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- $GABA_{B1a}$ contributes to memory maintenance.
- $GABA_{B1b}$ contributes to initial memory formation.
- Excessive $GABA_{B1b}$ may contribute to behavioral aspects of anhedonic phenotypes.
- Excessive $GABA_{B1a}$ may prevent an anhedonic phenotype.
- Both isoforms may be involved in the developmental trajectory of disorders.

Identifying the role of pre-and postsynaptic $GABA_B$ receptors in behavior

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Abstract

Although many reviews exist characterizing the molecular differences of $GABA_B$ receptor isoforms, there is no current review of the *in vivo* effects of these isoforms. The current review focuses on whether the $GABA_{B1a}$ and $GABA_{B1b}$ isoforms contribute differentially to behaviors in isoform knockout mice. The roles of these receptors have primarily been characterized in cognitive, anxiety, and depressive phenotypes. Currently, the field supports a role of $GABA_{B1a}$ in memory maintenance and protection against an anhedonic phenotype, whereas $GABA_{B1b}$ appears to be involved in memory formation and a susceptibility to developing an anhedonic phenotype. Although $GABA_B$ receptors have been strongly implicated in drug abuse phenotypes, no isoform-specific work has been done in this field. Future directions include developing site-specific isoform knockdown to identify the role of different brain regions in behavior, as well as identifying how these isoforms are involved in development of behavioral phenotypes.

Key words: $GABA_B$ receptor; isoform; knockout; cognition; depression; drugs of abuse

1. Introduction

The current literature review focuses on pre-clinical behavioral studies examining the differing roles between $GABA_{B1a}$ and $GABA_{B1b}$ receptors, which tend to be pre- and postsynaptic, respectively. This review addresses a critical gap in the current literature: whether in vivo studies have identified specific roles of $GABA_{B1a}$ and $GABA_{B1b}$ receptors in behavior. To date, multiple reviews have been published concerning the molecular differences between these receptors. Each of these reviews has called on the necessity of *in vivo* research to demonstrate behavioral differences attributed to the different receptors. *In vivo* studies using animal models have now been published, although there is no current literature linking the isoforms to behavior in humans. The current literature review synthesizes and critically analyzes the literature to-date, as well as elucidates gaps in the research and proposes future areas of study. We hypothesize that a review of this area will elucidate separate roles of $GABA_{B1a}$ and $GABA_{B1b}$ receptors.

2. Characterization of the $GABA_B$ receptor

2.1 General information

As the metabotropic component of the brain's major inhibitory system, $GABA_B$ receptors ($GABA_B$Rs) have been implicated in a wide range of human behaviors. These include development, substance abuse, and behavioral pathologies such as anxiety and depression (Agabio et al., 2012; Benke, 2013; Bowery, 2006; Kumar et al., 2013). $GABA_B$Rs fall into the class III metabotropic receptor category, along with metabotropic glutamatergic receptors (mGluR), calcium sensing receptors, and some pheromone and taste receptors. Class III receptors have a dynamic bilobate structure, where each subunit has a clamshell-like shape. When a ligand binds to a subunit, the clamshell "closes," causing the

receptor to activate (Pin et al., 2003). The $GABA_BR$ is a relatively consistent heterodimer. A functional receptor is comprised of a $GABA_{B1}$ (B1) and $GABA_{B2}$ (B2) subunit (Pin et al., 2004). Variation of the heterodimer is relegated to the B1 subunit. While multiple isoforms of B1 have been identified, the primary isoforms that are part of the central nervous system and are conserved across many species, including humans, rodents, and even cockroaches, are the B1a and B1b subunits (Blankenburg et al., 2015; Lee et al., 2010; Kaupmann et al., 1998; Pinard et al., 2010). Expression of a receptor into B1a or B1b is regulated by unique cAMP-induced binding in specific promoter regions, including interactions between cAMP response element-binding protein (CREB), activating transcription factor 4 (ATF4), and upstream stimulatory factors (USF). CREB acts as a promotor for both B1a and B1b, whereas overexpression of ATF stimulates B1a promotor activity but inhibits B1b promotor activity. In the absence of CREB binding, USF inhibits expression of B1b (Steiger et al., 2004).

First discovered in 1998 by Hawrot et al. (1998), the pair of sushi domains on the end of the N-terminal ectodomain that distinguishes the B1a subunit from the B1b subunit was structurally analyzed by Blein et al. (2004). Also known as a complement control protein, the presence of the sushi domain is believed to be responsible for the increased stability of B1a/B2 heterodimers as compared to B1b/B2 heterodimers. Further, inserting the B1a sushi domain into mGluR2, another Class III metabotropic receptor that has no endogenous sushi domain, increases surface stability (Hannan et al., 2012). As part of the complement control proteins, the sushi domains on B1a have long been proposed to be involved in protein-protein interactions that regulate the system (Marshall et al., 1999). The sushi domain of B1a has been shown to interact with the matrix protein fibrillin-2, an important scaffolding protein,

whereas B1b does not (Blein et al., 2004). This interaction with auxiliary proteins may lead to differences in receptor activity, pharmacology, and localization of receptors which cannot be completely modeled in *in vitro* studies (Marshall et al., 1999; Mohler & Fritschy, 1999). Further, Hawrot et al. (1998) suggest that the extracellular location of the sushi domains may indicate that they are important for regulating ligand binding other than GABA, although data supporting this theory have not yet been published.

2.2 Receptor location

The B1 isoforms are found widely across the brain and are located in most neurons, but not in non-neuronal cells, such as glial cells (Bischoff et al., 1999; Benke et al., 1999; Fritschy et al., 1999; Liang et al., 2000). It has been repeatedly demonstrated that B1a/B2 and B1b/B2 receptors localize on different areas of the neuron. Generally, B1a is located presynaptically on axonal terminals, while B1b is located postsynaptically on dendritic spines. However, both isoforms are present as autoreceptors and B1a receptors are also found on the dendritic branches (Bischoff et al., 1999; Kornau, 2006; Pinard et al., 2010; Waldmeier et al., 2008). Human B1a and B1b isoforms appear to follow this same pattern. Mammalian cells transfected with human B1a and B1b show cerebellar expression on granule and Purkinje cells, respectively (Kaupmann et al., 1998). Biermann et al. (2010) demonstrated that differential localization of the receptor isoforms is dependent upon the presence and amino acid sequence of the sushi domain. B1a receptors track to axonal locations, but when the amino acid sequence of the sushi domain is changed or deleted, axonal location is abolished. Further, inserting the sushi domain on B1b or mGluR1a, which respectively tend to have dendritic or somatodendritic locations, also causes them to move to axons. One notable exception to the pre- and postsynaptic classification of B1a and B1b

isoforms is the medial habenula, wherein B1b receptors appear to be presynaptically located on afferents to the interpeduncular nucleus (Bischoff et al., 1999). Exceptions such as the medial habenula highlight the importance of discerning between the roles of pre- versus postsynaptic and B1a versus B1b receptors and refraining from using such terms interchangeably.

Although both isoforms are prevalent across the central nervous system, the ratio of B1a to B1b varies based on cell type and anatomical structure. Many brain areas express similar levels of B1a and B1b mRNA, including the amygdaloid nuclei and substructures of the hippocampus. However, GABAergic cerebellar Purkinje cells express much higher mRNA levels of B1b than of B1a, whereas glutamatergic granular cells of the cerebellum display higher levels of B1a and negligible levels of B1b mRNA (Bischoff et al., 1999; Liang et al., 2000). This is not surprising, as B1a receptors tend to be presynaptically colocalized with mGluR2s (Kornau, 2006; Pinard et al., 2010; Ulrich & Bettler, 2007), and granular cells are the major excitatory input of the hippocampus and cerebellum. Conversely, Purkinje neurons are GABAergic and receive input from glutamatergic parallel fibers that originate in granular cells. Other anatomical areas of discrepancy include higher levels of B1a mRNA in the lateral nuclei of the amygdala, ventromedial hypothalamic nuclei, and all areas of the midbrain, including the ventral tegmental area (VTA). Higher B1b mRNA levels are notably expressed in the nucleus accumbens, ventral pallidum, and most areas of the thalamus (Bischoff et al., 1999). Although protein levels of B1a and B1b have not yet been investigated in such specific brain regions, Benke et al. (1999) investigated crude membrane protein levels of B1a and B1b in general brain regions. They found that whereas B1a and B1b protein expression is similar in the hippocampus, B1a shows greater expression in the

olfactory bulb and striatum, and B1b shows greater expression in the cerebral cortex, thalamus, cerebellum, and medulla.

2.3 In vitro *receptor roles*

At the cellular level, activation of pre- and postsynaptic $GABA_BRs$ leads to differing downstream effects. Kornau (2006) summarizes these effects as the following: presynaptically, receptors inhibit neurotransmitter release by inhibiting Ca^{++} channels via the β/γ subunits of the G protein. An emerging role of inhibited exocytosis following calcineurin release in response to increased cytoplasmic calcium levels has also been identified (McClure-Begley et al., 2014). Secondarily, at glutamatergic terminals, presynaptic receptors may retard synaptic vesicle recruitment following sustained activity of the system via the adenylate cyclase cascade activated by the i/oα subunit of the G protein. Postsynaptically, the β/γ subunits activate potassium class 3 (Kir3) channels, causing slow inhibitory postsynaptic currents (IPSC) and inducing long term potentiation at glutamatergic sites (Kornau, 2006).

Some of these effects have been directly linked to B1a or B1b receptor isoforms. Vigot et al. (2006) demonstrated that hippocampal B1b is involved in Kir3 channel modulated postsynaptic inhibition. Conversely, B1a located on dendritic branches inhibits cortical neurons via Ca^{++} channels, independent of the typical postsynaptic Kir3 channel mechanism (Perez-Garci et al., 2006). B1b also modulates postsynaptic inhibition of CA1 hippocampal cells in B1b knockout mice. B1b knockout, but not wild type or B1a knock out mice, show reduced baclofen-induced Kir3 currents (Vigot et al. 2006). B1a receptors control glutamate release in fibers projecting from the cortex to the thalamus (Ulrich & Bettler, 2007), and are mostly localized on glutamatergic terminals in the hippocampus (Shaban et al., 2006; Vigot et al., 2006). Further, B1a knockout mice show impaired excitatory

postsynaptic current (EPSC) amplitude reduction following activation of $GABA_B$ heteroreceptors, indicating impairment in regulating presynaptic inhibition. Both isoforms have been shown to act as autoreceptors, as baclofen reduces IPSC amplitude in both isoform knockouts to a similar extent (Vigot et al., 2006). On a larger systems level, Hannan et al. (2012) suggest that the stability of the B1a receptor at presynaptic locations may act as an inhibitory brake, reducing excessive releases of glutamate. B1a receptors have been shown to gate glutamatergic inputs to excitatory neurons of the amygdala (Pan et al., 2009), and that presynaptic receptor activation from cortical afferents is important for LTP induction in lateral amygdala principal neurons (Lange et al., 2014). The audiocortex to basolateral amygdala (BLA) pathway is also modulated by presynaptic $GABA_B$Rs (Cho et al., 2013). Conversely, the relative instability of the B1b receptor at postsynaptic locations may contribute to NMDA-mediated postsynaptic plasticity and in very severe conditions may lead to neurotoxicity.

It is greatly important to note that the roles of each receptor isoform are not constant, even within specific parameters. The roles of B1a and B1b may change based on structural location of the receptor, such as B1b modulating postsynaptic depression in the hippocampus, but B1a modulating postsynaptic depression in cortical neurons via different mechanisms (Perez-Garci et al., 2006; Vigot et al., 2006). Receptor roles, with and without discrimination between isoforms, have been shown to change even at the cellular level during pharmacological manipulation (Cruz et al., 2004; Labouebe et al., 2007; Michaeli & Yaka, 2010). These considerations reinforce the need for a site-specific method of isoform knockdown to investigate how the roles of B1a and B1b change across different brain structures (see section 3.3).

3. Identifying and modeling $GABA_B$ receptor isoforms

3.1 Indirect and non-causal isoform classification

As discussed in section 1.2, B1a/B2 receptors tend to locate presynaptically, whereas B1b/B2 receptors tend to locate postsynaptically. Therefore, some studies may infer whether a receptor is B1a or B1b by its electrophysiological properties. One commonly used technique is whole-cell patch clamp electrophysiology, which allows for stable intracellular recording to study the activity of ion channels within the membrane of pre- and postsynaptic cells. Vigot et al. (2006) used whole-cell patch clamp to demonstrate that B1a knockout mice hippocampal cells show robustly impaired inhibition of EPSC amplitude in the presence of baclofen, whereas B1b knockout mice hippocampal cells show a 60% reduction in outward current of IPSCs mediated by activating Kir3 channels. These results were specific to application of baclofen, suggesting that they are specific to $GABA_B$Rs. When not using isoform knockout mice, involvement of $GABA_B$R subunits can be extrapolated as B1a when impaired EPSC amplitude is present, or as B1b when alterations in Kir3-mediated IPSCs are seen (see section 3.2). Changes in pre- and postsynaptic architecture can also be looked at using techniques such as electron microscopy and immunogold labelling to observe changes in cellular structure. Increases in excitatory synapses or dendritic spines may imply a role for B1a or B1b receptors, respectively, as the isoforms are distributed in such a manner across the cell (Terunuma et al., 2014; Vigot et al., 2006). Alterations in B1a and B1b may also be identified in a direct, but non-causal manner via *in situ* hybridization. Site-specific labelled DNA or RNA tissue can be quantified and correlated with behavior or drug treatment (McCarson et al., 2006, 2005; Sands et al. 2004, 2003).

3.2 Direct genetic manipulation

As described in Vigot et al. (2006), the knockout mice were created via multiple intricate steps. BALB/C embryonic stem cells were made more permeable using an electrical field (electroporation), allowing introduction of constructs with mutated codons. The mutated codons selectively prevented the translation of B1a or B1b proteins by changing the initiation codons into stop codons. The BALB/C stem cells were then injected into C57BL/6 (B6) blastocysts to create a founder population. To ensure a BALB/C background for the mutant mice, the founder mice were crossed with BALB/C mice that expressed targeted Cre-recombinase, allowing site-specific recombination for the targeted B1a and B1b genes of interest. The knockout mice were reported to show no overt abnormalities in phenotype compared to the wild type mice. However, how the mice were phenotyped was not reported. mRNA levels for B1a and B1b were normal in both groups of mice, but B1a and B1b did not translate mRNA into B1a or B1b protein, respectively. This loss of translation confirmed that the change of the transcription initiation codon into a stop codon worked. Some apparent compensation occurred, as B1a knockouts showed an increase in B1b protein, and B1b knockouts showed an increase in B1a protein (129% and 115% of wild-type, respectively).

3.3 Considerations of identifying and modeling receptor forms

Currently, the only "direct" way of implicating B1a or B1b isoform subunits in behavior is by using isoform knockout mice. Other indirect, or correlational, methods exist, including electrophysiological recordings and *in situ* hybridization. Methods of directly and indirectly targeting the $GABA_B$ isoforms have both pros and cons. For indirect methods, the primary concern is that changes to the system are looked at secondary to behavior, making the isoform data correlative to the behavior. However, this also offers strength over the knockout methodology. Looking at the system secondary to behavior removes compensatory

mechanisms and alterations in development that may be present in knockout models. Contrary to *in situ* hybridization, electrophysiological, electron microscopy, and immunogold labelling methods do not directly identify the isoform. Whether it is B1a or B1b has to be inferred by identifying whether the receptor is located at the axon terminal or on the dendritic spine.

Use of genetic knockouts offers the ability to infer causation of behavioral changes via alterations in the $GABA_BR$ isoforms. Further, the cellular characteristics of these models have been well characterized and support the use of indirect models to identify the isoforms involved in behavior (Shaban et al., 2006; Vigot et al., 2006). As described in section 2.2, these models are not conditional knockouts, and the different isoforms are known to be involved in development. Fritschy et al. (1999) demonstrated that, relative to B1b protein expression levels in the adult brain, Sprague Dawley rats show double the amount of B1a from post-natal days (PND) 0-10. Expression levels then drop to around 50% of B1b levels at PND 21 and adulthood. Conversely, B1b remains at 50% of its adult levels at PND 0 and 5 then peaks at 150% at PND 10 and 21 before normalizing in adulthood. However, mRNA levels of B1a have been shown to be higher in adult brain (Bischoff et al., 1999; Liang et al., 2000). These levels suggest that B1a plays an important role in development before weanling age, whereas B1b becomes the predominant isoform at and after the time of weaning. The higher B1a mRNA levels during adulthood may indicate that, following weaning, B1a becomes important for interactions with other proteins that may further the development of the brain.

Behaviorally, the presence of B1a during development plays a role in stress resilience (O'Leary et al., 2014). Although Vigot et al. (2006) report no aberrant behavior between the

knockouts and the wild types, the types of behaviors monitored were not reported and this does not negate the possibility of developmental alterations, specifically system compensation for global knockout of each isoform. As previously mentioned, global knockout of the B1a and B1b isoforms resulted in upregulation of respective B1b and B1a protein levels compared to the wild type. However, Vigot et al. (2006) suggest a lack of compensation by the $GABA_B$ system, as baclofen binding is at half maximal levels in each of the knockouts compared to the wild type. This does not rule out compensation by other systems, specifically mGluRs which are often colocalized with B1a receptors (Kornau, 2006), or the fast-acting $GABA_A$ receptor system. Other experiments have demonstrated that direct manipulation outside of the $GABA_BR$ system directly alters function of $GABA_BRs$ and behavior. For example, knockout of semialdehyde dehydrogenase, which blocks downstream breakdown of GABA, causes epileptic seizures (Vardya et al., 2010). Replacement of serine 783 for an alanine residue reduces B2 receptor expression and leads to reduced contextual fear and impaired memory in the Barnes Maze task (Terunuma et al, 2014). Finally, knocking out B1 receptors specifically on orexin neurons alters sleep/wake patterns (Matsuki et al., 2009). Although these manipulations do not directly interfere with isoform expression, the authors suggest that part of the behavioral profile is due to alterations in postsynaptic $GABA_BR$ activity (Matsuki et al., 2009; Terunuma et al., 2014; Vardya et al., 2010). Therefore, if alterations in other systems affect the $GABA_BR$ system, it is not inconceivable to think that isoform knockout as described by Vigot et al. (2006) is affecting systems outside of the $GABA_BRs$.

Age is also an important factor to consider. It is not clear at what age point these analyses were done; Vigot et al. (2006) conducted most non-behavioral procedures before

adulthood; therefore, these results may be different or indicate compensational or developmental differences at alternative time points. The developmental role of B1 isoforms also presents a problem for electrophysiological methods. Often-times, cell recordings are taken from animals that have not yet reached adulthood, including many of the studies reviewed herein. As B1 isoform expression changes so rapidly over development, using electrophysiological data taken from young animals to support behavioral differences seen in adults may be confounded. An alternative direct method of isoform knock down is using a viral method, which is conditional and therefore avoids many pitfalls of the knockouts. In contrast to global knockouts, this method is brain-region specific which is often of interest in behavioral tasks (Cho et al., 2013; Shaban et al., 2006; Vigot et al., 2006).

In short, the genetic knockout isoform model is the most widely used across the studies discussed herein. However, this model may suffer from system compensation, loss of isoform-specific developmental roles, inability to make site-specific conclusions, and application of electrophysiological work done during adolescence to explain behavior in adulthood. To fully support the conclusions of the behavioral work in the next few sections, other methods of isoform identification must also be employed. These include *in situ* hybridization to understand how basic behavior alters isoform expression, and site-specific knockdown to understand the isoform-specific circuitry of these behaviors.

4. Existing *in vivo* work

4.1 Cognition: Learning and memory

4.1.1 Hippocampus-Based Tasks

Table 1 indicates a long list of cognitive tasks that have been employed to identify the role of each isoform in learning and memory. Novel object recognition, familiar object

recognition, and y-maze spontaneous alterations are considered to be hippocampal-based learning tasks. B1a and B1b are evenly distributed across the different hippocampal formations (Bischoff et al., 1999), however, B1a and B1b are more likely to be located on pyramidal and granular cells, respectively (Liang et al., 2000). This different cellular distribution may indicate different roles in development of memory and learning.

Novel object recognition tasks introduce an animal to two objects in the training phase, then replace one familiar object with a novel object. The expected behavior is for the animal to remember the familiar object, and therefore spend more time exploring the novel object. In a one-time novel object recognition task, Vigot et al. (2006) demonstrated no effect of genotype or time on object discrimination index between time-point 0 (initial object presentation) and reintroduction 24 hours later, indicating that isoform knockouts and wild type mice approached the object similarly at initial presentation and presentation 24 hours later. However, Jacobson et al. (2007a) showed that wild types, but not B1b knockouts, investigate the familiar object less 24 hours following initial presentation. In both experiments, only B1a knockouts showed no discrimination between a novel and a familiar object, and did not show recognition of the familiar object 10 minutes following the first presentation (Table 1). Vigot et al. (2006) attribute this loss of discrimination to reduced EPSPs and loss of GABAergic-mediated LTP in the hippocampus of B1a knockout mice. Again, these electrophysiological recordings were taken from adolescent brain slices, although the behavior was measured in adulthood. Electrophysiological recordings in adult brains may not demonstrate reduced EPSPs due to system compensation. If such were the case, Vigot et al.'s (2006) interpretation would not be supported. Cullen et al. (2014) also used an object discrimination task comparing only wild type and B1a knockout mice, but

included a longer period of training than one 3-minute session. Mice were given three daily 10-minute sessions with two identical glass beakers during which both genotypes explored each object to similar extents. Two and 24 hours following training, mice were given a 3-minute test session with a novel and familiar object. At two hours, both wild type and B1a knockout mice investigated the novel object more, but at 24 hours, the B1a knockouts failed to discriminate between the familiar and novel object. Immediately following the novel object tests, both familiar training objects were placed in the arena for a 3-minute session, but one object was placed in a novel location. Again, at two hours, both genotypes preferred the novel location object, but at 24 hours, the B1a knockout mice did not discriminate between the familiar and novel object locations. Cullen et al. (2014) suggest that these data represent the ability of B1a knockout mice to initially consolidate a memory, but an inability to maintain this memory (Table 1). The authors suggest that the primary mechanism of this memory decay may be the loss of B1a receptor-mediated inhibition in the hippocampus, causing over-excitation of the CA3 region, leading to a decline in memory maintenance (Figure 1).

The y-maze task is indicative of spatial working memory by measuring how apt an animal is at visiting unfamiliar arms of the maze. Correct alterations are counted when the mouse travels to each new arm of the maze in succession without visiting a familiar arm. Both knockout groups showed a decreased percentage of correct alterations in the y-maze compared to the wild type group, with only the B1b knockouts displaying increased locomotion (Jacobson et al., 2007a) (Table 1). Although locomotor activity was not quantified by Vigot et al. (2006), the number of initial stretch attend postures (SAPs) towards the object at time-point 0 was the same across all genotypes. However, in the Jacobson et al.

(2007a) study, B1b mice showed higher levels of SAPs towards the objects than other genotypes, suggesting an alteration in locomotion during both object recognition and the y-maze task. Object recognition does not inherently account for basal locomotion and it may be expected that increased locomotion would cause a general increase in exploratory behavior. This could explain the lack of "object recognition" in the B1b mice at 24 hours when only one object is available to explore if the B1b mice are more active in the apparatus. Although B1b knockout mice showed overall increased locomotion, the y-maze is less susceptible to differences in basal locomotion because the animal is simply asked to choose the correct arm over the course of one trial (Jacobson et al., 2007a).

These results suggest that B1a knockout mice display reductions in recognition of familiar and novel objects, whereas both B1a and B1b knockouts display reductions in spatial memory. The hippocampus has been implicated in both short-term and long-term object recognition. Specifically, the role of NMDA receptors in object memory via LTP is well recognized (Warburton et al., 2013). As B1a receptors colocalize with glutamatergic receptors and B1a knockouts also show a loss of LTP in the hippocampus (Vigot et al., 2006), it is reasonable to hypothesize that object memory is dependent upon synchrony of glutamatergic- and GABAergic-mediated LTP (Figure 1). Therefore, as demonstrated by the current studies, loss of object recognition should be present in B1a, but not B1b, knockout mice. Conversely, Jacobson et al. (2007a) suggest that both isoforms are involved in hippocampal spatial memory. Spatial memory is comprised of many different elements including place, route, item, and temporal organization coding (Eichenbaum & Cohen, 2014). The y-maze task used by Jacobson et al. (2007a) presents only one task of spatial memory, and a simple one at that. More complicated spatial tasks, such as the Barnes maze or radial

arm maze, may show isoform-specific disruptions in spatial memory that would allow for a greater understanding of how each isoform works at a circuitry level in spatial memory development. Further, as these studies employed the use of global knockouts, it is important to consider that brain regions outside of the hippocampus are involved in these results. Site-specificity could be directly investigated in the knockout animals. Other receptor systems could be site-specifically antagonized via microinjection prior to the behavioral tasks. For example, glutamate antagonists could be microinjected into the CA3 region of the hippocampus to investigate whether this antagonism reverses the impairments in memory retention observed by Cullen et al. (2014) in the B1a knockout mice. Conversely, the start codon for each isoform could be site-specifically knocked-in, to see if reintroduction of the receptor reversed behavior seen in the isoform knockouts. However, the preferred method would be a viral knockdown that is administered in adulthood. This method would be informative about the necessity and specificity of specific brain regions in behavior while also avoiding long-term developmental considerations of isoform knockout. It would also allow for isoform knockdown in multiple breeds, allowing for site-specificity to be investigated in lines which the behavior of interest is well characterized in or selectively bred for.

4.1.2 Amygdala-based tasks

In contrast to object and spatial memory, amygdala-dependent tasks, which require overt pairing of an aversive stimulus with an appetitive substance or cue, showed impairments in both knockout genotypes, whereas context learning is unaffected. Such tasks include conditioned taste aversion (CTA) where an appetitive substance, such as saccharin, is devalued through behavioral measures, such as pairing saccharin access with a shock, or via

appetitive measures, such as pairing it with lithium chloride to induce sickness (LiCl). Following devaluation, the reinforcing substance can be given in a period of extinction, where the aversive stimulus is not present, to measure how long the animal avoids the once appetitive reinforcer. Jacobson et al. (2006a) showed that, following one pairing with LiCl, B1a animals failed to acquire a CTA to saccharin solution, although 100% of the B1a animals showed sickness following the LiCl administration. BALB/C wild type animals showed extinction of CTA behavior on the 5th day of extinction testing. However, B1b knockouts remained at an aversion index of around 80% for the remainder of saccharin testing. Aversion persisted during a retest that took place a week after the end of extinction testing. A perseveration test was performed a week after the retest. The B1b animals continued to avoid the saccharin solution during the perseveration test, indicating that it was the association between the sickness and the taste, not the sickness and bottle location, which was persisting. CTA extinction was tested out to 4 weeks after the initial saccharin and LiCl pairing, at which point B1b animals still failed to extinguish saccharin aversion (Table 1). All tests were run in genotypes that were not given LiCl, and there were no specific genotypic effects on saccharin consumption. At all tests, total fluid consumption was comprised of about 80% saccharin intake. The CTA task employed by Jacobson et al. (2006a) represents an unconventional form of extinction for two reasons. The LiCl injection and extinction testing took place in the same context as the initial preference testing. CTA extinction testing assesses an animal's ability to increase responding to reacquire a previous behavior, whereas most extinction paradigms assess whether an animal begins to inhibit responding. Initial learning and extinction learning are also thought to be dependent on different processes. It has been shown that the central nucleus of the amygdala is involved in learning the

reinforcer-sickness association, but that the BLA is necessary for extinguishing CTA as well as fear associations (Bahar et al., 2004). This may suggest that B1a and B1b containing receptors are differentially controlling processes of CTA. Although the basolateral and central nucleus of the amygdala have similar levels of B1a and B1b mRNA expression (Bischoff et al., 1999), they have different afferent and efferent projections (Zorrilla & Koob, 2013). The predominantly GABAergic central nucleus is the major output of the amygdala, and projects to the lateral hypothalamus and brainstem, among other areas. The predominately glutamatergic BLA feeds forward to the central nucleus and is reciprocally connected to the hippocampus. A majority of the amygdaloid projections to the ventral striatum, specifically the nucleus accumbens, originate in the BLA. Both the CeA and BLA are involved in reinforcement (Figure 2). Multiple studies have shown that reductions in dopaminergic excitation in the central amygdala reduce intake of reinforcers. Conversely, altering protein synthesis in the BLA prevents cue-induced reinstatement and maintenance of conditioned place preference, while inactivation of the BLA abolishes cue-induced reinstatement (Duvarci & Pare, 2014; Zorrilla & Koob, 2013). In light of the differing roles of the amygdala, Jacobson et al.'s (2006a) results could be interpreted as an indication that B1a and B1b receptors are working differently within the amygdala to regulate CTA. Loss of B1a receptors in the central amygdala could be reducing inhibition of excitatory output, resulting in a loss of control over reinforcer intake even following an aversive pairing (Figure 2A). Yet loss of postsynaptic B1b receptor inhibition in the BLA may contribute to the animal's inability to learn that it is safe to consume the saccharin solution. Although ablation of the amygdala has been shown to abolish fear behavior (Zorrilla & Koob, 2013), loss of B1b receptor inhibition may lead to over-activity of the amygdala by reduced control of

excitatory output, thereby heightening fear levels and inhibiting the animal from sampling the saccharin solution (Figure 2B).

Short-term fear conditioning is also impaired in isoform knockout mice. Shaban et al. (2006) demonstrated that, when a 0.9 mA foot shock was given, B1a mice were unable to distinguish between two conditioned stimuli (CS). A CS^- tone indicated safety from shock and a CS^+ tone indicated shock, but B1a knockout mice showed the same amount of freezing during each tone without basal differences in locomotion or freezing behavior. However, the B1a mice had no differences in shock threshold compared to the wild type mice and learned the safety cue when a lower intensity shock (0.6 mA) was given. In contrast to B1a mice, B1b mice were completely unable to learn an association between the CS^+ and foot shock, although their threshold for foot shock-induced movements or vocalization was not different than wild types (Table 1).

A longer contextual conditioned fear study was conducted in only B1a knockout and wild type mice (Cullen et al., 2014). Mice were given five 1-second shocks (0.8 mA) in context A and freezing behavior was assessed. Mice were then tested for freezing behavior in Context A, or a new Context B, 2 hours, 24 hours, or 5 days following the training session. B1a and wild type mice show similar levels of freezing in Context A during training and at all subsequent test points. In Context B, B1a knockout mice show low levels of freezing similar to that of wild type mice at the 2 hour post-training point. However, at 24 hours and 5 days post-training, the B1a knockout mice show increased freezing in Context B (Table 1). Taken with the findings of Jacobson et al. (2006a), the results of Vigot et al. (2006) may show that B1b mice are able to form implicit associations between a reflexive response to

LiCl and an appetitive substance, but unable to form an overt cognitive association between a tone and a foot shock.

Cullen et al. (2014) interpret their results as showing that B1a knockout mice are initially able to consolidate the contextual memory, but are unable to maintain that memory over longer periods of time. These results are in opposition to the interpretation of Shaban et al. (2006), who attribute their short-term fear conditioning results to loss of glutamatergic-related LTP in the amygdala of B1a knockout mice. Shaban et al. (2006) attribute their results to an inability of B1a knockout mice to make a tone-shock association. However data from Cullen et al. (2014) indicate that at 24-hours post-training, the B1a knockout mice are showing impaired contextual fear that is not present in the wild type animals. As Shaban et al. (2006) trained their animals in Context A, but tested them in Context B 24 hours later, this may indicate that freezing behavior in their mice may also be due to impaired contextual association. If re-tested in Context A 24-hours later, it is possible that the B1a mice would not have shown impaired tone-associations. Shaban et al. (2006) attribute behavioral findings in adult mice to neurological changes observed in adolescent brains. This explanation is broad, overlooks the developmental role of the isoforms, and neglects to examine the behavior of the B1b knockouts. An alternate interpretation for both Shaban et al. (2006) and Cullen et al. (2014) is that the B1a knockouts are showing greater stimulus generalization than the wild type mice. Shaban et al. (2006) suggest that stimulus generalization may be responsible for their results. Cullen et al. (2014) did not find immediate stimulus generalization at 2 hours and 24 hours post-training, although they also used one training session. However, during the day 5 retention test, both genotypes show more freezing in Context B, but the B1a knockouts show significantly more than the wild

types. This may indicate B1a knockouts are less susceptible to contextual-cue generalization, but that this generalization still develops more rapidly than in wild type mice.

Failure to learn the tone-shock association in the B1b knockouts may be due to the necessity of the lateral to central amygdala pathway in forming CS associations (Duvarci & Pare, 2014). As the central amygdala is comprised of primarily GABAergic neurons, loss of postsynaptic B1b receptors to regulate the information being transmitted by the lateral amygdala may cause the loss of tone-shock association, thereby explaining the lack of freezing in response to the CS^{+} tone in the B1b knockout mice (Figure 3B). The mechanisms controlling non-discriminate B1a freezing may arise in the lateral amygdala. At a cellular level, tone-fear associations are more readily induced when GABAergic inhibition is reduced in the lateral amygdala (Duvarci & Pare, 2014). Loss of presynaptic B1a-mediated inhibition in the lateral amygdala may indiscriminately increase tone-shock associations at the higher shock level in B1a knockout mice. As B1b receptors are still present in the downstream central amygdala of B1a knockouts, this increase in tone-shock association may lead to over-expression of freezing behavior (Figure 3A). A secondary explanation would be that tone discrimination and tone-shock pairing takes part in different areas of the brain. Although Shaban et al. (2006) observe differences in cellular function in the amygdala of B1a and B1b knockout mice, these differences do not provide causality. An amygdala-focused explanation diminishes the role of brain regions known to be associated with the amygdala. Reciprocal connections exist between areas of the amygdala and the prefrontal cortex, hippocampus, ventral striatum, and the brain stem, which play a major role in behavioral "fear output" (Duvarci & Pare, 2014; Zorrilla & Koob, 2013). Loss of tone discrimination within the B1a knockouts could result from aberrant connections between the auditory cortex, which initially

processes the tone cue, and the amygdala. Behavior of the B1b mice may represent an atypical connection between the amygdala and the brainstem, wherein the amygdala fails to process the tone-shock association, thereby failing to produce a behavioral freezing response (Figure 3B). As discussed in section 4.1.1, these areas could be site-specifically targeted via receptor antagonism or isoform knock-in in the global isoform knockouts, or viral isoform knockdown in adult mice. These site-specific studies are necessary to investigate the way B1 isoforms are involved in circuitry of these behaviors.

Neither knockout genotype shows impairments in spatial association with a foot shock in the one-trial passive avoidance learning task. Jacobson et al. (2007a) placed animals in the light side of a light-dark box. Once animals traveled so far into the dark side, they were given one 0.5 mA foot shock until they returned to the light side, for a maximum of 5 seconds. Twenty-four hours later, a retention test was given, where latency to enter the dark side of the box was tested. All genotypes showed an initial short latency to enter the dark box on the training trial and vocalized when receiving the shock. All genotypes learned the association of the dark side with the shock, showing a longer latency to enter the dark side on the retention trial (Table 1). Interestingly, the authors note that passive-avoidance learning is completely abolished in global B1 knockouts (see Schuler et al., 2001), indicating that a functional $GABA_B$ heterodimer is necessary for learning in this task, but learning is not specific to either of the receptor isoforms. Global knockout of the B2 subunit also abolishes passive avoidance learning (Gassman et al., 2004). This indicates a necessity of $GABA_B$Rs, but a nonspecific isoform role, in passive avoidance learning. This may indicate that $GABA_B$ autoreceptor function maintains this behavior, as both isoforms act as autoreceptors and presence of only one isoform is necessary to maintain behavior.

Most notably, all cognitive tasks were impaired apart from passive avoidance learning. This impairment was overwhelmingly a result of loss of the B1a receptor, except for spontaneous alteration and conditioned fear learning, which were respectively impaired and abolished in B1b mice (Jacobson et al., 2007a; Shaban et al., 2006). Many of the cognitive tasks used were relatively simple. Familiar object recognition, novel object recognition, and spontaneous alteration tasks simply consist of introducing the animal to the context and quantifying exploration over one session (Jacobson et al., 2007a; Vigot et al., 2006). Only CTA and Cullen et al.'s (2014) contextual fear task represent long-term behavioral assays with repeated testing sessions. Even within the short cognitive tasks reviewed within this section, no information was given on within session learning. As B1a animals failed to learn CTA, it is unclear how B1a is involved in the CTA extinction process, although it may be assumed that it plays a complementary role to B1b. Cullen et al. (2014) would suggest that B1a is necessary in maintaining memory by providing inhibitory input to neural structures involved in memory tasks. Although Cullen et al. (2014) examined long-term contextual memory, extinction should also be monitored in more traditional tone + shock associated tasks. A significant amount is known about how the amygdala and connected regions regulate consolidation, extinction, and reconsolidation of auditory-cued fear (Duvarci & Pare, 2014), which may allow for more complete interpretation of the roles of each receptor across a learning assay. Further, it would be of interest to examine the genotypes in long-term retention tasks, such as the radial arm maze or Barnes maze, to observe if memory beyond 24 hours is affected in B1b mice as compared to wild type, or if there are impairments in short-term memory load. As B1b knockout mice show increased levels of immature, proliferating, and newly matured cells in the hippocampus compared to

wild type mice (O'Leary et al., 2014), it is important to test B1b knockout mice in long-term cognitive tasks to observe if their performance is similar or enhanced compared to wild type animals.

4.2 Stress, Depression, and Anxiety

Tables 2-4 detail the role of B1 isoforms on tests of unconditioned anxiety (Jacobson et al., 2007b) and anxiety following stress (O'Leary et al., 2014). O'Leary and colleagues employed two different forms of stress. The first was social stress, in which adult animals underwent 10 consecutive days of social defeat. The second was maternal separation with unpredictable stress (MSUS), in which dams were separated from their pups by a Plexiglas wall in the cage for 3 hours a day from PNDs 1-14. Separation happened at different times each day during the light cycle or early dark cycle. During separation the dam was stressed by 6 minutes of a forced swim task or 20 minutes of plastic tube restraint stress. Non-stressed pups and dams were left undisturbed in their cages during the stress sessions. At weaning on PND21, the pups were group housed with pups from other litters to avoid maternal litter effects and behaviors were tested in adulthood. O'Leary et al. (2014) chose the MSUS model to attempt to invoke depressive and anxiogenic phenotypes in adult mice, which they note is hard to achieve in mice following stress paradigms, and that much of their own work using this model has been done in rats. Previous studies have shown sex and genotype-specific effects of MSUS in B6 mice (Kundakovic et al., 2013; Weiss et al., 2011), which will be discussed alongside the results of O'Leary et al. (2014). The exact MSUS paradigm used by O'Leary et al. (2014) has not been characterized in BALB/C mice, which are the background strain of the isoform knockouts. This makes it difficult to compare results of their studies to the previous literature. Following MSUS, all animals underwent the same battery of tests in

the following order: ultrasonic vocalizations, stress-induced hypothermia, open field locomotor test, tail suspension test, elevated plus maze, saccharin preference (females only), female urine sniffing test (males only), and the forced swim test. Apart from ultrasonic vocalizations, all behaviors were conducted in adulthood and were separated by a one-week period.

4.2.1 Null Results

Isoform knockout confers no effects on a variety of anxiety assays, including stress-induced hyperthermia with or without the presence of a previous stressor, the staircase test, the marble burying test, and the elevated plus maze with or without a previous stressor (Jacobson et al., 2007b; O'Leary et al., 2014) (Table 2). These tasks represent a range of anxiety behaviors. Stress-induced hypothermia is independent of locomotion, whereas the staircase test characterizes locomotor versus anxiolytic responses. Increased locomotion can be characterized in the elevated plus maze by observing number of open arm entries, but passive avoidance can also be observed in a mouse that enters a dark arm and remains there. Finally, marble burying acts as a quantifier of active anxiety behavior by observing defensive burying response. Interestingly, global B1 knockouts display less investigative behavior and more freezing in the staircase test (Mombereau et al, 2004). As O'Leary et al. (2014) show no effects of individual isoform knockouts in the staircase task (Table 2), this indicates a necessary, but non-specific role of $GABA_BR$ inhibition in regulating behavior in that task. O'Leary et al. (2014) found no differences between the controls and MSUS wild type animals in the elevated plus maze (Table 2), although using the same MSUS paradigm Weiss et al. (2011) found that stress reduced anxiety in males in females in the elevated plus maze. This may be due to O'Leary et al.'s (2014) controls being BALB/C mice, whereas Weiss et

al. (2011) used B6 mice. Differences in behavior between these strains have been observed following maternal stress (Kundakovic et al., 2013). Lack of MSUS effects on anxiety behavior in the elevated plus maze may also be indicative of the sensitive nature of MSUS paradigms in mice (O'Leary et al., 2014). Alternative stress paradigms may be more appropriate for inducing behavior in BALB/C mice.

4.2.2. Non-Stress-Dependent Results

A few behavioral tasks exhibited non-stress-dependent results. These included activity in the light/dark box, elevated zero maze, maternal care during MSUS, and the tail suspension test following MSUS (Table 3). Wang et al. (2011) have previously shown that maternal separation without stress does not alter activity of BALB/C mice in the light/dark box, indicating that only an isoform- and/or sex-dependent result would likely be present in this task. Both the light/dark box and elevated zero maze compare the amount of time spent in the light versus dark areas of the apparatus to assess levels of anxiety. Basal anxiety differences were relegated to females, although anxiety levels were bidirectional in the female B1b knockouts, which showed reduced anxiety in the light/dark box but increased anxiety in the elevated zero maze without being subjected to a previous stressor (Table 3). The bidirectional nature of results in the females is unexpected, as these two tasks are considered to test similar behavioral constructs aimed at the internal conflict of exploring a novel environment versus escaping an open area (Razafsha et al., 2013). Male B1b mice made fewer transitions between the light and dark compartments, but unlike the female B1b knockouts, there was no change in total time spent in the light side (Table 3). This indicates that male B1b mice may show reduced passive anxiety; each time they enter the light side of the box they spent more time in it (Jacobson et al., 2007b).

In the elevated zero maze, mice were initially placed into a dark quadrant of the maze. Male B1a and B1b isoform knockouts showed no alterations in anxiety, but this may have been due to high basal levels of anxiety in the wild type males. Female B1b mice showed an increased latency to enter a light quadrant and spent significantly less time in the light quadrants and made significantly less transitions between quadrants (Jacobson et al., 2007b) (Table 3). However, non-stressed female B1b knockouts show increased open field locomotion (O'Leary et al., 2014) (see Table 4). Results of the light/dark box and elevated zero maze interpreted with high basal activity in mind may indicate that female B1b mice actually show an increase in anxiety in both tasks, demonstrated by the latency times to enter the dark side of the light/dark box and a light quadrant of the zero maze. Such an interpretation removes the conflict of results in these similar tasks. Given their increased basal locomotor levels in the open field, it would be expected that female B1b animals would show a reduced latency in both tasks. Exploratory behavior inhibition and freezing when introduced into a new environment are often used to quantify anxiety (Palanza, 2001), and both of these behaviors appear to be occurring in female B1b mice during the light/dark box and zero maze assay, indicating that presence of B1b in females is protective in these two assays. Although latency to move was not measured, increases in total immobility are also seen during the forced swim task for all female B1b knockouts, possibly indicating an anxious response (O'Leary et al., 2014) (see Table 4). However, there are no effects of female B1b knockout on immobility in the tail suspension test (O'Leary et al., 2014), potentially suggesting that these two tasks designed to measure behavioral "despair" are intrinsically different than simple anxiety tasks, as they take place in an inescapable situation (Razafsha et al., 2013). Unfortunately, other tasks by Jacobson et al., (2007b) did not include

latencies in their analyses. Therefore, we are unable to speak to whether female B1b animals show this exploratory and freezing inhibition in other anxiety or non-anxiety tasks. Freezing activity could easily be looked at in the elevated plus maze, Barnes maze, and in the fear tasks described in section 4.1, which did not use female mice. Further, freezing time following placement in the apparatus could be measured in all of these tasks by a trained observer or photobeam locomotor activity apparatuses. Looking at various specific behaviors within all of these tasks, including latency to transition, transitions made, total time moving, etc. is important in parsing out the known sex differences that are part of anxiety behavior. It is currently unknown what specific parameters of anxiety that sex affects, primarily because of the lack of preclinical research done in females. Organizational and activational effects of steroid hormones are often implicated in the different prevalence and expression of depressive and anxiety behaviors across males and females, and many stress paradigms that induce depressive or anxiogenic phenotypes in males are unreliable in females (Palanza, 2001). Although it is commendable that Jacobson et al. (2007b) and O'Leary et al. (2014) included both male and female isoform knockouts, it difficult to begin to interpret sex-dependent isoform differences in these tasks because basic sex-dependent differences have not been fully identified.

Maternal care and the tail suspension test, which were done during or after MSUS, did not show stress-dependent effects on behavior, although some isoform- and sex-specific effects existed. Dams of B1a knockout pups showed higher maternal care on PND7 regardless of whether pups and dams endured MSUS (O'Leary et al., 2014) (Table 3). However, this did not confer a protective factor against stress effects on the pups (see Table 4). The tail suspension test, along with the forced swim task (see Table 4), measure learned

helplessness as the amount of time spent immobile during the task. Learned helplessness, otherwise termed "despair," is considered to be a hallmark of depressive symptomology. Two criticisms of these assays are that they are only acute stressors versus the pervasive, long-term stressors seen in human depression, and that the behavior being modeled is not specific to depression; it may also be an expression of anxiety (Razafsha et al., 2013). In the tail suspension test, loss of B1a increased "despair" in male and female B1a mice, regardless of stress status. However, loss of B1b in male mice decreased "despair" regardless of stress status (O'Leary et al., 2014) (Table 3). Therefore, the isoforms appear to confer a bidirectional effect on behavioral despair in male mice only. The authors caution that decreased immobility in the male B1b knockouts may be due to increased activity seen in the open field. However, non-stressed female B1b knockouts also show this increased open field activity, although they do not display decreased immobility like the male mice do. Further, the same alterations in despair activity are not seen in the forced swim test (see Table 4), although the forced swim test is often used interchangeably with the tail suspension test to measure despair. These results may suggest that despair measures in the isoform knockouts under these paradigms are unreliable, and results of both the tail suspension test and forced swim test should be interpreted with caution for how isoform expression affects behavioral despair. Part of the unreliability in the current study may be due to order effects; the tail suspension test was always the third assay run, whereas the forced swim test was always the sixth assay run. By the time of the sixth behavioral test, the B1a knockouts may have lost their susceptibility for increased immobility that was seen in the tail suspension test. Although counterbalancing the order of tests may get around order effects, it may blunt true isoform differences in initial stress-susceptibility during the final tasks in a set of six

behavioral assays. Order effects have previously been shown by Hohmann et al. (2013) using a modified version of maternal separation from PND2-7 in BALB/C mice, which includes stressing the pups, but not the dam. The authors demonstrated that increases in aggression seen in the stressed pups is present when the resident/intruder task is run first, but it is not present when following an open field task. The strongest way to determine if there are isoform-specific differences in initial stress-susceptibility would be to run task-naïve animals through each behavioral assay. Following this initial assessment of each task, the animals could be run through batteries of other tests to see if initial stress-susceptibility is diminished over exposure to multiple behavioral tasks.

4.2.3 Stress-Dependent Results

Some behavioral assays used by O'Leary et al. (2014) do appear to show stress-dependent results. Apart from the ultrasonic vocalizations before weaning and the open field locomotion, these tasks are considered to model different aspects of depressive phenotypes. Although discussed as a measure of anxiety, O'Leary et al. (2014) did not consider time spent in the middle of the field during their open field test, thereby characterizing it only as a measure of locomotor activity. As previously mentioned, the forced swim test, along with the tail suspension test, is widely used to characterize behavioral "despair" by looking at time spent immobile while placed in water. However, this task also models aspects reported by those suffering from anxiety, including a focus on harm and threats. Social interaction, saccharin preference, and urine sniffing are considered to model anhedonia that is often seen in depressive phenotypes. These tests are not considered to be "inescapable," and therefore do not model "despair." Rather, the animal is given a choice between investigating something that is typically thought of as preferable, or taking no action. Loss of preference

for social interaction, a sweet reinforcer, or female urine sniffing for male mice is considered to model loss of pleasure that characterizes anhedonia (Razafsha et al., 2013).

How isoform knockout affects behavioral "despair" is currently unclear. Considering only the forced swim test, it would appear that B1a and B1b isoform knockout confers a protective factor against despair in all animals apart from the stressed female B1a and stressed male B1b knockouts (O'Leary et al., 2014) (Table 4). However, as discussed in the previous section, results of the forced swim test are hard to interpret for multiple reasons. Firstly, other behavioral assays run by O'Leary et al. (2014) do not fully support this interpretation. Results of the tail suspension test suggest that all B1a knockouts, regardless of stress- or sex-status, should show increased immobility whereas only male B1b knockouts should show decreased immobility in the forced swim test because the forced swim test and tail suspension test are believed to measure the same phenotype. O'Leary et al. (2014), interpret results of the tail suspension test by citing that all male B1b knockout mice show increased open field activity. However, decreased activity in the B1a knockouts and wild type mice compared to B1b knockouts might also be reflected in the forced swim task if it is relevant to the tail suspension task. It is important to keep in mind that the forced swim and tail suspension tests are not interchangeable. There is no change of hypothermia in the tail suspension test, and animals resume normal activity immediately following the assay, suggesting that tail suspension is less stressful or invasive than forced swim (Castagné et al., 2011). Secondly, order effects have been shown when multiple behavioral tasks are run in succession following maternal separation (Hohmann et al., 2013). Therefore, results of the forced swim test could be confounded, as the forced swim test was always run last by O'Leary et al. (2014). Thirdly, whether maternal stress alone causes increased immobility

during forced swim test is debatable (Mehta & Schmauss, 2011; Wang et al., 2011). Finally, Mombereau et al. (2005) have shown that loss of global B1 and B2 receptor expression leads to decreased activity in the forced swim task, therefore loss of activity may be due to loss of B2 receptors that colocalize with B1a and B1b subunits. The conclusion that B1b presence is protective against despair in females, whereas B1a presence is protective against despair in males may not be fully supported.

The effects of stress and isoform knockout on anhedonic behavior are also unclear. O'Leary et al. (2014) generally suggest that their results show that presence of B1a is protective against a depressive phenotype, and B1b leads to susceptibility for a depressive phenotype. However, out of the 15 tasks characterized by Jacobson et al. (2007b) and O'Leary et al. (2014), only six behaviors show a "protective" effect of the loss of B1b irrespective of stress status. One of these tasks is simply increased open field activity (Table 4). Increased activity directly confounds interpretation of the tail suspension test, which is another of the six tasks used to identify a "protective" factor. The remaining four tasks are those that characterize anhedonia. Following social defeat, wild type mice showed reduced social interaction compared to their control counterparts, which has been previously demonstrated in BALB/C mice (Savignac et al., 2011). B1b knockout mice showed the same pattern of reduced social interaction, although non-stressed B1b knockouts showed significantly less interaction than non-stressed wild type mice. Stressed B1b knockout mice did not show different levels of interaction than their non-stressed counterparts. Non-stressed B1b knockouts were also not different from non-stressed wild type mice (Table 4). Saccharin preference following social defeat was decreased in B1a knockouts. This same reduction was seen in females B1a knockouts exposed to MSUS compared to wild type animals exposed to

MSUS (Table 4). Males were not tested for saccharin preference following MSUS (O'Leary et al., 2014). Interestingly, Kundakovic et al. (2013) showed that their version of MSUS increased saccharin preference in female BALB/C mice, but this increase is not seen in the control mice exposed to MSUS by O'Leary et al. (2014), suggesting that this behavior may be very sensitive to slight differences in maternal stress paradigms. In male wild type mice, MSUS equalized preference for water versus female urine. Male B1a knockouts did not show a preference for female urine regardless of stress condition, whereas male B1b knockouts showed a preference for female urine regardless of stress condition, suggesting that their preference is not susceptible to stress-status (O'Leary et al., 2014) (Table 4).

These should be interpreted with caution considering the phenotypes produced by MSUS that have been previously demonstrated (Kundakovic et al., 2013; Weiss et al. 2011). O'Leary et al. (2014) found no differences in open field distance travelled, immobility time in the forced swim test, and saccharin preference between male and female control and MSUS-exposed wild type mice (Table 4). A lack of difference in open field distance travelled and immobility during the forced swim test following simple maternal separation in BALB/C males is supported by Wang et al. (2011). However, Mehta and Schmauss (2011) have shown that simple maternal separation using the same parameters as Wang et al. (2011) does cause increased immobility in male BALB/C mice during the forced swim task. Further, Kundakovic et al. (2013) have demonstrated that sucrose preference is increased in BALB/C males but decreased in females following maternal separation and pup stress. It is important to keep these results in mind, as these behaviors have not been well-investigated in BALB/C mice following MSUS. As previously mentioned, very little is known about what sex-status confers on anxiety and depressive phenotype susceptibility. Further, in regards to maternal

stress models, many of these models are poorly characterized and very little is known about how the model used by O'Leary et al. (2014) affects anxiety and depressive phenotypes in different breeds of male and female mice. Although O'Leary et al. ran a large set of tasks using appropriate stressed and non-stressed wild type controls, these tasks have been previously shown to be susceptible to order effects. As previously discussed, a better design would be to run these studies in sets of behaviorally naïve stressed and non-stressed males and females so that effects of stress-status and B1 isoforms can be better understood.

4.2.4 Neurobiological markers

The role of stress and genotype on corticosterone levels in isoform knockouts has also been investigated. O'Leary et al. (2014) took plasma corticosterone levels from stressed and non-stressed isoform knockouts and wild type mice 30 minutes after the forced swim test within a four-hour period. All male B1b animals showed elevated corticosterone levels, whereas all B1a male knockouts showed reduced corticosterone levels, regardless of stress, compared to wild type mice. There were no stress or isoform effects on corticosterone levels in the female mice. Jacobson et al. (2007b) found no effect of genotype on corticosterone or adrenocorticotropic hormone levels, which they only assessed in experimentally naïve males during a one-hour period. This suggests that markers of HPA-axis activation are increased in response to the behavioral assays in the B1b knockout males, but reduced in B1a knockout males, regardless of stress condition. Activation of the HPA-axis in B1b male mice may contribute to their decreased anxiety profile in many of the behavioral tasks used. However, female B1b knockouts show no differences in corticosterone levels, although they do show a decreased anxiety profile similar to the males (Table 2, 3). This may indicate that males are more susceptible to the stress of behavioral tasks, or that other parameters apart from forced

swim test-induced stress are influencing male isoform knockout corticosterone levels. Alterations in corticosterone levels to stress are highly susceptible to the duration and type of stress used, especially when considering maternal stress, as well as other factors, such as housing conditions, time of corticosterone testing, and other behavioral manipulations (Nishi et al., 2013).

In adulthood, following MSUS, female stressed and non-stressed isoform knockouts were subjected to 2 hours of restraint stress and c-Fos levels were subsequently determined across different brain regions. O'Leary et al. (2014) demonstrated an increase in c-Fos in all B1b knockout mice in the dorsal and ventral dentate gyrus, the CA3 of the hippocampus, the paraventricular nucleus, and the dorsal raphe nucleus compared to wild type and B1a knockouts. Increased neuronal activation in the nucleus accumbens of B1b mice was dependent on the presence of stress. Stress also reduced c-Fos in the wild type dorsal dentate gyrus compared to their non-stressed counterparts. Non-stressed B1a knockout mice showed reduced c-Fos compared to wild type in the CA3 and the VTA. There were no differences in regions of the cortex or amygdala. The c-Fos results suggest that the genotypes show complex patterns of neuronal activation. Most importantly, the B1b knockouts show increased neuronal activation in areas of the hippocampus regardless of stress status, indicating that the hippocampal activation of B1b knockouts is aberrant compared to B1a knockouts and wild type mice regardless of stress condition.

Although c-Fos data was collected in females and the behavioral tasks detailed in Table 1 were completed only using males, it is important to point out that B1a knockouts were impaired in familiar and novel object recognition, which are hippocampal-based tasks. However, c-Fos differences between the B1a knockouts and wild type were only seen in the

CA3 region, where non-stressed B1a knockout females showed reduced activation compared to wild type. The CA3 region is proposed to be important in integrating information relevant to memory encoding received directly from the entorhinal cortex and indirectly from the amygdala and cortex via the perforant path through the dentate gyrus (Palmer & Good, 2011). Therefore, reductions in c-Fos in this area may be related to the decreased performance in memory-based tasks in the B1a knockout mice (Table 1). As previously mentioned, B1b knockout mice show increased c-Fos expression but not heightened performance in the short-term memory tasks, suggesting that there may not be a direct relationship between c-Fos expression and memory performance.

In the hippocampus, non-stressed B1b knockouts show increased total cell proliferation in the subgranular zone of the dentate gyrus and in the granular layer of the ventral dentate gyrus compared to wild type and B1a knockouts. Wild type and B1a knockouts showed no differences, and stress in the B1b animals normalized cell proliferation to levels of wild type animals. Cell survival was increased in the ventral subgranular zone of the dentate gyrus in the non-stressed B1b knockout animals. In the stressed B1b knockout animals, cell survival was increased in the subgranular and granular layer of the dentate gyrus, whereas wild type animals showed a reduction in these areas following stress. Non-stressed B1b knockout mice also showed an increase in immature cells in the dorsal hippocampus and dentate gyrus (O'Leary et al., 2014). These results suggest protective factors within the hippocampus of the B1b knockout mice, which fit well with proposed mechanisms of stress and neurodegeneration. Bao et al. (2008) put forth a model wherein the hippocampus regulates HPA-axis response that creates a spiral of stress-induced neurodegeneration. Neurodegeneration of the hippocampus, either preceding or following

stress, reduces control of the HPA-axis, leading to increased stress and depressive responses, thereby leading to hippocampal neurodegeneration, and so on. As O'Leary et al. (2014) suggest, knockout of B1b receptors may lead to resilience in assays of anhedonia. Regardless of stress condition, B1b knockout mice show increase neuronal generation and activation within the hippocampus, potentially negating the effects of increased levels of HPA-axis activation, which may suggest an underlying mechanism of their behavioral resilience.

However, this explanation may be over-reaching. O'Leary et al. (2014) suggest that their results show that reductions in B1b receptors lead to stress resilience, whereas reductions in B1a receptors lead to stress susceptibility at a behavioral and neuronal level. Yet out of fifteen tasks looked at in the Jacobson et al. (2007b) and O'Leary et al. (2014) studies, only six assays show a "protective" effect of the loss of B1b regardless of stress status. One of these behaviors is simply increased activity in an open field, and another behavior, the tail suspension test, is directly confounded by increased activity in the B1b knockout mice. Further, much of the B1b knockout c-Fos data show increases in neuronal activity regardless of stress status (O'Leary et al., 2014). Increased levels of cell proliferation and immature cells do exist in the non-stressed B1b knockouts, but these levels are normalized following stress exposure and virtually unchanged in wild type and B1a knockouts following stress (O'Leary et al., 2014). If loss of B1b conferred a protective factor, instead of proliferation and immature neuron levels being normalized against wild type and B1a knockouts after stress, one may expect that wild type and B1a knockouts would also show decreases in cell proliferation and immature neuron levels following stress. It is also of interest to note that the increases in immature neurons and cell proliferation in non-stressed animals do not appear to contribute to associative learning, as B1b animals are

unable to extinguish CTA or learn a tone-shock association in conditioned fear (Jacobson et al., 2006a; Shaban et al., 2006) (Table 1; discussed in section 4.1).

As shown in Table 5, antidepressant treatment upregulates mRNA expression of the B1a receptor. Baclofen binding was also increased in every case, except for in the hippocampus when fluoxetine was administered. Table 5 is important, as it indicates that antidepressant treatment upregulates B1a expression, and O'Leary et al. (2014) show that presence of only B1a increases c-Fos expression, number of immature cells, and immature cells in the hippocampus. Yet O'Leary et al. (2014) demonstrated an increase in B1b expression in the hippocampus of a model of selectively bred helpless mice compared to their non-helpless counterparts (H/Rouen and NH/Rouen, respectively). However, the studies reviewed in Table 5 demonstrate that antidepressants work to increase B1a expression, not to decrease B1b expression (Sands et al., 2004, 2003; McCarson et al., 2006). From a treatment standpoint, it is especially important to explore whether animals that show increased depressive and anxiogenic symptomology would have increased B1b expression that is attenuated by antidepressants, or if antidepressants would increase expression of B1a to normalize the system. Further, as indicated by increases in baclofen binding, the expression ratio of B1a to B1b may not be as important to the behavioral outcomes as changes in functional binding that increase stability of the GABAergic inhibitory system. Therefore, levels of baclofen binding during baseline and following antidepressant treatment should be looked at in models like the H/Rouen and NH/Rouen mice, as well as animals exposed to MSUS and other stress paradigms, and animals exposed to behavioral tasks that characterize depression and anxiety.

5. Drugs of Abuse

5.1 Cocaine

Not surprisingly, the $GABA_BR$ system has been linked to cocaine self-administration and reinforcement in pre-clinical models. The earliest study to show involvement of presynaptic $GABA_BRs$ in cocaine-response was Shoji et al. (1997). Following chronic cocaine injections, regulation of GABA and glutamate release in the dorsolateral septal nucleus was interrupted due to diminished activity of presynaptic $GABA_BRs$. Shoji et al. (1997) also suggested that chronic cocaine administration would alter dopaminergic transmission in other nuclei, such as the VTA, for which the dorsolateral septal nucleus is a relay center between the VTA and the hippocampus. Recent studies have supported Shoji et al.'s (1997) assertion that presynaptic $GABA_BRs$ are involved in dopaminergic output, primarily by working in tandem with other receptor systems. Williams et al. (2014) demonstrated that corticotrophin releasing factor receptor 2 (CRF-R2) mediates GABA release from GABAergic-VTA interneurons, thereby activating nearby presynaptic $GABA_BRs$ located on glutamatergic neurons, causing a decrease in glutamate release. This glutamate release is necessary for excitatory VTA output via dopaminergic projections. In times of stress, activation of this CRF-R2/presynaptic $GABA_BR$ response would be expected to decrease excitatory output from the VTA. However, during chronic cocaine self-administration and extinction, CRF-R2s lose their response to pharmacological agonism while presynaptic $GABA_BRs$ become tonically activated. Williams et al. (2014) suggest that this loss of presynaptic $GABA_BR$-mediated glutamate release following cocaine self-administration would lead to an increase of excitatory VTA dopaminergic output following stress, instead of a decrease. Indeed, animals with a loss of B1a receptors do appear to be more susceptible to stress (see Table 3 for review). The VTA also has higher levels of B1a

mRNA expression compared to B1b and levels of B1a in other brain areas (Bischoff et al., 1999), which indicates a strong role of B1a in mediating VTA excitatory output.

This increase in GABAergic interneuron/presynaptic $GABA_BR$-mediated dopaminergic output has also been demonstrated in mice that were prenatally exposed to cocaine (Wang et al., 2013). Interestingly, Wang et al. (2013) also showed that prenatal cocaine caused a 25% increase in B1a surface protein expression in the striatum in adulthood, but that B1b and B2 protein levels were not affected. Thus, prenatal cocaine exposure may interfere with the typical trajectory of B1 isoform expression during development, as it has been previously shown that the striatum expresses more B1b mRNA but similar levels of B1a and B1b protein in adulthood (Bischoff et al., 1999; Fritschy et al., 1999). Prenatal exposure to cocaine may cause a significant increase of B1a protein compared to B1b, although this cannot be concluded definitively based on Wang et al.'s (2013) findings. Some evidence of interference in B1 levels following drug use has also been seen in humans. Human cocaine addicts, as well as human alcoholics and alcohol-naïve alcohol preferring (P) rats, show post-mortem decreases in overall B1 mRNA levels (Enoch et al., 2012). Although the findings of Enoch et al. (2012) are not isoform specific, they are important to consider. Overall reductions in B1 mRNA would suggest that human cocaine addicts and alcoholics do not show increased B1a mRNA levels or increased B1b expression levels during adulthood that would be expected based upon animal research (Bischoff et al., 1999; Fritschy et al., 1999; Liang et al., 2000). This suggests that these two drugs of abuse may disrupt normal brain function by reducing B1a mRNA levels as well as the number of B1b/post-synaptic $GABA_BRs$ (see section 2.3). Loss of B1a mRNA in drug addicts may lead to disruption in maintenance of brain function via loss of protein-protein interactions and

continued developmental maintenance. Loss of B1a/B2 and B1b/B2 receptor expression may lead to impaired association, contextual, and aversive memories (see Table 1). Loss of the ability to form or maintain contextual associations seen in B1b and B1a isoform knockouts, respectively (Cullen et al., 2014; Shaban et al., 2006) may indicate that those who continuously use drugs may begin to lose the association between drug abuse and aversive situations, whereas reductions in B1a/B2 surface expression may prevent the drug abuse/aversive situation association from forming during the first instances of drug use. As alcohol-naïve P-rats also show reductions in overall B1 mRNA expression, this may indicate that low B1 mRNA expression is an indicator of drug abuse susceptibility, and may contribute to drug use and abuse for the reasons mentioned above.

Postsynaptic $GABA_BRs$ have also been implicated in cocaine-response. Hearing et al. (2013) demonstrated that Layer 5/6 glutamatergic neurons of the mPFC, involved in the behavioral response to cocaine, are inhibited by $GABA_B$ neurons. This inhibition is mediated primarily by postsynaptic Kir.3 channels and repeated cocaine administration suppresses inhibition of the glutamatergic neurons via this pathway. This increased excitation is behaviorally related to enhanced cocaine-induced locomotor activity and reduced locomotor sensitization to cocaine across trials. Locomotor sensitization to cocaine can also be attenuated by pharmacologically targeting the $GABA_BR$ (Lhuillier et al., 2007). The mechanism by which cocaine induces alterations in neuronal excitation is via a reduction of GIRK2/B1 surface trafficking to the dendritic spine, due to a reduction of phosphorylation of serine 783 (S783). S783 phosphorylation leads to surface expression of B2, which is necessary for surface expression of B1 (Hearing et al., 2013). Terunuma et al. (2014) have demonstrated that replacement of S783 with an alanine increases Kir3 channel activation,

which leads to impaired contextual fear, familiar object recognition, and Barnes maze performance, which may not be specific to the $GABA_BR$ system. Hearing et al. (2013) also demonstrated that Ser-9, which is associated with GIRK2 phosphorylation, does not change following cocaine treatment. Taken together, these results may again suggest that increased Kir3 activity outside of the $GABA_BR$ system leads to alterations in cognitive processes and behavioral responses to drugs of abuse, and that this increased activity can be mitigated via increased activity of the postsynaptic $GABA_BR$ system.

Interestingly, cocaine treatment does not appear to regulate LTP response. Although B1a appears to regulate LTP in the hippocampus and amygdala, which are associated with loss of novel object recognition and conditioned fear, respectively (Shaban et al., 2006; Vigot et al, 2006), $GABA_BRs$ may not be involved in cocaine-induced LTP. Huang et al. (2007) demonstrated that antagonizing $GABA_BRs$ blocks induction of LTP in mPFC pyramidal neurons of saline-treated rats, but that it does not reduce the increased LTP seen in cocaine-treated animals. Conversely, antagonism of the $GABA_A$ receptor system increased LTP in saline-treated animals to levels seen in cocaine-treated animals without further increasing LTP in rats that received cocaine. Increased LTP in cocaine-treated rats was blocked by agonism of the $GABA_A$ receptor system. Thus, alteration of GABA-mediated LTP by cocaine is regulated by the $GABA_A$ receptor system. This may suggest that the role of $GABA_BRs$ in drug seeking, intake, and behavioral changes in response to drugs may lie outside of the realm of cellular "learning," which is associated with presynaptic B1a receptors, and may instead lie with postsynaptic mechanisms.

Multiple experiments would be needed to investigate the role of pre- versus postsynaptic $GABA_BR$ involvement in drug reinforcement. As knockout of B1a or B1b

appears to show different behavioral profiles, it would be of interest to see whether these profiles are altered with drugs of abuse on board. Long-term abuse of drugs such as cocaine in humans often leads to cognitive deficits in areas such as attention, working memory, and response inhibition (Sofuoglu et al., 2013). Aspects of these deficits can be modeled in animals by using the behavioral assays detailed in Table 1, as well as the radial arm maze, serial reaction time task, and delayed discounting tasks. As loss of B1a impairs object recognition, memory maintenance, and contextual fear, it may be expected that having cocaine on board during these tasks would further reduce performance in B1a knockout mice, whereas B1b knockout mice may perform similar to control animals with cocaine on board. The isoforms could also be investigated in conditioned place preference, conditioned aversion, and drug administration assays to observe how they regulate different aspects of drug reinforcement and seeking, which is currently unknown. Finally, considering the possible developmental role of the isoforms in drug addiction, the isoforms could be conditionally knocked out at different times throughout adolescence and adulthood to observe effects on drug seeking and intake at different time periods. Another method to investigate adolescent abuse time-course would be to expose wild type animals to cocaine starting a different age periods, then using *in situ* hybridization to quantify changes in isoform mRNA or DNA expression levels.

5.2 Ethanol

The $GABA_BR$ system is well-known to be involved in ethanol consumption, reinforcement, and withdrawal (Agabio et al., 2012). Most work looking at presynaptic $GABA_BR$ and ethanol-mediated inhibition has been carried out in the primarily glutamatergic BLA, which has a small but important population of GABAergic interneurons (Silberman et

al., 2009). It has been suggested that presynaptic $GABA_B$ autoreceptors work to mediate ethanol's enhancing inhibitory effect at classic local interneurons, while ethanol-enhanced inhibition in GABAergic lateral paracapsular interneurons is adrenoceptor-dependent (Silberman et al., 2012, 2009, 2008; Zhu & Lovinger, 2006). Therefore, ethanol's effects within the BLA are not specific to $GABA_B$Rs. Further, as both B1 isoforms act as autoreceptors, these results do not indicate a $GABA_B$R isoform-specific role in ethanol-induced system inhibition. However, a postsynaptic $GABA_B$R mechanism mediated through Kir3 channels has been identified. Federici et al. (2009) demonstrated that ethanol application increases $GABA_B$-, but not $GABA_A$-, mediated IPSPs in postsynaptic dopaminergic neurons of the VTA and substantia nigra pars compacta. Simultaneous application of baclofen and ethanol demonstrated that outward K^+ currents induced by baclofen were potentiated by alcohol, producing a stronger postsynaptic inhibition of dopaminergic neurons. This potentiation is of interest to drug abuse researchers, as baclofen has been repeatedly demonstrated to reduce ethanol intake in both clinical and pre-clinical models (see Agabio et al., 2012 for review). Whereas the data presented by Federici et al. would suggest that, if ethanol-induced $GABA_B$R-mediated postsynaptic depression of dopaminergic output within the ventral tegmental area is an underlying cause of ethanol reinforcement, then $GABA_B$ agonists such as baclofen would amplify this reinforcement instead of alleviating it. One explanation may be developmental. It is important to note that slices used by Federici et al. (2009) were taken during a wide range of the rat adolescent period (PND 14-35), during which $GABA_B$R surface expression levels are rapidly changing (Fritschy et al., 1999). Based on data discussed within this section and throughout the review, it is possible that adult slices would show decreased or even abolished postsynaptic

$GABA_BR$/ethanol inhibition, and that instead postsynaptic inhibition may be regulated by the $GABA_AR$. Secondarily, fully developed brains may not show the postsynaptic inhibitory effects of ethanol that are demonstrated in the developing brain. One theory may be that, in adolescence, heightened B1b/B2 receptor surface expression may augment responses to ethanol intake, and that this increase in postsynaptic $GABA_BRs$ and ethanol sensitivity may lead to altered reinforcement of ethanol intake. In the developed brain, lower surface expression of B1b/B2 receptors and/or altered sensitivity to alcohol may create a system where $GABA_BR$ agonism works against ethanol, making it a valuable pharmacological treatment for alcohol use disorders. Behaviorally, this could be investigated by placing adolescent animals in an operant self-administration paradigm in which they receive ethanol. Following acquisition of response and relevant levels of intake, baclofen would be administered and ethanol intake would be monitored. An increase of responding for/intake of ethanol could be interpreted as a synergistic effect of baclofen + ethanol inhibition of dopaminergic VTA neurons in adolescents. Due to the short period of adolescence, it may be more favorable to use a short-term design that monitors intake but not perceived reinforcement. One such model is drinking-in-the-dark (DID), which leads to high ethanol intakes in a brief period of time that does not require training (Rhodes et al., 2005). As in the operant paradigm, baclofen could be administered on the final day of drinking and ethanol intake would be observed. A DID design would allow for monitoring over very brief periods of adolescence to observe whether there is a change in baclofen sensitivity that is associated with adolescent development.

Other studies have contradicted the findings of Federici et al. (2009). Theile et al. (2011) demonstrated that ethanol increases DA neuron firing in the VTA in more tightly regulated

adolescent slices (PND 21-28), and that the effects of ethanol can be inhibited by both $GABA_B$Rs and $GABA_A$Rs. Xiao et al. (2009) have also demonstrated that ethanol increases DA neuron firing in the VTA of rat brain slices aged PND 22-32 by potentiating presynaptic glutamate release. Importantly, it has been demonstrated that ethanol self-administration during adolescence increases excitatory postsynaptic NMDA receptor levels in the VTA of adult rats (Stuber et al., 2008). These results highlight the necessity of considering developmental factors and using tightly controlled age groups in experiments.

As demonstrated in animals prenatally exposed to cocaine (Wang et al., 2013), presynaptic $GABA_B$Rs may also play a critical role during adolescent ethanol exposure. Mishra and Chergui (2013) stimulated glutamatergic fibers of the nucleus accumbens core of adult and adolescent (PND22-30) mice and observed field EPSPs and population spikes. Ethanol reduced postsynaptic glutamate responses to a greater degree in adolescent slices. This reduction was blocked by antagonism of both $GABA_A$Rs and $GABA_B$Rs in adolescent slices. Due to the minimal postsynaptic depression in adult slices, GABA receptor antagonism in the presence of ethanol was not carried out in adult slices. However, agonism of the $GABA_A$Rs and $GABA_B$Rs alone caused a much more pronounced reduction in postsynaptic activity in adolescent compared to adult slices, whereas $GABA_A$R antagonism alone caused a more pronounced increase in glutamatergic postsynaptic activity in adult slices, and $GABA_B$R antagonism alone had no effect. Mishra and Chergui (2013) suggest that their results show a presynaptic GABAergic mechanism of postsynaptic glutamatergic excitation. In adult mice, it appears that GABA is released and binds to $GABA_A$Rs, causing inhibition of glutamatergic activity. However, in adolescent mice, GABA may be overflowing to presynaptic $GABA_B$Rs, which also work to modulate glutamatergic activity.

Although there is more B1b than B1a surface expression during the age at which Mishra and Chergui (2013) took their adolescent slices (Fritschy et al., 1999), there is no indication whether the nucleus accumbens of an adolescent mouse has a greater $GABA_BR$ to $GABA_AR$ ratio than the adult mouse.

These results support well-established findings that the $GABA_BR$ system is a target for alcohol use disorder intervention. Although the current research does not support a need for development of isoform-specific drugs, not enough investigation has been done in this area to be conclusive. As with the cocaine data, these results potentially implicate the $GABA_BR$ system as an early marker of abuse liability as well as a potential treatment target for adolescents involved in misuse and abuse of drugs. B1 isoforms could be investigated in multiple ways related to ethanol intake. These include how ethanol affects the memory impairments present in B1a knockout mice, how isoforms are involved ethanol seeking and reinforcement, and how the isoforms are involved in the developmental trajectory of ethanol intake and reinforcement. Studies could incorporate isoform knockouts to observe direct influence of overall or site-specific knockout on future ethanol intake, or they may expose the animal to ethanol and behavioral tasks then run *in situ* hybridization to characterize how ethanol alters baseline levels of isoform mRNA and DNA expression.

5.3 Isoform-Specific Pharmacological Interaction

Research investigating differential pharmacological profiles of each isoform is limited and does not support a role of differential action for treatments that target $GABA_BRs$. Many drugs that interact with $GABA_BRs$ are positive allosteric modulators, which bind to the B2 subunit of the receptor (Pin et al., 2004). As all functional receptors include this B2 subunit, it is unlikely that positive allosteric modulators themselves would have differing actions at

B1a/B2 or B1b/B2 receptors. Further, auxiliary proteins, which interact with the $GABA_BR$ complex, have not yet been shown to affect GABA affinity or drug efficacy at native receptors. Complement control protein 1, which is present only on B1a receptors, is able to bind to the matrix protein fibulin-2. Fibulin-2 can bind to extracellular ligands and calcium, but binding of fibulin-2 to the B1a receptor is not specific to the presence of complement control protein 1, indicating a non-specific effect (Hannan et al., 2012; Blein et al., 2004). Rajalu et al. (2015) demonstrated that different K^+ channel tetramerization-domains, which bind to postsynaptic B1b/B2 receptors and the associated G-protein, do not alter basal GABA affinity or GABA affinity of native cells in the presence of the positive allosteric modulator GS39783.

Cell-type and receptor-location effects of $GABA_BR$ agonists have been shown. Yu et al. (1999) determined that the $GABA_BR$ agonist CGP44533 is less efficacious and potent at GABAergic interneurons compared to glutamatergic neurons. CGP44533 also works less potently and efficaciously at autoreceptors than at heteroreceptors. However, as both isoforms act as autoreceptors, this does not speak to a diverging pharmacological profile. Although each isoform produces different behavioral profiles, R-baclofen similarly mediates locomotor and endurance behaviors as well as temperature changes in both isoform knockouts (Jacobson et al., 2006b). These results are not surprising, as many studies have reported that each isoform shows similar levels of agonist and antagonist potency, as well as the same rank-order of potency (Bräuner-Osborne & Krogsgaard-Larsen, 1999; Green et al, 2000; Malitschek et al. 1998). However, Malitschek et al. (1998) demonstrated that R-baclofen is much more potent in the adult brain. Compared to PND 4, mid-range baclofen doses bind 40% more to B1a and B1b receptors in the adult brain. Although potency

normalizes across adolescence, at PND 28 there are still lower levels of baclofen binding compared to the adult brain (Malitschek et al., 1998). Once again, these results suggest that adolescents and adults show a different $GABA_BR$ pharmacological profile that may be of important consideration for the etiology of disease. Although current research does not suggest isoform-specific roles for pharmacological treatments that target $GABA_BRs$, it is possible that use of Ca^{++} or K^+ channel drugs may independently regulate behaviors that have been shown to be isoform specific.

6. Conclusions

In conclusion, the initial hypothesis of this review was confirmed. $GABA_B$ isoform knockout mice show a broad spectrum of isoform-specific behaviors. Global B1 and B2 subunit knockout studies have indicated many deficits in animals that do not express $GABA_BRs$. At times these deficits are isoform specific, such as the role of B1a in hyperactivity, seizure activity, protection of depressive phenotypes, and memory maintenance (Cullen et al., 2014; Jacobson et al., 2007a; O'Leary et al., 2014; Vienne et al., 2010), whereas B1b is involved in susceptibility to depression-like phenotypes and impaired memory formation (Cullen et al., 2014; O'Leary et al., 2014; Shaban et al., 2006). In other behavioral assays, such as seizure activity, tests of anxiety, and impaired passive avoidance learning, the presence of functional $GABA_BRs$ containing either B1 isoform rescues the aberrant behavior seen in B1 and B2 subunit knockout mice (Gassman et al., 2004; Jacobson et al., 2007a; 2007b; Mombereau et al., 2005, 2004; O'Leary et al., 2014; Schuler et al., 2001; Vienne et al., 2010). Although pre- and postsynaptic $GABA_BRs$ may play specific roles in controlling differing aspects of addictive drives and behavior, the necessity of development of isoform-specific drugs to treat drug use disorders is not currently supported.

However, it appears that $GABA_BRs$ may be involved in development of drug-use disorders and the isoforms may act as biomarkers for such disorders. Isoform-specific drug development may be useful for cognitive disorders, such as short-term memory loss or Alzheimer's disease. Treatments that target B1a may aid in memory maintenance, whereas treatments that target B1b may aid in initial memory consolidation. Although more research needs to be done, it is possible that development of a B1a-specific drug may work well as an anti-depressant treatment, as presence of B1a may be protective against symptoms of anhedonia following stress. As isoforms play a role in development of the central nervous system, their roles in behavior may develop over time and developmental considerations are important when considering etiology and treatment of behaviors and disease.

Although the studies reviewed herein confirm an isoform-specific role in behavior, there are a lot more studies that need to be done. A current issue is the lack of behavioral tests done in female animal models. Section 4.2 discusses how sex differences are known to be involved in phenotypes of depression and anxiety as related to isoform expression. However, few behaviors are well-characterized in males versus females, and this work must be done as well as including females in all future experiments. It is possible that there are sex-specific isoform roles in cognitive tasks, development of drug-use disorders, and response to pharmacological treatment. Further, the isoforms may play different roles in the course of basic male and female development, which need to be investigated to better understand how the B1 isoforms may be playing a sex-specific role in adult behavior.

The studies discussed within this review also implicate different brain areas for each behavioral deficit. However, all of these studies used global knockouts, making it impossible to directly implicate specific brain regions in all of the current behavior. In global isoform

knockouts, brain-region specificity could be investigated by site-specifically antagonizing receptor systems that are over-excited by loss of each isoform, such as the glutamatergic system. Conversely, the isoforms could be knocked-in to the region of interest. Both of these designs would "normalize" the over-excitation caused by isoform loss. However, the best study design would be to use a site-specific viral isoform knockdown in adulthood. Such a design would bypass the concern of compensation in the isoform knockouts, thereby giving a better indicator of how the isoform is site-specifically involved in behavior.

Finally, the $GABA_BR$ system is known to be strongly involved in drug abuse. However, no studies have been carried out to look at the role of isoform knockout in development or maintenance of drug addiction. As it appears that the isoforms may play a developmental role in addictive behavior, isoform knockouts could be used in studies that look at adolescent pre-exposure to drugs and how such pre-exposure may alter abuse liability in adulthood via free-choice drinking and operant drinking. Neurobiological studies could also be conducted to look at how isoforms and pre-exposure alters neurotransmitter signaling of dopamine, glutamate, and other systems of interest. Using viral knockdown of each isoform at specific time-points during adolescent drug-intake would give a clear idea of when isoforms are playing a role in drug susceptibility. How behavioral tasks alter baseline levels of isoform expression is also of importance, especially when considering drug treatment for such disorders. As discussed in section 4.2.4, antidepressant treatment alters isoform mRNA expression and baclofen bindings. However, how a battery of tasks that are thought to mimic depressive and anxiety-like phenotypes alters isoform expression is unknown, making it difficult to extrapolate how behavior, treatment, and isoform expression are inter-related. Looking at changes in mRNA and protein expression of each isoform using *in situ*

hybridization following drug administration and treatment would also be informative. Basic *in situ* hybridization studies would allow for a basic understanding of how isoforms are expressed across the brain following behaviors of interest and drug treatment, allowing for better designed knockout studies and interpretation of behavioral data. Although the current research supports an isoform-specific role in behavior, the suggested studies would better inform how these isoforms are involved in sex- and isoform-specific behavior as well as trajectory of drug abuse.

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Table 1: Contributions of the $GABA_BR$ isoforms to cognition[1]

Behavior	**Direction**	**Knockout Genotype**	**Publication**
Familiar object recognition	Impaired	B1a	Vigot et al., 2006; Jacobson et al., 2007a
Novel object recognition	Impaired	B1a	Vigot et al., 2006; Jacobson et al., 2007a
Novel object recognition – consolidation	No effect	n/a	Cullen et al., 2014*
Novel object recognition – maintenance	Impaired	B1a	
Spontaneous alteration	Impaired	B1a, B1b	Jacobson et al., 2007a
Conditioned taste aversion – learning	Abolished	B1a	Jacobson et al., 2006a
Conditioned taste aversion – extinction	Impaired	B1b	
Conditioned fear – learning	Impaired, abolished	B1a, B1b	Shaban et al., 2006
Conditioned fear – same context	No effect	n/a	Cullen et al., 2014*
Conditioned fear – different context	Impaired	B1a	
Passive avoidance learning	No effect	n/a	Jacobson et al., 2007a

1. "Direction" indicates in comparison to wild type mice. Asterisk (*) indicates that Cullen et al., (2014) only used wild type and B1a knockout mice. "n/a" refers to "not applicable" in cases where no significant results were reported.

Table 2: Null Results[2]

Behavior	**Direction**	**Knockout Genotype**	**Publication**
Stress-induced hypothermia	No effect	n/a	Jacobson et al., 2007b
MSUS stress-induced hypothermia	No effect	n/a	O'Leary et al., 2014
Staircase test	No effect	n/a	Jacobson et al., 2007b
Marble burying	No effect	n/a	
Elevated plus maze	No effect	n/a	
MSUS elevated plus maze	No effect	n/a	O'Leary et al., 2014

2. These behavioral tasks showed no effect of isoform on basal anxiety or anxiety measures following maternal separation unpredictable stress (MSUS). "n/a" refers to "not applicable" in cases where no significant results were reported.

Table 3: Non-Stress-Dependent Results[3]

Behavior	**Direction**	**Knockout Genotype**	**Publication**
MSUS maternal care	Increased	All FB1a	O'Leary et al., 2014
Light/dark Box	Reduced anxiety*	FB1b	Jacobson et al., 2007b
Elevated zero maze	Increased anxiety	FB1a, FB1b	Jacobson et al, 2007b
MSUS tail suspension test	Increased immobility	All FB1a, All MB1a	O'Leary et al., 2014
	decreased immobility	All MB1b	

3. All "directions" are compared to wild type mice in the same task unless marked by an asterisk (*), thereby indicating the direction is compared to the other isoform knockout. "MSUS" in the behavior column indicates that the task took place following unpredictable maternal stress. In the genotype column, "FBla" and "FB1b" indicate female mice. "MB1a" and "MB1b" indicate male mice. "NMS" indicates no stress, and "MS" indicates maternal stress. "All" indicates a main effect of genotype – results were not specific to stress condition. O'Leary et al. 2014 tested MSUS saccharin preference only in female mice, and MSUS female urine sniffing only in male mice.

Table 4: Stress-Dependent Results[4]

Behavior	**Direction**	**Knockout Genotype**	**Publication**
Social interaction following social defeat	Increased	B1b	O'Leary et al., 2014
	Decreased	B1a	
Saccharin pref. following social defeat	Reduced	B1a	
MSUS saccharin preference	Reduced	MS FB1a	
MSUS female urine sniffing	Increased	MS MB1b	
MSUS PND7 vocalizations	Increased	MS B1b	
MSUS Open field locomotion	Increased	NMS FB1b, All MB1b	
MSUS forced swim task	Decreased immobility	NMS FB1a, All FB1b, All MB1a, NMS MB1b	

4. All "directions" are compared to wild type mice in the same task unless marked by an asterisk (*), thereby indicating the direction is compared to the other isoform knockout. "MSUS" in the behavior column indicates that the task took place following unpredictable maternal stress. In the genotype column, "FBla" and "FB1b" indicate female mice. "MB1a" and "MB1b" indicate male mice. "NMS" indicates no stress, and "MS" indicates maternal stress. "All" indicates a main effect of genotype – results were not specific to stress condition. O'Leary et al. 2014 tested MSUS saccharin preference only in female mice, and MSUS female urine sniffing only in male mice.

Table 5: Anti-depressants and isoform mRNA expression[5]

Drug	Region	Direction	Isoform	Publication
Tranylcypromine (10 mg/kg)	Hippocampus	Increased	B1a, B2	Sands et al., 2003
Phenelzine (10 mg/kg)	Hippocampus	Increased	B1a	
Fluoxetine (5 mg/kg)	Hippocampus	Increased	B1a	
Fluoxetine (5 mg/kg)	Lumbar spinal cord	Increased; Decreased	B1a; B2	McCarson et al., 2006
Desipramine (15 mg/kg)	Hippocampus	Increased	B1a	Sands et al., 2003
Desipramine (15 mg/kg)	Spinal cord dorsal horn	Increased	B1a, B2	Sands et al., 2004
Amitriptyline (10 mg/kg)	Lumbar spinal cord	Increased; Decreased	B1a, B1b; B2	McCarson et al., 2006

5. Table 5 indicates the effects of different antidepressants on $GABA_BR$ subunit and isoform expression. All results were gathered using *in situ* hybridization, and direction indicates a comparison to baseline. Tranylcypromine and phenelzine are MAOIs, fluoxetine is an SSRI, and desipramine and amitriptyline are tricyclics.

Figure 1 Figure 1 details the hippocampal circuitry believed to be involved in memory maintenance. Panel A details typical function, in which a B1aRs and mGluRs are co-localized on the projection from the dentate gyrus and entorhinal cortex to the CA3 region of the hippocampus. Binding of GABA to the B1aR regulates glutamate release, thereby regulating LTP in the CA3. Panel B shows this same circuitry following loss of the B1aR on the afferent projections. Loss of presynaptic GABAergic control increases release of glutamate, resulting in unregulated LTP in the CA3 and loss of memory maintenance.

Figure 2 Figure 2 details the circuitry of the central and basolateral amygdala regions (CeA and BLA, respectively) in conditioned taste aversion. Panel A shows the circuitry of a B1a knockout, where the B1aR on the glutamatergic projection from the BLA to the CeA is lost. This loss results in decreased presynaptic inhibitory regulation of the glutamatergic projection, leading to increased excitatory input to the CeA. This may contribute to loss of fear inhibition. Panel B shows the circuitry of the B1b knockout, where B1bRs located in the BLA, which regulate excitatory input to and from the hippocampus and to the ventral stiatum and CeA. This loss of postsynaptic inhibition leads to excessive excitatory output, contributing to loss of extinction.

Figure 3 Figure 3 details the circuitry of the amygdala believed involved in conditioned fear response. Panel A shows the circuitry of B1a knockout mice. Loss of B1a presynaptic inhibition of the GABAergic projection from the auditory cortex to the lateral amygdala (LA) leads to loss of tone discrimination. This loss of discrimination is relayed to the central amygdala (CeA) and results in decreased inhibition of the brainstem, leading to increased freezing during the session. Panel B shows the circuitry of B1b knockout mice. Tone discrimination is relayed from the auditory cortex to the LA. However, loss of postsynaptic B1bRs in the CeA results in loss of tone information relay from the LA to CeA and results in increased CeA excitation. This increased excitatory input results in increased brainstem inhibition, which results in loss of freezing behavior in the B1b knockout mice.

Figure 1

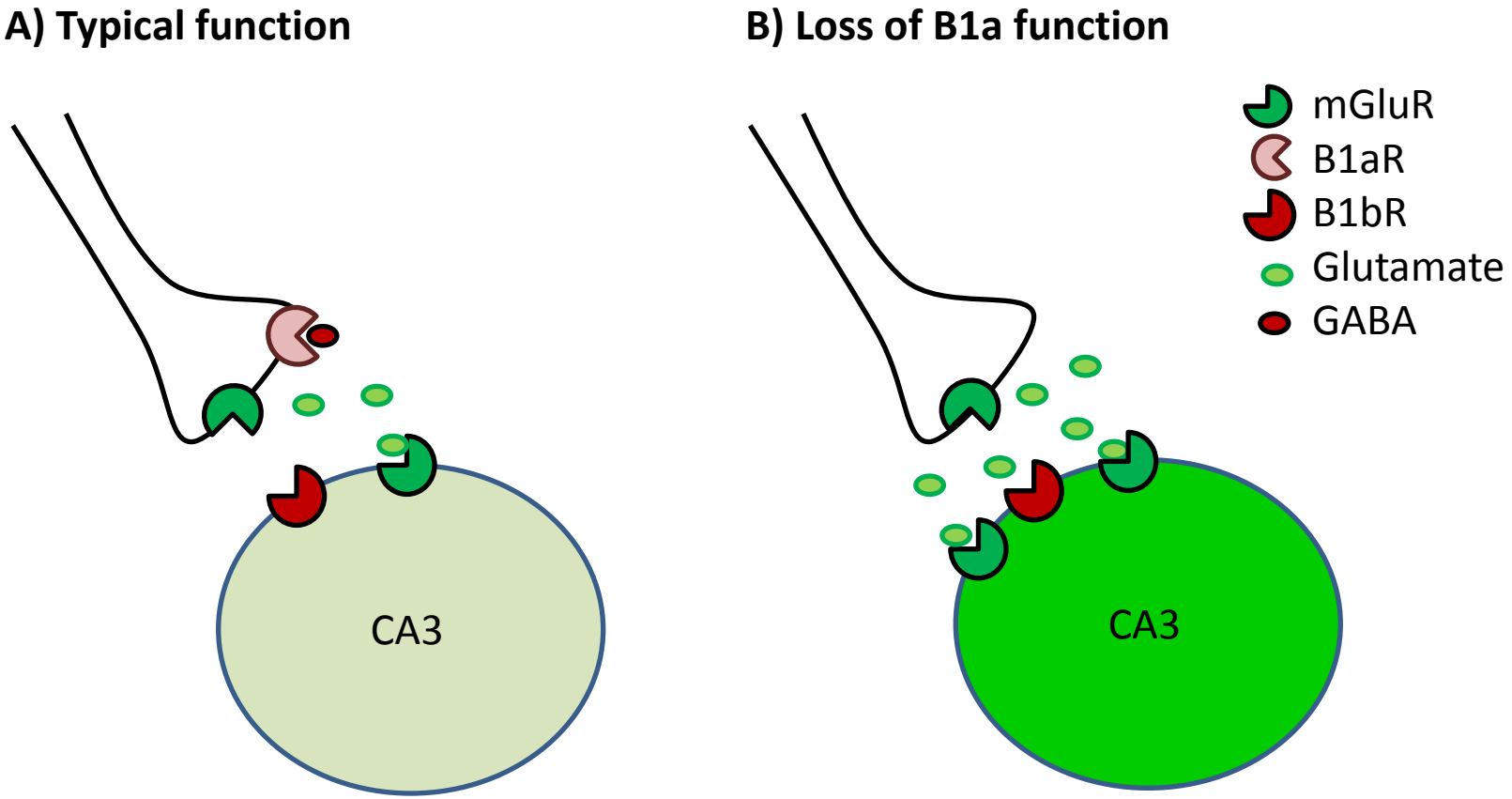

A) Typical function
B) Loss of B1a function
mGluR
B1aR
B1bR
Glutamate
GABA
CA3
CA3

Figure 2

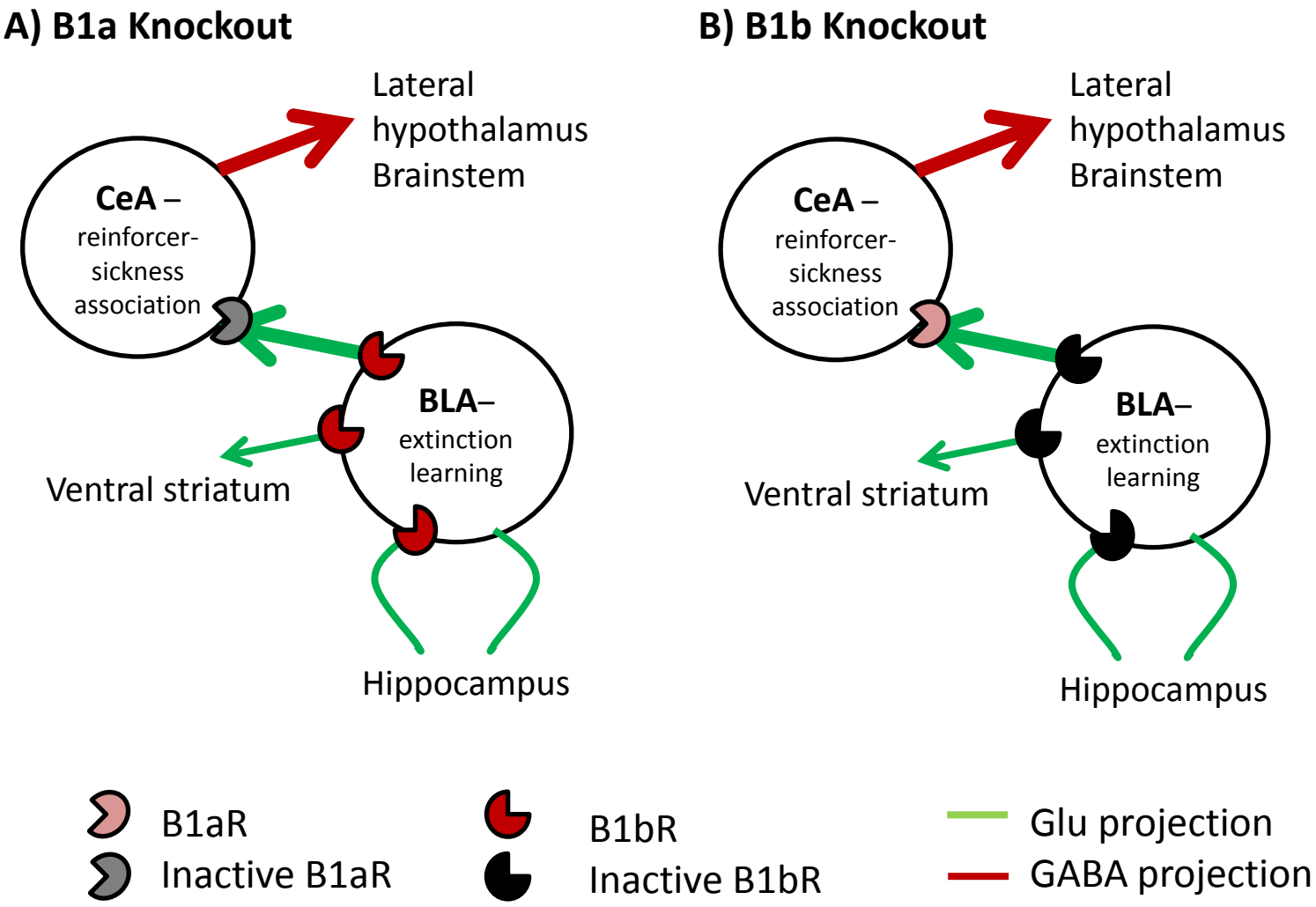
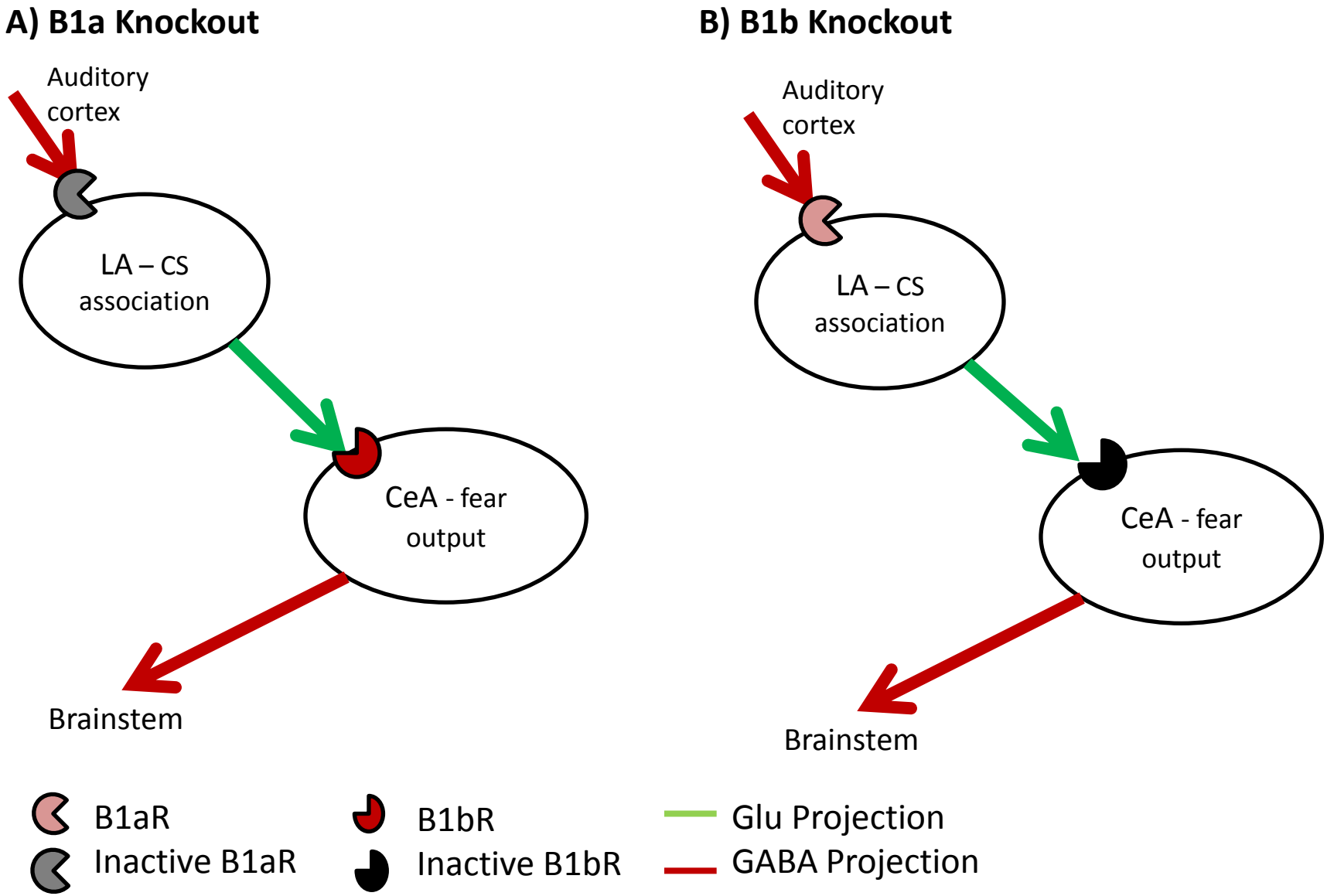

A) B1a Knockout
Lateral hypothalamus Brainstem
CeA – reinforcer-sickness association
BLA– extinction learning
Ventral striatum
Hippocampus
B) B1b Knockout
Lateral hypothalamus Brainstem
CeA – reinforcer-sickness association
BLA– extinction learning
Ventral striatum
Hippocampus
B1aR
Inactive B1aR
B1bR
Inactive B1bR
Glu projection
GABA projection

Figure 3


A) B1a Knockout
Auditory cortex
LA – CS association
CeA - fear output
Brainstem
B) B1b Knockout
Auditory cortex
LA – CS association
CeA - fear output
Brainstem
B1aR
Inactive B1aR
B1bR
Inactive B1bR
Glu Projection
GABA Projection