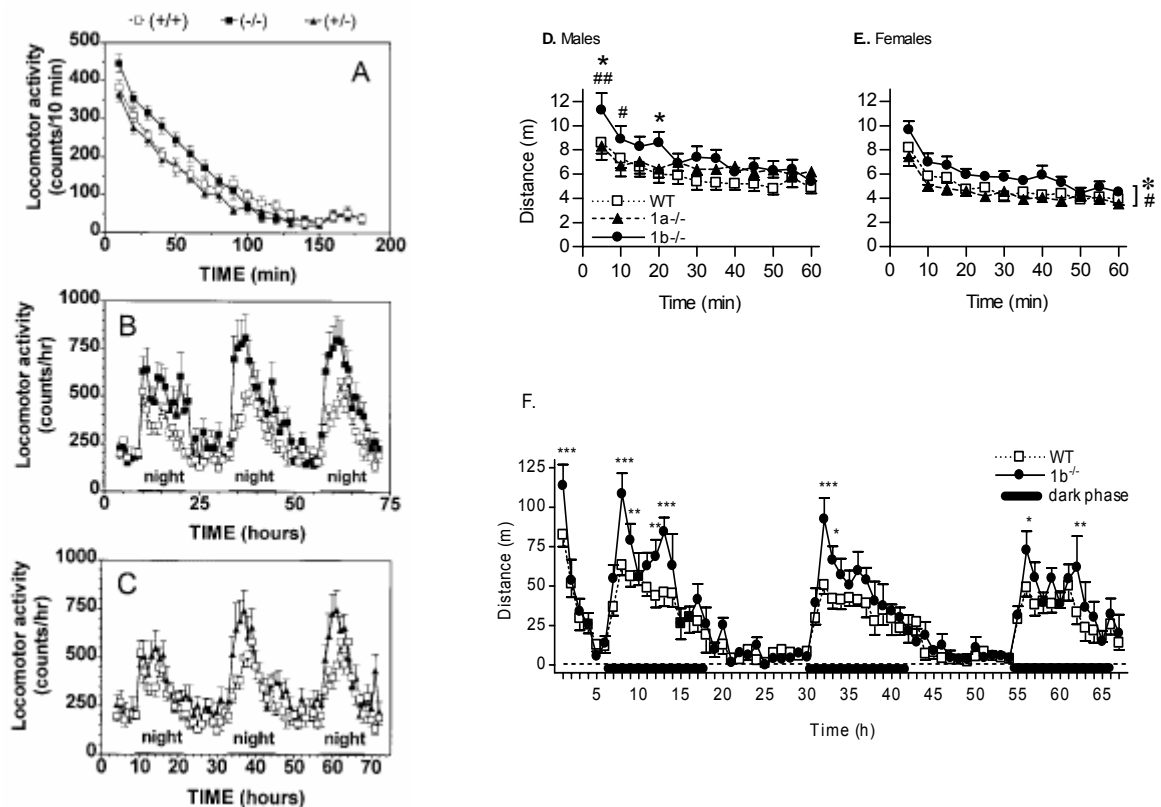


Fig. 1. K_{ir}3.2^{-/-} mice are hyperactive in a novel environment (A), and during the dark phase (B). K_{ir}3.2^{+/-} mice were also hyperactive in the dark phase (C). From Blednov et al., *Psychopharmacology* (2002) 159:370–378. Similarly, GABA_{B(1b)}^{-/-} mice are hyperactive in a novel environment (males, D; females, E); and during the dark phase (males only, F). From Chapter 3; Jacobson et al., *J Pharmacol Exp Ther* (2006) 319 (3):1317-1326.

Further investigations are clearly needed to characterise the influence of the GABA_{B(1)} isoforms on the dopaminergic system. In addition, research into the influence of either isoform in addiction related research, in which the GABA_B receptor has been firmly implicated (for a recent example see (Lhuillier et al. 2006), and see (Bettler et al. 2004; Bowery et al. 2002; Roberts 2005) for reviews), should yield interesting results.

8.3 GABA_{B(1)} Isoforms, Anxiety and Cognition

Aversive Learning and Memory

GABA_{B(1a)}^{-/-} and GABA_{B(1b)}^{-/-} mice showed profound differences in aversive learning and memory, as assessed in a conditioned taste aversion test (CTA; Chapter 4). GABA_{B(1a)}^{-/-} mice failed to acquire CTA, which is probably not entirely surprising given the phenotype of these mice in other cognitive tasks. For example, in an object recognition task, GABA_{B(1a)}^{-/-} mice were unable to remember familiar objects or to distinguish between novel and familiar objects (Chapter 7, Table 1). Therefore their acquisition failure in the CTA task could reflect either a failure to make, or consolidate, an association between the saccharin (the conditioned stimulus; CS) and the malaise (the unconditioned stimulus; US), or indeed to recognize the saccharin as familiar at all.

GABA_{B(1b)}^{-/-} mice, in contrast, showed a profound failure to extinguish CTA. One factor which may influence the rate of extinction is the strength of the original association (Nolan et al. 1997; Reilly and Bornovalova 2005). As a constitutive germline mutant, loss of the GABA_{B(1b)} isoform in GABA_{B(1b)}^{-/-} mice was also present at CTA acquisition. It could therefore be posited that the extinction failure of the GABA_{B(1b)}^{-/-} mice may have origins from a stronger acquisition, rather than a failure in extinction *per se*. GABA_{B(1b)}^{-/-} mice however, did not show a stronger aversion to saccharin at acquisition testing than wild-type mice, although as both were near ceiling aversion levels any such differences may not have been apparent. There was certainly no indication that GABA_{B(1b)}^{-/-} mice showed enhanced learning in other cognitive tasks (for example in working spatial or 24 hour consolidation of object recognition memory, both of which were impaired in GABA_{B(1b)}^{-/-} mice; Chapter 7, Table1).

To test the possibility of an enhanced CTA acquisition in GABA_{B(1b)}^{-/-} mice, a LiCl dose response experiment could be conducted, as different LiCl doses influenced the rate of extinction in the background BALB/c strain (Chapter 4). Alternatively a step-wise CTA acquisition protocol could be used to by using repeat CS-US pairings with low levels of LiCl. The rate of CTA acquisition could then be compared with wild-type mice. The ideal method for testing the impact of the GABA_{B(1b)} isoform on extinction learning alone would be selective antagonism of the GABA_{B(1b)} isoform after a successful CTA acquisition but before extinction training. In the absence of isoform selective antagonists, this could be addressed by either cre-lox conditional mutation techniques (Haller et al. 2004) or isoform-specific RNAi (Thakker et al. 2004; Thakker et al. 2005). CTA would be the ideal partner in such approaches, as in the absence of unreinforced CS exposures (i.e. extinction training), CTA shows remarkable

persistence (sometimes even for the lifetime of an animal) (Bures 1998a). This would allow sufficient time to produce a level of protein knockdown between CTA acquisition and the initiation of extinction training without greatly compromising the level of pre-extinguished aversion.

The alternative hypothesis for the extinction failure of $GABA_{B(1b)}^{-/-}$ mice is of course that they were unable to learn extinction, or perhaps to modify the original aversive trace through an extinction learning processes. A specific aversive memory extinction failure *per se* would represent a strong case for the promotion of the $GABA_{B(1b)}$ isoform as a target in anxiety disorders where persistence of aversive memory prevails, such as PTSD and specific phobia (Davis and Myers 2002; Davis et al. 2006; Ressler et al. 2004).

Other methods for investigating aversive memory have been examined with $GABA_{B(1)}$ isoform-deficient mice. In a conditioned fear paradigm, $GABA_{B(1a)}^{-/-}$ mice demonstrated a modest acquisition of conditioned freezing to a tone paired with a 0.6 mA footshock (CS+) versus an unpaired tone (CS-) (~38% versus ~20% freezing, respectively) (Shaban et al. 2006). This contrasts with the failure of $GABA_{B(1a)}^{-/-}$ mice to acquire CTA (Chapter 4). Indeed as $GABA_{B(1a)}^{-/-}$ mice were also impaired in object recognition and discrimination (Chapter 7, Table 1), it would therefore not have been surprising if $GABA_{B(1a)}^{-/-}$ mice were unable to discriminate between two tones. Tones by themselves can induce freezing in mice in the absence of associative learning (Kamprath and Wotjak 2004). It would have been interesting to determine whether or not the two tones used in the Shaban study produced variable levels of freezing when presented in the absence of conditioning, in case this may have contributed to differences seen between the CS+ and CS- induced freezing. Alternatively, it may also have been informative to compare the acquisition of fear conditioning using either of the two tones as the CS+. When fear conditioned with a 0.9 mA footshock however, $GABA_{B(1a)}^{-/-}$ mice no longer distinguished between the CS+ and CS-, although when the shock intensity was further increased to 1.35 mA, wild-type mice also failed to discriminate between the CS+ and CS- (Shaban et al. 2006). This indicated that $GABA_{B(1a)}^{-/-}$ mice generalized at a lower shock intensity than wild type mice. It is unclear at the present time precisely how shifting of the generalization threshold in $GABA_{B(1a)}^{-/-}$ mice relates to the accompanying loss of associativity of cortico-LA presynaptic LTP also seen in this mutant (Shaban et al. 2006). Further studies to fully investigate this phenomenon, perhaps in combination with *in vivo* recordings, promise to be very enlightening.

$GABA_{B(1b)}^{-/-}$ mice, in contrast to their successful acquisition of a CTA (Chapter 4), failed to acquire conditioned freezing at all (Shaban et al. 2006). However, as $GABA_{B(1b)}^{-/-}$

mice have a hyperlocomotor phenotype (Chapter 3), it is possible that this may interfere with the behavioural expression of freezing.

Innate Anxiety

Given the differential influences of the GABA_{B(1)} isoforms on conditioned aversive learning in CTA (Chapter 4), and previous findings that mice deficient in GABA_{B(1)} or GABA_{B(2)} subunits have a highly anxious phenotype in exploratory paradigms (Mombereau et al. 2004a; Mombereau et al. 2005; Mombereau et al. 2004b), it was of interest to examine unconditioned anxiety in GABA_{B(1a)}^{-/-} and GABA_{B(1b)}^{-/-} mice. However, in a comprehensive battery of unconditioned anxiety tests, including autonomic, active and passive behavioural readouts, there was no evidence for a specific influence of either isoform (Chapter 5). This indicated that the GABA_{B(1)} isoforms themselves do not have a defining role in innate anxiety.

One of the main differences between the GABA_B subunit- and GABA_B isoform-deficient mice is that GABA_{B(1)}^{-/-} and GABA_{B(2)}^{-/-} mice show a loss of all GABA_B receptor function (Gassmann et al. 2004; Schuler et al. 2001; Shaban et al. 2006), whereas residual agonist-induced function remains, albeit blunted, in both the GABA_{B(1a)}^{-/-} and GABA_{B(1b)}^{-/-} mice (Chapter 3; Perez-Garci et al. 2006; Shaban et al. 2006; Vigot et al. 2006). In particular, GABA_B autoreceptor function is preserved in GABA_{B(1a)}^{-/-} and GABA_{B(1b)}^{-/-} mice in the hippocampus and amygdala, but not in GABA_{B(1)}^{-/-} mice (Shaban et al. 2006; Vigot et al. 2006). Both the (ventral) hippocampus and amygdala are strongly implicated in anxiety and fear, (see (Bannerman et al. 2004) for a review). Although it is currently unknown what influences GABA_B autoreceptor function in these structures may exert on anxious behaviours, these findings suggest that further investigations into this area may be warranted.

Finally, it is worth noting that other endophenotypes of anxiety disorders have yet to be investigated in GABA_{B(1)} isoform-deficient mice (see Table 2, General Introduction). These include panic behaviour in response to a predator as a model of the “sudden onset of intense fearfulness” characteristic of panic disorder; examination of social interactions with unfamiliar conspecifics, to model “anxiety provoked by social situations, leading to avoidance behaviour” in social phobia; and ultrasonic vocalizations in pups separated from their mothers, to model separation anxiety. Examining these endophenotypes may be instructive avenues of study in future research.

Cognition

Studies with $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice have demonstrated that $GABA_{B(1)}$ isoforms have distinctly different roles in specific cognitive tasks.

$GABA_{B(1a)}^{-/-}$ mice were impaired in a range of tasks reliant on working memory (Chapter 7, Table 1). In tasks requiring long term memory, CTA acquisition was profoundly impaired, although passive avoidance was spared in these mice. Passive avoidance however is reliant on many different cognitive components, including spatial, procedural and associative elements (Crawley 2000), and thus there may have been compensation in these different processes to facilitate passive avoidance. It is interesting to note that in the studies by Vigot et al., (2006), $GABA_{B(1a)}^{-/-}$ mice were impaired in hippocampal LTP, and showed evidence for a loss of AMPA silent synapses. Long-term maintenance of LTP is dependent, at least in part, on the recruitment of additional AMPA receptors containing the GluR1 subunits into activated synapses (see (Malinow and Malenka 2002)). Of note, mice deficient in the GluR1 subunit of the AMPA receptor show a loss of hippocampal LTP, profound deficits in working spatial memory, but preservation of spatial reference memory (for examples see (Reisel et al., 2002; Schmitt et al., 2003; Zamanillo et al. 1999), and see (Bannerman et al. 2006) for a review). It may therefore be of interest to broadly assess both spatial working and reference memory in $GABA_{B(1a)}^{-/-}$ mice, to determine whether or not a similar phenotype to $GluR1^{-/-}$ mice is demonstrated in that spatial reference memory is spared.

$GABA_{B(1b)}^{-/-}$ mice show a different cognitive phenotype to that of the $GABA_{B(1a)}^{-/-}$ mice. $GABA_{B(1b)}^{-/-}$ mice, like $GABA_{B(1a)}^{-/-}$ mice, are moderately impaired in spontaneous alternating behaviour, and in long term familiar object recognition. However, they differ from the $GABA_{B(1a)}^{-/-}$ in preservation of short term familiar object recognition and novel object discrimination, and in CTA acquisition. Clearly CTA extinction cannot be compared with $GABA_{B(1a)}^{-/-}$ mice. However, a question that arises with the failure of CTA extinction in $GABA_{B(1b)}^{-/-}$ mice is whether or not it may also be accompanied by broader deficits in reversal learning. This would certainly be of great interest to evaluate from a cognitive science basis, and could be assessed in tests of reversal learning in spatial reference memory tasks.

The mechanisms underlying the cognitive phenotypes of $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice have yet to be investigated. It will be interesting to examine the influence of these isoforms, and in particular the postsynaptically expressed $GABA_{B(1b)}$ isoform, on downstream signalling pathways related to memory. $GABA_B$ receptors couple to adenylyl cyclase (Bettler et al. 2004) and influence the mitogen-activated protein kinase (MAPK) pathway (Ren and

Mody 2003). GABA_B receptors may therefore modulate cognitive processes via the cAMP-PKA-CREB or MAPK signalling pathways, both of which are strongly implicated in learning and memory (see (Abel and Kandel 1998; Arnsten et al. 2005; Berman and Dudai 2001; Silva et al. 1998)). This is supported by the finding that the GABA_B receptor antagonist SGS742 improved hippocampal-dependant spatial reference memory and reduced basal hippocampal CRE-binding (Helm et al. 2005). Clearly this will be an important series of studies for future investigation.

Table 1. Summary of cognitive phenotypes of GABA_{B(1a)}^{-/-} and GABA_{B(1b)}^{-/-} mice

Paradigm	Classification	GABA _{B(1a)} ^{-/-}	GABA _{B(1b)} ^{-/-}
Continuous spontaneous alternating behaviour	Working memory Spatial	Impaired	Moderately impaired
10 minute delay familiar object recognition	Working memory Recognition	Impaired	OK
Novel object discrimination	Working memory Recognition	Impaired	OK
24 hour delay familiar object recognition	Reference memory Recognition	Impaired	Impaired
Passive Avoidance	Reference memory Conditioned place avoidance	OK	OK
CTA acquisition	Reference memory Associative aversive learning	Impaired	OK
CTA extinction	Reference memory Extinction learning	?	Impaired

GABA_{B(1)} Isoforms: Emotional Learning & Memory Versus Learning & Memory

As GABA_{B(1a)}^{-/-} or GABA_{B(1b)}^{-/-} mice showed no innate anxiety phenotype, and a range of different cognitive impairments, the possibility that deficits in conditioned aversive learning and memory could be product of a cognitive phenotype, rather than a deficit in emotional learning and memory *per se*, bears some consideration. In aversive learning and memory paradigms however, evidence exists both “for” (CTA) and “against” (passive avoidance)

specific roles for the GABA_{B(1)} isoforms in emotive cognition. At least four considerations arise from this observation:

- 1) If GABA_{B(1)} isoforms play specific roles in emotional learning and memory, that are distinct from their effects on non-emotional learning and memory, they are not unique. Indeed, mice deficient in the cannabinoid receptor CB1 which show impairments in extinction of conditioned freezing (Kamprath et al. 2006; Marsicano et al. 2002) are not impaired in appetitive (Holter et al. 2005) or spatial learning (C.T. Wotjak, Max Planck, Munich, pers. comm.);
- 2) If GABA_{B(1)} isoforms do have a specific role in emotional learning and memory, then the specific processes of acquisition (GABA_{B(1a)}^{-/-}) and extinction (GABA_{B(1b)}^{-/-}) of aversive memories can occur independently of innate anxiety (at least with regard to the GABA_{B1} receptor isoforms);
- 3) In mutant mice which have deficits in both non-emotional and emotional aspects of cognition, it will be difficult to comprehensively dissect the former from the later, given the multimodal cognitive constructs in many of these tasks;
- 4) A specific role in non-emotional learning and memory processes does not preclude an important impact in the modulation of aversive memory. The most well-known example in this category is a pharmacological agent: D-cycloserine, a partial agonist at the NMDA receptor strychnine-insensitive glycine site and a cognitive enhancer, which speeds extinction learning in both animal models and in clinical studies, although it is without intrinsic anxiolytic activity in itself (Ressler et al. 2004; Walker et al. 2002).

8.4 GABA_{B(1)} Isoforms and Depression

The GABA_{B(1a)} isoform demonstrated a clear and specific involvement in depression-related behaviour (Chapter 6). This was illustrated by an antidepressant-like phenotype in the FST and by profound desensitization of presynaptic 5-HT_{1A} receptors. Desensitization of 5-HT_{1A} autoreceptors has been strongly implicated in the action of antidepressants, as evidence in many animal studies and in clinical trials (Artigas et al. 1996; Blier et al. 1997; Blier and Ward 2003; Cryan and Leonard 2000; De Vry 1995; Hensler 2003). Intriguingly, the expression of the 5-HT_{1A} receptor itself was unaffected in GABA_{B(1a)}^{-/-} mice. Immunohistochemistry, autoradiography and *in situ* hybridisation studies suggest that GABA_{B(1a)} isoforms are probably colocalised on 5-HT cell bodies in the raphe nuclei (Abellan et al. 2000a; Abellan et al. 2000b; Bischoff et al. 1999; Serrats et al. 2003). Together with the demonstration of reduced baclofen-stimulated G-protein binding in 5-HT transporter-deficient (5-HTT^{-/-}) and 5-HT_{1A}^{-/-}

mice (see (Fabre et al. 2000; Mannoury la Cour et al. 2001; Mannoury la Cour et al. 2004)), these findings may support a dysregulation in G-protein cross-talk between GABA_{B(1a)} and 5-HT_{1A} receptors on serotonergic cell bodies as a mechanism underlying the 5-HT_{1A} receptor desensitisation in GABA_{B(1a)}^{-/-} mice. Alternatively, or additionally, contributions to the 5-HT_{1A} desensitisation could arise from a loss of GABA_{B(1a,2)} receptor-mediated inhibition of 5-HT release, leading to increased 5-HT at the level of the DRN (and, for that matter, in postsynaptic structures), and thus a direct 5-HT-mediated desensitisation of DRN 5-HT_{1A} receptors (Adell et al. 2002). This latter mechanism is supported by evidence for postsynaptic 5-HT_{1A} receptor desensitisation in GABA_{B(1a)}^{-/-} mice (Chapter 6). This included a loss of hypolocomotor, ataxic and serotonin syndrome responses. Interestingly, attenuated *in vivo* responses to 5-HT_{1A} receptor activation has also been demonstrated following chronic antidepressant treatment in the clinic (Lesch et al. 1990; Rausch et al. 2006).

GABA_{B(1a)}^{-/-} and GABA_{B(1b)}^{-/-} mice are ideally suited for use in further studies to elucidate the mechanisms involved in interactions between the 5-HT and GABA_B receptors. Such studies would ideally involve an ultrastructural analysis of the GABA_{B(1)} isoform expression in the raphe nuclei, the influence of GABA_{B(1a)} isoform deletion on 5-HT_{1A} receptor G-protein coupling, and the baseline and stimulated release profile of 5-HT in GABA_{B(1a)}^{-/-} mice.

There is growing evidence that abnormalities in the 5-HT_{1A} receptor system in early life is responsible for the expression of pathological behaviour in the adult, rather than dysregulation of the adult 5-HT_{1A} receptor *per se* (Ansorge et al. 2004; Gross and Hen 2004). For example, neonatal rescue of 5-HT_{1A} receptor function in conditional 5-HT_{1A} receptor knockout mice reversed the anxiety phenotype normally seen in these mutants, but not when the rescue was performed in adult mice (Gross et al. 2002). In addition, the abnormal sleep architecture of adult 5-HTT^{-/-} mice, which show high levels of extracellular serotonin and a profound desensitization of presynaptic 5-HT_{1A} receptors, was rescued by transient treatment of the neonate with the 5-HT synthesis inhibitor pCPA, or the 5-HT_{1A} receptor silent antagonist WAY100635 (Alexandre et al. 2006). In this regard, it is interesting to note that the GABA_{B(1a)} isoform is highly expressed in the neonate and subsequently decreases during development (Fritschy et al. 1999; Malitschek et al. 1998). This raises the possibility that the GABA_{B(1a)} isoform may have a developmental role in the maturation of the 5-HT receptor system. Given the efficacy of serotonin depletion and 5-HT_{1A} receptor blockade on neonatal rescue of 5-HTT^{-/-} mice, it may therefore be of interest to conduct a similar study in GABA_{B(1a)}^{-/-} mice. Analysis of sleep architecture of GABA_{B(1)} isoform-deficient mice has not

been conducted to date. However, GABA_B receptors influence circadian rhythm (for examples see (Colwell et al. 1993; Gillespie et al. 1997; Moldavan et al. 2006; Ralph and Menaker 1989)), and GABA_{B(1a)}^{-/-} mice demonstrated a subtle increase in circadian locomotor activity during the light-phase (Chapter 3) – which, although not a measure of sleep *per se*, at least indicated that while moving, these mice were not asleep.

Altered sleep patterns are an endophenotype of anxiety and a classic diagnostic criterion in depression. Furthermore, in humans, sleep deprivation is one of the few fast acting antidepressant treatments available, and paradoxical (REM) sleep is altered by many antidepressant medications (Adrien 2002; Wirz-Justice and Van den Hoofdakker 1999). In a recent study, mice selectively bred for high levels of immobility in the tail suspension test were found to display lighter and more fragmented sleep and decreased REM sleep latency, abnormalities that resemble those observed in depressed patients (El Yacoubi et al. 2003). Sleep disturbances in fear conditioned rats (Jha et al. 2005; Pawlyk et al. 2005) and mice (Sanford et al. 2003a; Sanford et al. 2003b) have also been reported. These studies suggest that investigating sleep patterns in mice will be of great use in anxiety- and depression-related research. Further studies on the impact of GABA_B receptor manipulations on sleep and depression-related behaviours in mice are an interesting direction for future investigations.

Studies on the effects of GABA_{B(1a)} isoform deletion on brain-derived neurotrophic factor (BDNF) may also be warranted. GABA_B receptor antagonists have been shown to increase the concentration of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) in the hippocampus and cortex (Froestl et al. 2004; Heese et al. 2000). BDNF in particular has been proposed to contribute to the actions of antidepressants (see (Duman et al. 1997) and (Berton and Nestler 2006) for reviews). As such it is plausible that induction of BDNF could contribute to the antidepressant effects of GABA_{B(1a)} isoform deletion.

Both anxiety disorders and major depression (see Tables 1 and 2, General Introduction) are characterised by deficits in attention. Given the impact of the GABA_{B(1a)} isoform in particular on tests of working memory, and in depression-related behaviour and serotonergic neurotransmission, assessment of attention in GABA_{B(1a)}^{-/-} mice would be of interest to investigate in the future. This would probably be best assessed in the 5-choice serial reaction time task, in which mice must pay attention to five holes into which they can poke their nose to obtain a reward when, and only when, a hole is (randomly) illuminated. This task has recently been validated in C57BL/6 and DBA/2 mice, which were both able to do the task, although strain differences were apparent (Patel et al. 2006), and was applied in a study of galanin-

overexpressing transgenic mice to demonstrate that the known cognitive deficits in these mice were not a product of deficits in attention (Wrenn et al. 2006).

Finally, with regard to depression-related behaviour, $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice have yet to be examined in tests that model the induction of depression-related behaviour. Perhaps the most ideally suited test, despite its criticisms (Cryan and Mombereau 2004; Cryan and Slattery 2007), would be the chronic mild stress assay. In a shuttle-box learned helplessness paradigm, propensity to develop a state of behavioural despair *per se* may be difficult to delineate from specific and differential cognitive impairments in the isoform-deficient mice (for example in spatial working memory). A test such as olfactory bulbectomy, in contrast, may be biased by the hyperlocomotor phenotype of the $GABA_{B(1b)}^{-/-}$ mice. Chronic mild stress, in comparison, would allow assessment of the propensity of the isoform-deficient mice to develop stress-induced, depression-related behaviours relatively independently of overt spatial memory impairments or hypolocomotion. Furthermore, the background strain for the $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice, BALB/c, respond sensitively to chronic mild stress with alterations in fur state, grooming and increased conspecific aggression ((Ducottet and Belzung 2004; 2005; Mineur et al. 2003; Pothion et al. 2004). The inclusion of these additional, sensitive parameters to the normal hedonic endpoint in chronic mild stress (sucrose preference), may add considerably to the power of this test for the detection of real differences between $GABA_{B(1)}$ isoform mutants and wildtype controls (see (Jacobson and Cryan 2007) for a review).

Studies presented in this thesis represent the ‘tip of the iceberg’ with regard to the $GABA_{B(1)}$ isoforms in depression-related research. There is much work yet to be done in order to further define the underlying mechanisms and the full extent of the impact of the $GABA_{B(1a)}$ isoform, in particular, in endophenotypes of depression. However, these findings clearly identify a strong interaction of $GABA_B$ receptor heterodimers containing the 1a isoform with the serotonergic system and suggest that the $GABA_{B(1a)}$ isoform may be a potential therapeutic target for the development of novel antidepressants.

8.5 Perspectives

Research presented in this thesis demonstrated that the two isoforms of the $GABA_{B(1)}$ receptor subunit, $GABA_{B(1a)}$ and $GABA_{B(1b)}$, are functionally distinct molecular variants of the $GABA_B$ receptor. Furthermore, these isoforms have particular and differential relevance in the

formation and persistence of aversive memory and depression-related behaviour and neurobiology.

Overt phenotypes of mice deficient in the GABA_{B(1a)} isoform included: failures in the acquisition of aversive memory, antidepressant-like phenotype in the mouse FST, pre- and postsynaptic desensitization of 5-HT_{1A} receptors including attenuated HPA responses to 5-HT_{1A} receptor stimulation, and deficits in working spatial and recognition memory and in reference recognition memory.

The phenotype of GABA_{B(1b)}^{-/-} mice included novelty-induced and circadian psychomotor disturbances, profound failures in the extinction of aversive memories, impaired spatial working memory and deficits in the consolidation of recognition memory.

These phenotypes provide convincing evidence that GABA_{B(1)} receptor isoforms are specifically and differentially implicated in endophenotypes of depression, and indeed, despite a lack of impact on innate anxiety, in endophenotypes of anxiety-related disorders. It is of further interest to note that many of these endophenotypes presented in isoform-deficient mice are common to both depression and anxiety-related disorders. Future studies will also be able to focus on using these mice to delineate the contribution of the isoforms to other pathologies where GABA_B has been implicated, such as addiction, epilepsy, schizophrenia, pain, food intake and gastroesophageal reflux disease (Bowery 2006; Buda-Levin et al. 2005; Foltin 2005; Lhuillier et al. 2006; Mizukami et al. 2002; Patel et al. 2001; Paterson et al. 2004; Slattery et al. 2005b; Treiman 2001).

Clearly, the influence of the GABA_B receptor on physiology and behaviour are defined by the location of the receptor, which includes heterosynaptic, postsynaptic and autoreceptor locations. It is this differential expression profile which determines the functions that may be ascribed to the GABA_B receptor. This is delineated to a great degree by the differential trafficking of the GABA_{B(1a)} and GABA_{B(1b)} isoforms.

Perhaps the greatest challenge ahead for the GABA_{B(1)} receptor isoforms will be the development of isoform specific ligands. Given that the ligand binding domain for the GABA_{B(1a)} and GABA_{B(1b)} isoforms are both coded from the same region of the *Gabbr1* gene, it seems unlikely that targeting this site will yield selective ligands. The molecular architecture of the two isoforms themselves suggest the sushi domains, or their interacting proteins, as a possible targets for manipulating GABA_{B(1)} isoform trafficking. As pre- and postsynaptic GABA_B receptors, by nature of their location, have different interacting proteins and effector systems, targeting of proteins interacting at the GABA_B receptor C-terminus may provide a amenable therapeutic target for the GABA_{B(1)} isoforms (Marshall 2005). Finally

siRNA treatments may ultimately offer a method for specific targeting GABA_{B(1)} isoforms (Hoyer and Dev 2006). Although GABA_{B(1)} isoforms will be a complex target for the development of pharmacotherapies, data generated in this thesis demonstrate that these efforts should be worth it. It is hoped that one day the GABA_{B(1)} isoforms may contribute in the development of treatments for psychiatric disorders such as depression and anxiety, and the cognitive dysfunctions associated with them.

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Appendix I: Associated Publications

Differential compartmentalization and distinct functions of GABA_B receptor variants (2006) Neuron, 50 (4): 589-601

Vigot, R.; Barbieri, S.; Brauner-Osborne, H.; Turecek, R.; Shigemoto, R.; Zhang, Y. P.; Lujan, R.; Jacobson, L. H.; Biermann, B.; Fritschy, J. M.; Vacher, C. M.; Muller, M.; Sansig, G.; Guetg, N.; Cryan, J. F.; Kaupmann, K.; Gassmann, M.; Oertner, T. G.; Bettler, B.

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Differential Compartmentalization and Distinct Functions of GABA_B Receptor Variants

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Summary

GABA_B receptors are the G-protein-coupled receptors for the main inhibitory neurotransmitter in the brain,

γ-aminobutyric acid (GABA). Molecular diversity in the GABA_B system arises from the GABA_{B1a} and GABA_{B2a} subunit isoforms that solely differ in their ectodomains by a pair of sushi repeats that is unique to GABA_{B1a}. Using a combined genetic, physiological, and morphological approach, we now demonstrate that GABA_{B1a} isoforms localize to distinct synaptic sites and convey separate functions in vivo. At hippocampal GAB-to-GAI synapses, GABA_{B1a} assembles heteroreceptors inhibiting glutamate release, while predominantly GABA_{B1b} mediates postsynaptic inhibition. Electron microscopy reveals a synaptic distribution of GABA_{B1a} isoforms that agrees with the observed functional differences. Transfected GAD neurons selectively express GABA_{B1a} in distal axons, suggesting that the sushi repeats, a conserved protein interaction motif, specify heteroreceptor localization. The constitutive absence of GABA_{B1a} but not GABA_{B1b} results in impaired synaptic plasticity and hippocampus-dependent memory, emphasizing molecular differences in synaptic GABA_B functions.

Introduction

GABA_B receptors are considered promising drug targets for the treatment of neurological and mental health disorders (Bettler et al., 2004; Cryan and Kaupmann, 2005). Presynaptic GABA_B receptors are subdivided into auto- and heteroreceptors that control the release of GABA and other neurotransmitters, respectively. They restrict neurotransmitter release either by inhibiting voltage-sensitive Ca²⁺ channels or through a direct modulation of synaptic vesicle priming (Mintz and Isaac, 1993; Poncelet et al., 1997; Sakaba and Reher, 2002). Postsynaptic GABA_B receptors induce slow inhibitory potentials by gating Kir3-type K⁺ channels (Lüscher et al., 1997). Considerable evidence has accumulated over the years, using a variety of preparations and techniques, to support the notion that multiple subtypes of GABA_B receptors exist (Serrano and Palset, 1993; Sotocillo et al., 2002; Cunningham and Sirois, 1996; Debo et al., 1997; Gentry-Ram et al., 1994; Lal and McIlain, 2003; Mohler and Fritschy, 1999; Pozza et al., 1999; Yamada et al., 1999). The predicted receptor heterogeneity is not readily supported by molecular studies (Bettler et al., 2004). GABA_B receptors are heterodimers composed of GABA_{B1} and GABA_{B2} subunits, which are both required for normal receptor functioning (Marshall et al., 1999; Mohler and Fritschy, 1999). Accordingly, mice lacking GABA_{B1} (referred to as *l¹* mice) or GABA_{B2} subunits show a complete absence of typical GABA_B responses (Gassmann et al., 2004; Prosser et al., 2001; Schuler et al., 2001). The only firmly established molecular diversity in the GABA_B system arises from the GABA_{B1a} and GABA_{B1b} subunit isoforms (Kaupmann et al., 1997). However, no unique pharmacological or functional properties could be assigned to GABA_{B1a} or GABA_{B1b}. Most, if not all neurons coexpress GABA_{B1a} and GABA_{B1b}, which are generated by differential promoter usage from the GABA_{B1} gene (Blanchoff et al., 1999; Blazer et al., 2004). GABA_{B1a} and GABA_{B1b}

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Feeling Strained? Influence of Genetic Background on Depression-Related Behavior in Mice: A Review (2007). *Behavior Genetics*, 37 (1): 171-213

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REVIEW PAPER

Feeling Strained? Influence of Genetic Background on Depression-Related Behavior in Mice: A Review

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Abstract Depression is a growing pandemic in developed societies. The use of inbred mouse strains in pre-clinical psychiatric research has proven to be a valuable resource. Firstly, they provide the background for genetic manipulations that aid in the discovery of molecular pathways that may be involved in major depression. Further, inbred mouse strains are also being used in the determination of genetic and environmental influences that may pre-dispose or trigger depression-related behavior. This review aims to highlight the utility of inbred mouse strains in depression research, while providing an overview of the current state of research into behavioral differences between strains in paradigms commonly used in the field. Neurochemical differences that may underlie strain differences are examined, and some caveats and cautions associated with the use of inbred strains are highlighted.

Keywords Mouse strains · Depression · Animal models · Antidepressants

Edited by Andrew Holmes

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Introduction

"Strains every nerve to gain your point."
Marcus Tullius Cicero (106-43 BC)

Depression is one of the most incapacitating and debilitating disorders in developed nations (Kessler et al. 1994). The World Health Organisation predict that unipolar depression will be the second most common cause of debilitating disease by 2030, with an estimated cost in the US of about US\$80 billion per year. The most widely used antidepressants in clinical use today are based on the original serendipitously discovered tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors (MAOIs), and either block monoamine reuptake or degradation (Fraser 1997b; Slattery et al. 2004). Although these pharmacotherapies are reasonably efficacious (Fraser 1997b), against the background of increasing medical need, modern antidepressant therapies are still far from ideal, with a slow onset of action for all antidepressants and relatively high proportion of non-responders (about 25–35%). Additionally, although side effect profiles have been improved with the advent of selective reuptake inhibitors, it is clear that improvements are still needed (Fraser 1997a).

It is becoming very evident that two of the major contributing factors to the onset of major depression include life stress and genetic pre-disposition (see Charney and Maj 2004). Genetic factors have been estimated to account for 40–70% of the risk for developing major depression (Mailli et al. 2000; Lesch 2004). It is also clear that depressive disorders can result from interactions between a genetic pre-disposition and environmental stimuli (Clayton et al. 2003;

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- 1990-1993 Master of Agricultural Science (Hons)
Massey University, Palmerston North, New Zealand.
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Previous Employment:

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- 1999-2002 Researcher, Animal Stress and Welfare Team, AgResearch Ltd, Hamilton, New Zealand
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- 2006 Travel Award, ECNP Workshop on Neuropsychopharmacology for Young Scientists in Europe, Nice, France, 9-12 March 2006
- 2002-2003 Foundation for Research, Science and Technology: Bright Futures "Enterprise" Scholarship
- 1989 Helen E Akers Scholarship
- 1989 Johannes August Anderson Scholarship
- 1989 Farmers Union Scholarship

Professional Society Memberships:

European College of Neuropsychopharmacology
Society for Neuroscience
European Neuropeptide Club
Serotonin Club

Ad Hoc Reviewer:

Pharmacology, Biochemistry and Behaviour
European Journal of Pharmacology
Neuropharmacology
International Journal of Neuropharmacology
Neuroscience and Biobehavioural Reviews

Publications

Invited Lectures:

Molecular dissection of the role of GABA_{B1} receptor isoforms 1a and 1b in anxiety-related behaviour. 19th ECNP Congress, Paris, France, 16-20 September 2006.

GABA_{B(1)} receptor isoforms in animal models of depression and anxiety. Centre for Chronobiology, Psychiatric University Clinics, Universität Basel. 6 June 2006.

Molecular dissection of the role of GABA_{B1} receptor isoforms 1a and 1b in anxiety-related behaviour. ECNP Workshop on Neuropsychopharmacology for Young Scientists in Europe, Nice, France, 12 March 2006.

Submitted Manuscripts:

L.H. Jacobson, D. Hoyer, D. Uzunov, B. Bettler, K. Kaupmann, J.F. Cryan. Antidepressant-like effects and blunted 5-HT_{1A} receptor responses in mice lacking GABA_{B(1a)} but not GABA_{B(1b)}. *Neuropsychopharmacology*

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Jacobson, L.H., Bettler, B., Kaupmann, K., Cryan, J.F. Specific roles of GABA_{B(1)} receptor isoforms in cognition. *Behavioural Brain Research*. (In Press).

Fendt, M.[†], Schmid, S.[†], Thakker, D.R.[†], Jacobson, L.H.[†], Yamamoto, R., Mitsukawa, K., Maier, R., Kelly, P.H., McAllister, K.H., Hoyer, D., van der Putten, P.H., Cryan, J.F., Flor, P.J. mGluR7 facilitates extinction of aversive memories and controls amygdala plasticity. *Molecular Psychiatry*. (In Press). [†] THESE AUTHORS CONTRIBUTED EQUALLY TO THIS MANUSCRIPT.

Jacobson, L.H., Bettler, B., Kaupmann, K., Cryan, J.F. 2007. Behavioural evaluation of mice deficient in GABA_{B(1)} receptor isoforms in tests of unconditioned anxiety. *Psychopharmacology*. 190 (4): 541-53.

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Abstracts (Since Jan 2004):

L.H. Jacobson*, K. Kaupmann, B. Bettler & J.F. Cryan. GABA_{B(1)} receptor subunit isoforms: a role in anxiety? Poster, *Society for Neuroscience*, Atlanta, U.S.A., Oct. 2006.

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M. Fendt*, S. Siegl, S. Schmid, D.R. Thakker, L.H. Jacobson, R. Yamamoto, K. Mitsukawa, R. Maier, P.H. Kelly, K.H. McAllister, D. Hoyer, P.H. van der Putten, J.F. Cryan & P.J. Flor. mGluR7 facilitates extinction of aversive memories and controls amygdala plasticity. Poster, *Society for Neuroscience*, Atlanta, U.S.A., Oct. 2006.

L.H. Jacobson*, H.C. Neijt, B. Bettler, K. Kaupmann & J.F. Cryan. Molecular dissection of the role of GABA_{B1} receptor isoforms 1a and 1b in anxiety-related behaviour. Oral presentation. *European Neuropsychopharmacology* 16 Supplement 4, Abs S.04.05: S171. *Papers of the 19th ECNP Congress*, Paris, France, 16-20 Sept 2006

J. Guan*, R. Zhang, C. Chen, L.H. Jacobson, S. Mathai, D. Elliffe, P. Gluckman & D. McCarthy. GMPE mediated aging associated neurogenesis and astrogliosis in aged rats. XIVth IVBM 6th-10th June Noordwijkerhout, The Netherlands.

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