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An Investigation of the Role of GABAb Ligands on Cued and Contextual Fear Conditioning

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AN INVESTIGATION OF THE ROLE OF GABA$_B$ LIGANDS ON CUED AND

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by

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ABSTRACT

An Investigation of the Role of GABA$_B$ Ligands on Cued and Contextual Fear
Conditioning

by

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GABA is the primary inhibitory neurotransmitter in the brain and mediates several processes, including learning and memory. Activating or inhibiting GABA receptors allows for the examination of the effects of altered GABAergic signaling on these processes. The two main receptors, GABA$_A$ and GABA$_B$, each have a different mechanism of action when activated, thus they may contribute differentially to learning and memory. The metabotropic GABA$_B$ receptor responds with the activation of several intracellular signaling cascades, which provide long-lasting inhibitory effects that primarily mediate network function. Conversely, the GABA$_A$ receptor is an ion channel that contributes more immediate inhibitory effects through the movement of ions across the cell membrane. While there is more research regarding the role of the GABA$_A$ receptor in learning and memory because it was discovered first, the data on the role of the GABA$_B$ receptor in learning and memory are more varied and inconsistent. Because of the discrepancies in the literature, it is necessary to better characterize the effects contributed by the GABA$_B$ receptors to learning and memory. We examined the effects of a GABA$_B$ agonist (baclofen) and a GABA$_B$ antagonist (phaclofen) on the associative learning and memory task, cued and contextual fear conditioning, as well as the extinction of the learned associations. Using two protocols that vary in complexity and

differentially recruit brain regions to learn the associations, we were able to evaluate whether the $GABA_B$ ligands produce different behavioral effects based on task in our first experiment. In a second experiment, we then investigated whether the results seen in the previous two experiments could be attributed to how well the task was learned initially by delaying the onset of ligand administration. Further, we investigated whether administration of the ligands altered GABA receptor protein levels in the neurological regions associated with the behavioral tasks. While baclofen treatment impaired the extinction of both the cued and contextual fear associations in both experiments, phaclofen treatment did not alter the acquisition or extinction of any of the associations. Interestingly, we found task-dependent shifts in $GABA_B$ receptor protein levels in both baclofen- and phaclofen-treated animals in several brain regions. In some instances, significant differences in protein levels were found in delay-trained groups that were not evident even in a non-significant trend in the trace-trained groups. These protein differences suggest that the administration of $GABA_B$ ligands alters behavior and neurological protein levels in a differential manner. Further study on both components (behavior and cellular effects) is warranted to help elucidate the role of $GABA_B$ receptors in learning and memory.

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CHAPTER 1

INTRODUCTION

The processes that underlie learning and memory are important for the survival of any organism. Animals must be able to adapt based on previous experiences and also detect patterns in stimuli in order to avoid predators, collect food, and even battle the elements. Because there are many diseases and disorders that disrupt these processes, it is important to understand the mechanisms by which they normally occur. Insight into normal functioning may then allow the development of treatments directed at improving deficits in learning and memory. There are several of types of learning (e.g. associative and non-associative) and memory (e.g. short-term and long-term), which makes the study of the mechanisms simultaneously easier (because we can focus on a single aspect of the process) and more difficult (because each process may rely on multiple independent and/or related mechanisms).

Of particular interest is Pavlovian cued and contextual fear conditioning, a type of associative learning, in which a neutral tone stimulus (conditioned stimulus, CS) is paired with and cues the unconditioned stimulus (US), a mild foot shock, in a particular environment, or context. The US produces the unconditioned fear response (UR), which manifests as freezing in rodents. Once the associations between the US and both the CS and context are learned and produce behavior similar to the UR, the response becomes the conditioned response (CR). The strength of these associations can then be tested later by either presenting the CS in a novel context without the US or by returning the subject to the original context without the US and monitoring the proportion of time the subject spends expressing the CR.

This type of learning lends itself to being investigated experimentally because the associations are easily learned, readily quantifiable, and decrease over time without further US presentations (extinction). Extinction is the process of learning that the previously learned CS or context no longer accurately predict the US; this mechanism is thought to be inhibitory in nature, and is not analogous to forgetting. Further, the difficulty of this task, as well as the brain regions recruited to learn the associations, can be manipulated through procedural variation. The two variations commonly used are delay and trace cued and contextual fear (CCF) conditioning. Both procedures utilize the same CS and US, however in delay CCF the CS and US overlap in time, where in trace CCF, the stimuli are separated by a brief time interval. It has been demonstrated that the trace CCF procedure is more difficult to learn than the delay CCF procedure due to this time interval separating the CS and US.

While considerable research has been conducted regarding the brain areas that have been shown to mediate Pavlovian learning, some of the cellular mechanisms responsible for the learning are less well understood. The excitatory signaling component is well characterized for the cellular mechanisms that drive learning and memory; however, the brain also largely utilizes inhibitory signaling. While it has not been as extensively investigated as excitatory signaling, inhibitory signaling may also play a role in learning and memory. For instance, inhibitory systems can modulate excitatory processes that are known to be involved in learning and memory, such as theta and gamma oscillations. Further, it has been suggested that the process of extinction may rely on inhibitory signaling in order to alter the previously learned associations, allowing

an organism to be adaptable. The role of inhibitory systems, therefore, appears to be an important aspect of learning and memory, and requires further investigation.

Research Questions

In order to examine the role of altered $GABA_B$ receptor function in fear conditioning, we tested the effects administering two ligands, baclofen (a $GABA_B$ agonist) and phaclofen (a $GABA_B$ antagonist), throughout delay and trace cued and contextual fear conditioning (CCF) and extinction. We also investigated whether the differences seen by administering the ligands throughout the entirety of the delay and trace CCF procedures could be, in part, due to altered acquisition of the associations. Finally, we examined the effects of $GABA_B$ ligand administration on GABAergic proteins in brain regions implicated in learning and extinguishing the associations of delay and trace CCF.

CHAPTER 2

REVIEW OF RELATED LITERATURE

Inhibitory Drive in the CNS – GABA

Gamma-aminobutyric acid (GABA) was discovered in 1950 and has since been characterized as the primary inhibitory neurotransmitter in the central nervous system (CNS; Roberts, 1956). GABA cannot cross the blood brain barrier and must be synthesized in the brain via the decarboxylation of glutamate (Roberts, 1956; Olsen, 2001; Watanabe et al., 2002). There are two major subtypes of GABA receptors (GABA$_A$ and GABA$_B$), and while the primary mechanism of activation of both receptor subtypes is to hyperpolarize cells, they produce this effect through very different mechanisms (Olsen, 2001; Watanabe et al., 2002; Enna, 2007). The individual and collective action of neurons relies on periodic depolarizations and hyperpolarizations to generate the action potentials that are required for signaling (Hodgkin & Huxley, 1952; Miledi, 1967). Hyperpolarization typically leads to inhibition because a cell generally requires a net positive effect on its membrane potential in order to create an action potential (Bean, 2007).

GABA$_A$ receptors are proteins that span the cellular membrane four times and have several distinct subunits that associate heterogeneously into pentamers (Olsen, 2001; Enna, 2007). The receptors can be made up of any of the subunits, but the major subtypes include α, β, and γ, with the bulk of the receptors being made up of at least one α and one β subunit (Olsen, 2001). The composition of the subunits of the GABA$_A$ receptor dictates which ligands, such as barbiturates or benzodiazepines, can bind to that

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particular receptor, in addition to determining where the receptors will be located (Olsen, 2001; Mohler, 2009).

Functionally, the GABA$_A$ receptors are ligand-gated ionotropic chloride channels (Olsen, 2001; Enna, 2007). An ionotropic receptor is typically activated when a ligand binds and causes a conformational change in the protein. The conformational change creates an opening between the intra- and extracellular environments through the cell membrane, allowing ions to pass into or out of the cell (Olsen & Tobin, 1990). In the case of GABA$_A$ receptors, as soon as GABA binds, the channel opens and chloride rushes into the cell (Gahwiler & Brown, 1985; Bowery, 1993; Olsen, 2001; Watanabe et al., 2002). Because chloride carries a negative charge, it creates a net negative effect on the cell's membrane potential, causing the cell to hyperpolarize and thus to be inhibited. The GABA$_A$ receptors are very fast acting because as soon as the ligand (i.e. GABA) binds, the channel opens immediately; however, while the mechanism of action of this receptor is instantaneous, the effects of these channels are very short-lived because as soon as the ligand is dislodged, the channel immediately closes again (Watanabe et al., 2002). Thus, GABA$_A$ receptors are responsible for the fast-acting inhibitory currents within the CNS (Hevers & Luddens, 1998; Watanabe et al., 2002).

GABA$_A$ receptors are generally found on postsynaptic cell membranes (Watanabe et al., 2002; Enna, 2007) with some presynaptic occurrences (Kullmann et al., 2005). While GABA$_A$ receptors are found throughout the entire CNS, there are high levels of the receptor located within the frontal cortex and thalamus, with lower concentrations in the amygdala and hippocampus (Bowery, Hudson, & Price, 1987; Chu et al., 1990; Olsen & Tobin, 1990; Hevers & Luddens, 1998; Enna, 2007). Although there are generally more

GABA$_A$ receptors than GABA$_B$, GABA$_B$ receptors operate via a different mechanism of action and typically have a stronger affinity for GABA than GABA$_A$ (Bowery, Hudson, & Price, 1987; Chu et al., 1990; Isaacson, Solis, & Nicoll, 1993).

GABA$_B$ receptors are ligand-gated metabotropic G-protein coupled receptors. In contrast to an ionotropic receptor, a metabotropic receptor is not directly coupled to a channel; instead, when a ligand binds, the conformational change of the receptor effects a change in the G-protein with which it is associated (Enna, 1997; Brown & Sihra, 2008). G-proteins are tetramers that consist of three major subunits (α, β, and γ) that act together as a functional unit (Brown & Sihra, 2008). The change in the G-protein typically results in the intracellular dissociation of the α subunit from the cell-membrane-bound tetramer, which can initiate different cascade sequences inside the cell (Brown & Sihra, 2008). The intracellular effects of cascade sequences can, among other cellular functions, cause channels to open, cause the activation of several second messenger systems, and cause the initiation or silencing of transcription within the nucleus (Brown & Sihra, 2008). The β and γ subunits stay bound together and attached to the cell membrane, but also are capable of effecting changes within the cell (Brown & Sihra, 2008). Metabotropic receptors are slower acting compared to ionotropic because instead of a channel pore immediately opening in response to a ligand binding, a cascade sequence must be activated by the α or β and γ subunits of the G-protein before a channel is opened (Brown & Sihra, 2008). However, the effects of metabotropic receptors are long lasting compared to ionotropic receptors. When the ligand is dislodged from the receptor's binding site, the intracellular signal cascade persists until it is inactivated within the cell, unlike an ionotropic receptor. As a part of the signal cascade, the signal transmitted by

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the ligand is amplified and transduced within the cell (Brown & Sihra, 2008). Thus, even once the ligand is dislodged and is no longer providing input from outside of the cell, the signal continues to propagate within the cell. So while the $GABA_B$ receptors are responsible for a slow inhibitory current, the magnitude of effect is enhanced and longer lasting due to the signal cascade (Couve, Moss, & Pangalos, 2000; Bettler et al., 2004).

$GABA_B$ receptors are seven-transmembrane proteins and have two classes of receptor subtypes, $GABA_{B1}$ and $GABA_{B2}$; additionally, the $GABA_{B1}$ receptor subtype has two isoforms, $GABA_{B1a}$ and $GABA_{B1b}$ (Couve, Moss, & Pangalos, 2000; Bowery et al., 2002; Enna, 2007; Kohl and Paulsen, 2010). The subunits couple together to form a heterodimer, and each subunit demonstrates a unique role to help the functioning of the receptor (Enna, 1997; Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998; Villemure et al., 2005; Pinard, Seddik, & Bettler, 2010). The main difference between the two $GABA_{B1}$ isoforms appears to be related to the receptor's location. Receptors composed of $GABA_{B1a/2}$ generally inhibit presynaptically, and should act to prevent neurotransmitter release, whereas those composed of $GABA_{B1b/2}$ subunits appear to primarily inhibit postsynaptically through inhibitory postsynaptic currents (Perez-Garci et al., 2006; Vigot et al., 2006; Ladera et al., 2008; Kohl & Paulsen, 2010).

The $GABA_{B1}$ subunits appear to contain the binding site for GABA, as well as for agonists and antagonists, whereas the $GABA_{B2}$ subunits couple the receptor complex to G-proteins, as well as bring the heterodimer complex to the cell surface from the endoplasmic reticulum (Galvez et al., 2001; Robbins et al., 2001; Bowery et al., 2002; Kohl & Paulsen, 2010; Pinard, Seddik, & Bettler, 2010). If two $GABA_{B2}$ subunits or any combination of two $GABA_{B1}$ subunits bind together, the resultant $GABA_B$ receptor is

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dysfunctional. In $GABA_{B1}$-knockout mice, the typical G-protein-linked current is absent; $GABA_{B2}$-knockout mice demonstrate "atypical $GABA_{B1}$-mediated responses," suggesting that $GABA_{B1}$ is capable of coupling to other G-proteins in the absence of $GABA_{B2}$ (Pinard, Seddik, & Bettler, 2010).

As previously alluded to, $GABA_B$ receptors are found both pre- and post-synaptically, though there may be different mechanisms of action depending on location (Misgeld, Bijak, & Jarolimek, 1995; Watanabe et al., 2002; Enna, 2007; Kohl & Paulsen, 2010). Presynaptic $GABA_B$ receptors may be participating in a feedback loop as autoreceptors (Davies et al., 1991; Misgeld, Bijak, & Jarolimek, 1995; Zarrindast et al., 2002; Kohl & Paulsen, 2010) by mediating the presynaptic release of GABA, or as heteroreceptors (Sakaba & Neher, 2003; Tiao & Bettler, 2007; Bowery, 2010; Kohl & Paulsen, 2010) by mediating the presynaptic release of other neurotransmitters such as glutamate (Sakaba & Neher, 2003) or acetylcholine (Morton et al., 2001). These receptors appear to need strong stimulation and large amounts of GABA in the synapse in order to be activated, suggesting some may be located extrasynaptically (Misgeld, Bijak, & Jarolimek, 1995; Ladera et al., 2008; Pinard, Seddik, & Bettler, 2010).

Perhaps the only clear functional distinction based on location is the previously described auto- and heteroreceptor function that, by definition, must occur presynaptically. While there appears to be a trend of certain functions based on location, research continually demonstrates that there are exceptions. For instance, presynaptic $GABA_B$ receptors can affect neurotransmitter release by inhibiting voltage-gated calcium conductance in the cell membrane at the synaptic terminal (Misgeld, Bijak, & Jarolimek, 1995; Bettler et al., 2004; Bowery, 2007; Kohl & Paulsen, 2010). Once the $GABA_B$

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receptor is activated, the β and γ subunits of the G-protein dissociate from the G-protein complex and bind to voltage-gated calcium channels, which leads to their inactivation (Couve, Moss, & Pangalos, 2000; Bettler et al., 2004; Padgett & Slesinger, 2010). A reduction in calcium entering the synaptic terminal (i.e. decreased calcium conductance) leads to decreased release of neurotransmitter from the presynaptic neuron into the synapse (Llinas, Steinberg, & Walton, 1981; Bean, 2007). A decreased amount of neurotransmitter in the synapse then leads to decreased postsynaptic receptor activation because less neurotransmitter is available to bind and activate receptors. While this may not necessarily lead to postsynaptic inhibition through hyperpolarization of the postsynaptic membrane, decreased signaling to the postsynaptic neuron sufficiently modifies the strength of the stimulus within the postsynaptic cell, preventing the firing or inhibition of an action potential, as well as possibly altering the firing rate of the postsynaptic neuron.

However, it also has been demonstrated that $GABA_B$ receptors can inhibit a postsynaptic cell through the decrease of calcium conductance (Misgeld, Bijak, & Jarolimek, 1995; Bettler et al., 2004; Kohl & Paulsen, 2010; Padgett & Slesinger, 2010). Like the decrease in presynaptic calcium conductance, the postsynaptic decrease can be achieved through the inactivation of voltage-gated calcium channels through G-protein coupling (Kohl & Paulsen, 2010; Padgett & Slesinger, 2010). In the postsynaptic neuron, however, inhibition occurs because calcium, one of the primary depolarizing ions, is no longer entering the cell, thus there is a decrease in excitatory input making the initiation of action potentials more difficult. So although this method can occur in the postsynaptic neuron, it appears to be more common presynaptically (Bettler et al., 2004).

In addition to inactivating voltage-gated calcium channels, a GABA$_B$ receptor can activate an inward-rectifying potassium channel (Dascal, 1997; Mark & Herlitze, 2000; Brown & Sihra, 2008). The inward-rectifying potassium channel is activated by the dissociated β and γ subunits of the G-protein complex (Dascal, 1997; Brown & Sihra, 2008; Padgett & Slesinger, 2010), which increase potassium conductance. Potassium then effluxes out of the cell, leading to hyperpolarization (Bettler et al., 2004; Pinard, Seddik, & Bettler, 2010). While the effects of GABA and inward-rectifying potassium channels are more typically found in postsynaptic sites (Luscher et al., 1997; Yamada, Inanobe, & Kurachi, 1998; Mark & Herlitze, 2000; Bettler et al., 2004), recent research suggests that these channels may also help to regulate neurotransmitter release presynaptically (Ladera et al., 2008; Fernandez-Alacid et al., 2009). The presynaptic inward-rectifying potassium channels may work by limiting the amount of calcium that is allowed to enter the neuron by altering the duration of an action potential by counteracting the depolarization with an efflux of potassium (Ladera et al., 2008).

Finally, in addition to modulating specific channels, GABA$_B$ receptors also may act by inhibiting adenylyl cyclase (Bettler et al., 2004; Enna, 2007; Padgett & Slesinger, 2010). The α subunit dissociates from the G-protein complex and inhibits adenylyl cyclase, which normally initiates a number of other intracellular cascades, including those that affect short- and long-term memory (Birnbaumer, 2007; Brown & Sihra, 2008; Vianna et al., 2000; Padgett & Slesinger, 2010). This mechanism of action may be present at both pre- and postsynaptic neurons, as it has been suggested that the cyclic adenosine monophosphate (cAMP) cascade (which is activated by adenylyl cyclase) may

play a role in synaptic signaling via neurotransmitter release, and neuronal excitation (Ulrich & Bettler, 2007; Padgett & Slesinger, 2010).

While all of these mechanisms of action (decreased calcium conductance, increased potassium conductance, and inhibition of adenylyl cyclase) have been discussed separately, they could work in conjunction with each other. For instance, because the α subunit works independently of the β and γ subunits, activation of the $GABA_B$ receptor complex could lead to the inhibition of adenylyl cyclase and decreased calcium conductance or increased potassium conductance, or even all three, at the same time. Because the juxtaposition of these mechanisms would be an additive inhibitory effect, one can see how the magnitude of the effect of an activated $GABA_B$ receptor is more robust than that of a $GABA_A$ receptor.

$GABA_B$ receptors are located throughout the entire CNS, and appear to have a high concentration in the frontal cortex, lateral amygdala, and the thalamus (Bowery, Hudson, & Price, 1987; Chu et al., 1990). Additionally, moderate levels of $GABA_B$ receptors are present in the hippocampus (Bowery, Hudson, & Price, 1987; Chu et al., 1990). As previously mentioned, $GABA_B$ receptors appear to have a higher binding affinity for GABA than $GABA_A$ receptors, which could explain the decreased number of $GABA_B$ receptors compared to $GABA_A$ receptors (Isaacson, Solis, & Nicoll, 1993). When a receptor has a higher affinity for a substrate, the substrate is more attracted to, and more likely to bind to, the high-affinity receptor than any other receptor. Therefore, fewer high affinity receptors are necessary to gain a particular effect since they are likely to bind the substrate faster than and before other receptors. Low affinity receptors would then need to compete with the higher affinity receptors, and may do so with increased

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numbers. Alternatively, because GABA$_B$ receptors have a larger and more prolonged effect compared to GABA$_A$ receptors, fewer GABA$_B$ receptors may be needed to have a comparable effect. Further, having an increased number of high affinity receptors could lead to excessive signaling that would disrupt normal functioning.

GABA in Learning and Memory

Long-term potentiation (LTP) and synchronous neural firing (gamma and theta wave oscillations) have been implicated in facilitating learning and memory formation. LTP is the strengthening of synaptic connections between two or more neurons that fire simultaneously (Hebb, 1949; Bliss & Lomo, 1973; Bliss & Collingridge, 1993), and though it is most well characterized in the hippocampus (Malenka & Bear, 2004), the process also occurs in the amygdala and elsewhere (Herry & Garcia, 2002; Sigurdsson et al., 2007). Altering GABAergic tone is capable of affecting LTP (Davies et al., 1991; Staubli, Scafidi, & Chun, 1999; Trepel & Racine, 2000). Administering GABA$_A$ or GABA$_B$ antagonists to hippocampal slices increases the magnitude of LTP (Olpe & Karlsson, 1990; Steele & Mauk, 1999), whereas GABA$_A$ agonists decrease LTP induction (Blitzer, Gil, & Landau, 1990; Steele & Mauk, 1999; Fujii et al., 2000).

While several neurotransmitters play a role in regulating oscillations (Boguszewicz et al., 1996), GABAergic interneurons are important to the formation and entrainment of gamma and theta wave oscillations (Gonzalez-Burgos, 2010). GABAergic interneurons can also modulate oscillations by altering the frequency and amplitude of inhibitory postsynaptic currents (Henderson & Jones, 2005). Decreasing the effects of GABA through antagonism enhances theta and gamma wave oscillations

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(Konopacki et al., 1997; Leung & Shen, 2007), whereas increasing GABAergic effects using agonists reduces theta and gamma wave oscillations (Bland & Oddie, 2001; Brown, Davies, & Randall, 2007).

High frequency stimulation can induce LTP (Bliss & Collingridge, 1993; Tang et al., 1999), and while the high frequencies used *in vitro* are uncommon *in vivo*, synchronous oscillations that are commonly observed in the CNS are sufficient to modulate LTP (Orr et al., 2001; Axmacher et al., 2006; DeCoteau et al., 2007; Jutras & Buffalo, 2010). In fact, oscillations correlate with memory encoding (Klimesch, 1999; DeCoteau et al., 2007; Sederberg et al., 2007). Further, research demonstrates that inhibiting LTP (Davis, Butcher, & Morris, 1992; Balschun & Wetzel, 2002) or oscillations (Hosseinzadeh et al., 2005) is detrimental to learning and memory.

Because GABAergic tone can affect both LTP and synchronous network activity, which both affect learning and memory, GABAergic tone likely also affects learning and memory. What remains to be clearly elucidated are the discrete roles of the GABA receptors in learning and memory.

The alteration of GABAergic tone by either $GABA_A$ or $GABA_B$ ligands does affect how an animal learns a task. Consistent with the data above regarding $GABA_A$ ligands and LTP, the administration of $GABA_A$ agonists typically produce impaired learning and memory (Castellano, Cabib, & Puglisi-Allegra, 1996; Majchrzak & Di Scala, 2000; Chapouthier & Venault, 2002; Myhrer, 2003), and $GABA_A$ antagonists and inverse agonists enhance learning and memory (Castellano, Cabib, & Puglisi-Allegra, 1996; Chapouthier & Venault, 2002; Myhrer, 2003; Collinson et al., 2006). A similar, yet inconsistent, pattern of impaired learning and memory has been found with $GABA_B$

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agonists in some studies (Castellano, Cabib, & Puglisi-Allegra, 1996; McNamara & Skelton, 1996; Myhrer, 2003; Stuchlik & Vales, 2009) and enhanced learning and memory after administering GABA$_B$ antagonists (Castellano, Cabib, & Puglisi-Allegra, 1996; Getova & Bowery, 1998). However, the results from GABA$_B$ investigations are not well replicated and are, at times, contradictory. In a review by Myhrer (2003), four studies attempting to demonstrate the effects of baclofen (a GABA$_B$ agonist) on the same passive avoidance task found that baclofen improves, impairs, and does not alter performance. Since these four studies utilized the same task in the same manner and all administered baclofen systemically, the differing results could be due to the dosages or strain of animal used (Castellano, Cabib, & Puglisi-Allegra, 1996; Myhrer, 2003).

Few studies have utilized GABA$_B$ antagonists in specific learning and memory tasks, and there are inconsistent results among those studies that have been conducted, as well. For instance, Mondadori, Mobius, and Borkowski (1996) administered a GABA$_B$ antagonist after a passive avoidance task and found enhanced memory (as measured by increased step-through latencies to enter the darkened chamber associated with a mild foot shock) for the task. However, Zarrindast et al. (2002) also administered a GABA$_B$ antagonist after a passive avoidance task and found no effect on step-through latencies at low doses, whereas animals administered high doses actually demonstrated impaired performance in the task by stepping through to the shock-associated chamber faster than controls. Differences between these two studies include route of administration (systemic versus intracranial), gender of the animals, and type of animals used (mice versus rats). Ultimately, these discrepancies in both the GABA$_B$ agonist and antagonist literature demonstrate that the effects of the GABA$_B$ receptors need to be better characterized.

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Additionally, the relationship between dose and effect may be very different between agonists and antagonists; in other words, the same concentration of an antagonist and an agonist may not produce the same "amount" of facilitation or loss of function as it relates to learning and memory.

In addition to the inconsistencies described above, the task used can also affect the results. For instance, Brucato et al. (1996) demonstrated that a single $GABA_B$ antagonist, while capable of suppressing the induction of LTP in the dentate gyrus, altered behavior in only one of the three tasks used to measure spatial learning. The task that did show altered behavior was a water maze task, whereas the other two tasks were variations on the eight-arm radial maze. Using $GABA_B$ ligands may possibly alter GABAergic tone differentially based on the task used, and some tasks may be more or less sensitive to a specific dose. It is also possible that altering GABAergic tone by way of $GABA_B$ ligands only affects specific types of memory. Because the tasks described above all produced differing results, it is important to continue characterizing the role of the $GABA_B$ receptors in learning and memory.

GABA in Pavlovian Conditioning

We used Pavlovian fear conditioning, a task with well-defined neuronal regions associated with learning the procedure. This task can be modified to increase how difficult it is to learn the associations; further, while the procedural variants rely on the same underlying neuronal regions, each variant recruits the brain regions differentially to learn the associations (Makkar, Zhang, & Cranney, 2010). In Pavlovian fear conditioning, an unconditioned stimulus (US; e.g. a shock) is paired with a conditioned

stimulus (CS; e.g. a tone), and an association between the two is made (Pavlov, 1960). The unconditioned response (UR; e.g. fear), which is elicited naturally from the US, becomes associated with the initially neutral CS; that is, after Pavlovian conditioning, presenting the CS alone is capable of producing the conditioned response (CR, which is almost always the same behavior elicited by the UR). An interesting aspect of this task is that is it possible to experimentally measure the extinction of the associations, as evidenced by decreased behavioral responses to a CS presentation.

Extinction occurs when the CS is repeatedly presented in the absence of the US, overriding the previously learned association that the CS always predicts the US (Bouton et al., 2006; Myers & Davis, 2007). This process is not simply forgetting the association, however, as demonstrated by spontaneous recovery. Spontaneous recovery is marked by the sudden reinstatement of the CR without additional CS-US pairings after a period of time wherein the CR had previously decreased over time with continual CS exposure (i.e. extinction occurs) (Myers & Davis, 2002; Bouton et al., 2006). If extinction were indicative of forgetting, then the association should not be able to be recovered. Instead, extinction is thought of as the formation of a new memory that is proposed to inhibit the previously acquired CS-US association (Bouton, 2004), and GABAergic neurotransmission is likely to play a crucial role in this change (Akirav & Maroun, 2007).

Pavlovian conditioning involving a tone that occurs in a specific context and predicts (or cues) a mild foot shock is called cued and contextual fear (CCF) conditioning (Phillips & LeDoux, 1992). As the name implies, there are two main aspects to the task; the cued fear is elicited from the tone (CS), and the contextual fear is elicited from being in the original context (or environment) of the CS-US pairings (Phillips & LeDoux,

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1992). This task allows the investigation of the association between the CS and the US (cued fear), as well as the association between the US and the original context in which the US was presented (contextual fear). Each association differentially relies on distinct brain regions, however the formation of each association is dependent upon the training protocol used. The traditional protocol is called delay CCF conditioning; in this version, the CS and US overlap in time and co-terminate; that is, the US is presented during the same time that the CS is being presented. Because the CS and US overlap, this association is simple and easy to learn. Studies demonstrate that in order for an animal to exhibit cued fear when trained using delay CCF conditioning, an intact amygdala is required, whereas contextual fear depends on the hippocampus (Phillips & LeDoux, 1992; Kim & Jung, 2006; Curzon, Rustay, & Browman, 2009; Makkar, Zhang, & Cranney, 2010).

In another variation called trace CCF conditioning, a time interval is inserted between the termination of the CS and onset of the US; that is, the CS and US do not co-terminate, nor do they overlap in time. As in delay CCF conditioning, previous research demonstrates that fear associated with the CS is mediated by the amygdala (Curzon, Rustay, & Browman, 2009; Makkar, Zhang, & Cranney, 2010); however, the time interval between stimuli requires hippocampal processing in order for the association between the CS and US to be formed (Beylin et al., 2001). Animals with hippocampal lesions exhibited a drastic decrease in freezing behavior to the CS in a trace cued fear conditioning task compared to control animals, thus demonstrating that the hippocampus is involved in processing this temporal gap between CS and US (Beylin et al., 2001). Further, Beylin et al. (2001) demonstrated that for control animals, the longer the interval

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is between the CS and US, the more difficult the association was to learn; the amount of freezing demonstrates the strength of the learned association, and longer trace intervals produced less freezing. Trace conditioning does not affect contextual fear since altering the CS-US overlap does not change all of the environmental cues, thus the hippocampus still mediates contextual fear as in delay CCF (Curzon, Rustay, & Browman, 2009).

The brain regions involved in Pavlovian conditioning have been demonstrated to involve the CS-activation of the amygdala, which in turns leads to the CR, behaviorally (Quirk et al., 2003; Pare, Quirk, & Ledoux, 2004; Kim & Jung, 2006; Hartley & Phelps, 2010; Makkar, Zhang, & Cranney, 2010). This association is aided by inhibitory circuitry in the amygdala via control the formation of fear memories (Bolshakov, 2009; Ehrlich et al., 2009). Information is thought to be sent to the hippocampus, which leads to the acquisition and initial storing of the contextual fear memory; additionally, information about the expression of fear in relation to the context may be routed from the hippocampus through to the amygdala (Hobin, Ji, & Maren, 2006; Kim & Jung, 2006; Hartley & Phelps, 2010; Makkar, Zhang, & Cranney, 2010). Finally, the prefrontal cortex has been recently implicated in extinction learning due to its inhibitory projections between the amygdala and hippocampus (Kim & Jung, 2006; Quirk & Mueller, 2008; Hartley & Phelps, 2010; Makkar, Zhang, & Cranney, 2010). Interestingly, a high abundance of $GABA_A$ and $GABA_B$ receptors are located in the areas just discussed (Bowery, Hudson, & Price, 1987).

Several studies have demonstrated that altered GABAergic tone in these structures can affect Pavlovian conditioning. Most notable is that a $GABA_A$ agonist, muscimol, is commonly used to reversibly inactivate different brain regions associated

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with conditioned fear. For instance, Wilensky, Schafe, & LeDoux (2000) infused muscimol into the lateral amygdala in order to demonstrate that inactivating the amygdala blocks acquisition, but not the consolidation, of Pavlovian conditioning. McEown & Treit (2010) infused muscimol into the ventral or dorsal hippocampus either pre- or post-training of a conditioned fear task. Inactivating the ventral hippocampus before training impaired the acquisition of the conditioned fear task as demonstrated by animals failing to freeze throughout training in response to continual CS-US pairings, whereas inactivating the dorsal hippocampus post-training impaired retention as demonstrated by animals failing to freeze to the CS 24 hours later (McEown & Treit, 2010). Finally, Akirav, Raizel, & Maroun (2006) infused muscimol into the prefrontal cortex in order to investigate the effects on extinction. The authors suggest that the $GABA_A$ agonist enhanced extinction; while this interpretation is debatable considering the experimental animals never initially demonstrated behavior similar to the control animals, it is important to note that the ligand did actually alter the experimental animals' behavior.

Numerous studies also have investigated the role of GABA ligands in Pavlovian conditioning per se, although a preponderance of the studies are done using $GABA_A$ ligands or specific $GABA_A$ receptor subunit mutants (see Makkar, Zhang, & Cranney, 2010). As demonstrated earlier with other learning and memory tasks, the $GABA_A$ antagonists tend to improve learning and memory and $GABA_A$ agonists impair learning and memory. Improvements or deficits caused by $GABA_A$ alterations are thought to occur by either shutting down specific brain regions or allowing the regions to be more active than typically observed. Though the effect of altering $GABA_A$ receptor function has been extensively evaluated, there are fewer data regarding the role of $GABA_B$

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receptors and how longer-lasting changes to neuronal functioning may affect learning and memory in Pavlovian conditioning.

The current data regarding the role of $GABA_B$ receptors in Pavlovian conditioning are rather inconclusive. For instance, Jacobson et al. (2006) used $GABA_{B1}$ receptor knockout mice in a conditioned taste aversion task, a type of Pavlovian conditioning. In this task, animals were introduced to saccharine-flavored water, which rodents typically enjoy due to its sweetness and will drink more of compared to unflavored water. The mice were later injected with lithium chloride, which made the animals feel sick, every time they were given the saccharine-sweetened water. The animals soon learn to associate feeling ill with the saccharine-flavored water and learn to stop drinking this preferred water. As previously mentioned, there are two isoforms of $GABA_{B1}$ receptors, $GABA_{B1a}$ (thought to localize presynaptically) and $GABA_{B1b}$ (thought to localize postsynaptically); each receptor subunit isoform create a heterodimer with the $GABA_{B2}$ receptor. Interestingly, the $GABA_{B1a}$ knockouts were impaired in acquiring the taste aversion, as demonstrated by a lack of decreased ingestion of the saccharine-flavored water, while the $GABA_{B1b}$ knockouts were impaired in the extinction of the aversion, as demonstrated by a lack of decreased aversion to the saccharine-flavored water in absence of the lithium chloride-induced malaise over time (Jacobson et al., 2006).

Shaban et al. (2006) also utilized $GABA_{B1}$ receptor knockout mice, but instead used a cued fear conditioning task. Animals were presented with two tones, only one of which was paired with a shock. Wild-type animals were able to discriminate between the two tones and only exhibited freezing behavior (i.e. the CR) to the tone that had been

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paired with the shock. $GABA_{B1a}$ knockout animals, however, froze to both tones, demonstrating an over-generalization of fear, or an inability to learn the association specific to the CS paired with the US; the $GABA_{B1b}$ knockout animals were unable to acquire the task at all, as demonstrated by a lack of freezing to either of the tones used in the task (Shaban et al., 2006). These results do not correspond to those of Jacobson et al. (2006) who found that the $GABA_{B1a}$ knockouts had difficulty acquiring the task. This discrepancy could perhaps be explained by the amount of stress each task induces (Brucato et al. 1996). However, more data are needed to elucidate the role of these receptors in Pavlovian conditioning. While knockout studies provide some insight to the function of the receptors, the utilization of $GABA_B$ ligands can provide valuable information pertaining to the effect that *altered signaling* of the receptors has on learning and memory.

Based on the differences in function, discrepancies in the behavioral data between $GABA_A$ and $GABA_B$ receptors, and the inconsistencies within the $GABA_B$ literature described above, a thorough investigation of the role of $GABA_B$ receptors in a precise learning and memory task is warranted. By being able to utilize training variations that recruit discrete brain regions where high concentrations of the receptor may indicate that $GABA_B$ function may be particularly relevant, cued and contextual fear conditioning is a task that may help provide crucial information regarding alterations of $GABA_B$ receptor tone on learning. With these issues in mind, the following experiments were conducted in order to help clarify the role of $GABA_B$ receptors in learning and memory and extinction.

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Hypotheses and Implications

Based on the previous research that indicate altered inhibition via $GABA_A$ receptors in learning and memory, we predicted that the $GABA_B$ agonist, baclofen, would impair the acquisition of the fear conditioning, but that it would enhance the extinction of the learned fear. Alternatively, we hypothesized that the $GABA_B$ antagonist, phaclofen, would impair the acquisition as well as the extinction of the conditioned fear. Because the trace CCF protocol is more difficult to learn and is mediated by a more complex network of neurological regions, we predicted that any deficits or enhancements caused by the $GABA_B$ ligands would be more pronounced in trace CCF as compared to delay CCF. We further hypothesized that any effects of the ligands on protein levels would be more prominent in the trace-conditioned animals than the delay-conditioned groups. Specifically, we predict to see a decrease in receptor protein levels in the baclofen-treated animals because an agonist should down-regulate proteins; and we predicted to see an up-regulation in protein levels in the phaclofen-treated animals because an antagonist should up-regulate proteins. Based on any behavioral deficits seen, we should see corresponding protein changes in the brain regions that mediate the disrupted behaviors.

CHAPTER 3

MATERIALS AND METHODS

Subjects

One hundred and twenty male Sprague-Dawley rats (Harlan, Indianapolis, IN) approximately three months of age and weighing 250-300g were used. Rats were housed in a temperature and humidity controlled facility ($22 \pm 1°$ C), and food and water were provided ad libitum. Animals were housed in pairs and kept on a 12:12 light:dark cycle, lights on at 7:00am. All procedures were approved by the Institutional Animal Care and Use Committee and followed NIH guidelines.

Drug Treatments

R(+)-Baclofen hydrochloride (Sigma-Aldrich, St. Louis, MO) was dissolved in 0.9% physiological saline vehicle at a concentration of 2mg/mL. Phaclofen (Sigma-Aldrich) was dissolved in 0.9% physiological saline vehicle at a concentration of 0.3mg/mL. Compounds were administered via intraperitoneal (i.p.) injection 15 minutes before behavioral testing at a volume of 1ml/kg; 0.9% physiological saline was administered as a control also at a volume of 1ml/kg. Animals were randomly assigned to one of three treatment groups (saline, baclofen, or phaclofen administration; n=10) in one of two experiments (drug administration given through delay or trace cued and contextual fear (CCF) conditioning, or drug administration starting on Day 2 of delay or trace CCF conditioning; for a total of n=30 per experiment). Each experiment contained the same treatment groups, but an individual animal only participated in one experiment.

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Cued and Contextual Fear Conditioning

Fear conditioning training and contextual fear testing were conducted in a 10" x 10" x 7.5" acrylic chamber (San Diego Instruments, San Diego, CA). The floor of the chamber consisted of a stainless steel grid made of 1/4" grids spaced at 9/16". In between animals, the chamber was cleaned using a common household cleaner, Formula 409 (Clorox Company, Oakland, CA).

Cued fear testing was conducted in an altered context chamber that consisted of 17" x 10.5" x 5" opaque plastic. In addition to differences in material, shape, and height from the floor (because the altered context chamber has tall opaque walls, the chamber was placed on the floor instead of a table in order to observe animal behavior), a novel scent cue (vanilla extract) was added to one of the walls. The chamber was cleaned between animals using a 1% ethanol solution to ensure no olfactory overlap with the training chamber.

Trials were programmed and run with Freeze Monitor (San Diego Instruments) using a Cobalt Instruments computer. Two researchers visually monitored the animals' freezing behavior and recorded the data manually. The data collected for each animal included whether the animal was freezing (determined every 10 seconds), as well as a qualitative description of the behavior exhibited by the animal if it was not freezing (e.g. grooming, rearing, sniffing, walking, moving head).

Experiment 1 – GABA_B Ligands Administered Throughout Cued and Contextual Fear

Conditioning

Delay CCF Conditioning Protocol

Fifteen minutes prior to testing, animals received an i.p. injection of either saline, baclofen, or phaclofen. Subjects were taken from the colony room individually to a dedicated testing room containing the fear conditioning chamber (which doubled as the contextual fear testing chamber) or the cued fear testing chamber, a table upon which the fear conditioning chamber was placed, a computer desk, and chairs.

For delay cued and contextual fear conditioning (Day 1), the animals were placed into the fear conditioning chamber and allowed to explore the chamber for two minutes. After the two-minute acclimatization period, the conditioned stimulus (CS), a 2.9kHz 88dB tone, was presented for 30 seconds. One second before the CS terminated, a one second unconditioned stimulus (US), a 0.5mA foot shock, was delivered and co-terminated with the conditioned stimulus. For a visual representation of this procedure, please see Figure 1. Once both the CS and US terminated, the animals were given another two minutes to explore the chamber. The CS-US pairing was presented once more, for a total of two pairings. The animals were given a final two minutes in the chamber. Freezing behavior was recorded during the first and last two minutes of the training session by visual inspection by researchers every 10 seconds. At the end of the training session, the animals were taken back to their home cage.

On Day 2, 24 hours post-training, the animals underwent a cued fear test session. They again received an i.p. injection 15 minutes before testing. Animals were taken from

the colony room to the dedicated testing room and placed into the altered context chamber. Animals were given two minutes to explore the altered context chamber; after the two-minute exploration, the CS was presented for one minute. The animal had another two minutes to explore before the CS was presented again. The CS was presented in this manner for a total of four presentations during the cued fear test session and freezing behavior was recorded during the entire session by researchers every 10 seconds. Once the cued fear test session was over, the animal was returned to its home cage.

On Day 3, 48 hours post-training, the animals underwent a contextual fear test session. They received an i.p. injection fifteen minutes prior to behavioral testing. Animals were taken to the dedicated testing room and placed into the original conditioning chamber for the contextual fear test session. The animals remained in the chamber for 10 minutes; neither the CS nor the US were presented during this time. Data was collected in an identical fashion as previous days and at the end of the contextual fear test session, the animals were returned to their home cage.

Cued fear test and contextual fear test sessions were repeated once more over successive days; test sessions were performed as described above, including i.p. injections. A reminder trial occurred immediately after the 10-minute contextual fear test session on Day 5 and consisted of a single presentation of the 30-second CS co-terminating with the one-second US. Behavior was monitored for freezing during the 10-minute contextual fear test session, during the presentation of the CS, as well as two minutes after the end of the reminder trial; freezing was recorded by researchers every 10 seconds.

Cued and contextual fear were tested 24 and 48 hours (respectively) post-reminder in the same manner as described above, including i.p. injections.

Trace CCF Conditioning Protocol

All conditions and procedures were identical to those in Experiment 1 except for the following changes. For initial training on Day 1, instead of co-terminating with the 30 second tone, the one second 0.5mA shock was presented 2.5 seconds after the conditioned stimulus terminated. For a visual representation of this procedure, please see Figure 1. Additionally, instead of two tone-shock presentations, the animals received a total of four tone-shock pairings because previous research has established that this CCF variation is more difficult to learn. The remainder of the protocol followed exactly the same as described for the Delay CCF conditioning protocol, except the reminder trial consisted of the 30-second conditioned stimulus, followed 2.5 seconds later by the one-second unconditional stimulus.
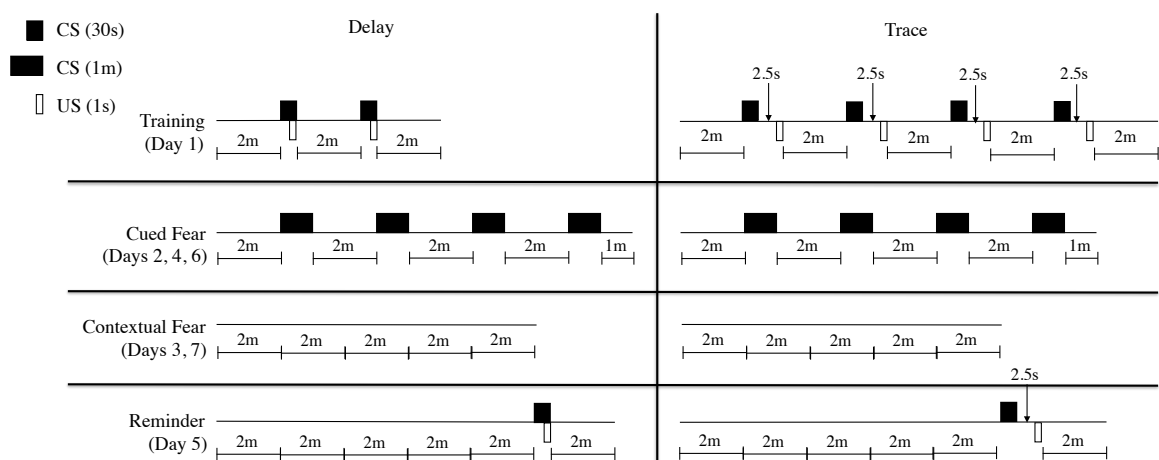

Figure 1. Visual Representation of Delay and Trace CCF Conditioning Protocols

Experiment 2 - GABA$_B$ Ligands Administered After Cued and Contextual Fear Conditioning Training

Delay CCF Conditioning Protocol

This experiment proceeded exactly like Experiment 1 Delay CCF Conditioning Protocol, with the exception that no drugs were administered on Day 1. Animals received i.p. injections 15 minutes before testing on all subsequent days.

Trace CCF Conditioning Protocol

This experiment proceeded exactly like Experiment 1 Trace CCF Conditioning Protocol, with the exception that no drugs were administered on Day 1. Animals received i.p. injections 15 minutes before testing on all subsequent days. For this portion of the experiment, the data of one baclofen animal was removed due to complications during the experiment, so saline n=10, baclofen n=9, and phaclofen n=10

Sensory and Analgesia Testing

In order to determine that the ligands administered to the animals did not alter sensorimotor functioning, nor produced an analgesic effect that could bias the measures of learning and memory, we conducted basic sensory and nociceptive tests on all groups. All control tests were completed 15 minutes after i.p. injections of the ligands administered during the cued and contextual fear procedures.

First, the animals were tested for their ability to respond to auditory stimuli. We tested startle amplitude using a chamber and the Startle software from San Diego Instruments (San Diego, CA); each group's startle response to 10ms white noise bursts

presented at 90, 100, 110, and 120dB was evaluated. This test was done 24 hours after the completion of the fear conditioning procedure.

After testing auditory functioning, we evaluated nociceptive sensitivity to ensure that the administered ligands did not produce an analgesic effect that may have altered learning and memory performance via a tail flick test. This procedure consisted of using water heated to 55°C and then placing the tip of the animal's tail into the water. This test has been used extensively to determine alterations in pain threshold without inducing any damage since the animals rapidly "flick" their tail from the water. Two researchers timed and recorded the latency for the animal to remove or "flick" its tail from the heat.

Tissue Collection

Fifteen minutes after the tail flick test, the animals were euthanized by CO_2 asphyxiation. Tissue was collected immediately afterwards. The animals had their frontal cortices, amygdalae, hippocampi, and cerebella dissected out. The tissue later underwent SDS-PAGE western blotting.

SDS-PAGE Western Blotting

Cortices, amygdalae, hippocampi, and cerebella were collected from rats by dissection and then flash-frozen in dry ice and stored at -80°C. Tissue was homogenized using RIPA buffer (20mM Tris-HCL (pH 7.5), 150mM NaCL, 1mM Na_2EDTA, 1mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5mM sodium pyrophosphate, 1mM β-glycerophosphate, 1mM Na_3VO_4, 1μg/ml leupeptin) from Cell Signaling (Danvers, MA) with 1mM DTT, 1mM PMSF, 20μg/ml aprotinin, and 0.1% SDS added.

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Homogenization was performed using a handheld Polytron (Kinematica Inc., Lucerne, Switzerland) tissue homogenizer. Tissue was then vortexed and centrifuged for 15 minutes at 15,000 x g at 4°C. The supernatant was collected and used to determine protein concentration via a bicinchoninic acid assay (BCA, Pierce, Rockford, IL).

Once sample concentration was known, all tissue was run via SDS-PAGE (Laemmli, 1970). Samples were loaded at a concentration of 20μg; samples contained protein from the sample of interest, a Laemmli buffer containing 1% SDS (BioRad, Hercules, CA), and distilled water for a total volume of 10μL. Samples were heat denatured at 100°C for five minutes, then buried on ice for five minutes before being loaded on an acryl gel. The gels were run on ice for 60 minutes at a constant 0.04A. Once the run was complete, the protein was transferred to a nitrocellulose membrane for one hour on ice at a constant current of 0.25A. Once the transfer was complete, the membranes blocked for two hours in blocking buffer (1x Tris-buffered saline with 0.05% Tween-20 (TBST), 5% BSA, and 0.01% NaN$_3$). The membranes were then incubated in 5% BSA TBST with a primary antibody (e.g. GABA$_{B1a/b}$, GABA$_{B2}$) dilution overnight at 4°C. The next morning, after a 30-minute warming-up period, the membranes were washed with TBST for 10 minutes, three times, for a total of 30 minutes. The membranes then were incubated in 5% milk TBST with the appropriate peroxidase-labeled secondary antibody for about 1.5 hours. After another 30-minute wash in TBST, the membranes were exposed to Amersham ECL Plus (GE Healthcare Life Sciences, Piscataway, NJ) and imaged using a Typhoon 9410 Variable Mode Imager (GE Healthcare Life Sciences). Protein quantities were analyzed using ImageQuant 5.2 software (GE Healthcare Life Sciences).

Statistical Analyses

Analyses were performed using SPSS. Data from Day 1 (training) were analyzed using paired t-tests and data from the reminder trial on Day 5 were analyzed using analysis of variance (ANOVA). Repeated measures ANOVA were run on data from all cued and contextual extinction sessions after both training and reminder. Data from startle amplitudes, tail flick latencies, and western blot analyses were also analyzed via ANOVA. Tukey post-hoc comparisons were performed following a significant result where applicable.

CHAPTER 4

RESULTS

Experiment 1 – GABA$_B$ Ligands Administered Throughout Cued and Contextual Fear

Conditioning

<u>Cued and Contextual Fear Conditioning</u>

In this experiment we tested whether the administration of the GABA$_B$ agonist baclofen or the GABA$_B$ antagonist phaclofen throughout the entirety of the Pavlovian delay or trace CCF protocols would alter performance in the tasks. For the delay CCF conditioning protocol, one-way ANOVA of each group's average proportion time freezing during the first two minutes of training revealed a significant difference between the groups ($F_{2,27}$=12.31, $p<0.01$; Tukey post-hoc revealed a significant difference between saline and baclofen only, $p<0.01$; see Figure 2a). There were no significant differences between groups during the last two minutes of training ($F_{2,27}$=0.263, $p>0.05$), demonstrating that each group responded equivalently to the training. Paired t-test analyses of PreCSUS freezing compared to PostCSUS freezing revealed that each group significantly increased freezing during the last two minutes of training (saline: t_9=-30.594, $p<0.01$; baclofen: t_9=-4.979, $p<0.01$; phaclofen: t_9=-17.321, $p<0.01$).

For the trace CCF conditioning protocol, one-way ANOVA of each group's average proportion time freezing during the first two minutes of training revealed no significant differences between groups ($F_{2,27}$=3.254, $p>0.05$; see Figure 2b). There were no significant differences during the last two minutes of training ($F_{2,27}$=1.259, $p>0.05$), demonstrating that each group responded equivalently to the training. Paired t-test analyses of PreCSUS freezing compared to PostCSUS freezing revealed that each group

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significantly increased freezing during the last two minutes training (saline: t_9=-20.846, p<0.01; baclofen: t_9=-5.596, p<0.01; phaclofen: t_9=-11.063, p<0.01).
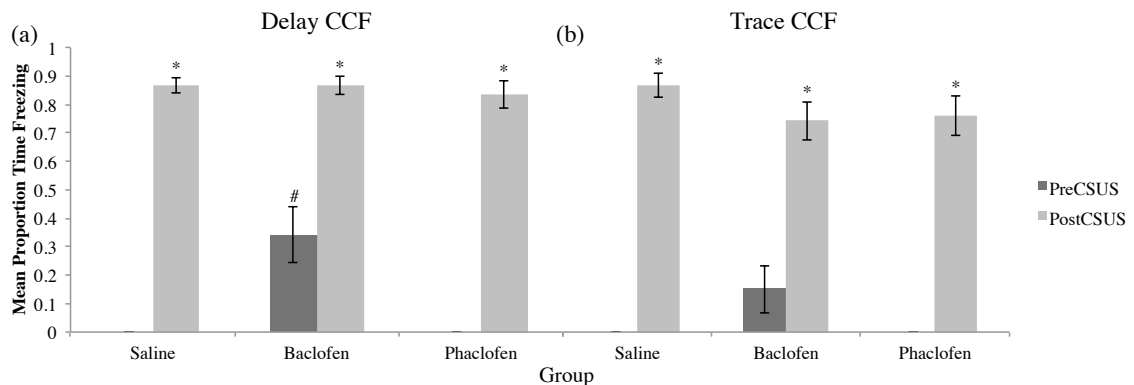

Figure 2. Experiment 1 Day 1 Training – Proportion of time freezing (±SEM) was determined for the first two minutes of training (PreCSUS) and the last two minutes of training (PostCSUS) for delay (a) and trace (b) CCF conditioning. # = Significantly different from saline, p<0.05; * = Significant difference between PreCSUS freezing and PostCSUS freezing, p<0.05

Cued fear was tested on Days 2, 4, and 6 in the altered context chamber to determine the strength of the association between the tone (the CS) and the mild foot-shock (the US). For the delay CCF conditioning protocol, repeated measures ANOVA revealed significant within-group effects for Days 2 ($F_{4,108}$=11.49, p<0.01), 4 ($F_{4,108}$=6.862, p<0.01), and 6 ($F_{4,108}$=10.251, p<0.01); only on Day 4 a significant difference between groups appeared ($F_{2,27}$=3.851, p<0.05). Although Tukey post-hocs revealed no significant differences compared to saline, the baclofen group did show a trend of increased freezing. A one-way ANOVA revealed no significant differences between groups during the reminder CS that was administered in the original training context ($F_{2,27}$=2.637, p>0.05; see Figure 3a).

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For the trace CCF conditioning protocol, repeated measures ANOVA revealed significant within-group effects for Days 2 ($F_{4,108}$=23.22, p<0.01), 4 ($F_{4,108}$=3.585, p<0.01), and 6 ($F_{24,108}$=4.699, p<0.01; see Figure 3b). Only on Day 4 a significant difference between groups appeared ($F_{2,27}$=5.622, p<0.01), and Tukey post-hocs revealed baclofen froze significantly more than saline (p<0.01). A one-way ANOVA revealed no significant differences between groups during the reminder CS that was administered in the original training context ($F_{2,27}$=0.883, p>0.05).

Contextual Fear was tested on Days 3, 5, and 7; the animals were placed back in the original training context and observed for 10 minutes, with no CS or US presentations. For the delay CCF conditioning protocol, repeated measures ANOVA revealed significant within-group effects only on Days 3 ($F_{4,108}$=2.992, p<0.05) and 7 ($F_{4,108}$=5.815, p<0.01), and significant differences between groups only on Day 5 ($F_{2,27}$=7.053, p<0.01; Tukey post-hocs revealed a significant difference between saline and baclofen, p<0.05). A one-way ANOVA revealed no significant differences between groups in the two-minute interval after the reminder CS-US pairing (PostReminder; $F_{2,27}$=0.551, p>0.05; see Figure 4a).

For the trace CCF conditioning protocol, repeated measures ANOVA revealed significant within-group effects only on Days 3 ($F_{4,108}$=5.186, p<0.01) and 7 ($F_{4,108}$=2.547, p<0.05), and significant differences between groups only on Day 5 ($F_{2,27}$=4.045, p<0.05; Tukey post-hocs revealed a significant difference between saline and baclofen, p<0.05). A one-way ANOVA revealed no significant differences between groups in the two-minute interval after the reminder CS-US pairing (PostReminder; $F_{2,27}$=1.069, p>0.05; see Figure 4b).

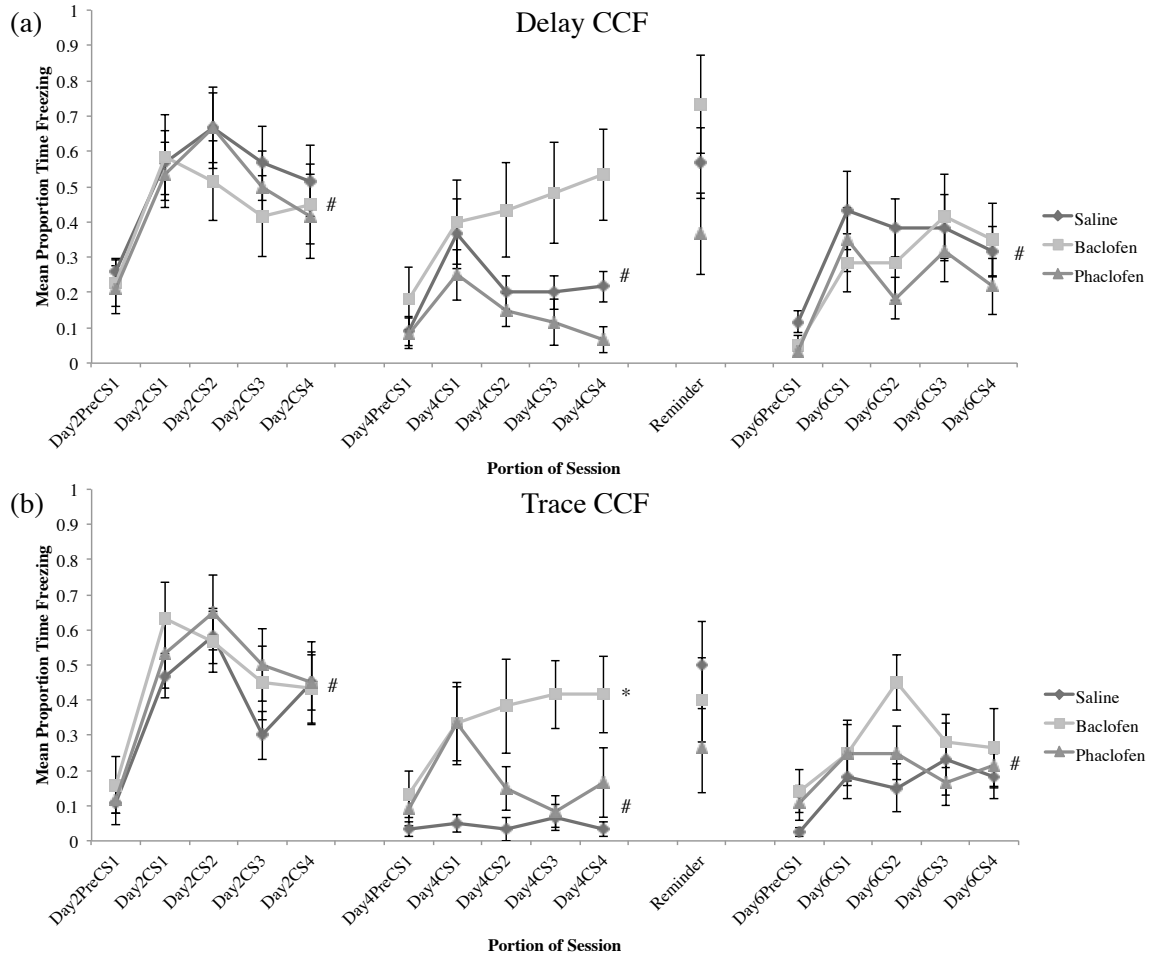**Figure 3.** Experiment 1 Cued Fear Extinction – Proportion time freezing (±SEM) was determined for the first two minutes the animal was placed into the altered context, as well as during each one minute presentation of the CS for delay (a) and trace (b) CCF conditioning. * = Significantly different from saline, $p<0.05$; # = Significant within-group effects, $p<0.05$
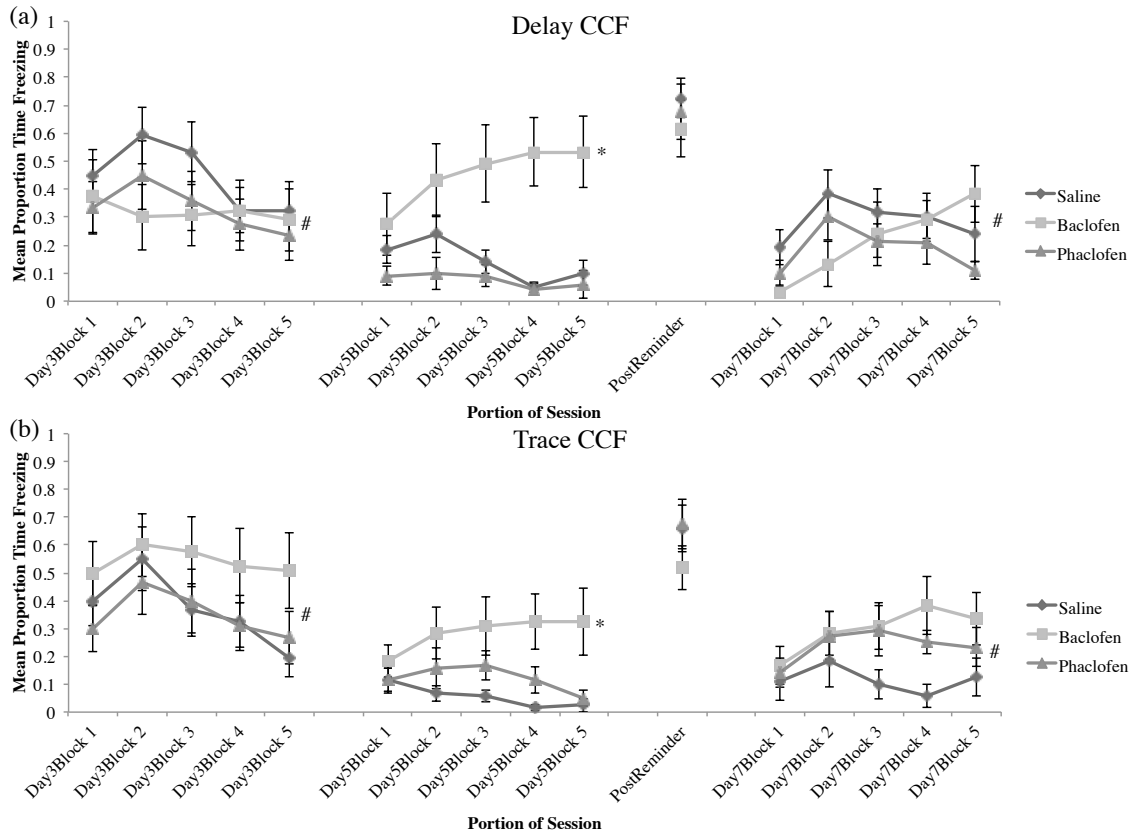
Figure 4. Experiment 1 Contextual Fear Extinction – Proportion time freezing (±SEM) was determined for each block of two minutes in the original training chamber for delay (a) and trace (b) CCF conditioning. * = Significantly different from saline, $p<0.05$; # = Significant within-group effects, $p<0.05$

Sensory Testing

To determine whether the $GABA_B$ agonist baclofen or the $GABA_B$ antagonist phaclofen altered the way the animals detected the stimuli, we tested their startle amplitudes to white noise bursts at several decibel levels. For the delay CCF conditioned animals, one-way ANOVA revealed no significant differences between groups at any decibel level ($p>0.05$; see Figure 5a). For the trace CCF conditioned animals, one-way ANOVA revealed significant differences between groups at 90 dB ($F_{2,117}=5.466$, $p<0.01$; Tukey post-hoc revealed that baclofen was significantly decreased compared to saline),

100 dB ($F_{2,117}$=12.726, p<0.01; Tukey post-hoc revealed that both baclofen and phaclofen were significantly decreased compared to saline), 110 dB ($F_{2,117}$=4.828, p<0.05; Tukey post-hoc revealed that baclofen was significantly decreased compared to saline), and 120 dB ($F_{2,117}$=5.428, p<0.01; Tukey post-hoc revealed that baclofen was significantly decreased compared to saline; see Figure 5b).
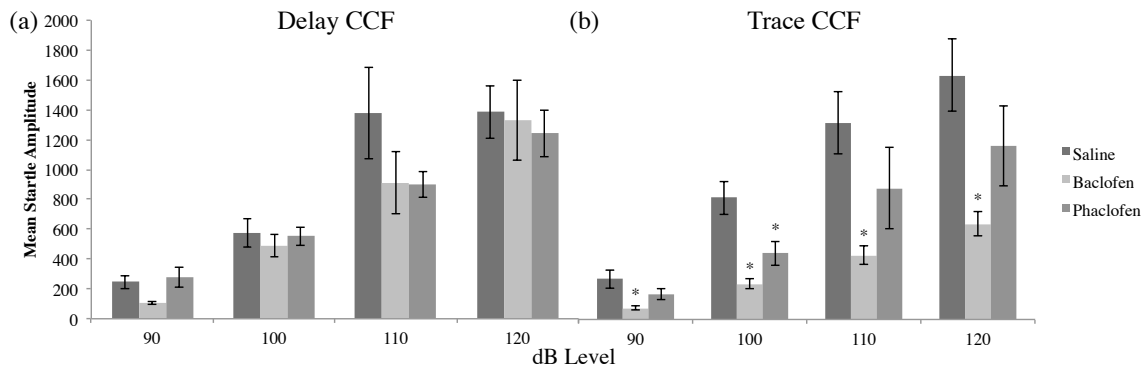

Figure 5. Experiment 1 Startle Amplitude – Average startle amplitude (±SEM) to several decibel levels for delay (a) and trace (b) CCF conditioned animals. * = Significantly different from saline

<u>Analgesia Testing</u>

In order to ensure that the administration of the GABA_B agonist baclofen or the GABA_B antagonist phaclofen did not alter the way the animals perceived pain, the tail flick test was used to measure analgesia. For the delay CCF conditioned animals, one-way ANOVA did not reveal any significant differences between the groups ($F_{2,27}$=2.653, p>0.05; see Figure 6a). For the trace CCF conditioned animals, one-way ANOVA did not reveal any significant differences between the groups ($F_{2,27}$=3.126, p>0.05; see Figure 6b).
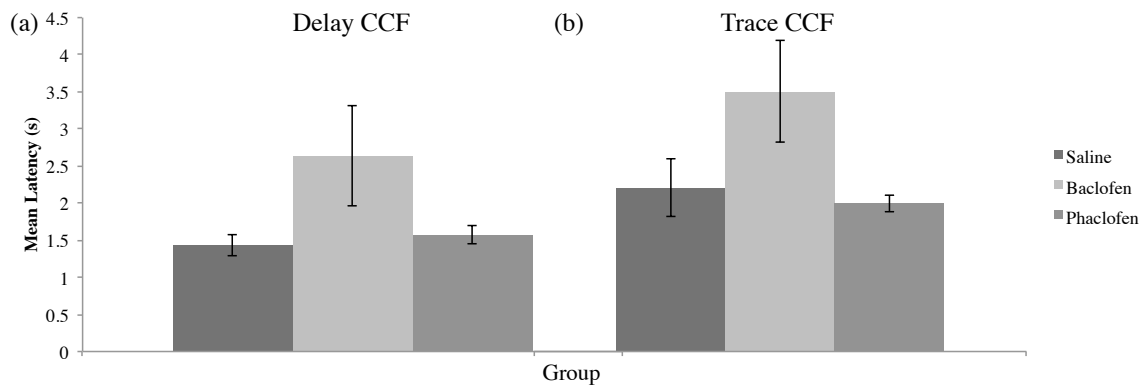
Figure 6. Experiment 1 Tail Flick Latency – Average tail flick latency (±SEM) for delay (a) and trace (b) CCF conditioned animals.

<u>SDS-Page Western Blotting</u>

For the delay CCF conditioned animals, one-way ANOVA of the normalized band densities compared to an average of the saline group revealed a significant decrease in the $GABA_{B1b}$ receptor subunit in the hippocampus in the phaclofen group compared to the saline group ($F_{2,27}=4.576$, $p<0.05$; Tukey post-hoc revealed a significant difference between saline and phaclofen, $p<0.05$; see Figure 7a), but no significant effects compared to the saline group for the $GABA_{B1a}$ receptor subunit ($F_{2,27}=4.005$, $p<0.05$; Tukey post-hoc did not reveal any significant comparisons with saline). While non-significant, there was a trend of increased protein levels for both $GABA_{B1}$ subunits in the baclofen group. While no significant differences were found between the groups for $GABA_{B1}$ receptor subunits in the cortex (see Figure 7e), there was a trend of decreased protein levels in the phaclofen group. Again, while non-significant, the baclofen group showed a slight trend of an increase in both receptor subunit protein levels.
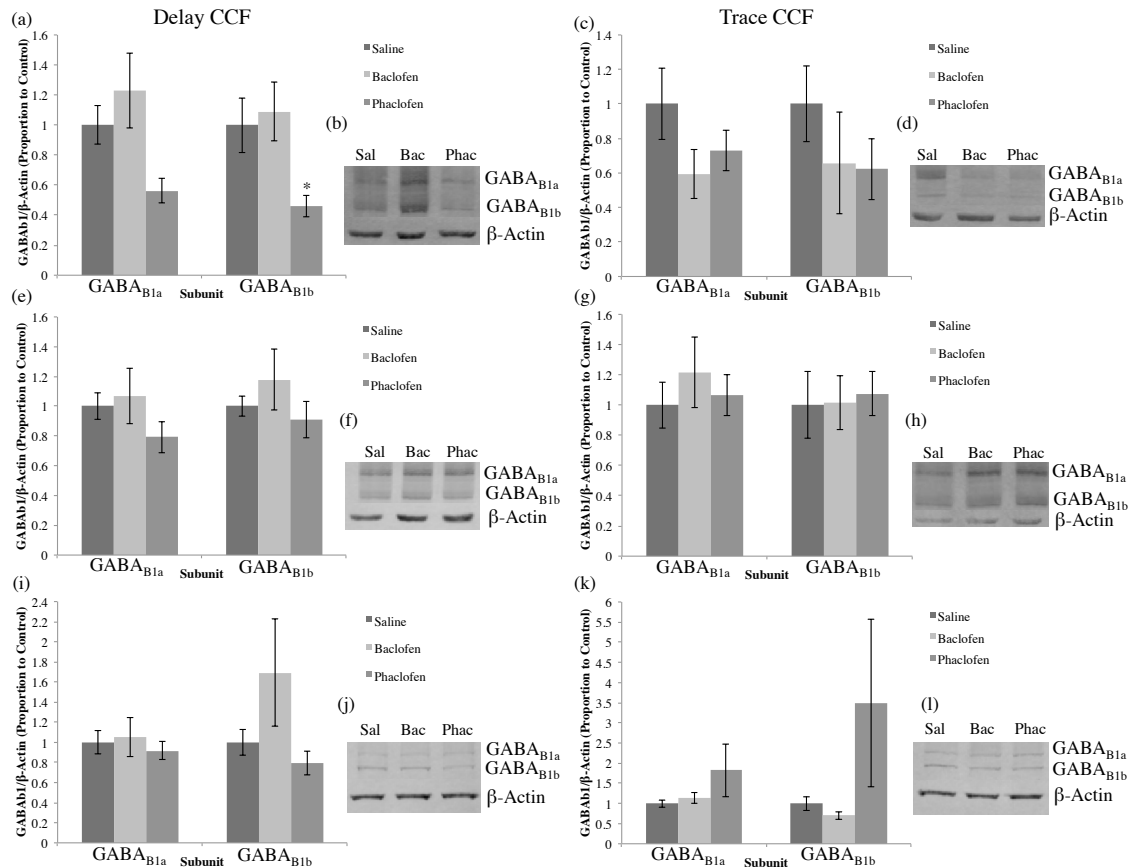
Figure 7. Experiment 1 $GABA_{B1}$ Western Blot Results – Average protein level (±SEM) in proportion to the saline control group for delay (a, b, e, f, i, j) and trace (c, d, g, h, k, l) CCF conditioned animals in hippocampal (a, c), cortical (e, g), and amygdalar (i, k) tissue, and representative western blot images (b, d, f, h, j, l). * = Significantly different from saline, p<0.05

While no significant differences were found between the groups for $GABA_{B1}$ receptor subunits in the cortex (see Figure 7e), there was a trend of decreased protein levels in the phaclofen group. Again, while non-significant, the baclofen group showed a slight trend of an increase in both receptor subunit protein levels. Further, no significant differences were found in the amygdala for the $GABA_{B1}$ receptor subunits (see Figure 7i); though, again there was an increase in the $GABA_{B1b}$ subunit in the baclofen-treated group, as well as a decrease in the $GABA_{B1b}$ subunit in the phaclofen-treated group.

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For the trace CCF conditioned animals, there was a non-significant decrease in hippocampal $GABA_{B1}$ protein in the baclofen and phaclofen groups compared to the saline group (see Figure 7c), though this difference was not evident in the cortex (see Figure 7g). Indeed, there is a slight increase in the $GABA_{B1a}$ protein in the baclofen-treated group. While not significant, there is an increase in both $GABA_{B1}$ subunit proteins for the phaclofen-treated group in the amygdala (see Figure 7k), and a decrease in the $GABA_{B1b}$ subunit for the baclofen-treated group.

Further, in the delay CCF conditioned animals, there was a significant decrease of the $GABA_{B2}$ receptor subunit in the phaclofen group compared to the saline group in the hippocampus ($F_{2,27}=9.374$, $p<0.01$; Tukey post-hoc comparison between phaclofen and saline $p<0.01$; see Figure 8a) and a significant reduction of the $GABA_{B2}$ receptor subunit in both the baclofen and phaclofen groups in the cortex ($F_{2,27}=15.187$, $p<0.01$; Tukey post-hoc comparisons for saline versus both baclofen and phaclofen $p<0.01$; see Figure 8e). No differences were found in the amygdala for the $GABA_{B2}$ receptor subunit (see Figure 8i).

For the trace CCF conditioned animals, there were no changes in the $GABA_{B2}$ receptor subunit in hippocampal tissue, but there was a trend of decreased protein levels in the baclofen group (see Figure 8c); no differences between groups in $GABA_{B2}$ protein levels were seen in the cortex either (see Figure 8g), although there was a slight increase in the phaclofen-treated group. In the amygdala, there was a significant increase in the $GABA_{B2}$ receptor protein in the baclofen-treated group ($F_{2,19}=5.58$, $p<0.05$; Tukey post-hoc comparison for saline versus baclofen, $p<0.01$) , and a non-significant increase in the phaclofen group (see Figure 8k).

Figure 8. Experiment 1 GABA$_{B2}$ Western Blot Results – Average protein level (±SEM) in proportion to the saline control group in delay (a, b, e, f, i, j) and trace (c, d, g, h, k, l) CCF conditioned animals in hippocampal (a, c), cortical (e, g), and amygdalar (i, k) tissue, and representative western blot images (b, d, f, h, j, l). * = Significantly different from saline, $p < 0.05$

Interestingly, a commonly implicated GABA$_A$ receptor subunit (GABA$_{A\alpha5}$) in learning and memory was not significantly changed between the delay CCF conditioned groups in the hippocampus (see Figure 9a) or in the cortex (see Figure 9e). Though non-significant, there was a slight increase in the protein levels in the baclofen group for the hippocampal tissue, and a slight decrease in the protein levels for both the baclofen and

phaclofen groups in the cortical tissue. A similar result was found for the trace CCF conditioned groups; the $GABA_{A\alpha5}$ receptor subunit was not significantly changed between the groups in the hippocampus (see Figure 9c) or in the cortex (see Figure 9g). There was a slight increase in the protein in the cortex for both the baclofen and phaclofen treated groups, but only a slight increase in the protein in the baclofen treated group in the hippocampus.
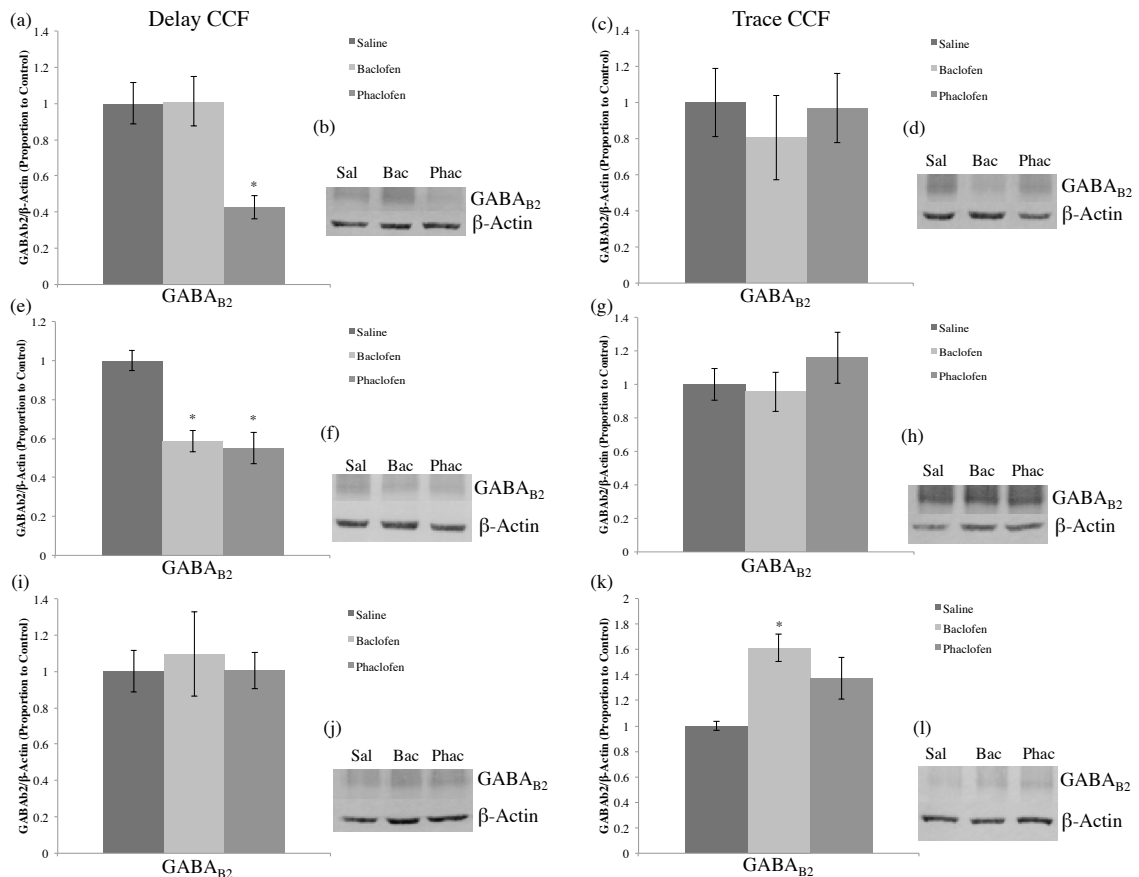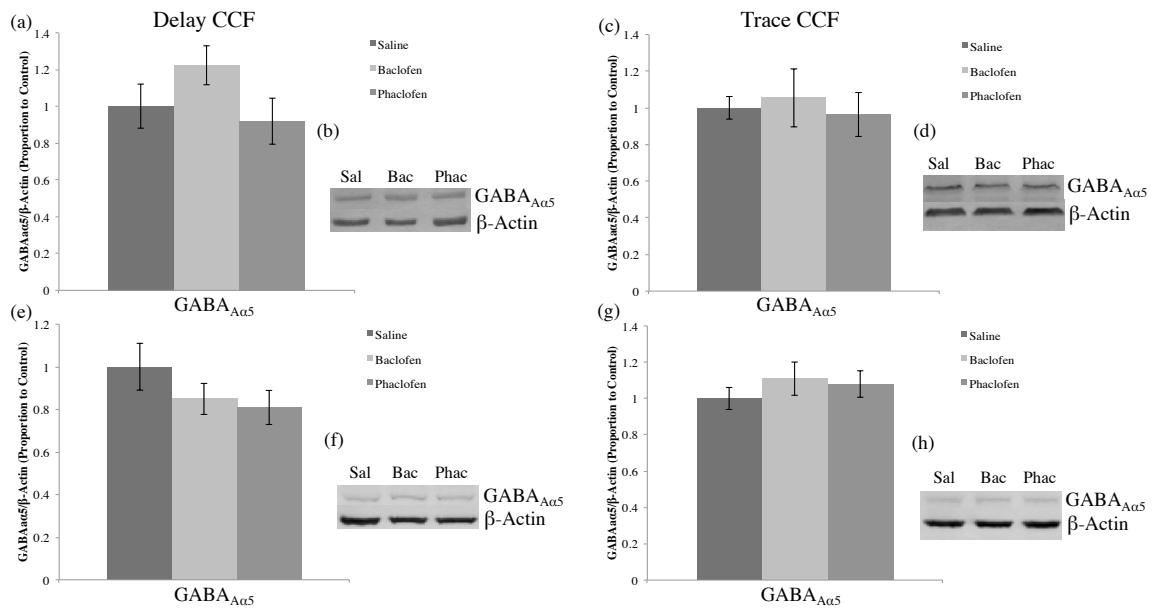

Figure 9. Experiment 1 $GABA_{A\alpha5}$ Western Blot Results – Average protein level (±SEM) in proportion to the saline control group in delay (a, b, e, f) and trace (c, d, g, h) CCF conditioned animals in hippocampal (a, c) and cortical (e, g) tissue, and representative western blot images (b, d, f, h).

Experiment 2 – GABA$_B$ Ligands Administered After Cued and Contextual Fear

Conditioning Training

Cued and Contextual Fear Conditioning

In order to verify that the results achieved in Experiment 1 were not a result of the GABA$_B$ ligands altering the way the animals acquired the task, we tested in Experiment 2 whether administering baclofen (GABA$_B$ agonist) or phaclofen (GABA$_B$ antagonist) 24 hours after all groups had been trained in and acquired either the delay or trace CCF conditioning task would alter performance during extinction. For the delay CCF conditioned animals, one-way ANOVA of each group's average proportion time freezing during the first two minutes of training could not be performed because no freezing was exhibited by any group; thus, there is no significant difference between the groups before training. Additionally, there were no significant differences during the last two minutes of training ($F_{2,27}$=1.588, p>0.05), demonstrating that each group responded equivalently to the training. Paired t-test analyses of freezing during the first two minutes of training (PreCSUS) compared to the last two minutes of training (PostCSUS) revealed that each group significantly increased freezing after training (saline: t_9=-15.523, p<0.01; baclofen: t_9=-37.315, p<0.01; phaclofen: t_9=-26.022, p<0.01; see Figure 10a).

For the trace CCF conditioned animals, the data of one baclofen animal was removed due to complications during the experiment, so saline n=10, baclofen n=9, and phaclofen n=10. One-way ANOVA of each group's average proportion time freezing during the first two minutes of training revealed no significant differences between groups ($F_{2,26}$=1.121, p>0.05). Additionally, there were no significant differences during the last two minutes of training ($F_{2,26}$=0.783, p>0.05), demonstrating that each group

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responded equivalently to the training. Paired t-test analyses of freezing during the first two minutes of training compared to the last two minutes of training revealed that each group significantly increased freezing after training (saline: t_9=-40.088, p<0.01; baclofen: t_8=-12.529, p<0.01; phaclofen: t_9=-15.377, p<0.01; see Figure 10b).
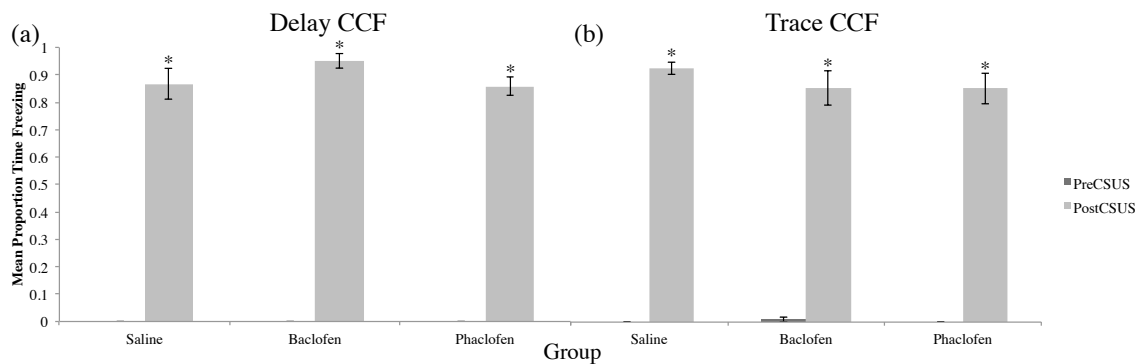
Figure 10. Experiment 2 Day 1 Training – Proportion of time freezing (±SEM) was determined for the first two minutes of training (PreCSUS) and the last two minutes of training (PostCSUS) for delay (a) and trace (b) CCF conditioning. * = Significant difference between PreCSUS freezing and PostCSUS freezing, p<0.05

Cued fear was tested on Days 2, 4, and 6 in the altered context chamber to determine the strength of the association between the CS and US. For the delay CCF conditioning protocol, repeated measures ANOVA revealed significant within-group effects for Days 2 ($F_{4,108}$=12.261, p<0.01), 4 ($F_{4,108}$=7.586, p<0.01), and 6 ($F_{4,108}$=6.603, p<0.01; see Figure 11a), as well as significant between group effects for Days 2 ($F_{2,27}$=9.351, p<0.01), 4 ($F_{2,27}$=14.726, p<0.01), and 6 ($F_{2,27}$=4.851, p<0.05). Tukey post-hocs revealed baclofen froze significantly more than saline (p<0.05 for all days). A one-

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way ANOVA revealed no significant differences between groups during the reminder CS that was administered in the original training context ($F_{2,27}=.159$, $p>0.05$).

For the trace CCF conditioning protocol, repeated measures ANOVA revealed significant within-group effects for Days 2 ($F_{4,104}=15.707$, $p<0.01$), 4 ($F_{4,104}=4.808$, $p<0.01$), and 6 ($F_{4,104}=7.978$, $p<0.01$; see Figure 11b), as well as significant between group effects for Days 2 ($F_{2,26}=5.338$, $p<0.05$) and 4 ($F_{2,26}=7.579$, $p<0.01$). Tukey post-hocs revealed baclofen froze significantly more than saline ($p<0.05$ for both days). A one-way ANOVA revealed no significant differences between groups during the reminder CS that was administered in the original training context ($F_{2,26}=0.511$, $p>0.05$).

Contextual Fear was tested on Days 3, 5, and 7; the animals were placed back in the original training context and observed for 10 minutes, with no CS or US presentations. Repeated measures ANOVA for the delay CCF conditioned animals revealed significant within-group effects only on Days 3 ($F_{4,108}=14.235$, $p<0.01$), 5 ($F_{4,108}=3.288$, $p<0.05$), and 7 ($F_{4,108}=5.606$, $p<0.01$), and significant differences between groups only on Day 5 ($F_{2,27}=18.898$, $p<0.01$; Tukey post-hocs revealed a significant difference between saline and baclofen, $p<0.01$). A one-way ANOVA revealed no significant differences between groups in the two-minute interval after the reminder CS-US pairing ($F_{2,27}=0.099$, $p>0.05$; see Figure 12a). For the trace CCF conditioning protocol, repeated measures ANOVA revealed significant within-group effects only on Days 3 ($F_{4,104}=7.034$, $p<0.01$), 5 ($F_{4,104}=3.233$, $p<0.05$), and 7 ($F_{4,104}=6.356$, $p<0.01$), and significant differences between groups only on Day 5 ($F_{2,26}=16.531$, $p<0.01$; Tukey post-hocs revealed a significant difference between saline and baclofen, $p<0.01$). A one-way

ANOVA revealed no significant differences between groups in the two-minute interval after the reminder CS-US pairing ($F_{2,26}=0.46$, $p>0.05$; see Figure 12b).

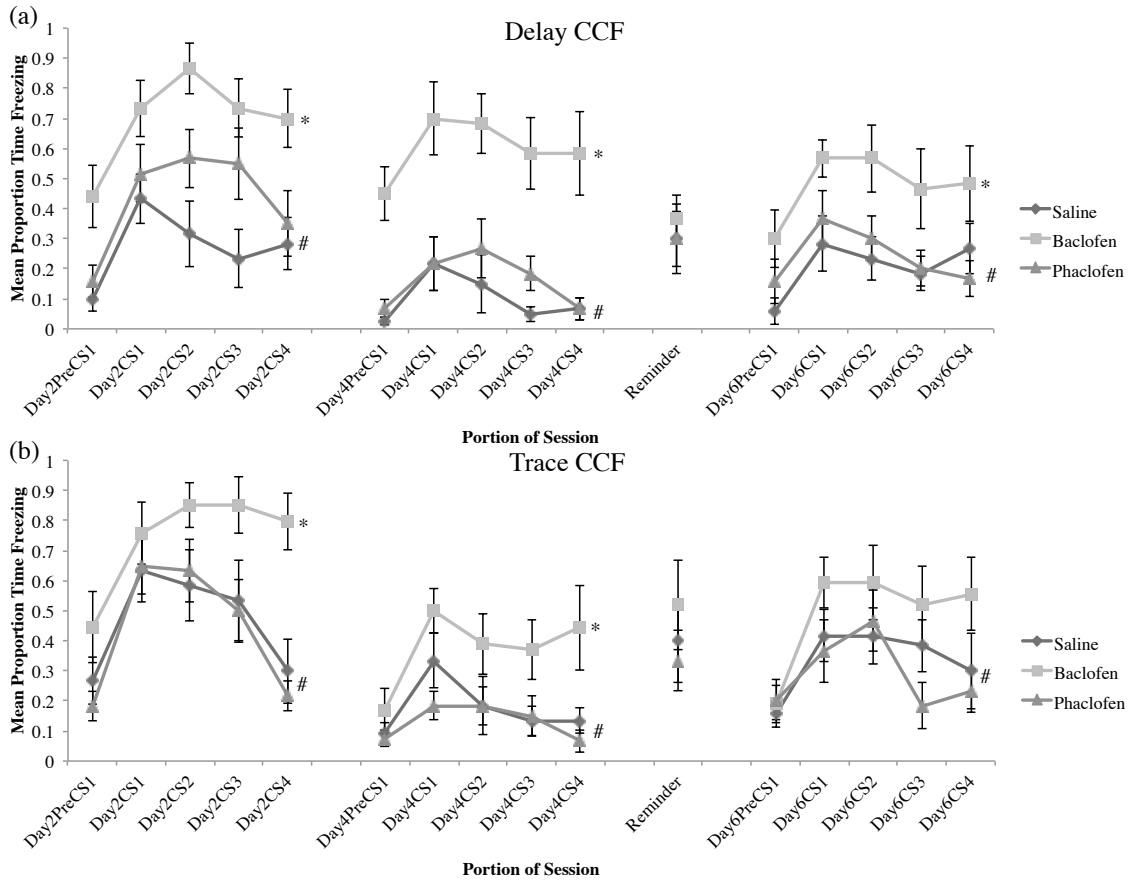**Figure 11.** Experiment 2 Cued Fear Extinction – Proportion time freezing ($\pm$SEM) was determined for the first two minutes the animal was placed into the altered context, as well as during each one minute presentation of the CS for delay (a) and trace (b) CCF conditioning. * = Significantly different from saline, $p<0.05$; # = Significant within-group effects, $p<0.05$
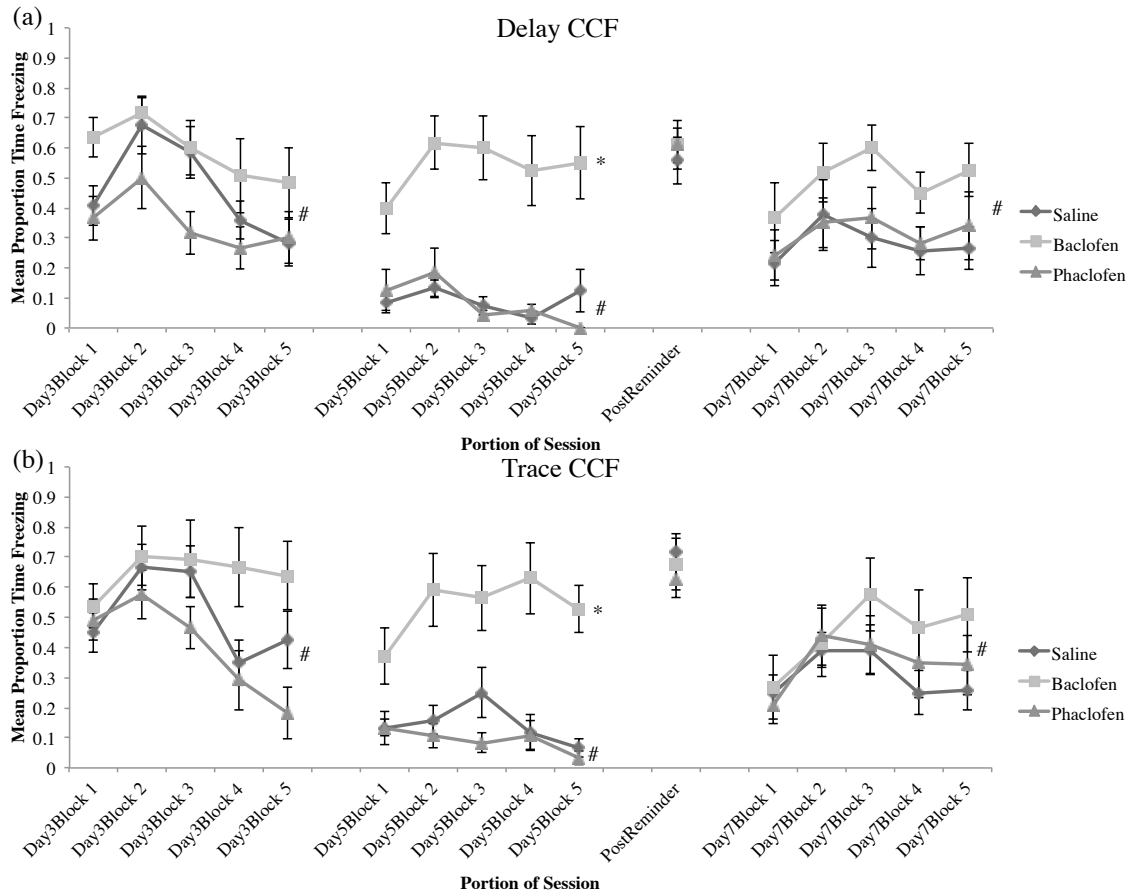
Figure 12. Experiment 2 Contextual Fear Extinction – Proportion time freezing (±SEM) was determined for each block of two minutes while the animal was in the original training chamber for the delay (a) and trace (b) CCF conditioning protocol. * = Significantly different from saline, $p<0.05$; # = Significant within-group effects, $p<0.05$

<u>Sensory Testing</u>

To ensure that the $GABA_B$ agonist baclofen or the $GABA_B$ antagonist phaclofen did not alter the way the animals detected the stimuli, we tested their startle amplitudes to white noise bursts at several decibel levels. For delay CCF conditioned animals, one-way ANOVA revealed no significant differences compared to saline ($p>0.05$; see Figure 13a). Likewise for the trace CCF conditioned animals, one-way ANOVA did not reveal a significant difference between groups ($p>0.05$; see Figure 13b).

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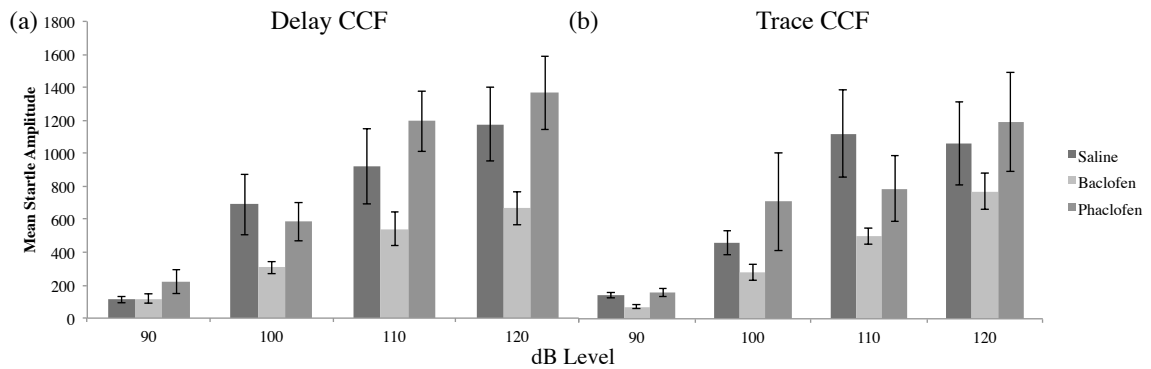
Figure 13. Experiment 2 Startle Amplitude – Average startle amplitude (±SEM) to several decibel levels for delay (a) and trace (b) CCF conditioned animals.

Analgesia Testing

To determine that neither the $GABA_B$ agonist baclofen nor the $GABA_B$ antagonist phaclofen altered the way the animals perceived pain, the tail flick test was used to measure nociception. For the delay CCF conditioned animals, one-way ANOVA did not reveal any significant differences between the groups ($F_{2,27}$=0.769, p>0.05; see Figure 14a). For the trace CCF conditioned groups, one-way ANOVA did reveal a significant difference between the groups ($F_{2,26}$=9.122, p<0.01); Tukey post-hocs revealed that baclofen had a significantly higher tail flick latency than saline (p<0.01; see Figure 14b).

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Figure 14. Experiment 2 Tail Flick Latency – Average tail flick latency (±SEM) for delay (a) and trace (b) CCF conditioned animals. * = Significantly different from saline
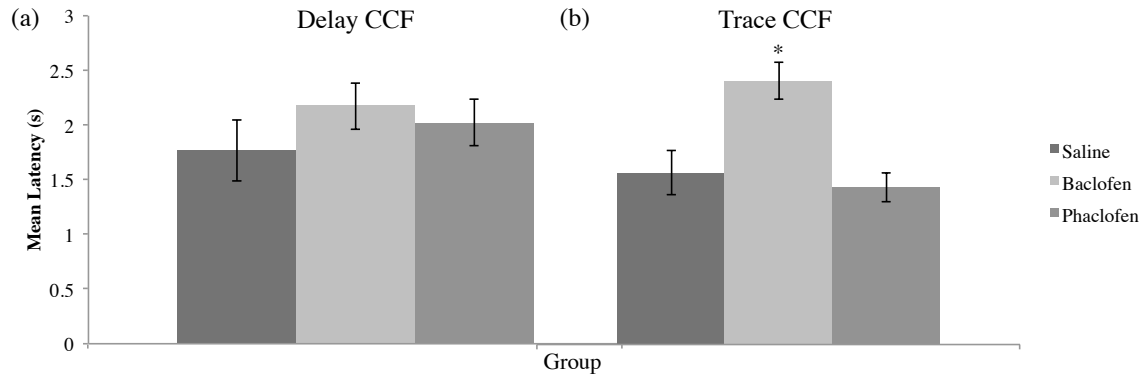
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CHAPTER 5

SUMMARY, CONCLUSIONS, AND FUTURE DIRECTIONS

While the administration of phaclofen did not alter performance in either the delay or trace CCF conditioning protocol compared to saline, baclofen administration did produce a consistent extinction deficit in both experiments. This deficit is evident in the lack of reduced freezing by the second cued (Day 4) and contextual (Day 5) extinction sessions compared to the controls. The saline and phaclofen-treated groups demonstrate typical extinction behavior as demonstrated by the reduction in freezing to both the tone and original training chamber over time as each is presented continually without further US presentations. This finding is surprising considering we had originally hypothesized that the administration of baclofen would enhance extinction (as evidenced by an increase in the rate of reduction in freezing) based on the current literature and theory of extinction that suggests that extinction is largely guided by inhibitory mechanisms (see Makkar, Zhang, & Cranney, 2010). Possibly because the bulk of the data are derived from research that used compounds that target $GABA_A$ receptors, $GABA_B$-driven alterations to extinction may just need to be characterized further.

Additionally, we hypothesized that phaclofen administration would impair extinction, which would be indicated by a lack of reduced freezing. Instead, the phaclofen-treated animals in both experiments were indistinguishable from the saline control groups. We also hypothesized that any behavioral differences would be more evident in the trace CCF conditioning protocol due to its increased difficulty compared to the delay task. However, all of the behavioral differences in the experiments mirror each other regardless of the protocol in which the animals were trained.

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We also originally hypothesized that baclofen administration would impair the acquisition of either delay or trace CCF task, as would be demonstrated by a reduction in proportion time freezing during the first test of cued (Day 2) or contextual (Day 3) fear. However, the administration of baclofen did not reduce freezing in either experiment at any point during Days 2 or 3. We also hypothesized that the administration of phaclofen would enhance acquisition of the tasks, which would be indicated by increased freezing throughout Days 2 or 3. Again, we saw no effect of the ligand during those two days.

While the animals treated with baclofen before training in both the delay and trace CCF conditioning protocols in Experiment 1 did show an increase in freezing behavior before any CS-US presentations, this difference was not present by the beginning of the session on Day 2, and did not reappear throughout the remainder of the experiment. Similarly, while freezing was increased during the first day of administration (Day 2) for the baclofen-treated animals in Experiment 2, these differences disappeared by the beginning of the second day of administration (Day 3). Therefore, any differences seen during Days 4 and 5 cannot be attributed to these initial drug effects.

Further, even though one of the baclofen-treated groups exhibited differences compared to the control group in each of the control tests, these differences cannot account for the extinction deficits we see in the experiments. For instance, a reduced startle response would suggest that administration of baclofen impaired detection of the CS. Additionally, an increase in tail flick latency would suggest that baclofen-treated animals had impaired nociception. Both of these cases should manifest as impaired acquisition (decreased freezing during Days 2 or 3 compared to controls) or enhanced extinction (an increase in the rate of the reduction of freezing during Days 4 or 5

compared to controls). However, all of the baclofen-treated groups showed no acquisition deficits and demonstrated extinction deficits. Therefore, any differences seen during Days 4 and 5 cannot be explained by altered CS detection or deceased nociception.

These experiments, therefore, demonstrate that baclofen impairs the extinction of both cued and contextual fear in both delay and trace CCF protocols. Extinction can be seen in the saline- and phaclofen-treated groups, as their freezing decreases similarly during Days 4 and 5. The baclofen-treated animals, however, lack this same reduction in freezing, demonstrating that they are not learning that the CS and the original context no longer predict the US.

Interestingly, the administration of either $GABA_B$ ligand produced task-dependent changes in the tissue. For instance, the baclofen-treated group that was trained in the delay CCF conditioning protocol showed a non-significant increase in the $GABA_{B1}$ subunits in the hippocampus, but in the trace CCF conditioned group, there was a non-significant reduction in the same tissue. Similarly, for the $GABA_{B1}$ subunits in the cortex, the delay CCF conditioned phaclofen group showed a non-significant reduction, whereas the trace CCF conditioned phaclofen group demonstrated an increase. We even found a drug-by-task difference in the amygdala; in the delay CCF conditioning protocol, the baclofen-treated group had increased levels, but the phaclofen-treated group had decreased levels of the $GABA_{B1b}$ receptor subunit. However this trend was reversed in the trace CCF conditioned groups, the baclofen-treated group had decreased levels, and the phaclofen-treated group had increased levels of $GABA_{B1b}$.

This pattern of task-dependent changes is evident for the $GABA_{B2}$ receptor subunit, as well. In the hippocampus of the delay CCF conditioned phaclofen-treated group, there is a significant reduction, but there is no evidence of a decrease in the protein in the trace CCF conditioned phaclofen group. Further, in the cortex, both the baclofen and phaclofen groups from the delay CCF conditioned animals show a significant reduction of the $GABA_{B2}$ protein, but these differences are non-existent in the trace CCF conditioned groups. Finally, while there were no changes to the protein in the amygdala of the delay CCF conditioned groups, there was a significant increase in the baclofen-treated group and a trend of increased $GABA_{B2}$ in the phaclofen-treated group of the animals trained in the trace CCF conditioning protocol.

These task-dependent protein changes do not, however, extend to other GABAergic proteins. The $GABA_{A\alpha5}$ receptor, a subunit that has the ability to alter learning and memory (Collinson et al., 2002), was not significantly changed between groups in the hippocampus or the cortex in either delay or trace CCF conditioned groups. Yee et al. (2004) demonstrated that a $GABA_{A\alpha5}$ knockdown mutant was resistant to extinction; the extinction deficits seen in these experiments, therefore, are not tied to changes in this particular receptor subunit.

While some of these protein changes do help to explain the baclofen-induced extinction deficits in both the delay and trace CCF conditioning tasks, others do not. Specifically, because the current theory suggests that increased inhibitory action facilitates extinction, the significant increase of $GABA_{B2}$ in the amygdala of the baclofen-treated animals that were trained in the trace CCF conditioning protocol does not support the behavioral extinction deficit. However, the significant reduction of the

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same protein in the cortex of the delay CCF conditioned animals in the baclofen group does support the behavioral data and fits with the current literature. Additionally, even though the administration of phaclofen did not alter behavior in either delay or trace CCF conditioning protocol compared to the saline group, it is very interesting that there are instances of altered protein levels in the these groups in several brain regions. Furthermore, we hypothesized that we would find more differences in the trace CCF conditioned groups because the task is more difficult than the delay protocol, however the most number of significant differences in protein levels were found in the groups that were trained in the delay CCF conditioning protocol.

In the trace CCF conditioned baclofen-treated group, there was a non-significant decrease in the hippocampus in both $GABA_{B1}$ subunits compared to the saline group. The $GABA_{B1a}$ receptor subunits are thought to mediate presynaptic inhibition, and the typical effect of baclofen is to presynaptically decrease neurotransmitter release by decreasing calcium conductance (Misgeld, Bijak, & Jarolimek, 1995). Possibly by increasing the amount of presynaptic inhibition (that leads to decreased neurotransmitter release) via the $GABA_B$ receptors, the receptors were down regulated to decrease the inhibitory effects. The effect of presynaptic inhibition on behavior depends on the neurotransmitter that was prevented from being released. Since the administration baclofen impaired extinction, it is possible that hippocampal glutamate is being affected. Indeed, Vigot et al. (2006) found that the $GABA_{B1a}$ receptor subunit localized at glutamatergic terminals in hippocampal tissue, and $GABA_{B1a}$ knockout mice demonstrated impaired LTP – a glutamatergic-dependent function. $GABA_{B1b}$ subunits are typically found on postsynaptic neurons, and the decrease in these subunits actually

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follows theoretical assumptions. Since baclofen acts to increase inhibition of the neuron, it makes sense that the neuron would down-regulate this protein to try to maintain homeostasis.

Phaclofen acts by antagonizing the $GABA_B$ receptor complex, so these decreased protein levels are paradoxical. A blocked and nonfunctional receptor should, theoretically, lead to an up-regulation of the receptor to help bring cellular functioning back to baseline. It is possible that up-regulating GABA receptors in certain brain regions poses risks to the cell, so the neuron may need to make the necessary changes by down-regulating the $GABA_B$ receptor complex and then use an alternative method to increase cellular inhibition. An alternative to increasing $GABA_B$ receptors could be increasing the inward-rectifying potassium channels (GIRK) associated with the $GABA_B$ receptors.

What is interesting is that phaclofen administration did not alter $GABA_{B1}$ receptors in the cortex, but there was a significant reduction in the $GABA_{B2}$ subunits in the cortex. The $GABA_{B2}$ subunit is the G-protein coupled receptor subunit and is the component needed to shuttle the receptor complex from the endoplasmic reticulum to the cell surface. Considering the SDS-PAGE western blots completed only detect total protein levels and not cell-surface-expressed protein levels, it is possible that these differences may disappear if an assay for surface-expressed proteins were done. Further, it is entirely possible that other differences between the control and baclofen-treated groups are being occluded by this western blotting procedure only being able to detect total protein levels. Future experiments could utilize cell surface biotinylation assays, quantitative polymerase chain reaction assays to examine mRNA differences, or western

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blots for phosphorylation targets like serine 783 that indicate cell-surface expressed GABA$_B$ receptors. Because the tissue analyses strongly indicate a task-dependent change in the proteins, it is also possible that more neurological resources are needed for the trace CCF conditioning task compared to the delay task, thus fewer alterations in the GABAergic proteins are necessary.

It is also possible that the administration of baclofen actually did alter these proteins. If the changes occurred earlier during the behavioral task than when we collected tissue, then we may have missed the ability to detect these differences. Based on the behavioral impairments, it was surprising not to see more protein changes in the baclofen-treated groups. Future experiments could examine the effect of baclofen administration on these protein targets at different time points throughout the delay and trace CCF conditioning protocols.

While the tissue analyses do not necessarily correspond to how typical G-protein coupled receptors (GPCRs) respond to agonist and antagonist mediation, they do fit in nicely with several *in vitro* GABA$_B$ receptor studies. For instance, the most robust changes to the GABA$_{B1}$ subunits were specifically to the preferentially postsynaptic GABA$_{B1b}$ isoform. Wetherington and Lambert (2002) demonstrated that GIRK-associated postsynaptic GABA$_B$ receptors rapidly desensitized to agonist treatment, whereas GIRK-associated presynaptic GABA$_B$ receptors failed to desensitize even after 24 hours of agonist treatment. Therefore, it is possible that presynaptic GABA$_B$ receptors are less sensitive to prolonged ligand treatment. Other research has demonstrated that GABA$_B$ receptors do no internalize in response to agonist treatment (Perroy et al., 2003; Fairfax et al., 2004; Mutneja et al., 2005), but that agonist treatment does produce a

decrease in cell surface-expressed receptors (Fairfax et al., 2004). These data suggest that $GABA_B$ receptors do not respond to ligand treatment like typical GPCRs. Couve, Moss, and Pangalos (2007) suggest that $GABA_B$ receptors may utilize a "new receptor model in which the levels of $GABA_B$ receptors are not correlated with receptor activity," and this conclusion can be logically extended to include behavioral activity, as well.

A consideration for future experiments would be the dose and route of administration of the compounds used in the current study. It is possible that the current dose of phaclofen is too low to effect a change in behavior, even if it is altering protein levels in several brain regions. A study of the effects of subcutaneous administration of these compounds could elucidate other effects on learning and memory. Additionally, since the current study simply examined the effects of a daily system injection of either baclofen or phaclofen on behavior, it would be very interesting to examine the effects of continual transcranial infusion of these compounds to specific brain regions. Further, by infusing the compounds transcranially, we could gain region specificity and would be able to examine the influence of altered GABAergic tone in a particular brain region on learning and extinction.

An open-field test would be an additional control test to consider for future experiments. This test would measure the distance an animal travels in a non-aversive environment; any differences in locomotion caused by the drugs administered would be easily detected. This control would best be utilized before the onset of the behavioral task.

Finally, tasks that utilize varying amounts of stress would also be interesting future experiments. Perhaps the drugs used would differentially affect performance

based on how stressed the animal is; the amount of stress may also relate to brain structures being used to complete a behavioral task. For instance, appetitive conditioning may not be mediated by the amygdala to the same degree as fear conditioning. It is possible that other tasks may be more sensitive to the effects caused by the ligands used in the current study.

Overall, the behavioral results of these experiments are quite consistent and demonstrate that baclofen administration hinders the extinction of both cued and contextual fear associations. While the tissue analyses do not necessarily demonstrate typical G-protein coupled receptor response to ligand administration, there are some very interesting protein level differences that strongly indicate a task-dependent change due to the ligands. Additionally, these results suggest that $GABA_B$ ligands alter behavior and cellular components differentially, meriting further research into these individual components. While rather interesting, these data need to be further clarified in future experiments to elucidate not only how altered $GABA_B$ receptor function affects the brain regions associated with fear conditioning, but to also determine if decreased $GABA_B$ receptor function can affect cued and contextual fear behavior.

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Publications
Di Pietro NC, Mashhoon Y, **Heaney C**, Yager LM, Kantak KM (2008) Role of dopamine D1 receptors in the prefrontal dorsal agranular insular cortex in mediating cocaine self-administration in rats. *Psychopharmacology* 200:81-91
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Manuscripts Under Review
Sabbagh JJ, **Heaney CF**, Bolton MM, Kinney JW. (2011) Efficacy of acute versus chronic administration of an NMDA receptor antagonist to induce an animal model of schizophrenia. Submitted to Progress in Neuro-Psychopharmacology and Biological Psychiatry

Manuscripts Under Review (continued)

Sabbagh JJ, **Heaney CF**, Bolton MM, Ure JA, Kinney JW. (2011) Donepezil
and Galanin Interactions in an Animal Model of Alzheimer's Disease. Submitted
to Behavioral Brain Research.

Bolton MM, **Heaney CF**, Sabbagh JJ, Murtishaw AS, Kinney JW. (2011) Deficits in
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Conference Presentations

Sabbagh JJ, **Heaney CF**, Bolton MM, Ambrose T, Kinney JW. (2009, Oct.) Efficacy of
acute versus chronic administration of an NMDA receptor antagonist to induce an
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Heaney CF, Sabbagh JJ, Bolton MM, Murtishaw AM, Santa Ana IK, Kinney JW.
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Sabbagh JJ, **Heaney CF**, Bolton MM, Ure JA, Kinney JW. (2010, Nov.) Donepezil and
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Heaney CF, Bolton MM, Murtishaw AS, Sabbagh JJ, Magcalas CM, Kinney JW. (2011,
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Bolton MM, **Heaney CF**, Sabbagh JJ, Murtishaw AS, Kinney JW. (2011, Nov.)
Comparision of an adult and developmental model of schizophrenia. Poster
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