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Alternations of NMDA and GABAB Receptor Function in Development: A Potential Animal Model of Schizophrenia

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ALTERATIONS OF NMDA AND $GABA_B$ RECEPTOR FUNCTION IN DEVELOPMENT:

A POTENTIAL ANIMAL MODEL OF SCHIZOPHRENIA

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ABSTRACT

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Schizophrenia is a debilitating mental disorder that affects up to 3% of the world population. The behavioral symptoms are categorized into positive and negative symptoms, which appear during late adolescence/early adulthood. Unfortunately, the underlying cellular and molecular mechanisms of the disease are poorly understood. Several hypotheses exist to explain mechanisms contributing to these behavioral alterations. One model proposes that a reduced function of the NMDA glutamate receptor on specific GABAergic interneurons may be responsible for deficits in schizophrenia. Post-mortem investigations provide evidence of reductions in both glutamate and GABA-related proteins in patients with schizophrenia. Further, GABAergic interneurons that are activated by glutamate via NMDA receptors are important for oscillatory activity involved with sensory processing and cognitive function. Alterations in the function of NMDA receptors on GABAeric interneurons are implicated in regulating neural network activity and, if disrupted, could potentially lead to altered brain function and deficits seen in schizophrenia. Several investigations have demonstrated reduction in NMDA receptor function or GABA receptor function induces deficits consistent with schizophrenia. Recent approaches have also focused on changes in NMDA or GABA function related to

schizophrenia as a neurodevelopmental disorder. This approach suggests that alterations in either system during brain development may result in behavioral deficits later in life. The purpose of the below studies was to determine if changes in NMDA receptor function or alterations in downstream GABA receptor function during development in rodent pups results in behavioral or biochemical alterations in adulthood that are relevant to schizophrenia. The data reveal that altering these receptor systems in development produce deficits in adulthood. Changes in sensorimotor gating, spatial learning and memory, and differential expression of multiple GABA related proteins in the brain tissue were observed in these animals.

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CHAPTER 1

INTRODUCTION

While the etiology of schizophrenia is not known, several behavioral symptoms exist that characterize the disorder. Patients suffer from a combination of symptoms including hallucinations and delusions (positive symptoms), as well as deficits in sensorimotor gating, learning, and memory (negative symptoms) and others. Although the symptoms appear during early adulthood or late adolescence, the disorder may originate from an unknown neurodevelopmental abnormality (Weinberger, 1987). Numerous studies have been carried out in order to better understand the cellular and molecular basis of this disease in order to effectively treat patients.

One of the current hypotheses to explain the pathophysiology of schizophrenia proposes that there is a reduction in glutamate and γ-aminobutyric acid (GABA) signaling in the brain. Specifically, a reduction of the N-methyl-D-aspartate (NMDA)-type glutamate receptor function on GABAergic interneurons produces altered network function and deficits associated with schizophrenia (Javitt, 2007). Administering drugs which block the NMDA receptor in healthy individuals produces behavior that is similar to the positive and negative symptoms observed in schizophrenic patients, and these drugs exacerbate symptoms in schizophrenic patients (Adler et al., 1999; Javitt & Zukin, 1991; Krystal et al., 1994; Lahti et al., 1995; Luby et al., 1962). Post-mortem investigations of schizophrenia populations also reveal a reduction in specific subunits of the NMDA receptors and several protein markers associated with GABAergic interneurons (Benes & Berretta, 2001; Bullock et al., 2008; Hashimoto et al., 2003; Mirnics et al., 2000; Torrey et al., 2005). These proteins include glutamate decarboxylase

67 (GAD67; enzyme required for GABA synthesis; Akbarian et al., 1996; Kaufman, Houser, & Tobin, 1991; Volk et al., 2000) and parvalbumin (PV; a calcium binding protein; Beasley & Reynolds, 1997). Many behaviors depend on the activation of GABAergic interneurons via glutamate binding to NMDA receptors including network oscillations involved in learning and memory and cognitive function, as well as the development of neural networks during brain development.

GABA signaling is imperative for initiating and maintaining neural network oscillations (Gonzalez-Burgos & Lewis, 2008). Data suggests that the negative symptoms and cognitive deficits seen in schizophrenic patients may arise from abnormal oscillatory activity (Gonzalez-Burgos, 2010; Lewis, Hashimoto, & Volk, 2005; Uhlhaas & Singer, 2010). NMDA receptor hypofunction on GABAergic cells could result in a reduced amount of GABA release. GABA release from these neurons inhibits a large population of downstream postsynaptic excitatory neurons, allowing them to become entrained into a synchronous firing pattern once the inhibition subsides (Roopun et al., 2008). GABA binds to both ionotropic (mainly $GABA_A$) and metabotropic ($GABA_B$) receptors. Binding to $GABA_A$ receptors results in fast-acting and short-lived inhibition (Macdonald & Olsen, 1994; Watanabe et al., 2002) whereas activation of $GABA_B$ receptors results in a slower onset but long-lasting inhibitory effect (Kerr & Ong, 1992). The $GABA_B$ receptors have been shown to be important in maintaining oscillatory activity due to their sustained inhibitory properties (Scanziani, 2000). A reduction of GABA release or binding to receptors could result in asynchronous firing and possibly the abnormal oscillatory activity seen in schizophrenia patients.

Animal models of schizophrenia provide valuable insight into the mechanisms that may be involved in the disorder. Administration of ketamine (an NMDA receptor antagonist) to adult rodents produces deficits in sensorimotor gating, spatial learning and memory, and fear associative learning similar to what is seen in the patient population (Bolton et al., 2012; Cilia et al., 1997; de Bruin et al., 1999; Mansbach and Geyer, 1991; Sabbagh et al., 2012). Drugs that alter $GABA_B$ receptor function are less well studied in learning and memory and have been largely excluded for any potential role in schizophrenia. A limitation in the above studies is that they rely on drug being active at the time of testing. An alternative approach that may also allow investigators to incorporate the developmental theory of schizophrenia would be to administer drugs that target this system during development and test for behavior in adulthood.

A wealth of data examining brain morphology, protein expression changes, genetics, environmental factors, and gene-environment interactions suggests that some of the pathology leading to schizophrenia occurs during early brain development (Fatemi & Folsom, 2009; Lewis & Levitt, 2002; Lewis et al., 2004; Rapoport et al., 2005; Weinberger, 1987; Weinberger & Lipska, 1995). NMDA receptors and GABA signaling contribute to major developmental processes including neuronal migration, synaptogenesis, and incorporation of neurons into neural networks (Komuro & Rakic, 1993; LoTurco, Blanton, & Kriegstein, 1991; Uhlhaas et al., 2010). An alteration in these receptor systems at critical brain developmental periods may have particular relevance to behavioral disturbances.

The purpose of this study was to determine if disrupting the function of NMDA or $GABA_B$ receptors in early life produces deficits consistent with schizophrenia in

adulthood. Our data demonstrate that a brief pharmacological alteration in NMDA receptor function and, separately, $GABA_B$ receptor function during early brain development of rat pups, results in altered behavior in adulthood in tasks that measure sensorimotor gating (using prepulse inhibition) and spatial learning in the Morris water maze. The most striking differences in these studies were differences between males and females receiving the same treatments, specifically the $GABA_B$ receptor antagonist (phaclofen) producing the most robust deficits. We also identified numerous changes in the tissue analysis in GABA related proteins as well as a marker for synaptic formation. These results suggest that alterations in of NMDA and $GABA_B$ receptors in brain development can produce lifelong impairments that may be related to neuropsychiatric disorders.

CHAPTER 2

REVIEW OF RELATED LITERATURE

Schizophrenia

Schizophrenia is a debilitating brain disorder which affects up to 1-3% of the population with symptoms that cause lifelong impairments and disability (Rossler et al., 2005). The clinical diagnosis is usually made in late adolescence or early adulthood when the symptoms typically first occur. These symptoms manifest into a constellation of behavioral abnormalities that are classified into either positive or negative subgroups. Characteristics of the positive symptoms include auditory hallucinations, delusions, and paranoia (Kay et al., 1987). Negative symptoms can be described as a deficit compared to the normal population. They leave patients with impairments in sensorimotor gating, attention, abstract reasoning, mental flexibility, learning and memory, affect, and certain aspects of information processing (Carter et al., 1996; Rushe et al., 1999; Swerdlow et al., 1994). Because of these deficits, patients are left with a lifetime disability in a variety of everyday functional and social domains (Burns, 2007; Green, Kern, Braff, & Mintz, 2000). Unfortunately, the etiology of schizophrenia is not known but there have been various cellular/molecular mechanisms proposed to account for the disorder.

Several neurochemical hypotheses have been suggested to explain the pathophysiology of schizophrenia. The discovery of dopamine antagonists' utility as an antipsychotic in the late 1950s suggested that dopamine has a role in the disorder. Dopamine is a monoamine neurotransmitter and has been shown to mediate multiple systems within the central nervous system (CNS); most importantly, it plays a major role in reward systems (Schultz, Dayan, & Montague, 1997) and motor function

(Korchounov, Meyer, & Krasnianski, 2010). The specific role of dopamine in schizophrenia remains to be identified; however, data indicate that antipsychotics alleviate positive symptoms by modifying dopamine receptor function, which suggests a dysregulation of dopamine in schizophrenia. Investigations of dopamine alterations in schizophrenia populations include the evaluation of post-mortem tissue, imaging in patient populations, and numerous animal models of dopamine dysfunction relevant to the disorder (Bird et al., 1977; Crow et al., 1979; Davis, Kahn, Grant, & Davidson, 1991; Elkashef et al., 2000; Haberland & Hetey, 1987; Howes & Kapur, 2009; Toru, Nishikawa, Mataga, & Takashima, 1982). Data from these studies have provided mixed evidence for altered dopamine in discrete brain regions as being associated with schizophrenia. These data also have provided the foundation for what is termed the dopamine hypothesis (Carlsson, 1988; van Rossum, 1966), which argues that elevated dopamine activity in subcortical regions (the striatum and basal ganglia) is associated with the positive symptoms of schizophrenia, while reduced dopamine activity within the cortex may be associated with the negative symptoms (Davis et al., 1991; Pycock, Kerwin, & Carter, 1980). However, limitations to this theory exist. For instance, the means by which dopamine tone is elevated in some regions and reduced in others has not been elucidated. Additionally, antipsychotic medications only moderately alleviate the positive symptoms and have limited efficacy for the negative symptoms (Javitt & Zukin, 1991). Although dopamine tone is altered in schizophrenia, the dopamine hypothesis does not account for the cause of these changes.

Glutamate Hypothesis of Schizophrenia

A more recent alternative model hypothesized to explain the cellular/molecular alterations in schizophrenia is based on a combination of data from drug abuse literature and separate post-mortem examinations of schizophrenia populations. The model proposes that there is a reduction in glutamate signaling within discrete circuits of the brain, which may account for the behavioral symptoms of schizophrenia and also may cause alterations in dopamine signaling. Glutamate is the main excitatory neurotransmitter in the brain. Activation of glutamatergic receptors results in excitation of both excitatory and inhibitory neurons through a variety of receptors, including the NMDA receptor. The initial investigation of the NMDA receptor as it relates to schizophrenia began with individuals who abused drugs that block these receptors (ketamine and phencyclidine; NMDA receptor antagonists). The effects of the abuse of these drugs as well as observations from previous studies with controlled administration of NMDA receptor antagonists to healthy volunteers showed that NMDA receptor antagonists are capable of mimicking the cognitive and behavioral symptoms of schizophrenia in healthy individuals (Adler et al., 1999; Krystal et al., 1994; Luby et al., 1962). Additionally, abuse of ketamine or phencyclidine (PCP) by schizophrenic patients has been demonstrated to exacerbate schizophrenia symptoms and produce psychosis in stable patients (Javitt, 1987; Lahti et al., 1995; Luby et al., 1962). These early observations provide support that the NMDA receptor may be involved in the disorder.

NMDA receptors play an important role in learning and memory, cognition, and sensory processing. In the hippocampus, NMDA receptors are necessary for long-term potentiation (LTP), a process in which the synaptic connections between two or more

neurons that fire together are strengthened (Bliss & Lomo, 1973; Bliss & Collingridge, 1993; Hebb, 1949). This process is hypothesized to be vitally important for learning and memory and is strongly linked to new memory formation. Pharmacologically blocking the function of NMDA receptors leads to impaired cognition, learning, immediate recall, and long-term retention (Lee et al., 1993; Ohno, Yamamoto, & Watanabe, 1994).

NMDA receptors are unique because they are both ligand and voltage-gated receptors. While the binding of glutamate and glycine to the NMDA receptor will induce a conformational change in the receptor, the presence of a magnesium (Mg^{+}) ion bound to the inside of the channel at resting membrane potential (-65 mV) prevents the passage of any ions through the channel. When the membrane is depolarized by non-NMDA glutamate receptors (AMPA and kainite), the Mg^{2+} ion is driven out of the channel by electrostatic repulsion, allowing the channel to open and calcium (Ca^{2+}) to enter (Mayer, Westbrook, & Guthrie, 1984; Nowak et al., 1984). The influx of Ca^{2+} into the cell leads to an increase in membrane potential, as well as the activation of a variety of Ca^{2+} dependent second messenger systems (Kleinschmidt, Bear, & Singer, 1987). Therefore, activation of the NMDA receptor requires a unique combination of ligand binding and membrane depolarization.

NMDA receptors are composed of subunits termed NR1, NR2, and NR3. All functional NMDA receptors contain one NR1 subunit and either one of several possible subunits of NR2 (NR2A-D) or NR3 (Monyer et al., 1992). Different subunit assemblies of NMDA receptors contribute different functional properties of the receptor, including the strength of the Mg^{2+} block, glycine sensitivity, and receptor occupancy (Ishii et al., 1992; Kutsuwada et al., 1992; Monyer et al., 1992). For example, NR1-NR2A and NR1-

NR2B channels are characterized by a stronger voltage sensitivity of the Mg^{2+} block (it takes a greater voltage change to dislodge the ion) compared to NR1-NR2C and NR1-NR2D channels (Monyer et al., 1992). Because of the weaker Mg^{2+} block seen in the latter receptor channels, their function may be to detect relatively small postsynaptic depolarizations. Another functional difference between subtype assemblies is that NR2A-containing receptor channels do not bind glutamate for very long (measured by offset decay time) compared to the other subtypes (Monyer et al., 1992). Consequently, NR1-NR2B, NR1-NR2C, or NR1-NR2D receptors mediate longer-duration excitatory postsynaptic potentials as compared to NR1-NR2A receptors. Throughout a lifespan, the assembly of NMDA receptors differs (Akazawa et al., 1994). During development, NR1 and all NR2 subunits are expressed at peak levels on postnatal day (PND) 7 in the rat brain (about third trimester for human brain development; Clancy et al., 2007); then, after PND 12, a distinct change in expression occurs for the NR2 subunits (Monyer et al., 1994). There is a shift from the expression of NR2B and NR2D subunits, both of which are most abundant in the neonatal brain, to the expression of NR2A in the forebrain and NR2C in the cerebellum (Akazawa et al., 1994; Cull-Candy, Brickley, & Farrant, 2001; Monyer et al., 1994). Since many important cognitive processes depend on the NMDA receptors, altering their assembly and thus their activity can cause devastating effects.

Alongside the previously mentioned drug abuse literature, complementary data from post-mortem examinations of schizophrenic patients have given further valuable insight into the possible glutamatergic changes that occur in the disorder. Not only is glutamatergic tone reduced in schizophrenia patients (Sherman et al., 1991; Squires et al., 1993), differential expression of mRNA for the NR2A (increased levels) and NR2D

(decreased levels) subunits has been observed in the prefrontal cortex (Akbarian et al., 1996). Additional changes include a reduction the NR1 subunit in the hippocampus versus healthy controls (Gao et al., 2010). These data suggest that not only is there an overall reduction in glutamate signaling, but there is also an alteration in the assembly and function of the NMDA receptor.

GABA Signaling

GABA is the main inhibitory neurotransmitter in the brain and is synthesized from glutamate by GAD67. GABAergic interneurons are responsible for inhibitory signaling that is vital for normal CNS function, including the regulation of network oscillatory activity. A necessary component of the activation of these GABAergic neurons comes from NMDA receptors. When glutamate binds, the receptors allow Ca^{2+} to enter causing the cell to depolarize and release GABA. These interneurons are very large compared to pyramidal neurons and connect onto many different excitatory postsynaptic neurons (e.g. dopamine, serotonin, glutamate). The rapid release of GABA onto the postsynaptic excitatory neurons produces a sustained inhibition. This inhibition entrains the postsynaptic neurons into a synchronous firing pattern and, once disinhibited (removal of inhibition), the neurons are then allowed to fire when receiving excitatory input from other cells. Based on the role of NMDA receptor function, it has been hypothesized that NMDA receptor-mediated excitation of GABAergic interneurons results in the inhibitory activity that drives network function. Numerous investigations have shown that NMDA receptors on inhibitory GABAergic interneurons are disproportionately more sensitive to NMDAR antagonists (Coyle, Tsai, & Goff, 2003; Grunze et al., 1996; Li, Clark, Lewis, & Wilson, 2002) and, more specifically, those that

contain PV may have relevance to schizophrenia (Beasley & Reynolds, 1997; Beasley e al., 2002; Coyle et al., 2003; Kinney et al., 2006; Morris, Cochran, & Pratt, 2005; Reynolds et al., 2001). These data suggest that a reduction in the function of NMDA receptors can cause a lack of inhibition in discrete brain regions that have PV+ GABAergic interneurons. The effect of this lack of inhibition is proposed to cause a disruption in the synchronous activity of downstream excitatory pyramidal neurons.

GABAergic interneurons play an important role in the generation and entrainment of oscillations (Gonzalez-Burgos, 2010); therefore, neuronal synchronization is thought to rely on GABA-mediated inhibition. Gamma- and theta-wave oscillatory activity is suggested to underlie working memory and information processing between cortical areas and is related to cognitive function (Bartos, Vida, & Jonas, 2007; Cardin et al., 2009; Fries, 2009; Gonzalez-Burgos & Lewis, 2008; Roopun et al., 2008; Salinas & Sejnowski, 2001; Sohal et al., 2009; Uhlhaas & Singer, 2010). Based on numerous studies, the cognitive deficits seen in schizophrenia may result from abnormal neuronal oscillatory activity (Gonzalez-Burgos, 2010; Lewis et al., 2005; Uhlhaas & Singer, 2010). Converging evidence suggests that the generation of oscillations, which are impaired during cognitive tasks in schizophrenic patients (Minzenberg et al., 2010; Spencer et al., 2004; Woo, Spencer, & McCarley, 2010), depends on NMDA receptor-mediated transmission on PV+ GABAergic interneurons (Phillips et al., 2012; Sohal et al., 2009). Each PV+ GABAergic interneuron projects onto many excitatory neurons and disinhibition from one interneuron can desynchronize the majority of a local network, leading to a disruption in cortical processing (Moghaddam et al., 1997; Phillips et al., 2012). Decreased expression of PV leads to increased asynchronous release of GABA

from PV+ GABAergic interneurons, which may result in a reduction in the ability of downstream excitatory neurons to integrate incoming stimuli, the functional consequence of which may be altered cognitive function and sensory processing (Javitt, 2009; Powell, Sejnowski, & Behrens, 2012; Uhlhaas & Singer, 2010). Although data provide evidence for a reduction in NMDA receptors and PV expression, the GABA receptors themselves may also play a role in desynchronization as it relates to schizophrenia.

GABA binds to both ionotropic and metabotropic receptors in the central nervous system. Ionotropic GABA receptors (mainly $GABA_A$) are ligand-gated ion channels permeable to chloride. Once GABA binds and activates these ionotropic channels, a fast acting inhibitory current is produced within the cell (Macdonald & Olsen, 1994). Conversely, the metabotropic GABA receptors ($GABA_B$) are G-protein coupled receptors that are located both pre- and postsynpatically and produce a slow onset but longer lasting inhibition (Couve, Moss, & Pangalos, 2000; Kerr & Ong, 1992). Presynaptic $GABA_B$ receptors suppress neuronal Ca^{2+} conductance, leading to the inhibition of neurotransmitter release of the presynaptic neuron. Postsynaptic $GABA_B$ receptors increase membrane conductance to potassium (K^+) leading to the hyperpolarization of postsynaptic neurons (Bettler e al., 2004; Bowery et al., 2002; Kohl & Paulsen, 2010; Misgeld, Bijak, & Jarolimek, 1995; Padgett & Slesinger, 2010). Functional $GABA_B$ receptors exist as heterodimers (Jones et al., 1998) comprised of a $GABA_{BR1}$ (contains binding site) and a $GABA_{BR2}$ subunit (contains regulatory G-proteins; Bowery et al., 2002; Galvez et al., 2001; Kohl & Paulsen, 2010; Pinard, Seddik, & Bettler, 2010; Robbins et al., 2001). Compared to the function of the $GABA_B$ receptor, the function of the $GABA_A$ receptor is better understood because of its direct activation and fast

inhibition properties. Both $GABA_A$ and $GABA_B$ receptors are able to modulate GABA oscillations and LTP, which results in altered learning and memory functions (Scanziani, 2000).

Although the distinct roles the GABA receptors play in cognitive functions such as learning and memory are unclear, *in vivo* and *in vitro* studies have shown they are able to modulate neuronal oscillations. For instance, simultaneously blocking $GABA_A$ and $GABA_B$ receptor function *in vitro* facilitates theta frequency oscillations induced by a cholinergic agonist (Konopacki et al., 1997). Data from transgenic animals and pharmacological studies demonstrate that the $GABA_B$ receptor is vital for the stability of cortical circuits, possibly due to the longer lasting inhibitory properties of these receptors. $GABA_{BR1}$ knockout mice develop spontaneous seizures and premature death (Prosser et al., 2001; Schuler et al., 2001); meanwhile, high doses of $GABA_B$ receptor antagonists administered to adult rodents induce hippocampal and neocortical seizures (Leung, Canning, & Shen, 2005; Vergnes et al., 1997). However, the data regarding cognitive task performance and learning and memory are limited and inconsistent. Several studies indicate that low dose $GABA_B$ receptor antagonists enhance (Getova & Bowery, 2001; Helm et al., 2005; Leung & Shen, 2007; Mondadori, Jaekel, & Preiswerk, 1993; Mondadori, Mobius, & Borkowski, 1996; Staubli, Scafidi, & Chun, 1999) or do not alter cognitive task performance compared to controls (Zarrindast et al., 2002). Studies using $GABA_B$ receptor agonists are similarly inconsistent and sometimes contradictory (Myhrer, 2003). The incongruent results from $GABA_B$ receptor ligands may be due to the fact that the receptors are located both pre, post, and extrasynaptically and any functional effect of the ligand may not produce straightforward results.

Similar to what is found in post-mortem investigations with NMDA receptor subunits, $GABA_B$ receptors are also decreased in the prefrontal cortex and hippocampus of schizophrenic patients (Ishikawa et al., 2005; Mizukami et al., 2000; Mizukami et al., 2002). Post-mortem analysis in schizophrenia populations have shown consistent alterations in GAD67 and PV expression in the hippocampus and prefrontal cortex (Beasley & Reynolds, 1997; Benes et al., 1991; Bullock et al., 2008; Hashimoto et al., 2003; Mirnics et al., 2000; Reynolds, Zhang, & Beasley, 2001; Torrey et al., 2005). Since both of these proteins are specific to GABAergic interneurons, these data suggest that GABA signaling may be altered in the disorder as well.

As previously discussed, the hypofunction of NMDA receptors may result in the disinhibition of GABAergic interneurons. It is also possible that the receptors to which GABA binds may be differentially expressed, such that a similar disinhibition occurs. If there is a reduction in pre- or postsynaptic $GABA_B$ receptor function, an alteration in the release or binding of GABA potentially results in asynchronous activity. Disrupting the function of either NMDA receptors or $GABA_B$ receptors could conceivably result in the same change in oscillatory activity that has been speculated to occur in schizophrenia.

Animal Systems to Model Mechanisms of Schizophrenia

Although schizophrenia is an inherently human disorder, certain symptoms can be tested in rodent models. For instance, prepulse inhibition to test for sensorimotor gating is often examined in animal models of schizophrenia, as well as to test the potential efficacy of novel antipsychotics (Swerdlow, Braff, & Geyer, 2000). Additionally, the Morris water maze is utilized to look for spatial learning and memory deficits and cued and contextual fear conditioning is used to investigate associative fear learning. As noted

above, schizophrenia patients exhibit deficits in all of these tasks, suggesting the impairments may have relevance to the disorder.

Sensorimotor gating can be described as the ability to filter extraneous noise from meaningful sensory inputs (Freedman et al., 1987). Patients have been characterized as being hypervigilant and sensitive to distracting sounds whereby their inability to gate stimuli makes them feel "flooded" by sensory stimulation (Venables, 1964). Sensorimotor gating can be measured in a task called prepulse inhibition (PPI) in both human and animals. PPI refers to the normal reduction of a startle response when a startling stimulus is preceded by a weaker, non-startling stimulus (prepulse). The theory is that the prepulse evokes an inhibitory response of the motor reflex that reduces the response to the startle stimulus (Braff & Geyer, 1990; Swerdlow, Geyer, & Braff, 2001). PPI is observed in neuropsychiatric disorders and can be modulated by drugs of abuse (Braff & Geyer, 1990; Geyer & Braff, 1987). Since PPI occurs on the first exposure of a prepulse-startle trial, it does not reflect a learned behavior but a reflexive response (Fendt, Li, & Yeomans, 2001; Graham, 1975; Hoffman & Wible, 1970; Ison, McAdam, & Hammond, 1973; Koch, 1999). Patients with schizophrenia startle similarly regardless of whether the startle stimulus is preceded by a non-startling, weaker prepulse, suggesting the prepulse does not inhibit this reflexive response as seen in healthy controls (Geyer & Braff, 1987). This disruption in PPI suggests a failure in sensorimotor gating that may result from stimulus over-flow either from the external environment and/or from within the subject itself.

Mediating brain regions for PPI of the startle reflex are found mostly within the brain stem. Briefly, the acoustic prepulse is relayed from the inferior and superior

colliculus to the pedunculopontine tegmental nucleus, with lesser contributions to the circuit from the laterodorsal tegmental nucleus and substantia nigra, pars reticulata. Activation of the midbrain nuclei by the prepulse is converted into long-lasting inhibition of the giant neurons in the caudal pontine reticular nucleus, with the involvement of $GABA_B$ receptors contributing to the inhibition of the startle response (Fendt et al., 2001; Swerdlow et al., 2001). Other regions are shown to regulate the brain circuitry responsible for PPI, including the hippocampus and the prefrontal cortex, which have subcortical projections to the nucleus accumbens and ventral tegmentum (Swerdlow et al., 2001). Both NMDA and $GABA_B$ receptors contribute to this circuit and altering these receptors produces PPI deficits. This task is foundational as a preclinical behavioral test for animal models of schizophrenia.

Another task used in research on animal models of schizophrenia to evaluate learning and memory deficits is the Morris water maze. Spatial learning and memory is disrupted in schizophrenic patients and it has been measured using a virtual, computer-based water maze task (Folley et al., 2010; Hanlon et al., 2006). The original Morris water maze was developed for rodents and consists of a circular tank filled with opaque water and a submerged escape platform that is not visible. In order to locate the hidden platform using a spatial strategy, the animal must use spatial cues that are located outside the maze. Across days, control animals begin to utilize a spatial strategy that depends on the extra maze cues, which results in decreased latencies (time) to find the platform. To determine how well the animals learned the task, a probe trial is conducted in which the platform is removed and animals swim for a full trial period. Depending on how well they learned the task, the animals should spend most of their time in the area of the maze

where the platform was located. Performance in the water maze is disrupted after hippocampal lesions (Amaral & Witter, 1991; Moser, Moser, & Andersen, 1993) where both NMDA and $GABA_B$ receptors are prevalent (Dutar & Nicoll, 1988; Monaghan, Andaloro, & Skifter, 1998; Sakurai, Penney, & Young, 1993).

Patients with schizophrenia exhibit deficits in emotional processing and emotional learning and memory (Hall et al., 2007). The deficits resemble those found in patients with amygdalae damage (Aleman et al., 2007; Hall et al., 2007; Kosaka et al., 2002; Whalley et al., 2009). For example, patients with schizophrenia have reduced activation of the amygdala when viewing emotional faces, as well as an alteration in hippocampal activity when viewing emotional scenes compared to control individuals (Hall et al., 2007). Pavlovian fear conditioning is a type of associative learning task that has been utilized to test for deficits in emotional learning and memory in animal systems (Bolton et al., 2012; Kinney et al., 2002; Phillips & LeDoux, 1992). By pairing a neutral stimulus such as a tone (conditioned stimulus; CS) with one that elicits a fear response such as a mild foot shock (unconditioned stimulus; US), the CS will eventually elicit a similar fear response (freezing behavior in rodents) without the presence of the US. This association can be quantified using the fear response to the CS and serve as a measure of how well an animal learns the association. After training, the animal can be placed in a novel environment with the presentation of the original CS to determine the cued fear association (association of fear to the tone) or the animal can be placed in the original training environment with no CS presentations to demonstrate contextual fear association (association of fear to the environment). Different neural mechanisms and regions govern the associations utilized in this task depending on how the CS and US are presented

during training. In the standard delay cued and contextual fear (CCF) procedure, the CS and the US overlap in time and co-terminate. This training protocol requires amygdala activation to make the cued fear association (Davis & Whalen, 2001; Kinney et al., 2002; LeDoux et al., 1990; Phillips & LeDoux, 1992; Schafe et al., 2001) and the contextual fear association is dependent on the hippocampus (Chen et al., 1996; Holland & Bouton, 1999; Kinney et al., 2002; Logue, Paylor, & Wehner, 1997; R. G. Phillips & LeDoux, 1992). Manipulating the training protocol by inserting a temporal gap between the cessation of the CS and the onset of the US (trace CCF procedure) makes the task more difficult and more presentations of the CS and US are required to learn the association (Beylin et al., 2001; Kinney et al., 2002). Also, both the hippocampus and amygdala become imperative to make the cued fear association (Bolton et al., 2012; Kinney et al., 2002; McEchron et al., 1998; 2000; Moyer, Deyo, & Disterhoft, 1990; Ryou, Cho, & Kim, 2001; Solomon et al., 1986; Sutherland & McDonald, 1990; Weiss et al., 1999) while contextual fear is still dependent on the hippocampus (Bolton et al., 2012; Chen et al., 1996; Holland & Bouton, 1999; Kinney et al., 2002; Logue et al., 1997; Phillips & LeDoux, 1992). Manipulations that result in differences in fear responses can reveal deficits in the mechanisms associated with different brain regions.

The above behavioral tasks are used to measure learning and memory deficits in rodents with relevance to those observed in the schizophrenia population. NMDA receptor antagonists have been used in animal models to investigate symptoms associated with schizophrenia. As previously mentioned, these antagonists include PCP, ketamine, as well as MK-801. Although PCP produces more potent and longer lasting effects than the other NMDA antagonists, low doses of ketamine (subanesthetic doses) induce

changes in cortical GABAergic activity that result in disinhibition (Behrens et al., 2007). Subanesthetic doses of ketamine in adult rodents produce sensorimotor gating deficits as measured by PPI (Bolton et al., 2012; Braff & Geyer, 1990; Sabbagh et al., 2012), as well as behavioral alterations in the Morris water maze (Becker et al., 2003; Moosavi et al., 2012; Sabbagh et al., 2012), and CCF (Bolton et al., 2012). Since blocking NMDA receptors on GABAergic interneurons produces a reduction in GABAergic tone due to diminished firing, an alternative approach to achieve a similar effect would be to alter GABA receptor function. As opposed to $GABA_A$ receptors whose responses are short lived, $GABA_B$ receptors provide the long lasting inhibition seen in oscillatory activity. However, drugs that target $GABA_B$ receptors are less utilized in schizophrenia research compared to NMDA receptor antagonists. As mentioned previously, $GABA_B$ receptor ligands administered to animals results in inconsistent behavior in learning and memory tasks (Myhrer, 2003). These inconsistencies may result because $GABA_B$ receptors are located pre, post, and extrasynaptically; further, the effect of the ligands may also rely on the task utilized. Our laboratory has demonstrated that administering a $GABA_B$ receptor agonist (baclofen) produces deficits in CCF (Heaney et al., 2012), and preliminary data suggest PPI deficits. While this approach to modeling schizophrenia has been productive in demonstrating potential mechanisms involved in the disorder, one drawback is that these deficits are only present when the drug is active in the animal's system. Therefore, it may be relevant to determine if alterations during development produce similar deficits without administering drugs at the time of behavioral testing.

Neurodevelopmental Approaches

Based on the typical age of diagnosis of schizophrenia and the overlap of this time point with several developmental processes, schizophrenia is suggested to be the result of disturbances in early brain development. Multiple proposed models argue that a change in developmental trajectory results in schizophrenia, which is not evident until early adulthood (Fatemi & Folsom, 2009; Lewis & Levitt, 2002; Lewis et al., 2004; Rapoport et al., 2005; Weinberger, 1987; Weinberger & Lipska, 1995). Studies that support this approach focus on post-mortem tissue analysis of patients with schizophrenia and the genetic and/or environmental stressors.

Post-mortem tissue analysis reveals that there are changes in specific circuits in the brain related to subtle variations during development. These investigations have found cytoarchitectural disorganization in the hippocampus with too few neurons in the superficial layers and too many neurons in deeper layers (Jakob & Beckmann, 1986). This study has since been replicated using similar methodology and extended into the prefrontal, temporal, and limbic cortices with the same results (Akbarian et al., 1996; Arnold et al., 1991; Benes et al., 1991). This cytoarchitectural disorganization suggests a failure of neuronal migration and inappropriate final placement of neurons, an event that occurs during early development. In normal brain development, the migration of neurons to these regions occurs during the second trimester of intrauterine development (Rakic, 1988). The disorganization of this process could potentially result in altered connectivity of these regions. Additionally, other post-mortem studies of patients with schizophrenia indicate there are no signs of neuronal damage (Falkai & Bogerts, 1986) and no sign of progressive worsening of the pathology over time, indicating that the cytoarchitecture

disorganization in schizophrenic patients must occur during development (Weinberger, 1987; Weinberger & Lipska, 1995). The cause of the alterations in post-mortem tissue is not known but several potential mechanisms have been proposed.

The initial disturbances during development might be triggered by a combination of genetic and/or environmental stressors. A number of susceptibility genes are associated with schizophrenia. For instance, the gene that encodes for the protein Reelin is reduced in the brains of patients with schizophrenia (Beffert et al., 2005; Impagnatiello et al., 1998). Evidence from animal studies shows that an appropriate amount of Reelin is necessary for proper neuronal migration (Fatemi et al., 1999; Goffinet, 1979), and a reduced amount of Reelin results in cytoarchitectural alterations similar to what is seen in schizophrenic patients (Fatemi, 2005). Additionally, the transcription factor Lhx6 is reduced in post-mortem schizophrenia brains (Volk et al., 2012). Lhx6 is specific to cells that are destined to be PV+ neurons and, during the prenatal period, it participates in the regulation of cell type specification, tangential migration, and maturation of these specific PV+ GABAergic neurons (Liodis et al., 2007; Volk et al., 2012; Zhao et al., 2008). Reduced Lhx6 in schizophrenic patients might explain the failure of PV+ neurons to appropriately express their phenotype. Changes in the expression of Reelin and Lhx6 in schizophrenia patients provide support for a developmental origin of the disorder.

Genetic factors may make the system more susceptible to stressors, but they do not guarantee that one will inherit the disorder. Monozygotic twin studies indicate that there is only a 40-48% chance of one twin being diagnosed with schizophrenia if the other is already diagnosed (Suddath et al., 1990). These data indicate that although a genetic disposition to the disorder exists, there are environmental risk factors that also contribute

to the disease. Some environmental stressors that have been suggested to be involved in changing the developmental trajectory include maternal stress and infection, prenatal malnutrition, obstetric complications, and urban living (McNeil & Kaij, 1978; McNeil, 1987; Vassos et al., 2012). Several large epidemiological studies indicate that prenatal exposure to viral infection increases the risk of schizophrenia due to the activation of the maternal immune response (Adams et al., 1993; Izumoto, Inoue, & Yasuda, 1999; Mednick, Huttunen, & Machon, 1994; Zuckerman et al., 2003). Stress in early life has been shown to produce behavioral differences in adult individuals and is complemented with data from rodent studies in laboratory settings (Gutman & Nemeroff, 2002). Although these studies inflict stress upon the developing fetus, the mechanism by which this would influence cytoarchitecture and molecular changes in the brain consistent with schizophrenia is not known.

Development of the brain relies on the tightly regulated processes wherein the coordinated activity of various mechanisms is essential. NMDA receptor-mediated synaptic activity is necessary for normal development of various brain regions, as well as for coordinated network activity due to their involvement in critical processes such as establishing synaptic contacts, neuronal migration, and synaptogenesis (Contestabile, 2000; Komuro & Rakic, 1993; LoTurco et al., 1991). However, GABA signaling precedes the development of NMDA receptor-mediated signaling and GABA plays a pivotal role in normal brain development as well. In early brain development, GABA acting on ionotropic $GABA_A$ receptors produces an excitatory, not inhibitory, response both *in vitro* and *in vivo* (Ben-Ari, 2007; Leinekugel et al., 2002; Sipila et al., 2006). This excitatory response is due to elevated intracellular chloride concentration in immature

neurons that begins to reduce to what is normally seen in mature cells around PND 5-7 in rodent pups and the effect of GABA acting on $GABA_A$ receptors becomes inhibitory around PND 10 (Ben-Ari et al., 1989; Ben-Ari et al., 2007). The early excitation provided by $GABA_A$ receptors aids in the membrane depolarization needed for NMDA receptors to expel the Mg^{2+} ion in order for the channel to be functional (Ben-Ari et al., 2007; Ben-Ari et al., 1997). Presynaptic $GABA_B$ receptors are functional at birth and provide inhibitory control of neonatal network activity at this early stage in development (Ben-Ari et al., 1997; McLean et al., 1996). Meanwhile, electrophysiological studies demonstrate that postsynaptic $GABA_B$ receptors on either pyramidal cells or interneurons are not functional during this time period (Caillard et al., 1998; Gaiarsa et al., 1995; Nurse & Lacille, 1999; Verheugen, Fricker, & Miles, 1999). Embryonic and neonatal rat hippocampal and cortical neurons do not exhibit postsynaptic $GABA_B$ receptor-mediated responses (the activation of potassium and the inhibition of Ca^{2+} currents) until after PND 7 (Ben-Ari et al., 1997) due to the lack of coupling between the receptors and the ion channels (via G-proteins) (Fukuda, Mody, & Princa, 1993). The balance between excitation and inhibition during early brain development is vital for normal neuronal network formation.

Since NMDA receptor activation is vitally important in development, and post-mortem analyses point to alterations during critical periods associated with schizophrenia, studies have combined these theories by administering NMDA receptor antagonists to rodent pups during development to evaluate if deficits consistent with schizophrenia are produced. The first two weeks of life for a rat pup (especially at PND day 7) are a vulnerable period of brain development that correlates with the second

trimester of pregnancy in humans (See Figure 1 below; Bayer et al., 1993; Clancy, Darlington, & Finlay, 2001; Clancy et al., 2007). Studies have found that administering NMDA receptor antagonists (PCP, MK-801, and few using ketamine) during early stages of brain development in rodents (PND 7-14) results in an increase of neurodegeneration in brain areas implicated in schizophrenia and that this neurodegeneration is consistent with normal apoptosis that occurs during development and, therefore, is not related to excitotoxicity (Ikonomidou et al., 1999; Wang et al., 2001; Wang & Johnson, 2005). As mentioned, neurodegeneration and excitotoxic effects are not seen in post-mortem analyses of schizophrenic patients. Additionally, studies have found behavioral deficits in adult rats that had previously been administered NMDA receptor antagonists during the first weeks of postnatal life (see Figure 1 below). These studies demonstrate deficits in sensorimotor gating and PPI, consistent with schizophrenia-like symptoms (Abekawa et al., 2011; Anastasio & Johnson, 2008; Beninger et al., 2002; Broberg et al., 2010; Harris et al., 2003; Rasmussen et al., 2007; Turner et al., 2010; Van den Buuse, Garner, & Koch, 2003; Wang et al., 2001; Wang & Johnson, 2005), with few using other behavioral tasks (Andersen & Pouzet, 2004). However, these previous studies use a moderate to high dose of drug that could possibly mimic a very large shutdown of the NMDA receptor system. It would be interesting to see if the effect of NMDA receptor antagonists administered at low doses produces similar learning and memory deficits in adulthood.

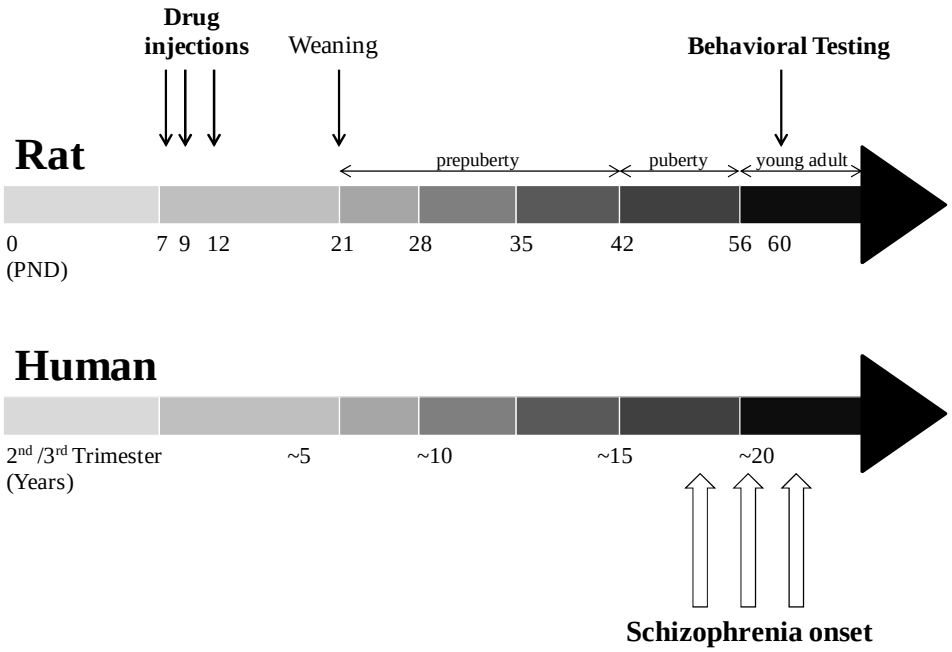

Figure 1. A comparison of rat and human brain development with a timeline of methods used in developmental approaches to schizophrenia. Derived from Tseng et al., 2009.

The use of ketamine compared to other NMDA receptor antagonists in schizophrenia research has advantages. It is commonly used in veterinary medicine (and to some extent, pediatrics) as a dissociative anesthetic; however, at subanesthetic doses, it displays different properties. Compared to the other NMDA receptor antagonists, ketamine has a lower potency, a shorter duration of action, a faster rate of induction, and is effective across different animal species (Hevers et al., 2008). In our laboratory, the administration of ketamine in adult systems has proven to model deficits seen in schizophrenia for several behavioral tasks such as PPI, the Morris water maze, and trace CCF (Bolton et al., 2012; Sabbagh et al., 2012). These features allow researchers to have better control of the drug *in vivo*, making it the optimal choice as an NMDA receptor antagonist. However, its utility at low doses in development to model deficits seen in schizophrenia has yet to be elucidated.

A more puzzling question in schizophrenia research is the effects of pharmacologically altering the $GABA_B$ receptor system in development. There has not yet been a study that examines administration of a $GABA_B$ receptor antagonist or agonist during development. Since NMDA receptors are centrally involved in the developmental pathogenesis of schizophrenia and rely on $GABA_B$ receptors during development, it is unknown what may occur if the $GABA_B$ receptor system is altered during this stage. As previously discussed, $GABA_{BR1}$ knockout mice display massive behavioral impairments, die prematurely, and may be a better model for epilepsy than schizophrenia (Prosser et al., 2001; Schuler et al., 2001). However, disturbing the $GABA_B$ receptors either by enhancing or inhibiting their function intermittingly could provide valuable insight into their role as it relates to this disorder.

Hypotheses and Implications

Building upon the wealth of research that implicates altered function of the NMDA receptor and changes in GABAergic signaling in schizophrenia as well as the importance of their role in brain development, we examined the behavioral and biochemical consequences of disrupting the functioning of either NMDA or $GABA_B$ receptors during critical time points in development.

Hypothesis 1:

We administered the NMDA receptor antagonist ketamine at a low dose (8 mg/kg) to rat pups at PND 7, 9, and 12. In adulthood, the animals should exhibit sensorimotor gating deficits measured by a reduction in PPI, impairments in spatial learning and memory demonstrated by the Morris water maze task, and deficits in fear associative learning seen in trace CCF compared to controls. The examination of brain tissue should result in alterations of proteins associated with NMDA receptors, GABA receptors, and GAD67, all of which have been found to be disrupted in schizophrenia.

Implications of Hypothesis 1:

If the low dose ketamine administration during development produces behavioral and protein expression changes consistent with what is observed in other schizophrenia models, then the data would indicate that alterations in NMDA receptors at the specific time points (PND 7, 9, and 12) induce a change in the system that results in deficits consistent with the disorder. Alternatively, if the dose and schedule of ketamine

administration does not induce the proposed deficits, it may be possible that a subtle, transient blockade of this receptor system is not enough to disrupt the developing network system and an increase in dose or multiple injection days may be considered.

Hypothesis 2:

In addition to the administration of ketamine to post-natal rat pups, we administered the $GABA_B$ receptor antagonist phaclofen and the $GABA_B$ receptor agonist baclofen to different groups of animals. The injections were on PND 7, 9, and 12 to determine if a change in GABAergic signaling during critical periods produces changes in behavior. There is a delicate balance in GABA signaling during development (as previously demonstrated) so the present study will determine if blocking or enhancing $GABA_B$ receptor function during this developmental period alters behavior in adulthood. We hypothesized that phaclofen and baclofen, each, would produce behavioral deficits (reductions in PPI and deficits in the Morris water maze and trace CCF) similar to ketamine deficits in Hypothesis 1. Blocking (with phaclofen) or enhancing (with baclofen) theoretically would alter connections in the developing brain and change network function. We also proposed that the tissue analyses would indicate alterations in proteins related to the GABAergic signaling.

Implications of Hypothesis 2:

If altering $GABA_B$ receptors, either by reducing or enhancing their function, during early brain development produces behavioral and/or

protein changes similar to what is seen in schizophrenia, then this experiment will provide evidence for the involvement of GABA signaling dysfunction potentially being involved in the disorder. If no deficits are detected with either phaclofen or baclofen, then alternate dosages and schedule of administration would be useful. The possibility exists that direct alterations of $GABA_B$ receptor function alone may be insufficient to alter developmental trajectories.

CHAPTER 3

MATERIALS AND METHODS

Subjects

Eight timed pregnant Sprague-Dawley dams were obtained from Charles River Laboratories (Hollister, CA). Dams were individually housed until parturition with a 12 hour light/dark cycle and with food and water available *ad libitum*. The day of birth was considered PND 0. Pups were sexed and randomly assigned to one of four treatment groups (saline, ketamine, baclofen, or phaclofen) on PND 2. All pups were weaned on PND 21 and pair housed throughout the remainder of the experiment with male (n = 55) and female (n = 35) animals housed in separate colony rooms. Behavioral testing began on PND 60 which corresponds to brain maturity in early adulthood for humans (Pignatelli et al., 2006). All procedures were performed in accordance with NIH guidelines for ethical treatment of research subjects, and approved by the University of Nevada, Las Vegas Animal Care and Use Committee.

Drug Treatments

All drugs were mixed with physiological saline (0.9% NaCl) to achieve a final concentration of 8 mg/ml ketamine (ketamine HCL from Henry-Schein, Indianapolis, IN), 2 mg/ml baclofen (baclofen hydrochloride from Sigma-Aldrich, St. Louis, MO), 0.3 mg/ml phaclofen (from Sigma-Aldrich, St. Louis, MO). The drugs were administered subcutaneously (SC) at a dose of 1 ml/kg. Animals were randomly assigned one of the three treatment groups: saline (n = 14 males; n = 6 females), ketamine (n = 14 males, n = 10 females), baclofen (n = 14 males; n = 9 females), and phaclofen (n = 13 males; n = 10 females).

Apparatus

Prepulse Inhibition of Acoustic Startle

Acoustic startle chambers (San Diego Instruments, San Diego, CA) measuring 28 cm (W) x 28 cm (H) x 28 cm (L) were used for the acoustic startle and PPI. Inside the chamber, the animals were individually placed in a transparent Plexiglas tube (10 cm wide and 20 cm long) that is mounted on an accelerometer which measures changes in movement. A Cobalt Instruments computer using Acoustic Startle software package (Startle, San Diego Instruments) recorded the data from the accelerometer.

Fear Conditioning Chambers

A Freeze Monitor chamber (San Diego Instruments) measuring 25.4 cm (W) x 19.05 cm (H) x 25.4 cm (L) with a stainless steel grid floor and Plexiglas walls was used to perform both the fear conditioning training and contextual fear test. The chamber was cleaned with Formula 409 (Chlorox, Oakland, CA) solution at the end of each session. An altered context chamber was used to test cued fear. This chamber consisted of an opaque plastic enclosure measuring 12.7 cm (W) x 26.67 cm (H) x 43.18 cm (L) with the addition of a vanilla scent on an inner wall to ensure a different olfactory background for the altered context. A solution of 0.1% ethanol was used to clean the chamber after each session. The chambers were connected to a Cobalt Instruments computer with the Freeze Monitor software (San Diego Instruments) to run each session. Freezing behavior (no movement besides breathing) was recorded every 10 seconds.

Morris Water Maze

The Morris water maze procedure was performed in a white polyethylene circular tank, 1.8 m (diameter), 76 cm (H), and 4.7 mm in thickness (San Diego Instruments). The tank was filled with tap water 48 cm deep and maintained at 25˚C. The addition of non-toxic paint made the water opaque to conceal the hidden platform (10 cm x 10 cm square platform made of clear plastic). The tank was divided into four quadrants, one in which the hidden platform was placed in the center 30 cm from inside the wall of the tank and 1.5 cm below the surface of the water. A 13 cm x 13 cm square white plastic cover, protruding 3 cm above the water, was placed on top of the platform during the visible training trials. The tank was positioned in the center of a training room (separate from the colony room) with large geometric shapes positioned on all four walls to serve as distal spatial cues for the animals to find the hidden platform. Trials were recorded and captured using a video tracking system (Smart, San Diego Instruments) from a Sony Handycam camera connected to a Cobalt Instruments computer. Latency to locate the hidden platform, speed of swimming, and thigmotaxis (proportion of time spend along the outer edge of the maze) data was collected for each trial. The amount of time subjects spent in each quadrant was recorded for the probe trial.

Tail Flick

The tail flick procedure was performed using a circular glass bowl, 20.32 cm (diameter) and 7.62 cm (H), filled with 1800 ml of water. The bowl was placed on a heating plate to maintain the water at 55˚C throughout each session.

Drug Administration

After the pups were sexed and randomly assigned to treatment groups on PND 2, they received 1 ml/kg subcutaneous injection of saline, ketamine, baclofen, or phaclofen on PND 7, 9, and 12 (Figure 1) then weighed every day until weaning (PND 21) to ensure proper growth and development.

Behavioral Testing

All behavioral testing began once animals reached PND 60 to ensure they reached early adulthood. All experimenters were blind to the subject's treatment group throughout testing.

Prepulse Inhibition of Acoustic Startle

Animals were taken into a separate training room and individually placed in the startle chambers. Animals were given five minutes to acclimate to the environment and presented with background noise (65 dB) throughout the session. Acoustic startle responses were examined after the presentation of 30 ms noise bursts of 90, 100, 110, and 120 dB. Sensorimotor gating was tested with auditory white noise prepulses of 74, 82, 86, or 90 dB presented 40 ms or 100 ms prior to the 120 dB startle pulse. Inter-trial intervals were randomized between 10 and 55 seconds. For each session, a random order of trial type presentations was presented 5 times. When evaluating the acoustic startle data, the first trial of each acoustic startle intensity response was discarded and averages of the remaining four trials were used for calculation. Percent PPI was calculated using the following equation: 100-[(average startle response with pre-pulse)/(average acoustic startle)]*100.

Cued and Contextual Fear Conditioning (CCF)

Subjects were individually taken from the colony room and placed in the fear conditioning chamber located in a separate training room. The animals were allowed to freely explore the chamber during the initial two minutes of the training session in which no stimuli were presented. Following the initial two minutes in the chamber, a 2.9 kHz 88 dB tone conditioned stimulus (CS) was presented for 30 s; 2.5 s following the termination of the CS, a 1 second 0.5 mA footshock (US) was administered. Following the termination of the US, a two minute interval elapsed prior to the next CS and US presentation. A total of 4 CS-US pairings were presented in the training session, each separated by two minute intervals. After the completion of the last pairing, the animals' behavior was observed for a final two minutes. The animals' freezing behavior was evaluated every ten seconds throughout the session by a trained experimenter blind to the experimental group. The criterion for freezing consisted of no movement of the animal other than breathing. Freezing behavior was recorded during the training session for the first two minutes (pre-training freezing) and for the final two minutes (post-training). Once the session was complete, the animal was returned to its home cage in the colony room and the chamber was cleaned.

Cued fear testing (fear to the CS) was examined 24 hours after training. Animals were individually placed in the altered context for a total of 13 minutes. The session consisted of an initial two minute interval, during which no stimuli were presented in order to evaluate freezing to the novel environment. A one minute presentation of the original CS used in training was presented, followed by a two minute interval between the onsets of the next CS tone, for a total of 4 CS tone presentations. Freezing behavior

was measured throughout the entire session. The animals were brought back to their home cage following the completion of the session and the chamber was thoroughly cleaned with 0.1% ethanol.

Contextual fear (fear to the training environment) was tested 48 hours after training. Animals were placed in the initial training chamber and allowed to freely explore for 10 minutes without any CS or US presentations. Freezing behavior was measured during the entire session in five, 2 minute bins. After the session, the animals were returned to their home cage and the chamber was cleaned.

A cued fear session (72 hours after training) and contextual fear session (96 hours after training) were administered in an identical fashion as previously described following the 48 hour contextual test. However, following the 96 hour contextual fear session, one CS-US pairing was presented (reminder trial) followed by a final two minute period of observation. An additional cued fear session (24 hours after reminder) and contextual fear session (48 hours after reminder) was carried out on subsequent days using the same protocols detailed above.

Morris Water Maze

Individually, animals were taken from the colony room into a separate testing room. A computer desk, a table with a heating cage, large geometric shapes on each wall, and the experimenter served as distal spatial cues as they can be seen from inside the maze. The animal was placed into the maze and into the water along the outer wall of one of the three randomly chosen quadrants that do not contain the hidden platform. The animal was allowed to swim until locating the hidden platform or for a maximum of 60 seconds. After 60 seconds, the experimenter guided the animal to the hidden platform.

Each animal was given 20 seconds to sit/stand on the platform to allow spatial orientation to distal extra-maze cues after which they were taken out of the maze and placed in a holding cage under a heat lamp for 30 seconds. Three additional trials were conducted using the same protocol for a total of four training trials per day. After the fourth interval under the heating lamp, the animal was gently dried with a towel and taken back to its home cage. The platform remained in the same location for each trial and across days. Training trials were conducted on successive days until the completion of either six days of hidden platform training or until the controls reached a latency of $\leq$ 15 seconds. The animals' path, latency, thigmotaxis, swim speed, and quadrant location was recorded.

Once the control animals reached the hidden platform latency criterion, a single probe trial was performed the following day. The hidden platform was removed from the maze and the animal was allowed to swim for 60 seconds with the tracking system measuring the amount of time the animal spent in each quadrant. To examine the ability of how well the animals learn a new location of the hidden platform, a reversal procedure was used in which the platform was moved to the opposite quadrant (180 degrees from original location) and the animals were given four trials with the same protocol as a hidden platform day. To test for visual and motor capabilities, a non-spatial test was performed using a visible platform. This task is similar to the hidden platform protocol except that the platform is visible to the animal and moved on successive trials to different quadrants for each trial.

Tail Flick

In order to ensure any differences that may be observed during the fear conditioning procedure are not tied to changes in nociception (i.e. analgesia), a standard

tail flick nociception test was performed on all animals. Subjects were individually taken from their home cage into a testing room. The initial 2–3 centimeters of the tip of the tail was placed in 55º C water and the latency for the animal to flick its tail out of the water was recorded. A maximum of 10 seconds was set as a criterion for withdrawal (flick).

Tissue Analysis

Tissue Collection

Following the tail flick test, all animals were humanely euthanized using CO_2 asphyxiation. Brains were immediately removed following CO_2 asphyxiation. The frontal portion of the cortex was dissected out, placed in microcentrifuge tubes, and kept frozen at -80˚C until tissue homogenization.

SDS-PAGE Western Blotting

Prefrontal cortices were homogenized using a RIPA buffer (20 mM pH 7.5 Tris-HCL, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 µg/ml leupeptin; Cell Signaling, Danvers, MA) with 1 mM DTT, 1 mM PMSF, 20µg/ml aprotinin, and 0.1% SDS added. Homogenization was performed using a handheld Polytron (Kinematica Inc., Luzern, Switzerland) tissue homogenizer. Homogenized tissue was centrifuged for 15 minutes at 15,000 x g at 4˚C. Supernatant was removed without disturbing the pellet and a protein concentration assay was performed using the bicinchoninic acid assay (BCA, Pierce, Rockford, IL).

Protein samples were loaded into 10% acryl gels at a concentration of 2 µg/µl with a mixture of Laemmeli buffer containing 2% SDS (BioRad, Hercules, CA) and DI water for a total of 10 µL volume. SDS-PAGE was held at a constant current 0.04 A for

45 minutes to separate protein samples according to molecular weight. Following the separation, the proteins were electrotransferred at 0.25 A for 1 hour to 0.2 µm nitrocellulose membranes (GE Water and Process Technologies, Feasterville-Trevose, PA). Membranes were placed in a blocking buffer with 5% BSA and TBST (1x Tris-buffered saline with 0.05% Tween 20) before being incubated overnight at 4˚C in primary antibody (anti-$GABA_A$α5 polyclonal rabbit, 1:750 (Millipore, Billerica, MA); anti-$GABA_{B1}$ polyclonal rabbit, 1:2000 (Cell Signaling Technology, Danvers, MA); anti-$GABA_{B2}$ polyclonal rabbit, 1:1000 (Cell Signaling Technology); anti-GAT1 polyclonal rabbit 1:1000 (Cell Signaling Technology); anti-NR2B polycolonal rabbit 1:100 (Cell Signaling Technology); anti-Kalirin polyclonal rabbit, 1:1000 (Cell Signaling Technology); anti-β-Actin monoclonal rabbit, 1:20,000 (ProteinTech, Chicago, IL)). Membranes were returned to room temperature the following day and washed with TBST 3 times for 10 minutes each. Following washes, membranes were incubated in secondary antibody (HRP-conjugated anti-rabbit, 1:5000, Vector Laboratories, Burlingame, CA) for 1.5 hours at room temperature. Following 3 additional 10 minute washes with TBST, the membranes were exposed to Amersham ECL Plus detection system (GE Healthcare Life Sciences, Pittsburgh, PA) and imaged using a Typhoon 9410 Variable Mode Imager (GE Healthcare Life Sciences). ImageQuant 5.2 software (GE Healthcare Life Sciences) was used to determine protein quantities. The proteins of interest were normalized to β-Actin density. The treatment group's normalized values were compared to the average control (saline) normalized values for each membrane. All SDS-PAGE experiments were all run in duplicate to ensure consistent data.

Statistical Analysis

Acoustic startle response and PPI data were analyzed via repeated measures analysis of variance (RM-ANOVA) across startle or prepulse intensities. One-way ANOVA for individual startle and prepulse intensities were performed after a significant RM-ANOVA. Mean latencies, swim speed, and thigmotaxis from the water maze for hidden platform days were analyzed across days using RM-ANOVA. Data from the time spent in each quadrant during the probe trial in the water maze were analyzed using one-way ANOVA (target versus other quadrants). RM-ANOVA were used for the CCF data except one-way ANOVA were used to determine differences between treatment groups in pre CS-US freezing and post CS-US freezing on the training day. Mean latencies for tail flick and mean densities for western blotting were analyzed using one-way ANOVA. Tukey post-hocs were performed following a significant ANOVA. To parse out the differences between the control group and either ketamine or the $GABA_B$ ligands, separate analyses were conducted. Males and females were analyzed separately except when comparing males versus females for the GAD67 protein.

CHAPTER 4

RESULTS

Behavioral Testing

Prepulse Inhibition of Acoustic Startle

A significant difference was observed in acoustic startle response (Figure 2) between treatment groups for the male animals (RM-ANOVA; $F_{(3,216)} = 3.425$, $p = 0.018$). The baclofen group exhibited a significant increase in startle response compared to saline (Tukey post hoc; $p = 0.013$). Analysis of individual startle intensities revealed that the significant increase in acoustic startle for the baclofen group occurred at 100 dB (One-way ANOVA; $F_{(3,216)} = 5.212$, $p = 0.002$; Tukey post hoc: baclofen versus saline, $p = 0.020$) and 110 dB (One-way ANOVA; $F_{(3,216)} = 4.842$, $p = 0.003$; Tukey post hoc: baclofen versus saline, $p = 0.002$) but not at 120 dB compared to saline. Conversely, significant reductions in startle responses were observed between the female treatment groups compared to saline (RM-ANOVA; $F_{(3,136)} = 6.942$, $p = 0.000$). The baclofen and phaclofen treated groups displayed a significant decrease in acoustic startle response compared to saline (Tukey post hoc: baclofen versus saline, p 0.019; phaclofen versus saline, $p = 0.009$). The decrease in acoustic startle response occurred at the 120 dB intensity (One-way ANOVA; $F_{(3,136)} = 5.920$, $p = 0.001$; Tukey post hoc: baclofen versus saline, $p = 0.010$; phaclofen versus saline, $p = 0.004$).

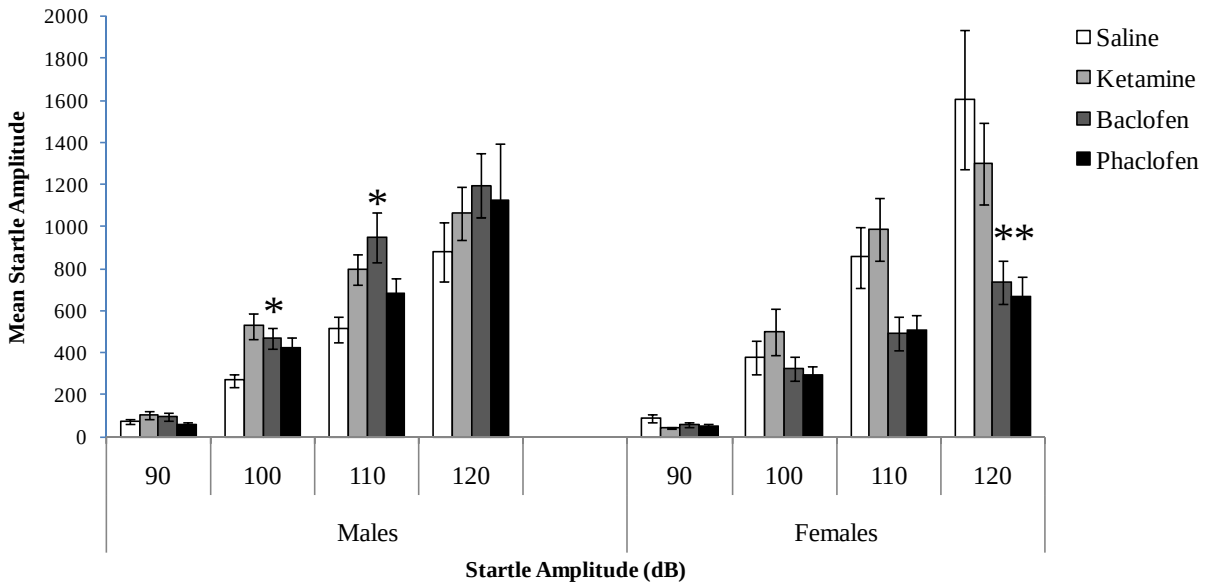

Figure 2. Acoustic Startle Responses. Mean startle amplitudes (±SEM) in response to noise bursts at multiple decibel intensities for males and females. Baclofen treated males exhibited a significant increase in startle response while the baclofen and phaclofen treated females displayed a significant reduced startle response. * = Significantly different from saline, $p < 0.05$.

For the PPI trials with a 100 ms inter-stimulus interval (ISI; Figure 3), no significant differences were observed between treatment groups compared to controls for the male animals across all prepulse intensities (RM-ANOVA; $F_{(3,216)} = 2.268$, $p = 0.082$). However, the female animals displayed a significant difference between treatment groups compared to controls (RM-ANOVA; $F_{(3,136)} = 8.586$, $p = 0.000$). Across prepulse intensities, the female baclofen group (Tukey post hoc, $p = 0.006$) and phaclofen group (Tukey post hoc, $p = 0.000$) exhibited a significant reduction in PPI. These reductions were observed at all of the prepulse intensities except for the 86 dB prepulse (One-way ANOVA; 74 dB: $F_{(3,136)} = 4.058$, $p = 0.008$, Tukey post hoc: baclofen versus saline, $p = 0.018$, phaclofen versus saline, $p = 0.010$; 78 dB: $F_{(3,136)} = 5.066$, $p = 0.002$, Tukey post hoc: balcofen versus saline, $p = 0.015$; phaclofen versus saline, $p = 0.020$; 82 dB: $F_{(3,136)}$

= 4.354, p = 0.006, Tukey post hoc: phaclofen versus saline, p = 0.004; 90 dB: $F_{(3,136)}$ = 3.571, p = 0.016, Tukey post hoc: phaclofen versus saline, p = 0.018)..

Separate analyses were conducted to compare only the ketamine and saline groups for the PPI trials with 100 ms ISI (Figure 3). A significant reduction in PPI was observed between the ketamine treated female group versus the female controls across prepulse intensities (RM-ANOVA; $F_{(1,62)}$ = 8.313, p = 0.005), significant reductions were observed at the 74 (One-way ANOVA; $F_{(1,62)}$ = 7.525, p = 0.008) and 86 dB ($F_{(1,62)}$ = 4.247, p = 0.044) prepulse intensities.

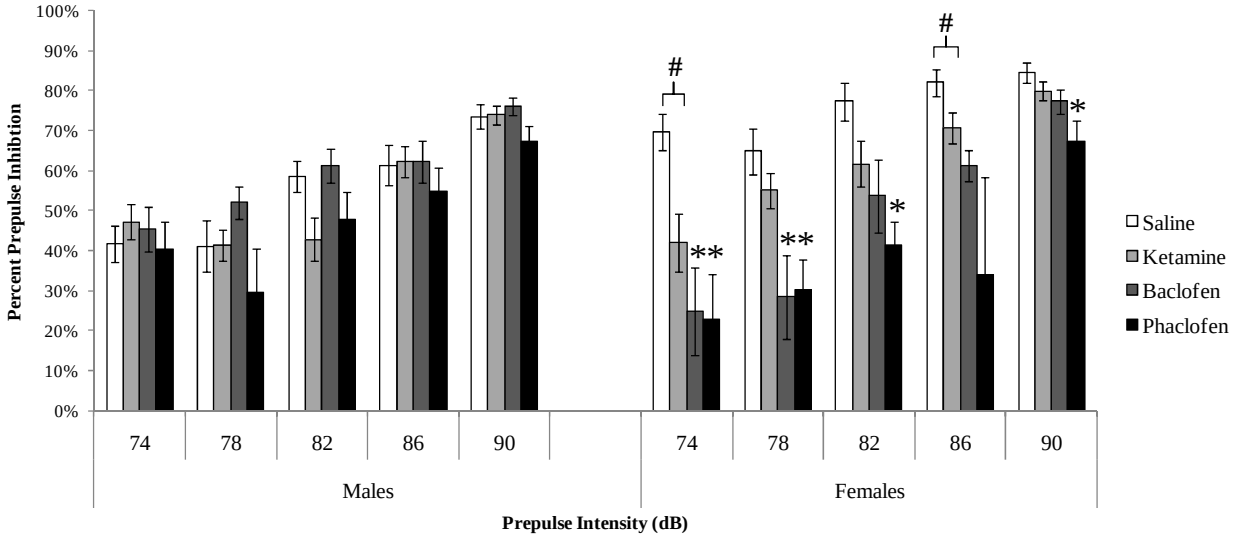

Figure 3. Percent Prepulse Inhibition with 100 ms ISI. Mean percent PPI (±SEM) at multiple prepulse intensities preceding 120 dB startle stimulus. No differences observed between the male treatment groups while significant reductions in PPI were seen for the baclofen and phaclofen treated females. # = Significantly differently from saline (ketamine analyzed in isolation), $p < 0.05$. * = Significantly different from saline, $p < 0.05$.

An evaluation of the PPI trials with a 40 ms ISI (Figure 4) revealed a significant difference between treatment groups across prepulse intensities in the male animals (RM-ANOVA; $F_{(3,161)}$ = 3.931, p = 0.010). Post-hoc analyses revealed a significant reduction

in PPI in the phaclofen group (Tukey post hoc: phaclofen versus saline, $p = 0.024$). One-way ANOVA for each prepulse intensity revealed a significant reduction only at the 74 dB prepulse ($F_{(3,161)} = 2.930$, $p = 0.035$; Tukey post hoc: phaclofen versus saline, $p = 0.039$). Female animals demonstrated a similar significant reduction in PPI across prepulse intensities ($F_{(3,101)} = 9.843$, $p = 0.000$) for the phaclofen group compared to saline (Tukey post hoc: $p = 0.000$). One-way ANOVA for individual prepulse intensities revealed the same significant reduction at the 74 dB prepulse intensity ($F_{(3,101)} = 5.439$, $p = 0.002$; Tukey post hoc: phaclofen versus saline, $p = 0.006$), 86 ($F_{(3,101)} = 4.322$, $p = 0.007$; Tukey post hoc: phaclofen versus saline, $p = 0.010$), as well as a significant difference at the 90 dB prepulse ($F_{(3,101)} = 3.145$, $p = 0.028$; Tukey post hoc: phaclofen versus saline, $p = 0.045$).

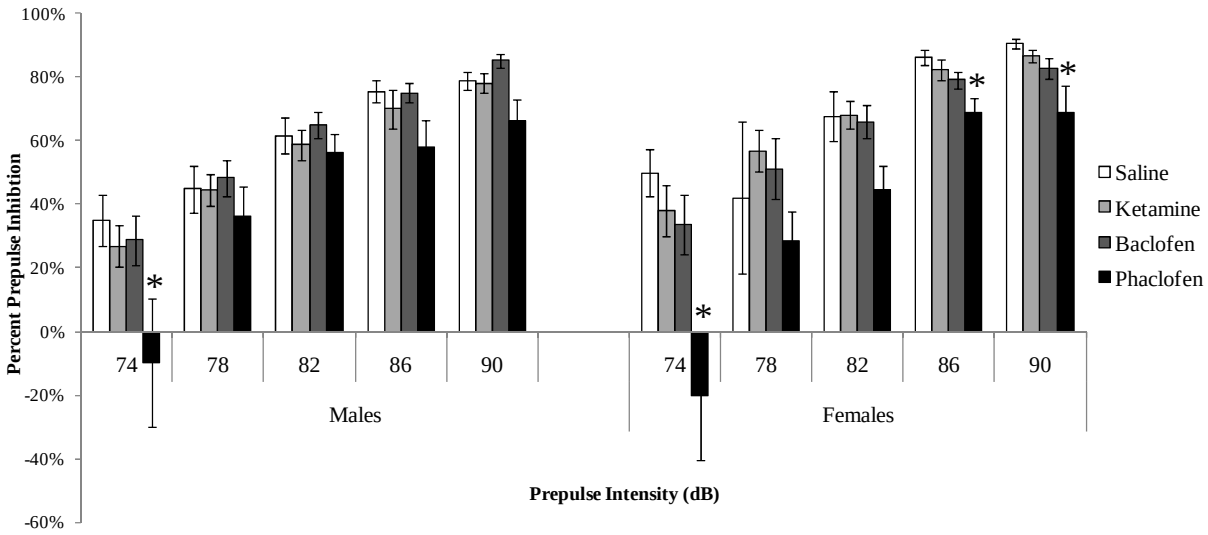

Figure 4. Percent Prepulse Inhibition with 40 ms ISI. Mean percent PPI (±SEM) at multiple prepulse intensities preceding 120 dB startle stimulus. Significant reductions in PPI were observed across prepulse intensities for both male and female phaclofen treated groups with a lack of PPI occurring at the 74 dB. * = Significantly different from saline, $p < 0.05$.

Cued and Contextual Fear Conditioning

No significant differences were observed in the freezing behavior of the males throughout training on Day 1 (see Figure 5). All groups froze equivalently during the two minute interval before the onset of the CS-US pairings (Pre CS-US; $F_{(3,51)} = 0.709$, $p = 0.551$) and during the two minute interval after the last CS-US pairings (Post CS-US; $F_{(3,51)} = 0.696$, $p = 0.559$). Similarly, the female animals were equivalent in their freezing behavior between groups during the Pre CS-US ($F_{(3,31)} = 0.908$, $p = 0.448$) and Post CS-US ($F_{(3,31)} = 0.536$, $p = 0.661$) as shown in Figure 5.

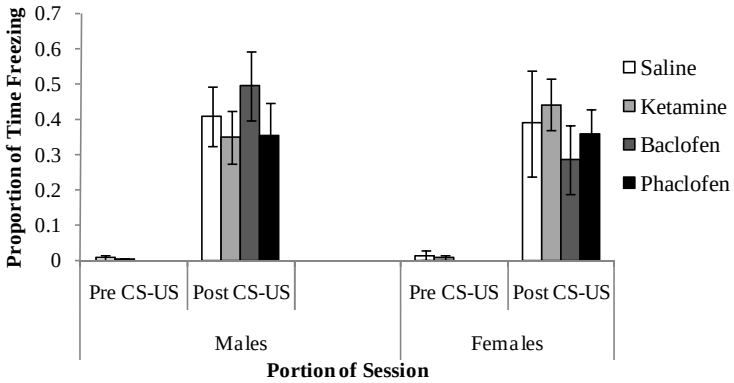

Figure 5. Day 1 CCF Training. Proportion of time freezing (±SEM) was determined for the first two minutes of training (Pre CS-US) and the last two minutes of training (Post CS-US) for the males and females. No differences were observed between treatment groups.

Following training, fear to the CS in a novel environment (cued fear) was tested (Cued Fear Day 2; Figure 6a). No differences were observed between the male treatment groups in freezing behavior during the first two minutes of the cued fear session (Pre CS1; $F_{(3,51)} = 0.985$, $p = 0.407$) and during the presentation of the cues (RM-ANOVA across CS1-CS4; $F_{(3,51)} = 0.767$, $p = 0.518$). The female animals also displayed no differences in freezing between treatment groups during Pre CS1 ($F_{(3,31)} = 2.847$, $p = 0.054$) and similar freezing behavior during cue presentations (RM-ANOVA across CS1-CS4; $F_{(3,31)} = 1.897$, $p = 0.151$).

Freezing to the training environment on (contextual fear) on Day 3 (Figure 6b) revealed no differences between the male treatment groups across the entire session (RM-ANOVA across Blocks 1-5; $F_{(3,51)} = 0.204$, $p = 0.893$) and, consistent with the males, no differences were observed for the female animals (RM-ANOVA across Blocks 1-5; $F_{(3,31)} = 2.626$, $p = 0.068$).

For Cued Fear Day 4 (Figure 7a), no significant differences were observed in freezing behavior between male treatment groups for Pre CS1 ($F_{(3,51)} = 0.885$, $p = 0.455$) or across cue presentations (RM-ANOVA across CS1-CS4; $F_{(3,51)} = 0.348$, $p = 0.791$). Female animals also displayed no significant differences between treatment groups for freezing behavior during Pre CS1 ($F_{(3,31)} = 0.670$, $p = 0.577$) and across cue presentations(RM-ANOVA across CS1-CS4; $F_{(3,31)} = 0.989$, $p – 0.411$).

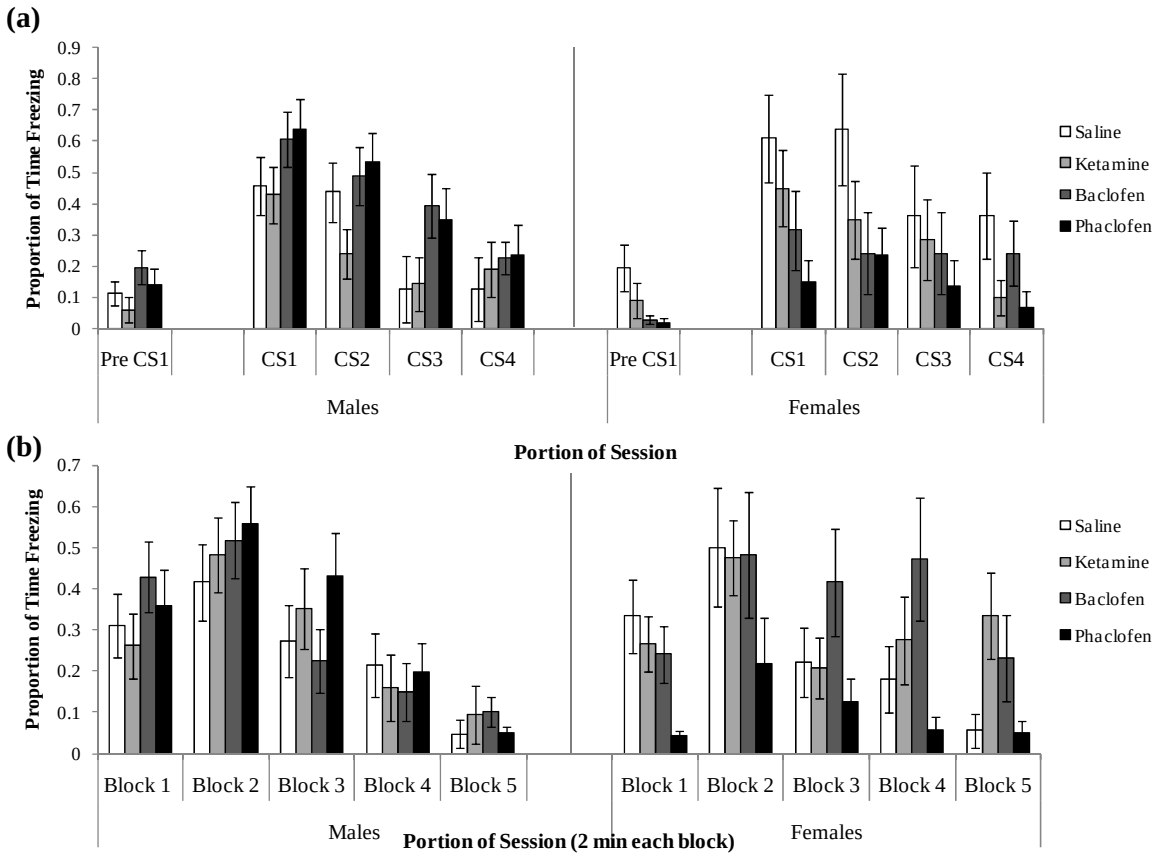

Figure 6. Cued Fear Day 2 and Contextual Fear Day 3. Proportion time freezing (±SEM) was measured for Cued Fear Day 2 (a) during the first two minutes before the presentation of the cues (Pre CS1) and during the cue presentations (CS1-CS4) and for Contextual Fear Day 3 (b) during each 5 two minute blocks. No significant differences were observed between treatment groups.

No differences in freezing were detected on Contextual Fear Day 5 (Figure 7b) between the male treatment groups (RM-ANOVA across Blocks 1-5; $F_{(3,51)} = 0.282$, $p = 0.838$) and female treatment groups (RM-ANOVA across Blocks 1-5; $F_{(3,31)} = 2.571$, $p = 0.072$) across the entire session. A single reminder trial (CS-US) was presented at the end of the session. During the two-minute interval following the reminder trial, neither male ($F_{(3,51)} = 1.031$, $p = 0.387$) nor female ($F_{(3,31)} = 0.369$, $p = 0.776$) animals displayed differences in freezing behavior.

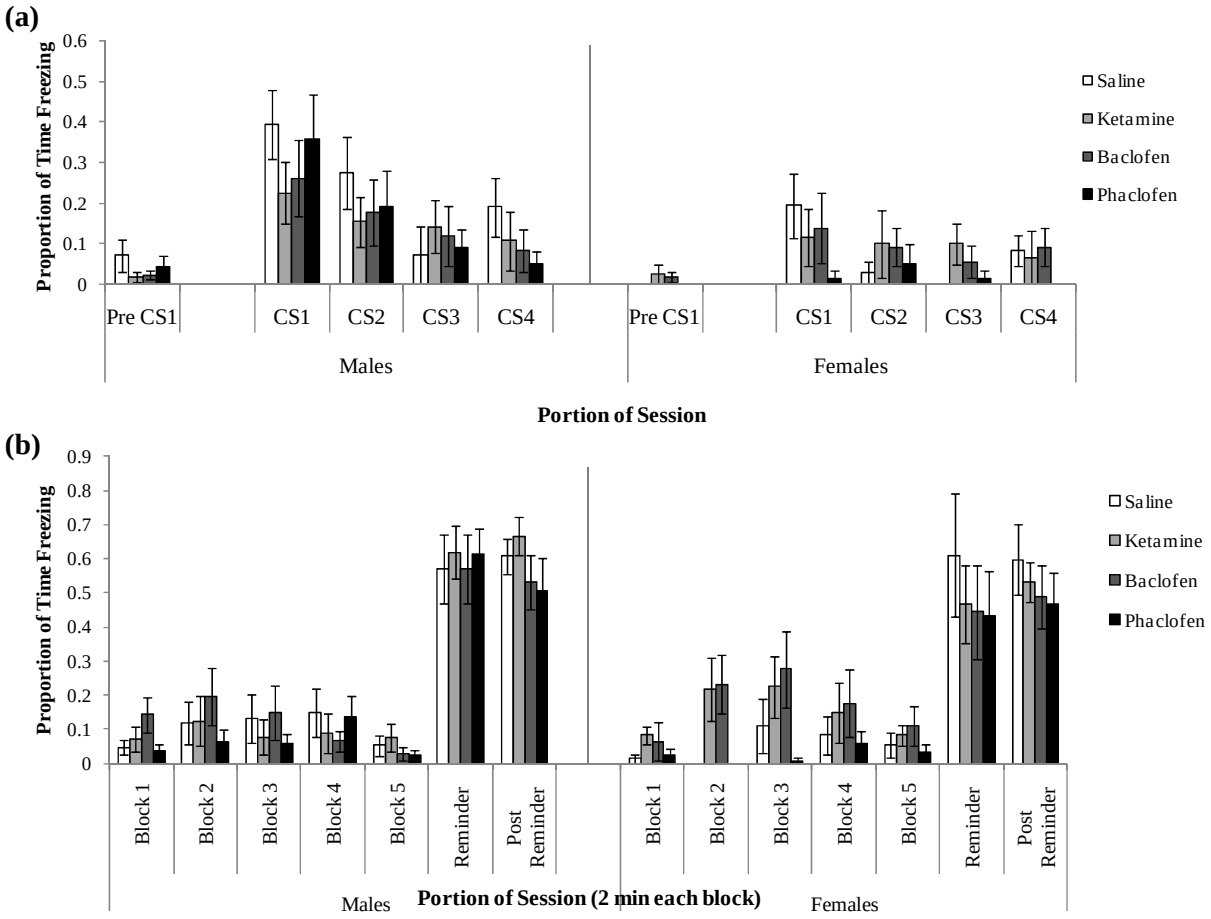

Figure 7. Cued Fear Day 4 and Contextual Fear Day 5. Proportion time freezing (±SEM) was measured for Cued Fear Day 4 (a) during the first two minutes before the presentation of the cues (Pre CS1) and during the cue presentations (CS1-CS4) and for Contextual Fear Day 5 (b) during each 5 two minute blocks. Freezing was measured during the cue presentation (Reminder) and the final two minutes after the CS-US presentation (Post Reminder).

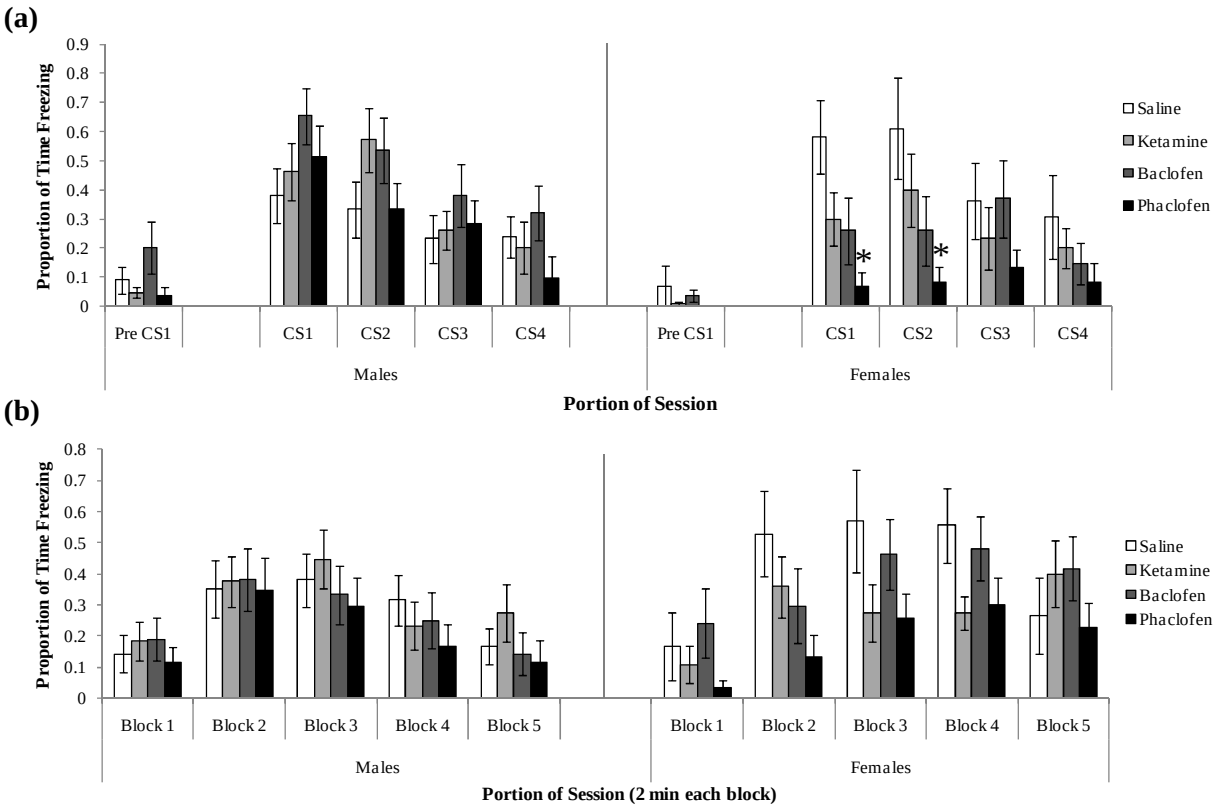

Figure 8. Cued Fear Day 6 and Contextual Fear Day 7. Proportion time freezing (±SEM) was measured for Cued Fear Day 6 (a) during the first two minutes before the presentation of the cues (Pre CS1) and during the cue presentations (CS1-CS4). The phaclofen treated females displayed a significant decrease in freezing during the CS presentations. Proportion time freezing (±SEM) was measured for Contextual Fear Day 7 (b) during each 5 two minute blocks. No differences between treatment groups were observed. * = Significantly different from saline, $p < 0.05$.

Freezing behavior on Cued Fear Day 6 Post Reminder (Figure 8a) revealed no significant differences between treatment groups for the male animals for Pre CS1 ($F_{(3,51)} = 1.989$, $p = 0.127$) and across cue presentations (RM-ANOVA across CS1-CS4; $F_{(3,51)} = 1.263$, $p = 0.297$). Although no significant differences among treatment groups in freezing behavior were seen for Pre CS1 for female animals ($F_{(3,31)} = 1.273$, $p = 0.301$), the phaclofen group displayed a significant decrease in freezing across cue presentations (RM-ANOVA; $F_{(3,31)} = 3.012$, $p = 0.045$; Tukey post hoc: phaclofen versus saline, $p = 0.028$). One-way ANOVA revealed the significant reductions occurred during CS1 ($F_{(3,31)}$

= 4.298, p = 0.012; Tukey post hoc: phaclofen versus saline, p = 0.006) and CS2 ($F_{(3,31)}$ = 3.308, p = 0.033; Tukey post hoc: phaclofen versus saline, p = 0.026).

For Contextual Day 7 Post Reminder (Figure 8b), no significant differences were found for the male animals across the two minute blocks (RM-ANOVA across blocks; $F_{(3,51)}$ = 0.386, p = 0.764). Similarly, no significant differences in freezing were observed between treatment groups for the female animals (RM-ANOVA across blocks; $F_{(3,31)}$ = 1.811, p = 0.166).

Morris Water Maze

Latency to reach the hidden platform across successive days was measured for six consecutive days (Figure 9a). No significant differences were observed in latency for males between treatment groups across hidden platform training (RM-ANOVA; $F_{(3,215)}$ = 2.079, p = 0.104). Similarly, latencies between treatment groups for the female animals were not significantly different across days (RM-ANOVA; $F_{(3,215)}$ = 0.017, p = 0.997). Swim speed across hidden platform training days (Figure 10a) revealed no differences between the male treatment groups (RM-ANOVA; $F_{(3,215)}$ = 1.989, p = 0.117) and the female treatment groups (RM-ANOVA; $F_{(3,79)}$ = 1.711, p = 0.172). However, thigmotaxis (proportion of time spent on the outer perimeter, Figure 10b) differed among the male treatment groups across hidden platform days (RM-ANOVA; $F_{(3,215)}$ = 2.882, p = 0.037) with the ketamine group spending significantly more time along the perimeter (Tukey post hoc, p = 0.049). The female treatment groups displayed no differences in time spent along the perimeter across hidden platform days (RM-ANOVA; $F_{(3,80)}$ = 2.342, p = 0.079). Separate analyses were performed comparing only male ketamine group to the male saline group. The male ketamine group displayed a significantly increased latency to find the hidden platform over successive days (RM-ANOVA; $F_{(1,38)}$ = 6.883, p = 0.012). Swim speed was not significantly different between the male saline and ketamine groups (RM-ANOVA; $F_{(1,109)}$ = 0.063, p = 0.802), the ketamine group did display higher thigmotaxis (RM-ANOVA; $F_{(1,109)}$ = 7.141, p = 0.009) which could be related to the longer latency during the hidden trials.

After completion of the last day of hidden platform training, a probe trial was performed (Figure 9b). The baclofen treated males failed to display a selective search as

measured by time spent in target quadrant versus all other quadrants ($F_{(3,51)} = 4.493$, $p = 0.007$; Tukey post hoc: target versus quadrant 1, $p = 0.118$; target versus quadrant 2, $p = 0.043$; target versus quadrant 3, $p = 0.006$). The male saline, ketamine, and phaclofen group each displayed a significant search during the probe trial (saline: $F_{(3,55)} = 15.015$, $p = 0.000$; Tukey post hoc, target versus quadrant 1, $p = 0.000$; target versus quadrant 2, $p = 0.000$; target versus quadrant 3, $p = 0.000$; ketamine: $F_{(3,55)} = 13.886$, $p = 0.000$; Tukey post hoc, target versus quadrant 1, $p = 0.008$; target versus quadrant 2, $p = 0.000$; target versus quadrant 3, $p = 0.000$; phaclofen: $F_{(3,51)} = 11.176$, $p = 0.000$; Tukey post hoc, target versus quadrant 1, $p = 0.005$; target versus quadrant 2, $p = 0.000$; target versus quadrant 3, $p = 0.000$). In the female animals, a selective search for the former platform location was observed for the baclofen ($F_{(3,32)} = 15.153$, $p = 0.000$; Tukey post hoc target versus quadrant 1, $p = 0.000$; target versus quadrant 2, $p = 0.000$; target versus quadrant 3, $p = 0.000$) and phaclofen ($F_{(3,36)} = 4.865$, $p = 0.006$; Tukey post hoc target versus quadrant 1, $p = 0.039$; target versus quadrant 2, $p = 0.008$; target versus quadrant 3, $p = 0.027$) treated female groups as measured by the proportion of time spent in the target quadrant. The female saline and ketamine treatment groups did not exhibit a selective search, failing to spend significantly more time in any quadrant (saline: $F_{(3,20)} = 1.362$, $p = 0.283$; ketamine: $F_{(3,36)} = 1.466$, $p = 0.240$).

Reversal training was carried out 24 hours following the probe trial. Neither the males nor female animals displayed any significant differences in latency to find the new platform location (Figure 9a; Males: $F_{(3,215)} = 1.079$, $p = 0.359$; Females: $F_{(3,136)} = 0.767$, $p = 0.514$) or swim speed differences (Figure 10a; Males: $F_{(3,215)} = 0.734$, $p = 0.533$; Females: $F_{(3,135)} = 1.740$, $p = 0.162$). However, the ketamine treated male group exhibited

a significant increase in thigmotaxis compared to the control group (Figure 10b; $F_{(3,215)} = 4.621$, $p = 0.04$; Tukey post hoc saline versus ketamine, $p = 0.010$). No significant differences in thigmotaxis were observed between the female treatment groups (Figure 10b; $F_{(3,136)} = 1.527$, $p = 0.210$).

Visible platform training was conducted to assess visuomotor abilities. Significant differences were found among the male treatment groups for latency to locate the visible platform (Figure 9a; RM-ANOVA; $F_{(3,215)} = 3.039$, $p = 0.030$); however, no significant differences between groups were detected in post hoc analyses (Tukey post hoc; saline versus ketamine, $p = 0.927$; saline versus baclofen, $p = 0.991$; saline versus phaclofen, $p = 0.069$). While no differences were observed between the male treatment groups for swim speed (Figure 10a; RM-ANOVA; $F_{(3,215)} = 0.817$, $p = 0.457$), the ketamine and phaclofen groups displayed an increase thigmotaxis across the two visible days (Figure 10b; RM-ANOVA; $F_{(3,215)} = 6.061$, $p = 0.001$; Tukey post hoc, saline versus ketamine, $p = 0.001$; saline versus phaclofen, $p = 0.028$). Based on the females' performance during the hidden platform and reversal training days, they received only one day of visible training. No significant differences were observed for the latency (Figure 9a; $F_{(3,136)} = 0.086$, $p = 0.968$) and swim speed (Figure 10a; $F_{(3,136)} = 2.424$, $p = 0.069$) between treatment groups; however, the ketamine treated female group displayed an increased thigmotaxis compared to saline (Figure 10b; $F_{(3,136)} = 3.165$, $p = 0.027$; Tukey post hoc saline versus ketamine, $p = 0.025$).

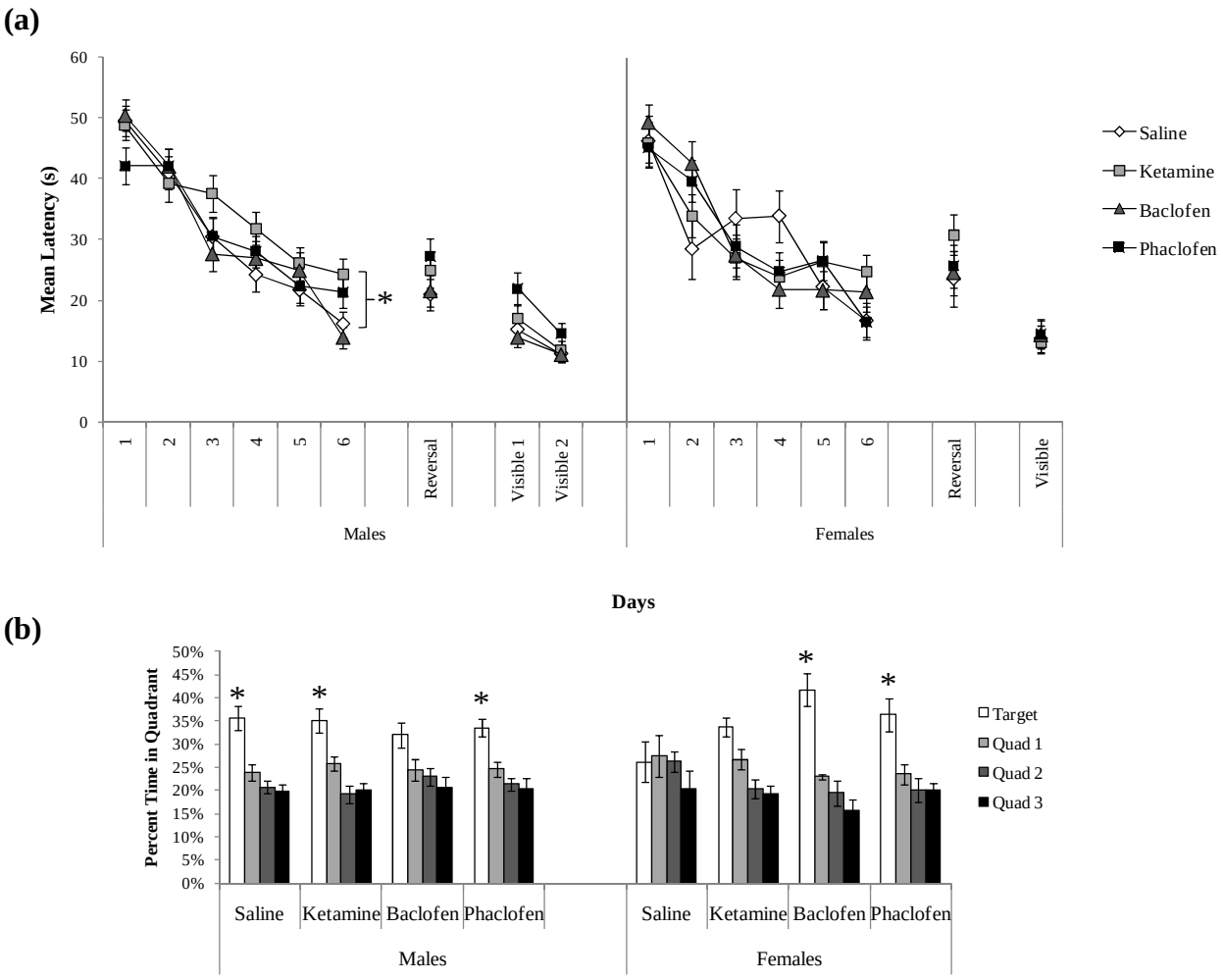

Figure 9. Morris Water Maze Latency and Probe Trial. (a) Mean latency (±SEM) across hidden platform days, reversal, and visible. Male ketamine group took significantly longer to find hidden platform across days. No differences were observed in the females. * = Significant difference between saline versus ketamine, $p < 0.05$. (b) Mean percent time in quadrant (±SEM) with target versus quadrants for each treatment group. Male saline, ketamine, and phaclofen groups and female baclofen and phaclofen group spent significantly more time in the target quadrant versus quadrant 1, 2, and 3. * = Significantly different from target quadrant, $p < 0.05$.

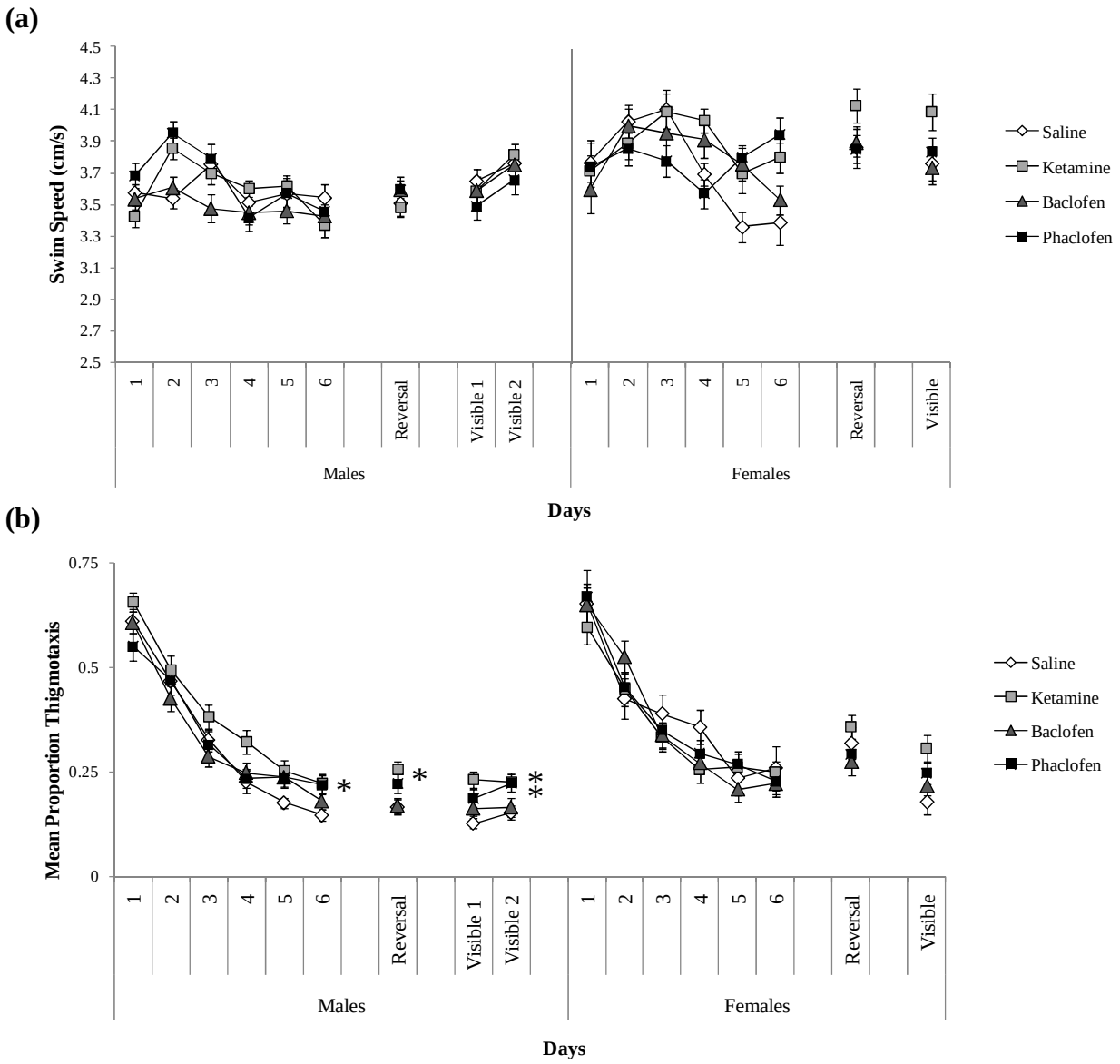

Figure 10. Swim Speed and Thigmotaxis. (a) Mean swim speed (±SEM) across hidden, reversal, and visible days for males and females. No significant differences were observed. (b) Mean proportion thigmotaxis (±SEM) across hidden, reversal, and visible days for males and females. Ketamine males displayed a significant increase in thigmotaxis for hidden, reversal, and visible days while the phaclofen males displayed a significant increase on visible days. No differences were observed in the females. * = Significantly different from saline, $p > 0.05$.

Tail Flick

Tail flick (Figure 11) was performed to examine nociception differences between treatment groups following the completion of the Morris water maze. No significant differences were observed between treatment groups for males (One-way ANOVA; $F_{(3,51)} = 0.903$, $p = 0.446$) or females ($F_{(3,31)} = 0.691$, $p = 0.565$) indicating no nociceptive changes may be related to any differences in the above data.

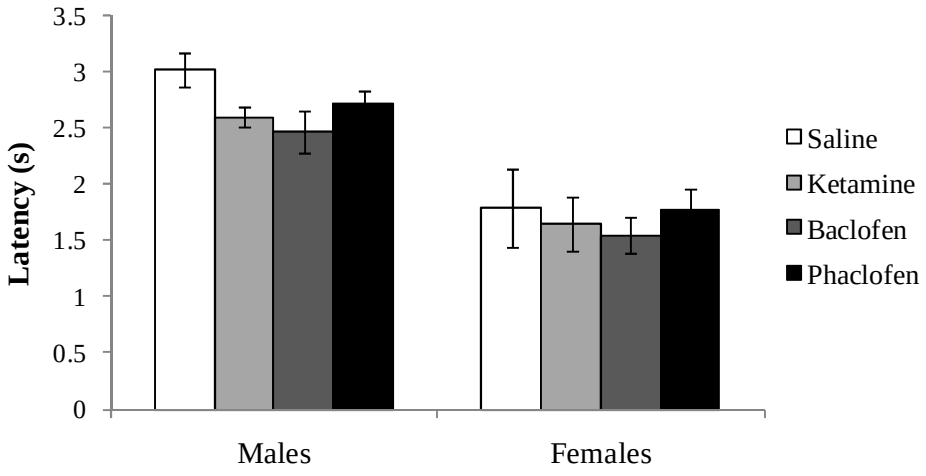

Figure 11. Tail Flick Latency. Mean latency (±SEM) to remove tail from hot water. No differences were observed between treatment groups for males or females.

Tissue Analysis

SDS-PAGE Western Blotting

Protein levels of multiple specific receptor subtypes relating to GABA and NMDA signaling in the cortex were analyzed via SDS-PAGE/Western blotting. No significant differences were observed between treatment groups in the protein levels of either of the identified subtypes of the $GABA_{B1}$ receptor subunit (1a or 1b; Figure 12a) for males (1a: $F_{(3,46)} = 1.090$, $p = 0.363$; 1b: $F_{(2,46)} = 0.591$, $p = 0.624$) or females (1a: $F_{(3,41)} = 0.863$, $p = 0.468$; 1b: $F_{(3,41)} = 2.639$, $p = 0.062$). Separate analyses were performed to examine differences in only the animals treated with the $GABA_B$ ligands compared to saline. Baclofen and phaclofen treated males did not exhibit any differences in protein levels of either of the $GABA_{B1}$ (1a and 1b) receptor subunit (1a: $F_{(2,33)} = 0.666$, $p = 0.521$; 1b: $F_{(2,32)} = 0.506$, $p = 0.607$). A significant increase was observed in the protein levels of the 1b receptor subunit in the phaclofen treated females ($F_{(2,33)} = 6.323$, $p = 0.005$; Tukey post hoc for saline versus phaclofen, $p = 0.004$) while no differences were detected for the 1b receptor subunit in the post hoc analyses for baclofen treated females (Tukey post hoc for saline versus baclofen, $p = 0.192$). Additionally, no differences were detected in the 1a receptor subunit for the baclofen or phaclofen treated females ($F_{(2,32)} = 0.564$, $p = 0.574$).

Evaluation of $GABA_{B2}$ receptor protein levels in the cortex (Figure 12b) did not reveal any significant differences in the males ($F_{(3,59)} = 0.930$, $p = 0.432$). However, the female phaclofen group displayed a significant increase in expression level $GABA_{B2}$ ($F_{(3,41)} = 4.919$, $p = 0.005$; Tukey post hoc for saline versus phaclofen, $p = 0.003$). No

differences were found for $GABA_{A\alpha5}$ protein levels (Figure 12c) between the treatment groups in males ($F_{(3,44)} = 0.523$, $p = 0.669$) or females ($F_{(3,38)} = 0.471$, $p = 0.704$).

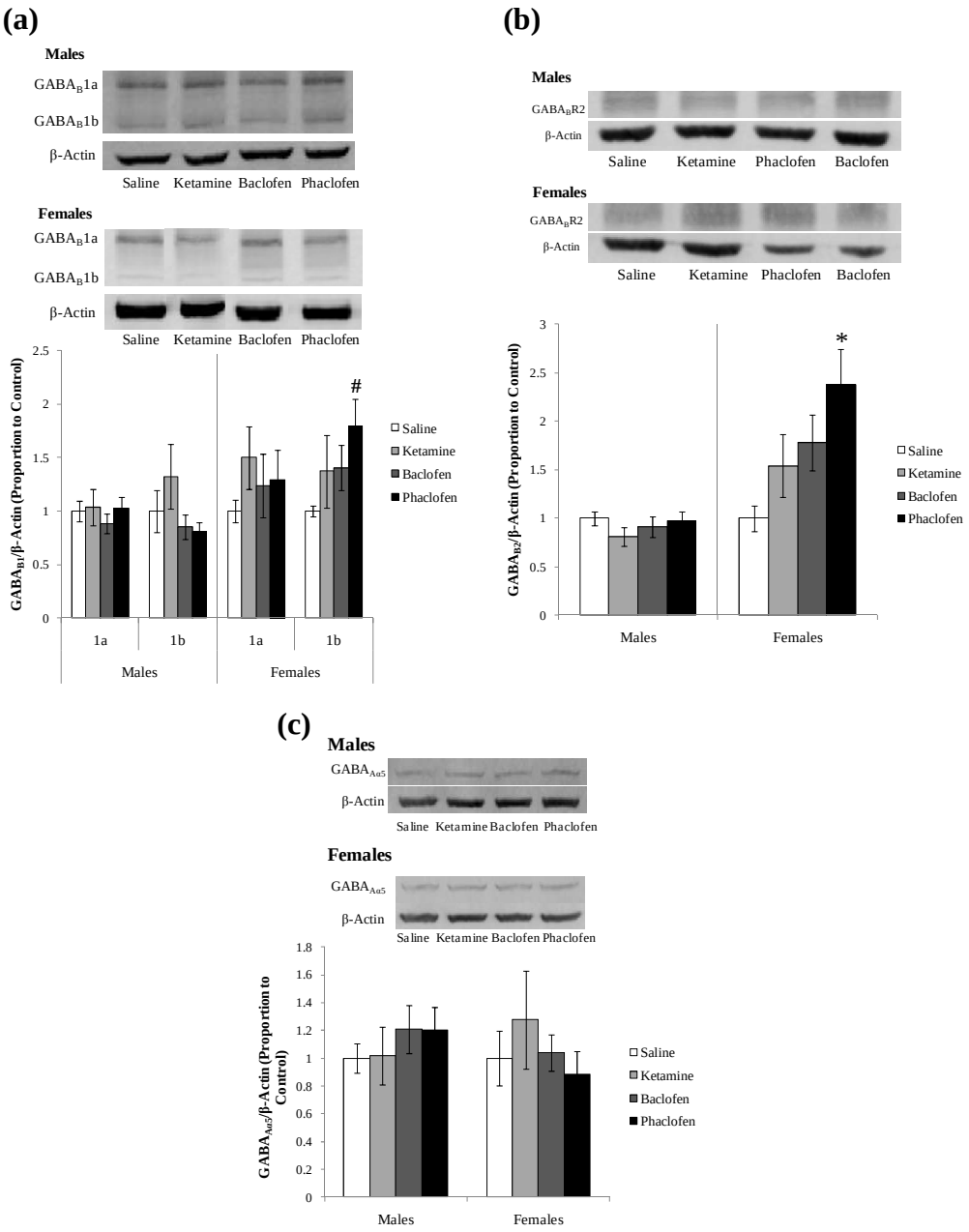

Figure 12. GABA Receptor Protein Levels. (a) Proportion (±SEM) and representative blots of $GABA_{B1a}$ and $GABA_{B1b}$ protein levels normalized to β-Actin from the cortex. No differences were observed between the male treatment groups. Phaclofen treated females displayed increased $GABA_{B1b}$ protein levels compared to controls.# = Significantly different from saline (baclofen and phaclofen in analysis only), $p < 0.05$. (b) Proportion (±SEM) and representative blots of $GABA_{B2}$ protein levels normalized to β-Actin from the cortex. No differences observed between male treatment groups. Phaclofen treated females displayed a significant increase in $GABA_{B2}$ protein levels. * = Significantly different from saline, $p < 0.05$. (c) Proportion (±SEM) and representative blots of $GABA_{\alpha5}$ protein levels normalized to β-Actin from the cortex. No differences were observed between males and females.

Protein levels for GAD67 (Figure 13a) were not significantly different between treatment groups for males ($F_{(3,50)} = 0.568$, $p = 0.639$) or females ($F_{(3,36)} = 0.611$, $p = 0.612$). However, the literature suggests a difference in GAD67 protein levels between males versus females related to developmental maturation, therefore additional analysis were conducted to directly compare GAD67 protein expression in males and females in the same treatment groups (Figure 13b). A significant increase in the GAD67 protein expression was observed for the phaclofen treated females compared to the phaclofen treated males ($F_{(1,20)} = 6.505$, $p = 0.019$) and for the ketamine treated females compared to the ketamine treated males ($F_{(1,22)} = 9.601$, $p = 0.005$). No differences were seen between the male and female saline groups ($F_{(1,18)} = 0.047$, $p = 0.831$) or between the male and female baclofen groups $F_{(1,22)} = 1.577$, $p = 0.222$).

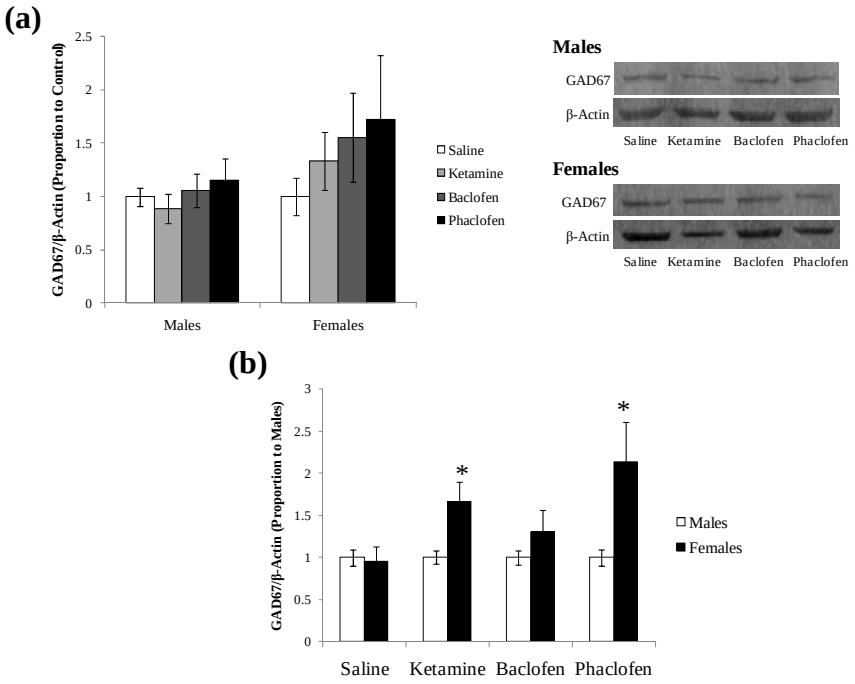

Figure 13. GAD67 Protein Levels. (a) Proportion (±SEM) and representative blots of GAD67 protein levels normalized to β-Actin from the cortex. No significant differences were observed between treatment groups for males or females. (b) Proportion (±SEM) of GAD67 protein levels normalized to the males. A significant increase was observed for the ketamine treated females compared to ketamine treated males as well as a significant increase for GAD67 in the phaclofen treated females compared to phaclofen treated males. * = Significantly different from males, $p < 0.05$.

The evaluation of the NR2B subunit of the NMDA receptor protein levels (Figure 14a) revealed no significant differences between treatment groups for males ($F_{(3,51)}$ = 0.387, p = 0.763) or females ($F_{(3,36)}$ = 1.151, p = 0.342). Phaclofen treated females did displayed a significantly higher level of the kalirin 5 protein isoform compared to the other female treatment groups (Figure 14b; $F_{(3,36)}$ = 4.354, p = 0.010; Tukey post hoc for saline versus phaclofen, p = 0.008). No significant differences were found in the other kalirin isoforms for the females (Figure 14b; kalirin 7: $F_{(3,36)}$ = 1.208, p = 0.321; kalirin 9: $F_{(3,36)}$ = 0.838, p = 0.482; kalirin 12: $F_{(3,36)}$ = 0.542, p = 0.656) and no differences were found in protein levels for any of the kalirin isoforms for the males (kalirin 5: $F_{(3,50)}$ =

0.880, p = 0.458; kalirin 7: $F_{(3,50)}$ = 0.465, p = 0.708; kalirin 9: $F_{(3,50)}$ = 0.497, p = 0.686; kalirin 12: $F_{(3,50)}$ = 1.255, p = 0.300).

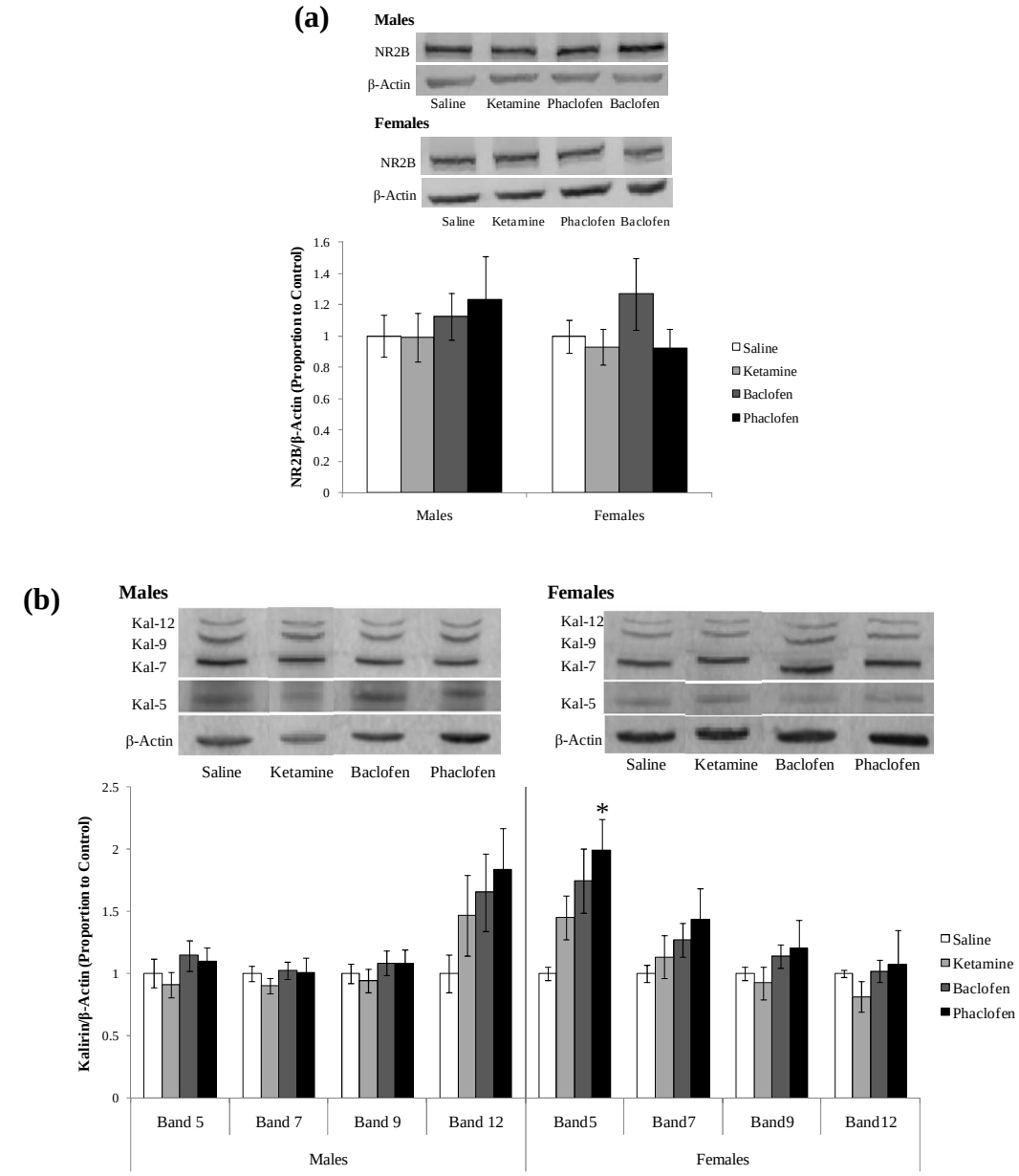

Figure 14. NR2B and Kalirin Protein Levels. (a) Proportion (±SEM) and representative blots of NR2B protein levels normalized to β-Actin from the cortex. No significant differences were observed. (b) Proportion (±SEM) and representative blots of kalirin protein levels normalized to β-Actin from the cortex. A significant increase was observed for kalirin 5 isoform in phaclofen treated females. * = Significantly different from saline, $p < 0.05$.

CHAPTER 5

DISCUSSION

Administration of ligands that alter the function of $GABA_B$ or NMDA receptor function during a critical period in early brain development produced a sizable change in behavior in adulthood. The early life alterations in $GABA_B$ and NMDA receptor signaling also produced a change in several proteins in the brain. Specifically, administering ketamine on PND 7, 9, and 12 (Hypothesis 1) disrupted responses in PPI and performance in the Morris water maze. Additional changes were observed in protein expression of GAD67 in males versus females. The administration of $GABA_B$ ligands (baclofen or phaclofen) in development (Hypothesis 2) altered acoustic startle responses, PPI, performance in the Morris water maze, and the expression of multiple proteins related to GABAeric signaling as well as a protein implicated in synapse formation. These data represent the first reports of a lifelong change in both behavioral and biochemical processes associated with early life $GABA_B$ receptor function. The deficits observed differed based on gender and the specific behavioral task or protein.

The data in these studies demonstrate behavioral alterations in acoustic startle response and PPI in adulthood from the transient enhancement or blockade of the $GABA_B$ receptor system during development. These findings also lend evidence to the importance of these receptors during early brain development. Inhibitory tone mediated through $GABA_B$ and NMDA receptors in adulthood is necessary for the prepulse to reduce the acoustic startle response in PPI. The acoustic startle response is a simple, sensorimotor reflex in the presence of fear (Koch & Schnitzler, 1997). In this experiment, the male and female treatment groups displayed different behavior in their acoustic startle

responses. The male baclofen group displayed an enhanced startle response across multiple startle stimuli whereas the female animals administered baclofen displayed a decrease in the startle response. The male ketamine group did not display an increase in startle response across startle intensities (RM-ANOVA not significant versus saline) and, therefore, we cannot state that they exhibited an increase in startle response overall despite an increase in startle for the 100 dB startle stimulus. While no differences were observed in the male phaclofen group, the female phaclofen group displayed a significant reduction in startle response across intensities and, in particular, at the 120 dB level. Decreases in acoustic startle response are typically observed after habituation or sensitization after repetitive presentations (Pilz & Schnitzler, 1996). In our experimental methods, the stimuli are only presented five times randomized throughout the session thus insufficient for habituation or sensitization. Based on this, the increase in acoustic startle response in the baclofen male group as well as the reduction seen in both the baclofen and phaclofen females likely represent a gender dependent disruption to GABA signaling involved in the sensorimotor circuitry.

Assessing the influence of multiple temporal gaps (interstimulus intervals; ISI) between the prepulse and the startling stimulus allows for the ability to parse discrete regions and transmitter systems involved in the multisensory PPI circuitry. The nonstartling prepulse inhibits the reflexive response to a startling stimulus if it precedes the startling stimulus between 10-1000 ms (Hoffman & Ison, 1980). By manipulating the interval between the prepulse and startle stimulus (ISI), differing neurotransmitter systems and brain regions are involved (Hoffman & Ison, 1980; Swerdlow et al., 2001; Yeomans et al., 2010). NMDA receptors located in the prefrontal cortex and the

hippocampus mediate the inhibition of the prepulse between ISIs of 100-500 ms (Swerdlow et al., 2001). Both ionotropic and metabotropic GABA receptors also influence the inhibition of the startle response. PPI occurring at shorter ISIs (starting at 20 ms) are principally governed by $GABA_A$ receptors due to the fast acting nature of these ionotropic channels (Yeomans et al., 2010). Metabotropic $GABA_B$ receptors exhibit a longer latency to activate but induce a longer lasting inhibitory effect that can impact PPI over a wide range of ISIs beginning at approximately 40 ms ranging to as much as 500 ms (Yeomans et al., 2010). The combination of $GABA_A$ and $GABA_B$ receptors allow for PPI over the entire range of intervals. The reduction in PPI in the 100 ms ISI trials in females administered baclofen, phaclofen, and ketamine with no difference in the male animals suggest that the females may be more sensitive to the subtle changes in these receptor systems during early brain development. Interestingly, the reduction in PPI across multiple prepulse intensities (see Figure 3) is typically seen in patients with schizophrenia and animal models of schizophrenia (Bolton et al., 2012; Cilia et al, 1997; Mansbach and Geyer 1991; Sabbagh et al., 2012; Swerdlow et al., 1998). One can argue that the deficits displayed by the female baclofen and phaclofen in the PPI with 100 ms ISI may be due to the reduction of the acoustic startle response. To a certain extent, the startle response might have contributed to the deficits seen in the PPI with 100 ms ISI but there were no differences in the female baclofen group in the PPI with 40 ms ISI making it unlikely that the PPI deficits are an artifact of the reduced startle response.

The lack of reduction and borderline enhancement of the startle response in PPI is extremely rare. More compelling is that at the 40 ms ISI for both the phaclofen treated males and females exhibited this deficit, and this is the only difference shared by the

males and females in this study. This indicates that, following $GABA_B$ receptor antagonism in early life, not only did the animals fail to display the normal reduction in startle response in the presence of a prepulse, but in some animals, there was an increase in the startle response on trials that contained the prepulse. Few reports indicate a change similar to those observed with limited data indicating only select stimulants can produce this effect (Ashare et al., 2010; Gould et al., 2005). Knockout mice with reduced expression of $GABA_{B1}$ receptors also display an enhanced response with the presence of the prepulse (Prosser et al., 2001). However, these mice also experience seizures and resemble a model of epilepsy. Further, homozygous knockout of $GABA_{B1}$ leads to premature death. It is plausible that the reduction of $GABA_B$ receptors in the knockout study may lead to a compensatory gain in systems that affect the PPI circuitry. Although our experiment only transiently blocked $GABA_B$ receptors at brief intervals in early brain development, it is clear that the alteration in $GABA_B$ receptor function did alter circuits involved in PPI in a similar way as in the knockout study. These data further imply the importance of normal functioning of the $GABA_B$ receptors during brain development.

The spatial learning and memory results from the Morris water maze revealed some interesting findings. The importance of NMDA receptors during adulthood in learning the hidden platform location using spatial cues have been observed in numerous studies (Amaral & Witter, 1991; Moser, Moser, & Andersen, 1993; Sabbagh et al., 2012). Multiple studies have demonstrated that the administration of NMDA receptor antagonists induce deficits in spatial learning and memory (Didriksen, Skarsfeldt, & Arnt, 2007; Moosavi et al., 2012; Sabbagh et al., 2012). In this experiment, the male ketamine group displayed deficits locating the hidden platform across training days indicating

impairment in spatial learning; however, the selective search during the probe trial suggests they did learn the location using a spatial strategy. There are multiple non-spatial as well as spatial strategies a subject can use to navigate this maze. The most efficient spatial strategy is to immediately orient to the spatial cues in the room once placed in the water and swim directly to the hidden platform location, an approach commonly seen in controls. A less efficient strategy is a mixture of procedural and spatial where the subject explores the maze before locating a specific position then orients itself to find the hidden platform location (Vorhees & Williams, 2006). Based on the male ketamine group latencies, the selective search during the probe trial, and the high thigmotaxis, a possibility exists that these animals did not immediately attend to the spatial cues once placed in the maze but instead swam around the perimeter before orienting to the general area where the platform was located. This indicates that postnatal administration of ketamine to male animals produced a subtle deficit compared to the controls in acquiring the location of the hidden platform location but based on the selective search during the probe, the animals were able to solve the task. Our data are consistent with previous studies administering an alternative NMDA receptor antagonist (PCP) during the same developmental time period (PND 7, 9, and 11) in which PCP administration demonstrated robust deficits acquiring the hidden platform location across days and significantly increased thigmotaxis for male animals but no differences were observed for the females (Andersen & Pouzet, 2004). PCP binds the NMDA receptor for a longer period of time compared to ketamine (Zukin & Zukin, 1979) which may account for more robust deficits observed in developmental PCP administration. Therefore, the

data from our experiment demonstrate that impairments in spatial learning and memory can result from a subtle disruption in NMDA receptor function during development.

Few studies have specifically investigated and used ligands that target $GABA_B$ receptors in spatial learning and memory tasks. Although there are no other studies to compare the effects of $GABA_B$ receptor alteration in development to adulthood performance in the Morris water maze, we did not find any differences in the acquisition of the hidden platform training for the baclofen or phacofen treated male animals. However, the male baclofen group failed to display a selective search during the probe trial. Equivalent performance throughout training and a lack of a selective search during the probe trial does indicate an impairment in learning the task (Beiko, Candusso, & Cain, 1997; Kinney, Starosta, & Crawley, 2003; Wolfer et al., 1998). One possibility is that the baclofen treated males were using a non-spatial strategy such as swimming a specific distance from the wall of the maze and eventually bumping into the hidden platform (Beiko, Candusso, & Cain, 1997; Kinney, Starosta, Crawley, 2003). Without adopting a spatial strategy using the cues to solve the task would explain the nonselective search. For the female data, the hidden platform training data suffered considerable variability which may have influenced the lack of a difference between treatment groups. Both the female saline and ketamine group did not display a selective search during the probe trial but it is difficult to interpret the data for the ketamine group when the controls failed to demonstrate learning the spatial task. Numerous studies have indicated greater variability in female animals performance in the water maze (Andersen and Pouzet, 2004; Beiko et al., 2004; Berger et al., 2006; Frick et al., 2000; Wang et al., 2001). Estrogen

levels have been demonstrated to influence learning strategy in tasks like the Morris water maze which may explain the females' behavior (Chesler & Juraska, 2000).

Emotional learning and memory was examined using trace cued and contextual fear conditioning. All treatment groups froze equivalently after the CS-US presentations on training day, indicating equivalent effects of training and no differences were observed during the initial cued fear or contextual fear sessions. All of the male and female groups displayed similar extinction to the fear associations as demonstrated by the reduction of fear behavior across days. Our laboratory has previously demonstrated alterations in ketamine or $GABA_B$ receptor function in adulthood alters the cued fear association (Bolton et al., 2012; Heaney et al., 2012). Additionally, following the administration of ketamine in adult animals an increase in protein levels specific to the postsynaptic subunit (1b) of the $GABA_B$ receptor was observed in the amygdala (Bolton et al., 2012). This suggests an interaction between changes in NMDA receptor function and $GABA_B$ receptors in fear conditioning (Bolton et al., 2012). Diminished NMDA receptor function results in decreased excitation of GABAergic interneurons thus diminished GABA release and an alteration in GABA receptors (Bolton et al., 2012; Zhang, Behrens, Lisman, 2008). A reduction in NMDA receptor activity is hypothesized to be involved in the pathophysiology of schizophrenia so it is logical to determine if a change in $GABA_B$ receptors produces similar behavioral alterations as a change in NMDA receptors as it relates to this disorder. Separate experiments performed in our laboratory investigating $GABA_B$ receptor function associated with CCF in adult animals revealed impaired extinction of the learned association in baclofen administered adult animals (Heaney et al., 2012). Therefore, it has been demonstrated that ketamine and

baclofen produce deficits in the trace cue fear association which relies on both the amygdala and hippocampus (Bolton et al., 2012; Heaney et al., 2012). Since NMDA antagonists induce a change in $GABA_B$ receptor levels and both NMDA antagonists and $GABA_B$ agonists produce deficits in fear learning, it is important to investigate the adulthood behavioral effects of postnatal alteration of these neurotransmitter systems as it relates to CCF. An interesting observation in this current experiment is that the phaclofen treated female group displayed a decrease in freezing to the CS following the reminder session compared to the controls (Figure 8a). Since there were no differences in freezing before the reminder trial, this may indicate that the phaclofen females did not exhibit equivalent reinstatement of the fear association compared to controls. Reinstatement is the recovery of the fear response following a post-extinction presentation of the CS-US (Kim & Richardson, 2007). In neurobiological models of extinction as well as studies that demonstrate impaired extinction in rats with medial prefrontal cortex (mPFC) lesions, it is theorized that the mPFC inhibits the amygdala by activating GABA interneurons during the expression of extinction (Hobin, Goosens, & Maren, 2003; Morgan, Schulkin, & LeDoux, 2003; Quirk et al., 2000; Santini et al., 2004; Sotres-Bayon, Cain, & LeDoux 2006). Adding to this, current neurobiological models of extinction maintain that the hippocampus modulates the expression of learned fear or extinction by inhibiting or exciting the mPFC (Hobin, Goosens, & Maren, 2003; Kim & Richardson, 2007). The CS-US reminder triggers the hippocampal inhibition of the mPFC, preventing the mPFC from inhibiting the amygdala following extinction. From this model, we can infer from the reinstatement deficit observed in the phaclofen treated females that disrupted GABA signaling within the hippocampus-mPFC-amygdala

network may have contributed to a reduction in reinstatement of the conditioned fear. While this change is subtle, it does indicate an alteration in Pavlovian learning as a result of phaclofen administration in early life.

In order to examine if alterations in specific proteins may be related to the behavioral changes observed in the present experiments, we performed SDS-PAGE directed at multiple GABA, NMDA, and associated targets in all groups. We also investigated if any changes were observed in the kalirin protein that is associated with synapse formation and remodeling and has also been found to be altered in schizophrenia. Cortex samples were evaluated based on previous literature indicating alterations in GABAergic function being related to deficits in behavioral tasks and patient post-mortem tissue in this region (Fatemi, Folsom, Thuras, 2011; Guidotti et al., 2000; Ishikawa et al., 2005; Swerdlow et al., 2001; Woolley et al., 2013). The $GABA_B$ receptor is composed two distinct subunits, $GABA_{B1}$ and $GABA_{B2}$. Distinct $GABA_{B1}$ isoforms have been determined to be specific to either the presynaptic (1a) or postsynaptic (1b) expression (Steiger et al., 2004). The phaclofen treated females displayed a significant increase in the postsynaptic (1b) isoform of the $GABA_B$ receptor. Combined with the increased expression in the $GABA_{B2}$ receptor subunit for the phaclofen treated females, these data indicate there may be an overall increased expression of postsynaptic $GABA_B$ receptors. This suggests that an increase in the expression of postsynaptic $GABA_B$ receptors resulted from the brief antagonism of $GABA_B$ receptors during development. An increase in postsynaptic receptors in the phaclofen treated females may be related to the deficits observed in the acoustic startle response and PPI at multiple intervals. The neurotransmitter acetylcholine is necessary to produce the startle response in both the

acoustic startle and PPI and is regulated by inhibitory circuits (Swerdlow et al., 200l; Yeomans et al,. 2010). The decrease in PPI observed indicates a lack of reduction in the startle response in the presence of the prepulse. These data, coupled with the increase in postsynaptic $GABA_B$ receptors, may reflect diminished GABA tone thus a lack of inhibition on cholinergic neurons. However, the extent to which this increase in protein resulted in functional $GABA_B$ receptors remains to be determined as well as the time point at which this increase occurred (in development, adolescence, or adulthood).

We investigated other GABA related proteins that have been implicated in PPI and schizophrenia as well as those that may be related to a change in GABA signaling. $GABA_{A\alpha}$ was examined for protein differences between treatment groups as a reduction has been demonstrated following PPI deficits, similar to the ones observed in this study (Hauser et al., 2005). However, no differences were seen in the $GABA_{A\alpha5}$ receptor, which indicates the early life treatment did not induce a change in all GABAergic signaling targets.

Evidence suggests that increases in GAD67 levels positively correlate with GABA release. Although no differences existed between treatment groups within each gender, we examined if the treatment had differential effects comparing genders. Changes in GAD67 mRNA are influenced by steroid hormones such as progesterone and estrogen (McCarthy et al., 1995; Perrot-Sinal, Davis, McCarthy, 2001; Searles et al., 2000). However, there are conflicting reports on GAD67 expression as either being increased or decreased in the presence of estrogen (McCarthy et al., 1995; Souza et al., 2009). Based on the sexually dimorphic expression of GAD67, we examined whether the effect of treatment may influence expression of this protein between genders. The data

indicated a significant increase in the females when normalized to males of the same treatment, indicating a difference between gender in the ketamine and phaclofen treated animals. Studies demonstrate that GABA exerts a negative feedback effect on the expression of GAD67 *in vivo* and *in vitro* and GAD67 is particularly sensitive to changes in GABA (Rimvall & Martin, 1994). An increased expression in GAD67 protein may indicate a reduction in GABA levels in the cortex. The increased expression of GAD67 in the ketamine treated females compared to ketamine treated males may explain the sex differences observed in PPI with 100 ms ISI. Since GABA is necessary for the inhibition of this task, altered GABA levels as indicated by increased GAD67 expression may account for the deficit. In addition, expression of GAD67 in the phaclofen treated females (compared to the phaclofen treated males) was increased. Based on data that indicate an inverse relation between GAD67 protein expression and GABA levels as well as our findings that $GABA_{B1b}$ and $GABA_{B2}$ protein levels are increased in phaclofen treated females, these results may indicate decreased GABA levels in the prefrontal cortex of these animals. Reduced GABA levels would explain the PPI with 100 ms ISI deficits and possibly the lack of reinstatement in CCF for the phaclofen treated females. The expression of GAD67 was not changed between genders for the saline and baclofen group which means the changes are specific to the phaclofen and ketamine treated groups. The data suggest that the blocking either the $GABA_B$ or the NMDA receptor during this developmental time point had a greater impact on the female developing nervous system compared to the male.

As $GABA_B$ has been implicated in numerous processes underlying neuronal migration and synapse formation (Ben-Ari et al., 2007; Huang, Di Cristo, & Ango, 2007;

Gaiarsa et al., 2011; Owens & Kriegstein, 2002; Represa & Ben-Ari, 2005;), we also investigated if changes in proteins associated with synaptogenesis and remodeling were observed in the treatment groups. One protein in particular, kalirin, has been found to serve a role in synapse formations and restructuring (Ma et al., 2008). Differential expression of kalirin isoforms has also been observed in post-mortem analysis of patients with schizophrenia. Some studies report an increase in expression (Deo et al., 2012) while others report a decreased expression (Hill, Hashimoto, Lewis, 2006; Penzes & Remmers, 2012) in the prefrontal cortex. In our experiment, the phaclofen treated females displayed an increase in protein expression levels for kalirin 5. An excess of kalirin indicates that the mechanisms involved in synapse formation are dysregulated, which may be associated with excessive attempts to build synapses (Penzes & Remmers, 2012). This may indicate a role for $GABA_B$ receptors in regulating synapse formation during early brain development. This can also be related to the increased expression in the other proteins examined (postsynaptic $GABA_B$ receptors and GAD67) in that altered function of $GABA_B$ receptors results in dysregulated synapse formation leading to behavioral deficits in the female animals. However, this theory requires further investigation.

The most apparent differences in the above study were reflected in the female treatment groups. These findings were unexpected as we did not anticipate the differences between the sexes. Few studies include females in both behavioral and biochemical experimental conditions, especially studies that focus on animal models of schizophrenia. Most animal studies utilize exclusively male rodents as opposed to females unless the researchers are interested in the influence of hormones (estrogens).

Interestingly, estrogen has been demonstrated to be neuroprotective against cellular dysfunction and damage, which has been shown *in vitro* and *in vivo* (see Singh, Dykens, Simpkins, 2006). Separately, studies by Woolley (1997, 1998) reveal that chronic administration of estradiol increase NMDA receptor expression and sensitivity to glutamate. During the proestrus phase of a rodent estrous cycle, when estrogen levels are at their peak, the number of synapses in the hippocampus increased by 30% compared to the estrous phase when estrogen levels are the lowest (Warren and Juraska, 1997). The drastic change in hippocampal morphology across the rodent estrous cycle is further complicated by the length of the cycle. The estrous cycle of a rodent is very short (four days) and influences their behavior in multiple tasks including PPI (Koch, 1998) and learning and memory tasks (for review, see Dohanich, 2002). Investigations using female rodents include initial tests for phase in the estrous cycle using a variety of methods. In this experiment, estrous phase was not examined; however, all females were housed in the same colony room. Based on previously established studies that mammalian females synchronize their ovarian cycles while living in close proximity (see McClintock, 1984), we elected to not examine the phase of estrous. Instead, we pseudo randomized the pair-housed cage assignments between treatment groups in the colony room. If there were differences in the phase of cycle between animals, we tried to minimize the effect it may have had in one treatment group versus another. Future studies performed in our laboratory with females will incorporate estrous phase testing to ensure all animals are in the same phase.

In this current experiment, the hormonal regulation of developmental processes may have played a larger role than we had initially hypothesized. Estrogen and

progesterone receptors are abundant in brain regions involved in reproduction but they are also found in smaller concentrations in regions such as the basal forebrain, hippocampus, and cortex. Estrogen and progesterone binding to these receptors affect the rates at which proteins are synthesized (Dohanich, 2003). For example, estrogen and progesterone secreted by the ovaries or exogenously administered can alter enzymes, receptors, transporters, and other proteins associated with glutamate and GABA signaling (Daniel & Dohanich, 2001). During development, the estradiol concentrations are the same in neonatal male and female animals (Amateau et al., 2004; McCarthy & Konkle, 2005; Nunez and McCarthy, 2008). Sexual differentiation appears during the first to second week of life due to a perinatal testosterone surge in male rodents (Ravizza et al., 2003; Galanopoulou, 2008). As it relates to the NMDA and GABA signaling, the sex dependent change that occurs during this early period in the rodent life is the differential expression of the neuronal Cl^--extruding K^+/Cl^- co-transporter (KCC2; promotes chloride extrusion). As outlined, GABA in development is excitatory when acting upon pyramidal $GABA_A$ receptors until after the end of first postnatal week of life in a rat (Ben-Ari et al., 1989). These effects are due to the relatively high expression of the $Na^{2+}/K^+/Cl^-$ co-transporter 1 (NKCC1; promotes Cl^- entry into the cell) and the low expression of the KCC2 which results in a high intracellular Cl^- concentration (Ben-Ari, 2002; Damborsy & Winzer-Serhan, 2012; Payne, Stevenson, Donaldson, 1996; Rivera et al., 1999). After the first postnatal week of life in a rat, NKCC1 is downregulated and KCC2 is upregulated, allowing Cl^- to be expelled out of the cells which results in a shift of GABA binding $GABA_A$ receptors to induce an inhibitory post synaptic potential (Ben-Ari, 2002; Rivera, 1999). This process is sex dependent as it occurs earlier in females compared to

males (Damborsky & Winzer-Serhan, 2012; Galanopoulou, 2008; Nunez & McCarthy, 2007). Therefore, the differences observed between males and females in the present experiments may be due to the administration of ligands during the time this shift occurs and the extended duration of GABA-mediated excitation in the multiple male brain regions (Nunez and McCarthy, 2007; Galanopoulou, 2008). The administration of $GABA_B$ and NMDA ligands during this postnatal time period would then produce differential effects in males and females. In the current investigation, we observed multiple deficits in female animals following changes in $GABA_B$ receptor function. Given that the switch from excitatory to inhibitory occurred during the same time frame as when these drugs were administered in females (as this switch happens earlier compared to males), it is possible that alterations in receptor systems from the drug administration presented substantial problems in the females. This may also account for the lack of deficits in the males as the drug administration occurred prior to this switch in depolarizing effects of GABA on $GABA_A$ receptors. The increase in inhibition during development as seen in the females during the drug administration period may represent a critical period that, when disrupted, results in lifelong alterations. By blocking $GABA_B$ receptors in females, the inhibition by which they rely may have greater effects. This may be a potential explanation for the deficits observed in this experiment and the changes in protein expression of multiple GABA related markers. Administering $GABA_B$ ligands at different developmental time points might further elucidate the effects of $GABA_B$ receptors and this excitatory to inhibitory shift in early brain develop.

These experiments were performed to determine if early life changes in NMDA or $GABA_B$ receptors would produce alterations with relevance to schizophrenia. Despite a

lack of a comprehensive demonstration of schizophrenia deficits in these animals, several groups did exhibit deficits related to the disorder. For the behavioral tasks, the phaclofen and baclofen treated females as well as the ketamine treated males exhibited deficits in the PPI with 100 ms ISI and only the ketamine male animals demonstrated subtle spatial learning and memory deficits. Although there were increases in the protein expression of multiple markers examined for the phaclofen treated females, our data do not agree with the protein changes observed in the post-mortem studies except for the increase in kalirin 5. Typically, the patient population exhibits a reduction in $GABA_B$ receptor proteins, $GABAA_{\alpha 5}$, GAD67, and NR2B (Fatemi, Folsom, Thuras, 2011; Guidotti et al., 2000; Hauser et al., 2005; Ishikawa et al., 2005; Mizukami, 2000, 2002; Zai et al., 2005) whereas the differences observed in this study demonstrated only increased expression in a subset of these proteins. In addition, sex differences exist in the schizophrenia patient population. These include men exhibiting more negative symptoms compared to women (see Leung & Chue, 2000). The results from our study comparing males versus females do not reflect what is seen in the patient population.

Regardless of how these results relate to schizophrenia, the deficits between the treatment groups are intriguing. A subtle alteration of these receptor systems in early brain development produced a lifelong behavioral change as evidenced by deficits in PPI, learning and memory, and changes in several protein levels. This study supports a role for precise signaling of the $GABA_B$ and NMDA receptors within specific time periods in mammalian brain development. These neurotransmitter systems are involved in establishing early neuronal networks (Komuro & Rakic, 1993; LoTurco, Blanton, & Kriegstein, 1991; Uhlhaas et al., 2010), and slight alterations in $GABA_B$ or NMDA

receptor mediated signaling may be sufficient to permanently alter connectivity that persists into adulthood. Although other neurodevelopmental studies administering NMDA receptor antagonist produced similar and differing deficits, most use more potent drugs such as PCP and MK-801 and some studies inject the drug for multiple consecutive days (Andersen and Pouzet, 2004; Broberg et al., 2010; Harich, Gross, Bespalov, 2007; Takahashi et al., 2006; Wang et al., 2001). Our study set out to examine subtle alterations in the NMDA receptor in development rather than a robust reduction in its function. To our knowledge, this study is the first to assess the effects in adulthood on behavioral and specific protein levels of early postnatal administration of a $GABA_B$ receptor agonist and antagonist. The deficits produced by both ligands establish the importance of the $GABA_B$ receptor and pave the way for future studies to further elucidate the role of $GABA_B$ receptors in development and their possible involvement in neurodevelopmental disorders.

Additional investigations are required to elucidate the mechanisms involved in disruption the $GABA_B$ and NMDA receptors in early brain development. Examining the effects of several concentrations and multiple time points in development would aid in the characterization of the role of these receptor systems in development. Further protein analysis of additional brain regions and protein markers may also provide insight into the mechanisms involved. The data from this study provide only a glimpse of the importance of normal function of the $GABA_B$ and NMDA receptors in early brain development.

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