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Gabab Regulation of Methamphetamine-Induced Associative Learning

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$GABA_B$ REGULATION OF METHAMPHETAMINE-INDUCED ASSOCIATIVE LEARNING

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LIST OF ABBREVIATIONS

A	Actin
aCSF	Artificial Cerebral Spinal Fluid
ANOVA	Analysis of Variance
AP	Anterior/Posterior
ATP	Adenine Tri-phosphate
Bac	Baclofen
BS^3	Bis(Sulfosuccinimidyl)suberate
Ca^{++}	Calcium
cAMP	Cyclic adenine mono-phosphate
CL	Cross-link
CPA	Conditioned place aversion
CPP	Conditioned place preference
CREB	Cyclic-AMP response element binding protein
DA	Dopamine
DAT	Dopamine transporter
DV	Dorsal/Ventral
Fend	Fendiline
GABA	γ-amino butyric acid
$GABA_BR$	$GABA_B$ receptor

GIRK	Inwardly rectifying potassium channel
HC	Hippocampus
I	Intracellular
i.p.	Intraperitoneal
i.v.	Intravenous
K^+	Potassium
Meth	Methamphetamine
MDT	Medial dorsal thalamus
Min	Minute
ML	Medial/Lateral
mPFC	Medial prefrontal cortex
NAc	Nucleus accumbens
NE	Norepinephrine
NET	Norepinephrine transporter
Non CL	Non cross linked
PAM	Positive Allosteric Modulator
pCREB	Phosphorylated cyclic-AMP response element binding protein
PFC	Pre-Frontal Cortex
PKA	Protein Kinase A
p.o.	Oral administration
rpm	Rotations per minute
S	Surface
Sal	Saline

s.c.	Subcutaneous
SEM	Standard error of the mean
SERT	Serotonin transporter
VMAT2	Vesicular monoamine transporter 2
VP	Ventral Pallidum
VTA	Ventral Tegmental Area
5-HT	Serotonin

CHAPTER I

INTRODUCTION

Addiction is a chronic, relapsing disorder for which strikingly few effective therapies exist, and there are no FDA-approved pharmacotherapies for psychostimulant addiction. There is an immense need to identify the neurobiological underpinnings of stimulant addiction and develop efficacious pharmacotherapies to compliment the current mainstay treatment of behavioral/cognitive therapy. Several theories of addiction exist. However, the propensity of drug-associated cues to increase neuronal activity (Childress et al., 1999; Hotsenpiller et al., 2001; Hotsenpiller and Wolf, 2002; Rebec and Sun, 2005; Maas et al., 1998) and elicit drug-craving and seeking (Grant et al., 1996; Bonson et al., 2002; Ehrman et al., 1992) suggests a considerable contribution of maladaptive associative learning processes in the underlying neuropathology of addiction. The association between the rewarding effects of abused substances and contextual cues can be profound and long-lasting. Even after long-periods of abstinence, cues associated with drug-use can precipitate drug-craving, drug-seeking and relapse to drug-taking (Ehrman et al., 1992; Hartz et al., 2001; O'Brien et al., 1992). In the laboratory, conditioned place preference (CPP) is demonstrated by rodents (Tzschentke, 1998; Tzschentke, 2007) and humans (Childs and deWit H., 2009) whereby subjects will choose to spend time in a

context previously associated with a rewarding substance; a behavior which is thought to reflect the increased salience attributed to drug-associated cues. Thus CPP is a valuable research tool to evaluate the neuronal adaptations associated with drug-induced associative learning and provides a means to evaluate the utility of potential pharmacotherapies to reduce the salience of drug-associated cues.

Methamphetamine (Meth) is a powerful psychostimulant which reliably produces CPP (Tzschentke, 2007; Tzschentke, 1998). This behavior reflects molecular, cellular, and circuit adaptations that occur as a consequence of repeated psychostimulant administration (Hyman et al., 2006; Nestler, 2004) as well as the adaptations that underlie acquisition, maintenance, and expression of the Meth-context association (Alberini, 2009; Bailey et al., 1996). Human imaging studies demonstrate that basal neuronal activity is decreased in the cortex after chronic cocaine use (Bolla et al., 2004; Goldstein and Volkow, 2002; Volkow et al., 1992). However, the brain is hyper-responsive to stimuli, including unconditioned stimuli (subsequent drug-injections) (Sax and Strakowski, 2001; Pierce and Kalivas, 1997; McDaid et al., 2007; McDaid et al., 2006b) as well as conditioned stimuli (e.g., drug-associated cues) (Hotsenpiller et al., 2001; Rebec and Sun, 2005; Rebec and Sun, 2005; Hotsenpiller and Wolf, 2002). This hyper-responsivity may be a mechanism underlying cue-induced drug-craving and -seeking in humans (Childress et al., 1999; O'Brien et al., 1998; Childress et al., 1999; Ehrman et al., 1992) and rodents (Crombag et al., 2008). While the

hyper-excitable brain state is likely the consequence of maladaptations in a number of neurotransmitter systems such as down-stream signaling proteins, gene expression patterns, and neuronal function, decreased function of the γ-amino butyric acid (GABA) system, the major inhibitory neurotransmitter in the central nervous system, may significantly contribute to the hyper-responsive brain state. The metabotropic $GABA_B$ receptor ($GABA_BR$) system is down-regulated by psychostimulant administration. Such down-regulation includes decreases in $GABA_BR$ expression (Frankowska et al., 2008b; Frankowska et al., 2008a), G-protein expression (i.e., $G_{i/o}$) (Nestler et al., 1990), and functional coupling of the receptor to the G-protein (Xi et al., 2003; Zhang et al., 2000). However, it is not clear if this down-regulation occurs at a time when Meth-conditioned behaviors are expressed or if a decrease in $GABA_BR$ signaling during withdrawal might contribute to the maintenance of the mal-adapted brain state and behaviors. **This dissertation project evaluated $GABA_BR$ expression and distribution at a time when rats demonstrate Meth-induced behaviors (e.g., CPP).**

The $GABA_BR$ system has recently emerged as a potential therapeutic target for drug addiction (Brebner et al., 2002; Cousins et al., 2002; Rose and Grant, 2008; Xi and Gardner, 2008). Pharmacological augmentation of $GABA_BR$ signaling inhibits the **development** and **expression** of many psychostimulant-induced behaviors including CPP (Li et al., 2001), conditioned locomotion (Hotsenpiller and Wolf, 2003), motor sensitization (Bartoletti et al., 2005; Bartoletti et al., 2004;

Frankowska et al., 2009; Lhuillier et al., 2007), and self-administration (Brebner et al., 2005; Campbell et al., 1999; Filip et al., 2007; Filip and Frankowska, 2007; Ranaldi and Poeggel, 2002; Roberts et al., 1996; Roberts and Andrews, 1997; Shoaib et al., 1998; Smith et al., 2004; Weerts et al., 2007). These results demonstrate a role of the $GABA_BR$ in drug-induced processes, but to better emulate the clinical scenario we wanted to determine the capacity of $GABA_BR$ ligands to modify the **maintenance** of a previously established psychostimulant-induced behavior. To date, only Bartoletti and colleges have demonstrated the capacity of the $GABA_BR$ agonist baclofen to inhibit the maintenance of a previously established psychostimulant-induced behavior; they demonstrated that 10 once-daily injections of baclofen inhibited the maintenance of amphetamine-induced motor sensitization (Bartoletti et al., 2004). **We proposed that pharmacologically augmenting $GABA_BR$ signaling would disrupt mnemonic processes necessary to maintain and subsequently express previously acquired Meth-induced CPP.** Furthermore, during the hours, days, and weeks following psychostimulant administration and memory acquisition, the brain is highly dynamic with withdrawal-time-dependent effects being observed in the $GABA_BR$ system (Zhang et al., 2000; Jayaram and Steketee, 2005) and withdrawal time-dependent behavioral effects are reported in humans (McGregor et al., 2005). **Thus, we hypothesized that withdrawal time dependent effects on $GABA_BR$ expression/distribution and/or function may be reflected as a change in the capacity of $GABA_BR$ ligands to disrupt the maintenance of Meth-induced associative learning.**

Baclofen is a direct-acting (i.e., orthosteric) $GABA_BR$ agonist, and it produces substantial undesirable side effects, including sedation and motor impairment, that may limit its clinical usefulness (Cryan et al., 2004; Jacobson and Cryan, 2005; Heinzerling et al., 2006; Ling et al., 1998; Shoptaw et al., 2003). Positive allosteric modulators (PAMs) of the $GABA_BR$ provide an alternative means to augment GABAergic signaling. $GABA_BR$ PAMs do not directly activate receptors; instead they increase the efficacy of endogenous GABA (Bettler et al., 2004; Urwyler et al., 2001; Urwyler et al., 2005; Gjoni et al., 2006). This dependence on endogenous GABA release makes PAMs regionally and temporally specific in contrast to direct acting $GABA_BR$ agonists which bind to and activate all $GABA_BRs$ regardless of endogenous GABAergic tone. These attributes of PAMs result in fewer unwanted side effects than those typically observed with direct acting agonists, like baclofen (Cryan et al., 2004). Substantiating the idea that these drugs may be useful for addiction therapy, PAMs have been shown in laboratory rodents to successfully reduce cocaine self-administration (Smith et al., 2004) and prevent molecular adaptations resulting from psychostimulant administration (Lhuillier et al., 2007). **We hypothesized that systemic administration of the $GABA_BR$ PAMs would antagonize the maintenance of Meth-induced CPP without the side effects associated with the $GABA_BR$ agonist baclofen.**

One region that might be particularly important in mediating the effects of systemically administered $GABA_BR$ ligands is the medial dorsal thalamus (MDT). The MDT expresses moderate to high levels of $GABA_BR$ (Margeta-Mitrovic et al., 1999; Charles et al., 2003) and *via* afferent and efferent projections modulates and is modulated by brain regions important for the regulation of psychostimulant-induced behaviors and mnemonic processes. Indeed, baclofen (Romanides et al., 1999) or lidocaine (Floresco et al., 1999) injected into the MDT inhibits working memory. The role of the MDT in the maintenance of psychostimulant-induced associative learning is unclear. **To fill this gap, the present dissertation project evaluated the role of $GABA_BRs$ in the MDT as critical regulators of the maintenance of Meth-induced CPP**.

A stand alone pharmacotherapy may not be the most effective approach to reduce the propensity of cue-elicited relapse; likewise, behavioral/cognitive therapy (i.e., extinction therapy) has not been particularly efficacious in reducing relapse in abstinent drug-dependent humans (Conklin and Tiffany, 2002) or in rodent models of addiction (Crombag and Shaham, 2002; Di Ciano P. and Everitt, 2004). There is convincing evidence which suggests that combining extinction therapy with a pharmacotherapy may be more efficacious in reducing cue-elicited responses in humans (O'Brien et al., 1990) and rodents (Heinrichs et al., 2010) than with either strategy alone. **Thus, we proposed that the $GABA_BR$ agonist, baclofen would promote extinction training**.

These experiments will 1) shed light on the role of $GABA_BRs$ in the maintenance of Meth-induced associative learning, 2) provide insight into the role of the MDT in the maintenance of associative memories, and 3) evaluate the utility of $GABA_BR$ activators to reduce the salience of psychostimulant-associated cues.

CHAPTER II

LITERATURE REVIEW

Methamphetamine Addiction

Meth, a highly addictive psychostimulant, is one of the most abused substances world-wide (United Nations Office on Drugs and crime, 2009) with Meth use disturbingly high in certain regions in the United States including Hawaii, San Diego, Los Angeles, San Francisco, and Phoenix (National Institute on Drug Abuse, 2009). The euphorogenic effects of amphetamines (including Meth), is the consequence of elevated extracellular concentrations of the monoamines serotonin (5-HT), dopamine (DA), and norepinephrine (NE) (Baumann et al., 2002b; Baumann et al., 2002a; Kuczenski et al., 1995; Melega et al., 1995; Rothman et al., 2001). The massive increase in synaptic monoamine concentrations is achieved *via* multiple mechanisms including inhibition of reuptake transporters for dopamine (DAT), norepinephrine (NET), and serotonin (SERT) as well as the vesicular monoamine transporter 2 (VMAT-2) (Amara and Kuhar, 1993; Rothman and Baumann, 2003; Rudnick and Clark, 1993; Sulzer et al., 2005). These mechanisms of Meth function to increase monoamine concentrations in the synapse. By virtue of Meth increasing the synaptic concentrations of the monoamines Meth can be considered an indirect agonist at DA, NE, and 5-HT receptors. In addition, Meth decreases monoamine

concentration of monoamines achieved as a consequence of these multiple mechanisms exceeds that achieved by, e.g., cocaine which only inhibits reuptake transporters (i.e., DAT, NET, SERT) (Hyman and Malenka, 2001). Thus, the effects of Meth are more profound and long lasting than those observed after cocaine administration (Fowler et al., 2008) which is thought to underlie the extraordinarily high abuse liability of Meth.

Withdrawal from psychostimulant administration

The massive efflux of synaptic monoamines observed during psychostimulant administration activates a number of neurotransmitter receptors to initiate a cascade of down-stream signaling events that result in neuronal adaptations which change over the course of hours, days, and weeks after exposure (Zhang et al., 2001; Ernst and Chang, 2008; McGregor et al., 2005; Camp et al., 1997; McDaid et al., 2006b; Zhang et al., 2000; Jayaram and Steketee, 2005). These adaptations can persist long after terminating psychostimulant use, and thus may underlie the subjective states (e.g., negative affect) that occur during withdrawal as observed by McGregor and colleagues in Meth withdrawn addicts (McGregor et al., 2005). Studies demonstrate that basal neuronal activity is decreased in the cortex during withdrawal from chronic cocaine use in humans (Bolla et al., 2004; Goldstein and Volkow, 2002; Volkow et al., 1992) and after psychostimulant administration in rodents (Kalivas and Hu, 2006), however the brain is hyper-responsive to stimuli including re-exposure to cocaine or cocaine-associated cues (Hotsenpiller et al., 2001; Rebec and Sun, 2005; Rebec and

Sun, 2005; Hotsenpiller and Wolf, 2002; Febo et al., 2004; Ferris et al., 2005; Sun and Rebec, 2006; Kalivas and Hu, 2006). As withdrawal time and contextual cues are important factors in determining neuronal function, brain state, and subjective behavioral effects, a better understanding of the underlying neuronal adaptations may aid in developing anti-addiction pharmacotherapies.

Psychostimulant-induced associative learning

Persistent adaptations in neuronal function likely contribute to the behavioral hallmarks of withdrawal from drug addiction including compulsive drug-seeking, the inability to stop using the drug even when desired, and high propensity to relapse to drug-use even after long-periods of withdrawal (American Psychiatric Association, 2000). There are several theories why individuals exhibit these behaviors; however, the association which forms between the drug (unconditioned stimulus) and contextual cues (conditioned stimulus) appears to be a credible candidate. Repeated administration of psychostimulants creates persistent molecular and cellular adaptations (Everitt and Wolf, 2002; Hyman and Malenka, 2001; Hyman et al., 2006; Nestler, 2004). The stimulant-induced effects are similar to those that are engaged during mnemonic processes (i.e., learning and memory) (Abel and Kandel, 1998; Bailey et al., 1996). These overlapping mechanisms (Hyman et al., 2006; Kelley, 2004) include effects on kinases (e.g., PKA) (Chen et al., 2009; Selcher et al., 2002) and transcription factors (e.g., CREB) (Carlezon et al., 2005; Lonze and Ginty, 2002). Adaptations such as these may contribute to cue-elicited neuronal hyper-responsivity

observed in brain regions such as the prefrontal cortex (especially the orbitofrontal and anterior cingulate cortices), amygdala, and thalamus (Childress et al., 1999; Childress et al., 2008; Kilts et al., 2001; Kilts et al., 2004; Brown et al., 1992; Zombeck et al., 2008; Ciccocioppo et al., 2001; Rhodes et al., 2005; Franklin and Druhan, 2000), cue-induced drug-craving (Ehrman et al., 1992; Hartz et al., 2001; O'Brien et al., 1992), and drug-seeking (Ciccocioppo et al., 2001; Filip and Frankowska, 2007). Drug-induced associative learning (i.e., drug-induced conditioned place preference, CPP) is the process where an association is learned between the unconditioned stimulus (e.g., the psychostimulant) and the context (e.g., place) where the drug was administrated, i.e., the conditioned stimulus) wherein drug-free laboratory animals (Tzschentke, 1998; Tzschentke, 2007) and humans (Childs and deWit H., 2009) tend to spend more time in a previously drug-paired context than in one not associated with the rewarding effects of abused substances. Conditioned place preference can be induced with a wide range of substances including the Meth (Tzschentke, 1998; Tzschentke, 2007). Thus, CPP is an efficient and effective means to model the enhanced salience of Meth-associated cues (i.e., the increased significance attributed to Meth-associated cues) that occurs during addiction.

Memory that results from associative learning processes (such as that engaged during CPP) can be divided into short-term (also known as working memory) and long-term memory. Short- and long-term memories involve distinct processes. Long-term memory requires a complex interplay of kinases and protein

transcription in order for learned events to be consolidated, retained, and subsequently recalled. Processes necessary for consolidation of long-term memory (events which occur soon after learning) and reconsolidation (events necessary to retain a memory following recall) also involve kinases (e.g., PKA) and protein expression in a *time dependent manner* within particular brain regions such as the hippocampus, frontal cortex, and amygdala (McGaugh, 2000). Recall (also known as memory expression) requires glutamate receptors. The maintenance phase of long-term memory is a relatively un-explored area, and while it is clear that memory maintenance is highly dynamic (Abel and Kandel, 1998; Alberini, 2009; Bailey et al., 1996), the exact mechanisms remain to be elucidated.

Associative learning established during CPP can be broken down into several distinct phases including development, maintenance, and expression. The development of CPP refers to the time when the association between the drug and the context memory is being acquired (i.e., during conditioning) and expression is when the learned association is recalled and is demonstrated behaviorally. The maintenance of CPP refers to the time between when the drug-cue memory is acquired and when it is subsequently recalled. This dynamic phase is a potential point of therapeutic intervention to disrupt unwanted memories after they have been established. This disruption can occur as a result of a pharmacologic intervention modifying processes that are necessary to maintain memory or *via* extinction training wherein an unwanted memory is

replaced or over ridden by a new memory. Applications of these memory maintenance disrupting approaches are considered in this dissertation.

The $GABA_B$ receptor and psychostimulant administration

GABA is the major inhibitory neurotransmitter in the central nervous system. Ionotropic $GABA_A$ receptors mediate fast, robust neuronal inhibition *via* activation of chloride channels whereas $GABA_B$Rs modulate neuronal activity *via* G-protein ($G_{i/o}$)-dependent mechanisms including inhibition of high voltage activated calcium (Ca^{++}) channels, activation of inwardly rectifying potassium channels (GIRKs), and inhibition of cAMP production (Bowery, 1993; Mott and Lewis, 1994). These mechanisms blunt both pre- and post-synaptic neuronal activity. In order for the $GABA_B$R to efficiently mediate these inhibitory effects, the $GABA_B$R must function as an obligate heterodimer complex containing both the $GABA_B$R1 and the $GABA_B$R2 receptor (Jones et al., 1998; Kaupmann et al., 1998; Kuner et al., 1999; White et al., 1998). The $GABA_B$R1 receptor subtype cannot be trafficked to the neuronal surface due to the presence of an endoplasmic reticulum (ER) retention signal. This 'signal' is found in both isoforms of the $GABA_B$R1, the $GABA_B$R1a and the $GABA_B$R1b, which differ from each other by the presence or absence of a sushi domain. Dimerization of the $GABA_B$R1 with the $GABA_B$R2 masks the ER retention signal which allows the receptor dimer to traffic to the neuronal surface (Jones et al., 1998; Kaupmann et al., 1998; Kuner et al., 1999; White et al., 1998). Following insertion, the $GABA_B$R1 is responsible for binding to the ligand whereas the $GABA_B$R2 is

responsible for binding of the receptor to the G-protein. Thus, both $GABA_BR$ subunits are necessary for ligand-mediated signal transduction (Bettler et al., 2004; Bowery et al., 2002).

Psychostimulant-induced brain adaptations are highly dynamic and contribute to withdrawal-time and context-dependent brain states, which involve multiple neurotransmitter systems and downstream signaling molecules (Nestler, 2001; Chao and Nestler, 2004). One component contributing to the maladapted brain state may be the $GABA_BR$ system. Specific adaptations are influenced by neuronal phenotype, number of drug exposures, and withdrawal duration; however, in general the $GABA_BR$ system is down-regulated after psychostimulant administration in a brain region and withdrawal-time dependent manner. The down-regulation includes decreased $GABA_BR$ expression (Frankowska et al., 2008b; Frankowska et al., 2008a), decreased G-protein expression ($G_{i/o}$) (Nestler et al., 1990; Striplin and Kalivas, 1993), and decreased $GABA_BR$/G-protein coupling (Kushner and Unterwald, 2001; Zhang et al., 2000). Furthermore, increased basal GABAergic tone (Jayaram and Steketee, 2005; Xi et al., 2003) and evoked GABA release (Bustamante et al., 2002) is observed after psychostimulant administration which would be consistent with the apparent down-regulation of the $GABA_BR$ system if the brain is attempting to blunt neuronal activity (i.e., return the brain to a pre-psychostimulant state). Decreased function of the $GABA_BR$ system may contribute to the hyper-excitable brain state observed in subjects during re-exposure to drug stimuli.

Withdrawal from psychostimulant administration results in region-specific changes in brain states that vary during the hours, days, and weeks following psychostimulant administration. Examples include, decreased $G_{i/o}$ G-protein expression at 14 days, but not at one day, of withdrawal from repeated cocaine administration (Striplin and Kalivas, 1993), functional coupling of the $GABA_BR$ to the G-protein is decreased in the cortex at 14 days withdrawal from repeated amphetamine, an effect that is not observed at one day withdrawal (Zhang et al., 2000), and extracellular GABA concentrations are elevated at one and seven, but not 28 days of withdrawal from repeated cocaine administration (Jayaram and Steketee, 2005). These studies demonstrate withdrawal time-dependent effects of psychostimulant administration on the $GABA_BR$ which may significantly alter neuronal excitability. Furthermore, changes such as these may render the brain more vulnerable to pharmacological interventions at certain withdrawal times than at others. The studies in this dissertation are designed to evaluate these withdrawal phase-dependent effects.

The $GABA_B$ receptor: drug-induced behaviors and mnemonic processes

Pharmacologically augmenting $GABA_BR$ signaling (*via* systemic administration of a $GABA_BR$ agonist) blunts psychostimulant-induced behaviors, including CPP (Li et al., 2001), motor sensitization (Bartoletti et al., 2004; Frankowska et al., 2009; Lhuillier et al., 2007; Hotsenpiller and Wolf, 2003), and self-administration (Ranaldi and Poeggel, 2002; Brebner et al., 2005; Campbell et al., 1999; Filip et

al., 2007; Roberts and Andrews, 1997; Smith et al., 2004; Weerts et al., 2007). These findings lend credibility to the idea that the $GABA_BR$ system influences psychostimulant-induced behaviors. Furthermore, systemic administration of $GABA_BR$ agonists negatively regulates mnemonic processes (Castellano et al., 1989; Levin et al., 2004; McNamara and Skelton, 1996; Nakagawa et al., 1995; Swartzwelder et al., 1987; Zarrindast et al., 2004; Zarrindast et al., 2001); however, memory-improving effects have also been observed (Georgiev et al., 1988; Saha et al., 1993). Therefore, it is not clear what effect pharmacological augmentation of $GABA_BR$ signaling might have on the maintenance of learned associations between Meth and the contextual cues.

The $GABA_BR$ modulates neuronal excitability, signal transduction, and protein expression; thus, changes in the $GABA_BR$ system will significantly affect brain function. Effects on neuronal excitability are mediated by G-protein-dependent activation of inwardly rectifying K^+ channels and inhibition of high voltage activated Ca^{++} channels (i.e., P/Q- and N-type) (Bowery, 1993; Mott and Lewis, 1994). The $GABA_BR$ is located both pre- and post-synaptically (Bowery, 1993; Chen et al., 2004; Wirtshafter and Sheppard, 2001) regulating neuronal activity and the release of other neurotransmitters including dopamine (Gong et al., 1998; Kalivas, 1993; Santiago et al., 1993a; Santiago et al., 1993c; Smolders et al., 1995), and glutamate (Bonanno et al., 1997; Huston et al., 1990). Dopamine and glutamate are critical regulators of drug-induced behaviors, and learning and memory. In addition, $GABA_BRs$ negatively regulate protein expression and

signal transduction *via* a G-protein-dependent mechanism (e.g., inhibition of cAMP) to regulate the activity of the transcriptional regulator cAMP response element binding protein (CREB) (Barthel et al., 1996; Bettler et al., 2004; Mott and Lewis, 1994; Lhuillier et al., 2007; Yin et al., 2006). In addition, a G-protein-independent mechanism exists wherein transcription factors (e.g., ATF4/CREB2) bind directly to the carboxyl terminus of the receptor (Bettler et al., 2004; White et al., 2000; Nehring et al., 2000). Thus, processes that are critical for the maintenance of Meth-induced behaviors may be disrupted by pharmacologically augmenting $GABA_BR$ signaling.

$GABA_B$ receptor positive allosteric modulators

Stimulation of the $GABA_BR$ by the direct acting agonist baclofen causes significant side effects including hypothermia, sedation, and motor impairment (Cryan et al., 2004; Jacobson and Cryan, 2005; Heinzerling et al., 2006; Ling et al., 1998; Paredes and Agmo, 1995; Shoptaw et al., 2003). Baclofen binds to the orthosteric site, which is located in the large extracellular region of the receptor, and activate all $GABA_BRs$ regardless of endogenous GABAergic tone. In contrast, positive allosteric modulators (PAMs) of the $GABA_BR$ bind to a transmembrane site of the receptor which is distinct from the orthosteric binding site. Binding of the PAM to the transmembrane site has minimal or no effect on $GABA_BR$ signaling in the absence of an agonist. Rather, PAMs induce a conformational change which enhances coupling of the receptor to the G-protein as well as increases the affinity of the receptor for the ligand (i.e., agonist

binding). Consequently, PAMs increase the potency and maximum efficacy of endogenous GABA (Bettler et al., 2004; Gjoni et al., 2006; Urwyler et al., 2001). The enhancement of $GABA_BR$ signaling only in brain regions / active synapses where GABA is endogenously released likely underlies the fact that these drugs have fewer side effects than baclofen and therefore may be an alternative to baclofen (Bettler et al., 2004; Cryan et al., 2004; Paredes and Agmo, 1995). Furthermore, the desensitization that can occur during baclofen administration is not observed with PAMs (Gjoni and Urwyler, 2009; Gjoni and Urwyler, 2008). Modulation of $GABA_BRs$ may be a way to modify the maintenance of CPP without the associated side effects of direct acting agonists such as baclofen.

The medial dorsal thalamus: drug-induced behaviors and mnemonic processes

Mnemonic processes engage numerous brain regions; however, those which have been very well characterized are the hippocampus (HC) and the prefrontal cortex (PFC). The HC is critically involved in the early phases of memory consolidation. Memories are subsequently transferred to the PFC for long-term storage. Other regions have also been implicated in learning and memory processes such as the amygdala and the more recently identified medial dorsal thalamus (MDT). The thalamus has typically been considered a relay region between the limbic system and the cortex; however, recently the role of the MDT has been recognized as an important regulator of learning and memory. The MDT is connected with the PFC, NAc, and VP *via* glutamatergic (Pirot et al.,

1994; Kuroda et al., 1995; Kuroda et al., 1998) and GABAergic projections (Churchill et al., 1996b; Groenewegen, 1988; Mogenson et al., 1987; Zahm et al., 1996) *via* efferent and afferent projections. A primary source of glutamatergic afferents to the PFC (a region important for executive control and memory) is the MDT (Giguere and Goldman-Rakic, 1988; Pirot et al., 1995). Based on this anatomy it is not surprising that the MDT is important for associative processes. Oyoshi *et al.* revealed that the MDT (particularly the medial portion) is engaged during conditioned associative tasks (Oyoshi et al., 1996). Furthermore, lesions of the MDT inhibit the acquisition of sucrose-induced CPP (McAlonan et al., 1993)

The thalamus, including the MDT, expresses moderate to high levels of $GABA_BRs$ (Margeta-Mitrovic et al., 1999; Charles et al., 2003). Following at least 10 days of stable cocaine self-administration, $GABA_BR$ expression is significantly reduced throughout the rat brain including in the MDT (Frankowska et al., 2008b; Frankowska et al., 2008a). Although this change occurs as a consequence of cocaine self-administration it is not clear if it is driving the self-administration behavior. Studies have shown that modifying $GABA_BR$ signaling within the MDT modifies behavior; augmenting $GABA_BR$ signaling within the MDT by intra-cerebral injections of baclofen dose-dependently increases spontaneous motor activity (Churchill et al., 1996a) and disrupts working memory (Floresco et al., 1999; Romanides et al., 1999). The status of $GABA_BRs$ in the MDT at a time when Meth-induced associative learning is expressed is unknown. Furthermore,

the effect of locally augmenting $GABA_BR$ signaling during the maintenance of Meth-induced associative learning has not been explored. The experiments in this project fill in these gaps in the literature.

Significance and therapeutic implications

Addiction is a chronic and relapsing condition. Costs associated with addiction are an enormous burden on society and the families of the addicted individual. Thus, there is a huge incentive to determine the underlying neuronal adaptations and develop effective therapies to reduce the propensity of cue-elicited relapse. Currently, there are no FDA-approved pharmacotherapies for Meth addiction and the incidence of relapse is remarkably high following behavioral/cognitive therapy. Relapse to using abused drugs remains a major challenge for abstinent addicts. Therefore, determining a way to reduce the salience of drug-associated memories will be of value to reduce cue-elicited relapse and we propose that the $GABA_BR$ may be viable pharmacotherapy target.

CHAPTER III

RATIONALE FOR EXPERIMENTAL DESIGN AND METHODOLOGY

The studies contained in this dissertation are designed to explore the role of the $GABA_BR$ in the maintenance of Meth-induced learned associations. Several different CPP paradigms were used to evaluate treatment duration and withdrawal-time dependent effects of $GABA_BR$ ligands on the maintenance of Meth-induced CPP. We further explored combining a $GABA_BR$ pharmacotherapy with extinction training as a potential means to rapidly mitigate the maintenance and subsequent expression of learned associations between the rewarding effects of Meth and contextual cues. To better understand the underlying adaptations contributing to Meth-induced behaviors, the expression and distribution of the $GABA_BR$ were evaluated at a time when Meth-induced behaviors were exhibited. An explanation of selected methodologies is provided below.

Associative learning is a biological imperative from an evolutionary standpoint. Animals and humans learn to avoid harmful and stressful situations (conditioned place aversion, CPA) and to seek out beneficial and pleasurable experiences (conditioned place preference, CPP). The CPP task measures the associations that develop between the unconditioned stimulus (e.g., rewarding properties of

psychostimulants) and the unconditioned stimulus (e.g., contextual cues) (Bardo et al., 1995; Tzschentke, 1998; Tzschentke, 2007). Place conditioning is a relatively easy, high-throughput method and it is becoming increasingly used for evaluations in laboratory rodents; however, the conditioning apparatus, methodological details, statistical analysis, and even terminology are highly variable. The merits of the selected CPP paradigms included in this dissertation, and why these paradigms were selected, are detailed below.

General Methods

Animals

Male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 250-300g at the start of the study were acclimated to the *vivarium* for at least five days prior to the onset of the experiments. Rats were housed in pairs in a climate-controlled environment (23-25º) on a 12 hr light/dark cycle, and allowed *ad libitum* access to food and water. Cage mates were given identical pharmacological treatments. Housing facilities are accredited through the Association for Assessment and Accreditation of Laboratory Animal Care, and all experiments were carried out in accordance with the conditions set forth by the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996) and with the approval of the Loyola University Medical Center or Rush University Medical Center Institutional Animal Care and Use Committee.

Drugs

(+)Methamphetamine HCl (Sigma, St. Louis, MO) was dissolved in 0.9% sterile saline and administrated as 1mg/ml/kg as the base. Saline was administered as 1ml/kg. All injections were given intraperitoneally (i.p.).

Apparatus for Assessing Conditioned Place Preference

The test room was dimly lit (54-108 lux) with white noise continuously present (white noise generator, San Diego Instruments, San Diego, CA). The CPP apparatus (63cm x 30cm x 30cm) consisted of three chambers divided by Plexiglas sliding doors (AccuScan Instruments, Inc., Columbus, OH); two large conditioning chambers (25cm x 30cm x 30cm) separated by a small center chamber (13cm x 30cm x 30cm). Each chamber had distinct, yet neutral, visual and tactile cues. Time spent in each chamber and motor activity was monitored *via* two sets of photobeams (24 in the horizontal and 12 in the vertical plane).

The rats were transported from the *vivarium* to the test room at least 30min prior to the start of the experiment. A 30min pre-test, was used to determine initial chamber bias and these data were used to assign a Meth-paired chamber (half were Meth-paired in the chamber in which the greatest amount of time was spent during the pre-test and half in the chamber in which the least amount of time was spent during the pre-test). Conditioning was initiated two to three days later. During the conditioning phase, Meth conditioned rats were given a Meth injection (1mg/kg, i.p.) every other day (days 1, 3, and 5) and immediately placed into the appropriate chamber for 45min. On the alternate days (5-day protocol: 2 and 4,

6-day protocol: 2, 4, and 6), rats were given a saline injection (1ml/kg, i.p.) and immediately placed into the opposite chamber for 45min. Saline conditioned rats were given a saline (1ml/kg) for all conditioning sessions. A drug-free CPP test was performed three days after the last conditioning session (5-day protocol, day 8; 6-day protocol, day 9; termed CPP Test 1). This was accomplished by placing rats into the center chamber and the sliding doors were immediately removed allowing free access to the entire CPP box. The test session lasted 30min and time spent in each chamber was determined.

Elevated Plus Maze

The elevated plus maze (EPM) consisted of two sets of perpendicular arms (50.2 x 10.8cm) located 91.4cm above the floor. The intersection of the arms was 10.8 x 10.8cm. Closed arms had black plastic walls that were 40cm in height on either side and open arms had no walls. Time spent in each arm and number of arm entries were (closed *vs.* open) over 5min were recorded by three trained observers which were averaged to obtain the value for each rat. EPM experiments were conducted under red light conditions.

In order to examine the effects of acute Meth (1mg/kg) administration on anxiety, a Meth injection was administered 30min prior to the EPM test session. Rats were then pre-tested, conditioned, and tested as described previously. One day later, rats were given a drug-free EPM test to determine if Meth-conditioning altered anxiety.

Statistics

Conditioned Place Preference: For the pre-test data, time spent in each chamber was compared using a Paired *t*-test. CPP was defined as spending significantly more time in the chamber paired with Meth- *vs.* time spent in the same chamber during the pre-test or spending significantly more time in the chamber paired with Meth- *vs*. time spent in the saline-paired chamber. These within group analyses were conducted using a Paired *t*-test. Between group analyses were conducted using a Student's *t*-test. Elevated Plus Maze: Time spent in each arm as well as number of arm entries were compared between Meth- and saline-rats using a Student's *t*-test.

All data are presented as mean ± standard error of the mean (SEM). Statistical outliers were determined as those rats that spent greater than two standard deviations above or below the mean time spent in any chamber or arm.

Results

Conditioned place preference apparatus: Non-biased *vs.* biased

Typically, a CPP apparatus consists of two- or three-chambers (three chambers includes a small, center chamber which connects the two conditioning chambers); each of which contain distinct contextual cues. Studies used in the current project used a three-chamber apparatus (Accuscan Instruments, Inc; Columbus, OH); opaque Plexiglas sliding doors separated the two large

conditioning chambers (25cm x 30cm x 30cm) from the small center chamber (13cm x 30cm x 30cm). For rodents, conditioning typically utilizes visual and tactile cues although other sensory modalities may be used (e.g., olfactory cues). The conditioning apparatus can be configured such that naïve animals spend approximately equal amounts of time in each chamber (unbiased apparatus) or unequal amounts of time in each chamber (biased apparatus). This determination is often made prior to initiating the study in order to establish 'baseline' behavior, and is termed the pre-test. There is some argument that pre-tested rodents are slower at acquiring the learned association (Tzschentke, 1998), as this presents an opportunity for the animals to become familiar with the environment; however, including a pre-test has not impacted the ability of rats to successfully acquire and express Meth-induced CPP (Chapters IV-IX).

The interpretation of the behavior observed during the pre-test deserves consideration. Typically, the rats initially explore the new environments presented by the CPP box, and then, as they become familiar with the surroundings, they may elect to spend more time in one environment over the other. The affinity for a particular context often can be ethologically explained, i.e., rats prefer dark places and avoid brightly lit places, and the literature typically refers to this as a "preference" for dark places and an "aversion" to the lit places. Thus, choice of contextual cues (aversive *vs.* neutral) may contribute to the development of a biased *vs.* a non-biased apparatus. Stimuli such as bright lights are typically avoided by rodents; light/dark box studies demonstrate that

rodents will chose to spend more time in a dark environment compared to one that is brightly lit (Bourin and Hascoet, 2003; Costall et al., 1989; Ramos, 2008). In a CPP apparatus with black and white walls (e.g., Med Associates CPP apparatus) the natural tendency of rodents to spend time in the chamber with the black walls is often deterred by the addition of a bright light in that chamber; thus, creating an apparatus in which subjects spend approximately equal amounts of time in each chamber (Carlezon, 2003). In this example, the addition of the aversive stimuli of the bright light produces a non-biased apparatus. We contend that use of aversive stimuli in the CPP paradigm may compromise the ability to interpret behavioral outcomes by introducing the confounding factor of anxiety; thus the preference that develops following conditioning may reflect a reduction in anxiety rather than rewarding properties of the drug *per se*. Thus, a better approach may be to use contextual cues which are inherently neither preferred nor aversive in rodents, termed neutral contextual cues.

The use of neutral cues (stimuli which are inherently neither aversive nor preferred by rodents) such as vertical *vs.* horizontal stripes and non-noxious textured floors (see Fig. 1), typically will not produce a group preference for either chamber; however, individual rats may have a preference for one chamber (Shen et al., 2006). This point is illustrated in Fig. 2. As a group, 55 rats did not demonstrate a significant preference for Chamber A (vertical stripes on walls with a textured floor with an overturned paint dish glued in the middle) or Chamber B (horizontal stripes on walls with a textured floor with a flat, rectangular piece of

Plexiglas glued to the center of the floor with only a minimal amount of time being spent in the center compartment (solid color walls with a smooth slightly raised platform floor) (Fig. 2A, 818±49s *vs.* 855±51s; paired *t*-test, p>0.05); however, individual rats tended to spend more time in one chamber relatively to the other (Fig 2B; approximately 65% *vs*. 27%). As the time spent in one compartment *vs*. another was random (i.e., some rats spent more time in chamber A and others in chamber B, Fig 2B), this behavior is difficult to interpret in ethological terms. One theory, is that the rats may be exploring the CPP apparatus until they are sufficiently habituated at which time they stop exploring resulting in significantly more time being spent in that chamber. While individual rats had a bias for one chamber or the other, as a group there was no chamber bias; thus, the CPP apparatus used for studies in this project were conducted in a non-biased apparatus in the sense that all rats do not prefer one chamber over the other.

The use of a non-biased *vs.* a truly biased apparatus can significantly impact behavioral outcomes. For example, Cunningham and colleagues manipulated the conditioning apparatus such that rats had either no preference (non-biased) or a *strong* preference (biased) for one chamber (Cunningham et al., 2003). The use of either the biased or non-biased apparatus can significantly influence behavioral outcomes. This factor is discussed below in the use of a non-biased or biased experimental design.

Conditioned place preference experimental design: Non-biased *vs.* Biased design and other considerations

Non-biased *vs.* biased CPP *design* refers to the chamber in which animals are paired with the drug based on initial chamber preference (determined by a pre-test). One method statistically 'neutralizes' chamber bias by using a counterbalanced drug-paired chamber assignment, wherein half of the animals are assigned to be drug-paired in the initially preferred **and** others in the non-preferred chamber (Carlezon, 2003). Counterbalancing creates approximately equal (not statistically different) times being spent in the drug-paired and non-drug-paired chamber prior to conditioning; however, this approach can introduce considerable variability when animals have strong unconditioned chamber bias making it difficult to assess small shifts in preference. In contrast, a biased design assigns all animals to be drug-paired in the initially preferred **or** non-preferred chamber. This methodology has been thoroughly discussed in several publications (Brielmaier et al., 2008; Cunningham et al., 2003; Roma and Riley, 2005; Hinson et al., 1991).

The use of a non-biased *vs.* a biased apparatus and experimental design can significantly impact behavioral outcomes. For example, Cunningham and colleagues manipulated the conditioning apparatus such that rats had either no preference (non-biased apparatus) or a *strong* preference (biased apparatus) for one chamber (Cunningham et al., 2003). Rats were randomly assigned to receive ethanol in the non-biased apparatus and either the initially preferred or

non-preferred chamber in the biased apparatus. This study determined that ethanol-induced CPP can be produced in the non-biased apparatus, however, when individual rats express a strong initial preference for a chamber during the pre-test (defined as a large difference in time spent in one chamber relative to the other) as occurs in a biased apparatus, significant CPP is achieved only in by pairing ethanol with the initially non-preferred chamber (Cunningham et al., 2003). This effect is likely due to the ceiling effect that can occur when a strong, initial preference is present prior to conditioning. Similar findings are reported with clonidine (Cervo et al., 1993), cocaine (Nomikos and Spyraki, 1988), cocaethylene (Schechter, 1995), heroin (Schenk et al., 1985), and nicotine (Calcagnetti and Schechter, 1994). Furthermore, conditioned place aversion (CPA) can only be produced when rats are paired with the hallucinogenic drug LSD in the initially preferred chamber (Meehan and Schechter, 1998). These findings have been corroborated by our laboratory with Meth-induced CPP. Although we do not have a true “biased apparatus” and the rats randomly spent more time in one chamber or the other (Fig 2B), thus individual rats have a strong unconditioned bias. Here, rats were pre-tested and assigned to receive Meth in a counterbalanced manner (wherein half of the rats were assigned to receive Meth in the initially preferred chamber and others in the initially non-preferred chamber) and subsequently conditioned for five-days with 1mg/kg Meth (Fig 3A). Using this non-biased design we observed that conditioning produced CPP when all rats (n=45) were evaluated as a group; time spent in the Meth-paired chamber was significantly greater during the CPP test compared to the

same chamber during the pre-test (Fig 3B, designated Non-Biased Counterbalanced; n=45, paired *t*-test, $p<0.001$). However, splitting these rats into those paired in the initially non-preferred and preferred chambers revealed that only those rats Meth-paired in the initially non-preferred chamber demonstrated significant CPP (time spent in the Meth-paired chamber during the CPP test compared to the same chamber during the pre-test) (Fig 3B; designated Biased Non-preferred; n=23; paired t-test, $p<0.001$). In contrast, evaluation of data collected from rats paired in the initially preferred chamber revealed that time spent in the Meth-paired chamber was decreased, not increased, after Meth-conditioning (Fig 3B; designated Biased Preferred; n=22; paired t-test, $p<0.05$) indicating a ceiling effect that may occur when a strong unconditioned bias is present. These results indicate that it is not appropriate to Meth-pair rats in the preferred chamber in a biased apparatus. It also should be noted that analyzing this same data set by comparing time spent in the saline- and Meth-paired chambers for the non-biased and biased (Biased Preferred and Biased Non-Preferred) all resulted in positive CPP (Fig 3C). However, it should be noted that this outcome likely does not reflect learning (CPP) in all treatment groups. Rather, the CPP data achieved for the Biased Preferred rats looks remarkably similar to that achieved during the pre-test thus this reflects the unconditioned preference rather than a learning phenomenon. Thus, this underscores the notion that multiple methods of analysis should be used to verify CPP results as the use of a non-biased vs. biased apparatus and design may influence the behavioral outcomes and appropriate methods of analysis.

A controversial aspect of the biased design is the potential contribution of anxiety associated with pairing in the initially non-preferred chamber may have on behavioral outcomes. These concerns arise as a result of complex issues regarding the motivational states associated with the initially non-preferred context (Schenk et al., 1985), especially if the non-preferred context can be ethologically defined (e.g., in a bright chamber). Data obtained from the biased design can be interpreted in two ways 1) the preference is the result of the association between the rewarding properties of the drug or 2) the preference is achieved as a result of anti-anxiety properties of the drug (i.e., initial anxiety for the non-preferred chamber is overcome). There are several lines of evidence that belay the concern that CPP is the result of anxiolytic, rather than rewarding, properties. Regarding the first concern, psychostimulants are not anxiolytic but rather are anxiogenic particularly after repeated administration (Cancela et al., 2001; Olausson et al., 2000). For example, the anxiogenic drugs methamphetamine, amphetamine, and MDMA induce CPP (Daza-Losada et al., 2007; Lin et al., 1999; Tzschentke, 1998). We observed that Meth (1mg/kg) administration did not alter anxiety as measured on the elevated plus maze (time spent in open *vs.* closed arms or number of open *vs.* closed arm entries, Fig 4B and 4C, time spent in each arm as well as number of arm entries were compared between Meth- and saline-rats using a Student's *t*-test, $p>0.05$) when administered 30min prior to the elevated plus maze test (time during peak psychomotor effects). Furthermore, assessment of anxiety on the elevated plus

maze failed to reveal an anxiogenic or anxiolytic effect following repeated administration of methamphetamine which was sufficient to produce a conditioned place preference when evaluated within 24hr of the drug-free conditioned place preference test (Fig 4D & 4E, time spent in each arm as well as number of arm entries were compared between Meth- and saline-rats using a Student's *t*-test, $p>0.05$). The second line of evidence to belay concerns about the contribution of anxiety is that anxiolytic drugs do not always produce a preference in the CPP paradigm. Variable doses and paradigms used make it difficult to directly compare across studies, but the benzodiazepines diazepam and alprazolam produce CPP in some cases (Gray et al., 1999; Le et al., 2002; Papp et al., 2002; Walker and Ettenberg, 2003; Walker and Ettenberg, 2001; File, 1986) but have no effect on conditioning in others (File, 1986; Matsuzawa et al., 2000; Meririnne et al., 1999). Additionally, serotonergic drugs (venlafaxine and paroxetine), which are commonly used to treat anxiety disorders (i.e. anxiolytic effects), do not produce CPP (Deslandes et al., 2002; Subhan et al., 2000; Tzschentke et al., 2006). Taken together, it is unlikely that a reduction in anxiety is the main cause of Meth-induced CPP in the protocol employed for the current studies.

Measuring preference

There are several methods to evaluate CPP data; including between and within group comparisons. The selection of a method of analysis and statistical approach are dictated by experimental design and hypothesis being tested. Between group comparisons allow for direct treatment effects to be evaluated. Illustrated in Figures 5 and 6 are data collected from a six-day conditioning paradigm (timeline illustrated in Fig 4A) wherein rats were conditioned with either Meth or saline. Between group analyses revealed the following. Time spent in the Meth-paired chamber (day 1, 3, 5 chamber for saline conditioned rats, termed "Meth-like chamber") was significantly greater in Meth-conditioned rats compared to saline-conditioned rats (Fig 5A; saline conditioned rats (white bar, n=12); Meth-conditioned rats (black bar, n=48); Student's *t*-test, $p<0.05$). The second method demonstrated that subtracting time spent in the saline-paired chamber (day 2, 4, 6 chamber for saline conditioned rats, termed "saline-like chamber") from the time spent in the Meth-like or Meth-paired chamber demonstrated significant between group differences as well (Fig 5B; saline-conditioned rats (white bar, n=12); Meth-conditioned rats (black bar, n=47); Student's *t*-test, $p<0.05$). Finally, time spent in the Meth-like or Meth-paired chamber minus time spent in the same chamber during the pre-test revealed significant between group differences (Fig 5C; saline-conditioned rats (white bar, n=11); Meth-conditioned rats (black bar, n=47); Student's *t*-test, $p<0.001$). While these methods of analysis are visually quite different, all demonstrate significant between group differences indicating that Meth conditioning resulted in significant CPP. Within group comparisons are generally employed for evaluating shifts in

preference across time. For example, time spent in the Meth-paired chamber before and after pharmacological treatments or before and after extinction training. Among the commonly used methods of analysis to evaluate preference within an experimental group include comparing time spent in the Meth-paired chamber *vs.* time spent in the saline-paired chamber (Fig 6A; n=50; Paired *t*-test, $p<0.001$) and time spent in the Meth-paired chamber across multiple tests for preference (e.g., pre-test *vs.* CPP test) (Fig 8B; n=50; Paired *t*-test, $p<0.001$).

The majority of studies in this project assess changes in preference across time (i.e., before and after pharmacological treatments in the home cage and before and after extinction training); therefore, we chose to evaluate data using a within subject analysis. For example, within group comparison of rats conditioned in a five-day conditioning paradigm (Fig 3A) revealed that the Biased Preferred rats spent significantly more time in the Meth-paired chamber than in the saline-paired chamber (Fig 3B; black bars; n=22; paired *t*-test, $p<0.01$); however, preference evaluated across time (pre-test *vs.* CPP test) demonstrated that time spent in the Meth-paired chamber *decreased* after conditioning (Fig 3A; black bars; n=22; paired *t*-test, $p<0.05$). This observation underscores the importance of validating behavioral outcomes with more than one statistical approach in order to avoid type I and type II error. While not presented in this dissertation, all data sets were analyzed with several different statistical methods to verify the behavioral outcomes.

Concluding Remarks

There is a place for the biased design in CPP assessments. Especially when using a CPP apparatus which engenders a strong, unconditioned bias which will reduce variability revealing CPP which might otherwise be undetected due to the ceiling effect which may occur. We contend, and here demonstrate, that the biased design demonstrates reward-mediated behavior and not necessarily an anxiety-reducing phenomenon and thus is appropriate to test the proposed hypothesis in the current project.

Rationale for doses used

Methamphetamine

In the current series of experiments, 1mg/kg Meth reliably produced persistent CPP in several different experimental paradigms independent of conditioning protocol used, withdrawal time imposed, or repeated CPP testing. This outcome is in agreement with published studies demonstrating that Meth-induced CPP is readily produced with 1mg/kg Meth in both two- and eight-day conditioning protocols (Kitanaka et al., 2010; Li et al., 2002; Herrold et al., 2009; Schindler et al., 2002). A dose-response evaluation for Meth-induced CPP was conducted and demonstrated that Meth-induced CPP was produced by 0.3mg/kg and 1mg/kg (as the base) but not 0.1mg/kg in a six-day conditioning protocol; but, the persistence of the behavior was more robust in the 1mg/kg conditioned rats (data collected by Amy A. Herrold). Indeed, studies contained in this dissertation demonstrate the enduring behavioral effects (i.e., CPP) established with 1mg/kg

Meth conditioning (see data contained in Chapters IV-VIII and especially Chapter IX). Furthermore, this dose and dosing paradigm is below that which induces neuronal toxicity (e.g., decreased DAT expression and glial fibrillary acid protein expression) (Krasnova and Cadet, 2009; McDaid et al., 2006a).

$GABA_B$ Receptor ligands

Baclofen is a well-characterized drug which has been clinically available since the 1970's. It has been widely employed as a $GABA_BR$ agonist ((-) baclofen IC_{50}=33μM; (+) baclofen IC50=0.04 μM) (Bowery et al., 1983). Baclofen has been tested as an anti-relapse medication in clinical trials (Brebner et al., 2002; Cousins et al., 2002; Heinzerling et al., 2006; Ling et al., 1998; Shoptaw et al., 2003); however substantial side effects including sedation and motor impairment limit the utility of baclofen (Cryan et al., 2004; Heinzerling et al., 2006; Jacobson and Cryan, 2005; Ling et al., 1998; Shoptaw et al., 2003). The dose selected for baclofen in the current study (2mg/kg) is below the threshold published in the literature to produce significant side effects in rodents; baclofen (2.5 & 5mg/kg) inhibits motor behavior, impairs memory, and induced hypothermia (Cryan et al., 2004). We have verified that motor performance assessed on the rotarod was significantly inhibited by 4mg/kg baclofen (For results see Chapter V). Additionally, while 3mg/kg baclofen produced an impairment in spontaneous motor activity, the motoric effects of 2mg/kg baclofen did not differ from that of vehicle (horizontal activity, photobeam breaks over 75min post-injection for

vehicle (n=7), 2422±189; baclofen 2mg/kg (n=6), 1728±388; baclofen 3mg/kg (n=6), 1067±258; ANOVA p=0.012, *post-hoc* Dunnett's).

The dose of 2mg/kg baclofen is within the range of doses found to successfully i) attenuate the *development* and *expression* of Meth-induced CPP when administered 30 min prior to each daily conditioning session or CPP test, respectively (1.25, 2.5, 5mg/kg, i.p.) (Li et al., 2001) ii) inhibit the *reinstatement* of nicotine-induced CPP and self-administration when administered 5 min prior to testing (0.612, 1.25, 2.5mg/kg, i.p.) (Fattore et al., 2009), iii) reduce cocaine self-administration when administered 10 min prior to the session (2.5mg/kg, i.p.) (Smith et al., 2004), iv) reduce amphetamine self-administration (i.e., break point) when administered 30min prior to testing (1.8, 3.2, 5.6mg/kg, i.p.) (Brebner et al., 2005), and v) inhibit the *maintenance* of amphetamine-induced motor sensitization (2mg/kg, i.p.) (Bartoletti et al., 2004). Plasma and brain concentrations of baclofen are reported to be relatively consistent for at least 180min after a single bolus intravenous (i.v.) injection of baclofen in the rat (50mg/kg) (Deguchi et al., 1995) which aligns with enduring effects of GABAergic ligands on drug-induced behaviors. Orally administered baclofen (5mg/kg) elicits centrally-mediated events including hypothermia (Cryan et al., 2004) within one hour after baclofen administration suggesting that the onset of action is relatively short.

To evaluate the involvement of $GABA_BRs$ in the MDT in the maintenance of Meth-induced CPP, baclofen was injected directly into the MDT. The dose of 0.5nmol/0.5µl/side baclofen was selected because it is within the range of MDT-injected doses found to be behaviorally relevant to inhibit working memory (doses used were 0.03-0.3nmol) (Romanides et al., 1999) and influence motor activity (doses used were 0.003-1.0nmol) (Churchill et al., 1996a). The behavioral effects (i.e., motor activity and memory retention) of locally injected baclofen can be antagonized by co-administration of $GABA_BR$ antagonists (i.e., CGP35348 or saclofen) (Zarrindast et al., 2002; Churchill et al., 1996a) indicating these behavioral effects are the consequence of baclofen acting at the $GABA_BR$.

Fendiline. Fendiline is an L-type Ca^{++} channel blocker that is used clinically as a coronary vasodilator (Bayer and Mannhold, 1987; Nawrath et al., 1998; Tripathi et al., 1993) has recently been discovered to function as a $GABA_BR$ PAM (Ong and Kerr, 2005; Ong et al., 2005). While radioligand binding studies to determine the affinity of fendiline for the $GABA_BR$ have not been published, fendiline was found to potentiate baclofen inhibition of neuronal activity (neuronal inhibition EC_{50}=20µM) (Chen et al., 2005; Ong et al., 2005). The $GABA_BR$ positive allosteric modulator fendiline modulates $GABA_BR$ signaling without the motor side effects associated with baclofen as we verified using the rotarod assessment for motivated motor behavior (Chapter V). While motor performance was significantly inhibited by 4mg/kg baclofen, the $GABA_BR$ PAM fendiline (5mg/kg) did not affect performance (For results see Chapter V). The ability of

fendiline to modify psychostimulant-induced behaviors remains unknown but the dose of 5mg/kg was selected based on studies which show peripheral cardiovascular effects (Bayer and Mannhold, 1987). Fendiline pharmacokinetics demonstrate a slow onset of action and a long half-life (Bayer and Mannhold, 1987). Adverse behavioral effects (including sedation) were observed with 30mg/kg fendiline (unpublished data) thus the dose used for the studies in the current project was 5mg/kg.

GS39783, and CGP7930. The $GABA_BR$ PAMs GS39783 and CGP7930 were selected based on several criteria: 1) They are positive allosteric modulators of the $GABA_BR$ but with distinct molecular structures. 2) While radioligand binding studies to determine the affinity of these ligands for the $GABA_BR$ have not been published, they both enhance the binding of $GABA_BR$ agonists (i.e., baclofen and GABA), decrease the binding of $GABA_BR$ antagonists, and potentiate the effects of baclofen on K+ currents, cAMP production, and neuronal activity (EC_{50}=1μM for both ligands) (Urwyler et al., 2004; Chen et al., 2005; Olianas et al., 2005). 3) Lack of significant side effects at even 200mg/kg (p.o.) (Cryan et al., 2004). 4) The dose of 30mg/kg (i.p.) GS39783 and CGP7930 successfully reduces cocaine self- administration when administered 10 min prior to the session (Smith et al., 2004). Finally, 5) while the pharmacokinetics of the PAMs GS39783 and CGP7930 have not been published, GS39783 (3mg/kg, i.p.), and CGP7930 (30mg/kg, i.p.) significantly reduce cocaine self-administration for at least 10hr, indicating that these ligands may actively alter brain processes for several hours

after a single administration (Smith et al., 2004). The dose of 30mg/kg for both ligands was selected based on their favorable side effect profile and ability to alter psychostimulant-induced behaviors.

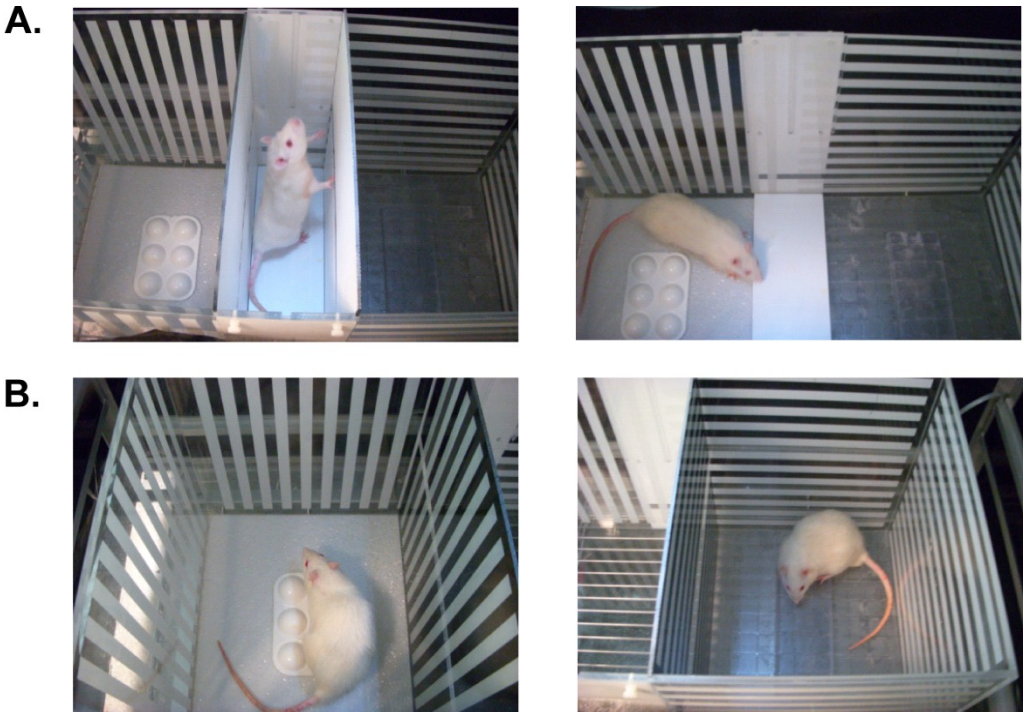

Figure 1. Illustration of three-chamber CPP apparatus used for studies in the current project. **A.** Shows the test procedure before (left) and after (right) the dividers have been removed. **B.** Shows the chambers (A & B) used for conditioning.

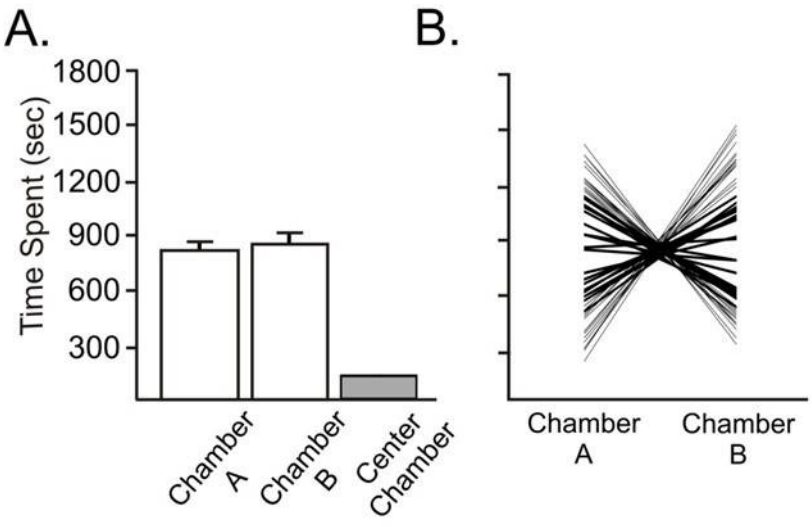

Figure 2. Demonstration of pre-test results generated in our three-chamber CPP apparatus. **A.** Time spent in each chamber (sec) as an average of a group of rats (n=55). Data analyzed as a paired t-test, $p>0.05$. Center chamber was not included for statistical analysis and is illustrated for qualitative purposes. **B.** The same data illustrated as individual rats.

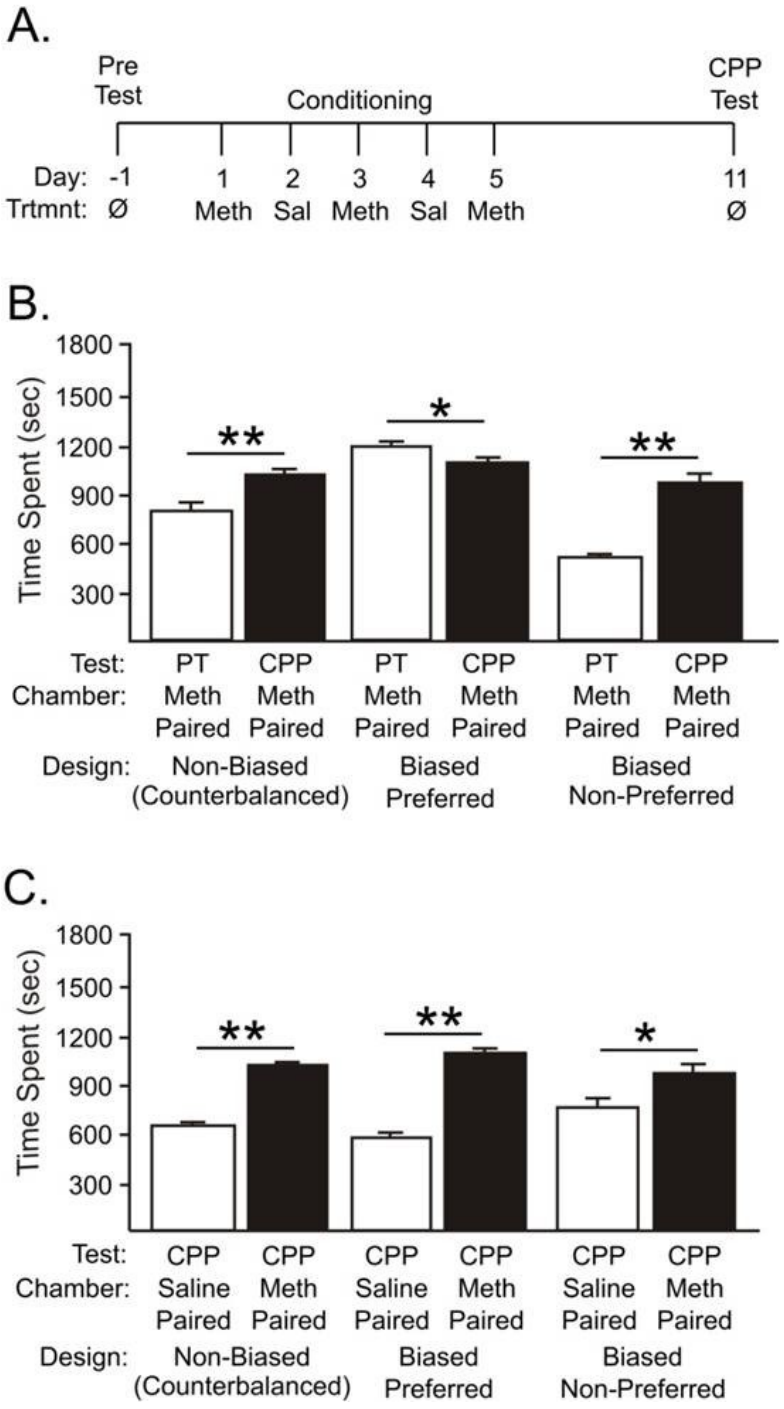

Figure 3. Non-biased *vs.* biased conditioned place preference experimental design. **A.** Timeline illustrating the five-day conditioning protocol. Conditioning was preceded by a drug-free pre-test followed by five days of conditioning which consisted of Meth (1mg/kg)-conditioning interposed by saline (1ml/kg)-

conditioning, concluding in a drug-free CPP test. Trtmnt, treatment; Meth, methamphetamine; Sal, saline; Ø, no drug. **B.** Within group comparison of time spent in the saline- and Meth-paired chambers during the CPP test. **C.** Within group comparison of time spent in the Meth-paired chamber during the CPP test compared to time spent in the same chamber during the pre-test. Non-biased Counterbalanced (n=45), Biased Preferred (n=23), Biased Non-Preferred (n=22). Data were analyzed with a paired *t*-test for each data set, * $p<0.05$, ** $p<0.01$.

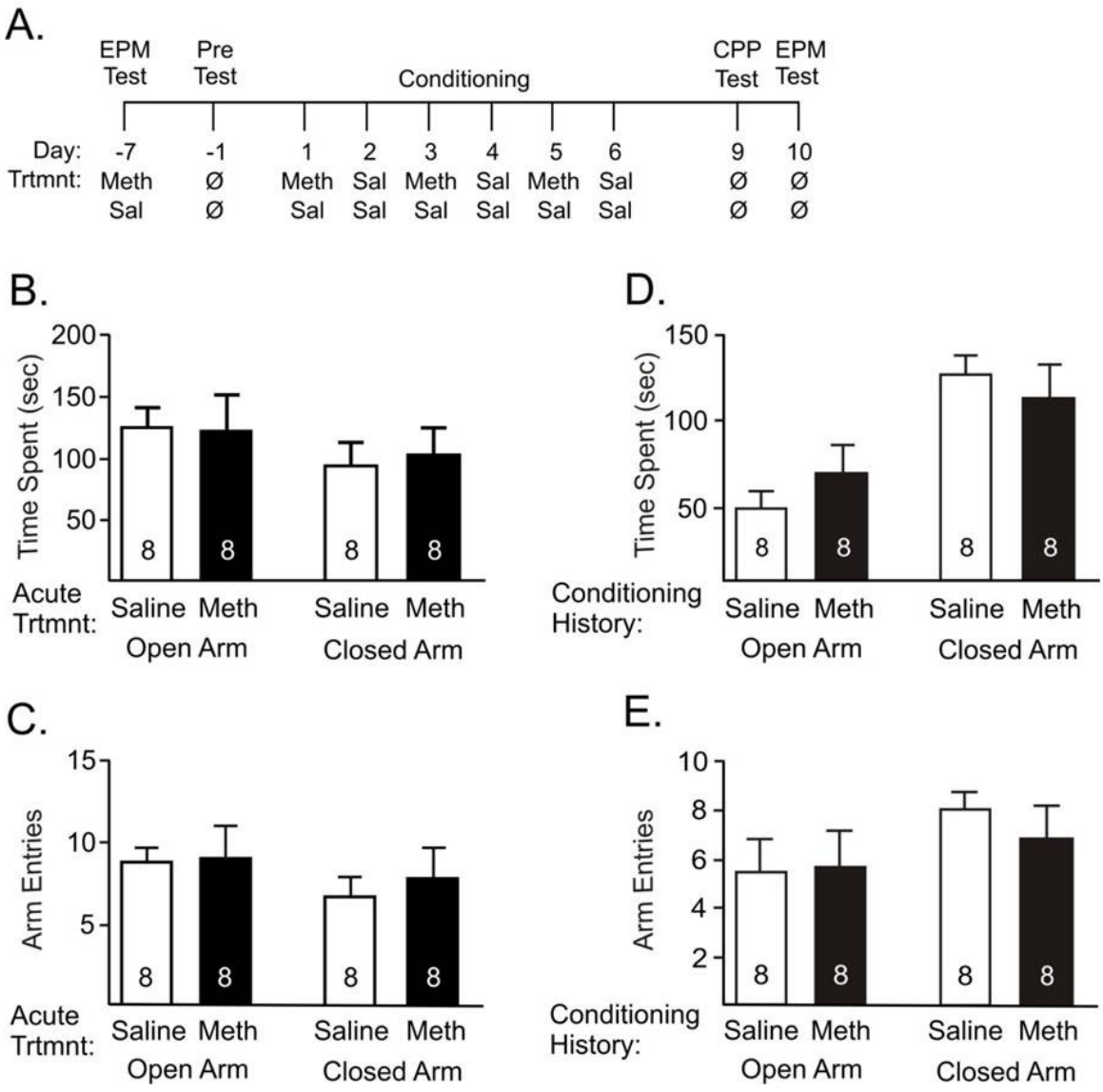

Figure 4. Neither acute Meth nor repeated Meth administration had any effect on anxiety measured in the elevated plus maze. **A.** Timeline illustrating testing and treatment protocol. **B & C.** Effect of acute saline (1ml/kg) or Meth (1mg/kg) administration on time spent in the open or closed arms **(B)** or number of arm entries into the open or closed arms **(C)**. **D & E.** Effect of repeated saline (1ml/kg) or Meth (1mg/kg) administration in the CPP conditioning paradigm when tested in a drug-free state 24hr after testing for CPP on time spent in the open

arms **(D)** or number of arm entries into the closed or open arms **(E)**. Data were analyzed with a Student's *t*-test, $p>0.05$.

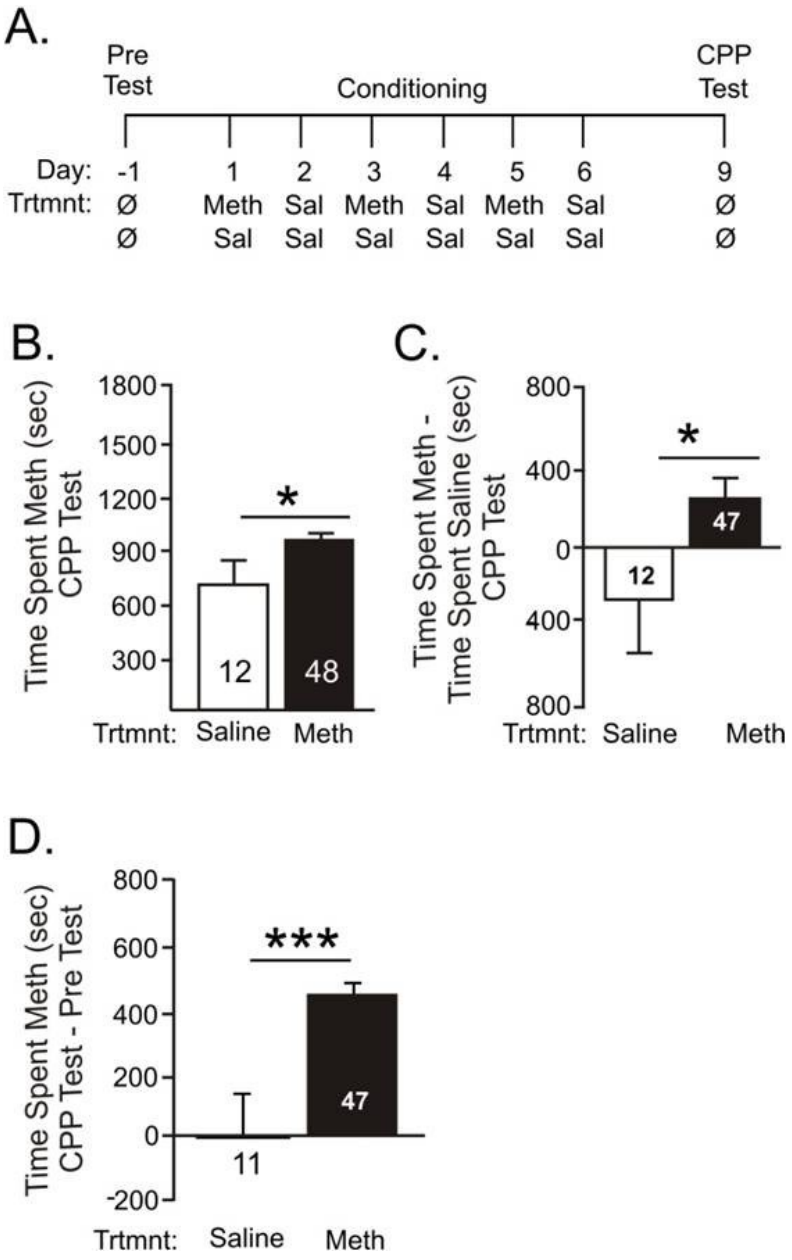

Figure 5. Between group analysis for Meth-induced CPP. **A.** Timeline illustrating the six-day conditioning protocol. A drug-free pre-test was followed by conditioning which consisted of Meth (1mg/kg)-conditioning interposed by saline (1ml/kg)-conditioning, concluding in a drug-free CPP test. Trtmnt, treatment; Meth, methamphetamine; Sal, saline; Ø, no drug. **B.** Illustrates time spent in the Day 1, 3, 5-paired chamber (saline or Meth) for each group during the CPP test.

C. Illustrates time spent in the Day 1, 3, 5-paired chamber minus time spent in the Day 2, 4, 6-paired chamber during the CPP test for each group. **D.** Illustrates time spent in the Day 1, 3, 5-paired chamber during the CPP test minus time spent in the same chamber during the pre-test for each group. Data were analyzed with a Student's t-test, * $p<0.05$, *** $p<0.001$. White bars indicate saline-conditioned rats (they received saline-pairings on all days); black bars indicate rats that were conditioned with Meth and learned to associate Meth with a particular chamber.

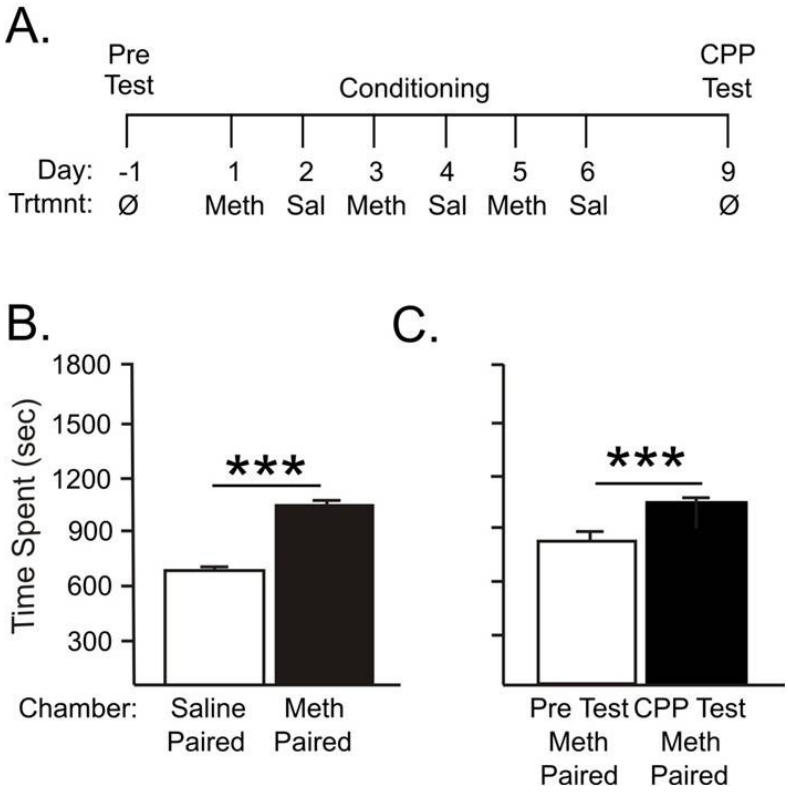

Figure 6. Within group analysis for methamphetamine (Meth) conditioned rats. **A.** Timeline illustrating the six-day conditioning protocol. Conditioning was preceded by a drug-free pre-test followed by six days of conditioning which consisted of Meth (1mg/kg)-conditioning interposed by saline (1ml/kg)-conditioning, concluding in a drug-free CPP test. Trtmnt, treatment; Meth, methamphetamine; Sal, saline; Ø, no drug. **B.** Illustrates time spent in the saline- and Meth-paired chambers during the CPP test. **C.** Illustrates time spent in the Meth-paired chamber during the CPP test compared to time spent in the same chamber during the pre-test. Data were analyzed with a paired t-test, $n=55$, $p<0.001$.

CHAPTER IV

REPEATED BACLOFEN ADMINISTRATION INHIBITS THE MAINTENANCE OF METHAMPHETAMINE-INDUCED CONDITIONED PLACE PREFERENCE

Abstract

Using conditioned place preference (CPP), the present study sought to determine if post-conditioning, home cage administration of the $GABA_BR$ agonist baclofen inhibited the maintenance of methamphetamine (Meth)-induced associative learning and if this effect depended upon the time period during which baclofen was administered. Male Sprague Dawley rats were conditioned with Meth (1mg/kg, i.p.) and saline. Baclofen (2mg/kg, i.p.) was subsequently administered and CPP was tested as follows: i) Baclofen given for two days during early withdrawal from Meth conditioning (protocol days 6 & 7); CPP tested one or 11 days later. ii) Baclofen administered for two days during more protracted withdrawal from conditioning (protocol days 16 & 17); CPP tested for CPP one day later. iii) Baclofen given for 10 days (protocol days 9-18); CPP tested 3 days later. These studies revealed that post-conditioning administration of 10 once-daily home cage injections of baclofen inhibited the maintenance and subsequent expression of Meth-induced CPP. This inhibitory effect was not observed when baclofen was administered for two days independent of when those two injections were administered. These data indicate that the maintenance of Meth-induced associative learning can be disrupted by

pharmacologically augmenting $GABA_BR$ signaling for 10 days. As a reduction in the significance of Meth-associated cues is a means to reduce relapse, baclofen may be of value for addiction therapy in abstinent Meth-abusing humans.

Introduction

Re-exposure to drug-paired cues increases neuronal activity in limbic brain regions in drug-withdrawn, drug-addicted/dependent humans (Childress et al., 1999; Childress et al., 2008) and rodents (Brown et al., 1992; Zombeck et al., 2008; Franklin and Druhan, 2000; Ciccocioppo et al., 2001; Rhodes et al., 2005), and can elicit drug-craving and drug-seeking even after long periods of withdrawal (O'Brien et al., 1992; Ehrman et al., 1992). Therefore, disrupting the maintenance of the drug-cue association may reduce the propensity of cue-induced relapse. Learned associations between the rewarding effects of abused substances (unconditioned stimulus) and the context in which the drugs were administered (conditioned stimulus) can be produced in the laboratory using conditioned place preference (CPP) in humans (Childs and deWit H., 2009) and rodents (Tzschentke, 2007). Thus, this behavioral paradigm has been widely employed to investigate processes engaged during associative learning as well as to help identify potential pharmacotherapies for relapse prevention. There are currently no FDA-approved treatments for methamphetamine (Meth) addiction and the mainstay behavioral/cognitive therapies might be improved by adding a pharmacotherapy. To best model the human scenario, the potential therapies should demonstrate the capacity to reduce/reverse the drug-conditioned effects.

Towards that end, we employed Meth-induced CPP in rats and subsequently administered repeated post-conditioning, home cage injections of baclofen, a $GABA_BR$ agonist, to determine if it would inhibit the maintenance of Meth-induced associative learning.

Baclofen has received considerable support as a viable therapy for psychostimulant abuse (Brebner et al., 2002; Xi and Gardner, 2008; Rose and Grant, 2008; Vocci and Appel, 2007; Cousins et al., 2002). Neuronal activity is negatively regulated by the $GABA_BR$ (Mott and Lewis, 1994; Bowery, 1993); the $GABA_BR$ decreases release of several neurotransmitters including glutamate (Giorgetti et al., 2002; Huston et al., 1990; Bonanno et al., 1997) and dopamine (Santiago et al., 1993a; Gong et al., 1998; Kalivas, 1993; Smolders et al., 1995; Santiago et al., 1993c). Moreover, $GABA_BRs$ modify downstream effectors, such as transcription factor activity (Bettler et al., 2004; Mott and Lewis, 1994; Barthel et al., 1996), which also underlie psychostimulant-induced neuroplasticity (Chen et al., 2009; Nestler, 2001) and mnemonic processes (Wang et al., 2006; Quevedo et al., 2004; Bailey et al., 1996; Berke and Hyman, 2000) Therefore the $GABA_BR$ is positioned to influence many systems critical for the maintenance of CPP behaviors.

The $GABA_BR$ is down-regulated after psychostimulant administration (Frankowska et al., 2008b; Kushner and Unterwald, 2001; Zhang et al., 2000) and pharmacologically augmenting $GABA_BR$ signaling inhibits the development

and expression of psychostimulant-induced behaviors, including CPP (Li et al., 2001), motor sensitization (Bartoletti et al., 2005), and self administration (Smith et al., 2004; Brebner et al., 2005; Filip et al., 2007; Filip and Frankowska, 2007; Ranaldi and Poeggel, 2002). However, to date only a report from Bartoletti and colleagues (Bartoletti et al., 2004) has demonstrated that baclofen inhibits the *maintenance* of a previously established psychostimulant-induced behavior; i.e., the maintenance of amphetamine-induced motor sensitization was inhibited by baclofen. Motor sensitization and CPP demonstrate different dose-related profiles for amphetamine and engage different brain substrates (Shen et al., 2006; Rademacher et al., 2006; Shoblock et al., 2003). Thus, to extend the current literature, we determined if baclofen would inhibit the maintenance of Meth-induced CPP.

The current study evaluated withdrawal time- and duration-dependent effects of baclofen on the maintenance of Meth-induced CPP. Withdrawal from psychostimulants is a dynamic process with different brain states being present during the hours, days, and weeks following psychostimulant exposure (Zhang et al., 2001; Ernst and Chang, 2008; McGregor et al., 2005; Camp et al., 1997; McDaid et al., 2006b; Zhang et al., 2000; Jayaram and Steketee, 2005); thus the brain substrate on which baclofen will act will vary greatly depending on the withdrawal time. This includes flux in a number of neurotransmitters (e.g., GABA and glutamate) and downstream signaling molecules (e.g., the transcription factor CREB). For example, functional coupling of the $GABA_BR$ is decreased in

the cortex at 14 days withdrawal from repeated amphetamine, an effect that is not observed at one day withdrawal (Zhang et al., 2000). Likewise, the transcription factor CREB is differentially regulated after a sensitizing regimen of Meth with CREB being elevated at three but not 14 days withdrawal (McDaid et al., 2006b). Thus, the brain state is highly dynamic and baclofen administered during one post-conditioning withdrawal time may not necessary be effective during another. The current study evaluated the possibility of "windows of opportunity" for baclofen to be effective as well as the possibility of treatment duration effects of baclofen on the maintenance of Meth-induced associative learning.

Materials and Methods

Animals

Male Sprague-Dawley rats (n=94) (Harlan, Indianapolis, IN) weighing 250-300g at the start of the study were acclimated to the *vivarium* for at least five days prior to the onset of the experiments. Rats were housed in pairs in a climate-controlled environment (23-25º) on a 12 hr light/dark cycle, and allowed *ad libitum* access to food and water. Cage mates were given identical pharmacological treatments. Housing facilities are accredited through the Association for Assessment and Accreditation of Laboratory Animal Care, and all experiments were carried out in accordance with the conditions set forth by the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*

(National Research Council, 1996) and with the approval of the Rush University Institutional Animal Care and Use Committee.

Drugs

(+)Methamphetamine HCl (Sigma, St. Louis, MO) was dissolved in 0.9% sterile saline and administrated as 1mg/ml/kg as the base; saline was administered as 1ml/kg; baclofen (2mg/kg; Sigma, St Louis, MO), and baclofen vehicle (0.9% saline; 1ml/kg). All injections were given intraperitoneally (i.p.).

The 2mg/kg dose of baclofen was selected because it was within the range of doses used in laboratory rats to successfully i) attenuate the development and expression of Meth-induced CPP when administered 30min prior to each daily conditioning session or CPP test, respectively (doses tested were 1.25, 2.5, 5mg/kg, i.p.) (Li et al., 2001), ii) inhibit the reinstatement of nicotine-induced CPP and self-administration when administered 5min prior to testing (0.612, 1.25, 2.5mg/kg, i.p. were used) (Fattore et al., 2009), iii) reduce cocaine self-administration when given 10min prior to the session (2.5mg/kg, i.p.) (Smith et al., 2004), iv) reduce amphetamine self administration when administered 30min prior to testing (doses tested were 1.8, 3.2, 5.6mg/kg, i.p.) (Brebner et al., 2005), and v) inhibit the *maintenance* of amphetamine-induced motor sensitization (2mg/kg, i.p.) (Bartoletti et al., 2004). In addition, we determined in a pilot study that 3mg/kg baclofen reduced motor activity, but no impairment was observed with 2mg/kg baclofen (e.g., horizontal activity, over 75min post injection for

vehicle (n=7), 2422±189; baclofen 2mg/kg (n=6), 1728±388; baclofen 3mg/kg (n=6), 1067±258; one-way ANOVA p=0.012, *post-hoc* Dunnett's showed significance for 3mg/kg).

Apparatus for Assessing Behavior

The test room was dimly lit (54-108 lux) with white noise continuously present (white noise generator, San Diego Instruments, San Diego, CA). The CPP apparatus (63cm x 30cm x 30cm) consisted of three chambers divided by Plexiglas sliding doors (AccuScan Instruments, Inc., Columbus, OH); two large conditioning chambers (25cm x 30cm x 30cm) separated by a small center chamber (13cm x 30cm x 30cm). Each chamber had distinct visual (vertical *vs.* horizontal lines on walls) and tactile (patterned floor with an overturned paint dish glued to the center of the floor or differently patterned floor with a smooth rectangle glued to the center of the floor) cues which were randomized for visual/tactile cue combinations. The center chamber had white, solid colored walls and a smooth slightly raised platform floor. Time spent in each chamber and motor activity was monitored *via* two sets of photobeams (24 in the horizontal plane and 12 vertical).

Conditioned Place Preference

Rats were transported from the animal housing room to the adjacent test room at least 30min prior to the start of the experiment. Rats were subjected to a 30min pre-test at least 72hr prior to initiating conditioning to determine unconditioned

preference. Pre-test results demonstrated that the box configuration resulted in rats spending unequal amounts of time in each chamber (n=94; time spent 727±38sec *vs.* 950±39sec, $p<0.05$); thus, rats were Meth-paired in the chamber in which they spent the least amount of time during the pre-test. As illustrated in the experimental timelines (Figs. 9A, 10A, & 11A), conditioning occurred over five days; Meth-conditioned rats were given a Meth injection (1mg/kg) every other day for three days (days 1, 3, & 5) and a saline injection (1ml/kg) on the alternate two days (days 2 & 4). This five-day conditioning protocol reliably produces amphetamine- (Shen et al., 2006; Rademacher et al., 2006) and Meth- (Chapters IV& VII and unpublished data) induced CPP. Moreover, alternating the order of Meth administration does not influence preference outcomes and the resulting CPP is similar to that obtained with 6-day conditioning protocol (Chapter VI). During conditioning, immediately after the injection rats were placed into the appropriate chamber of the CPP box for 45min. After conditioning, three experiment-specific protocols were used to determine withdrawal time and treatment duration effects of baclofen on Meth-induced CPP, as described below.

Experiment 1: Early Withdrawal (Short vs. Long-Term Maintenance)

This experiment was designed to ascertain the effects of baclofen on the short-term maintenance of Meth-induced CPP (see Fig. 9A for experimental time line). Rats were assigned to a treatment group based on pre-test outcomes such that time spent in each chamber prior to conditioning was approximately equal between treatment groups. Rats were administered two-once daily home cage

injections (protocol days 6 & 7) of baclofen (2mg/kg) or baclofen vehicle (1ml/kg); one day (protocol day 8) or 11 days (protocol day 18) after the last injection rats were tested for CPP in a drug-free state. For the CPP test, rats were allowed free access to the entire CPP box and time spent in each chamber was monitored for 30min.

Experiment 2: Protracted withdrawal (Long-Term Maintenance)

This experiment was designed to ascertain if baclofen effects on the maintenance of Meth-induced CPP were withdrawal-time dependent (See Fig. 10A for experimental time line). After conditioning, but before the home cage treatments were initiated, rats were given a drug-free CPP test to verify the development of the preference (Fig 10A; protocol day 11). Rats that did not increase time spent in the Meth-paired chamber on CPP Test 1 compared to the same chamber during the pre-test by at least 10% (180sec) were excluded from the study. Culling rats based on the strength of learning (Paolone et al., 2009), helps assure that only those rats that *clearly acquired the task* were used to determine the potential for $GABA_BR$ ligands to subsequently reduce the acquired preference. Rats were assigned to a treatment group such that the magnitude of CPP expressed during CPP Test 1 was approximately equal for both treatment groups. Rats were subsequently administered two once-daily home cage injections (protocol days 16 & 17) of baclofen (2mg/kg) or baclofen vehicle (1ml/kg). One day after the last home cage injection (protocol day 18), rats were

tested in a drug-free state in order to ascertain if preference demonstrated during CPP Test 1 could be disrupted.

Experiment 3: Sub-Chronic Baclofen Administration & the Long-Term Maintenance of Meth-induced CPP

This experiment was designed to ascertain if a longer duration baclofen treatment would inhibit the maintenance of Meth-induced CPP (see Fig. 11A for experimental time line). Rats were tested in a drug-free state three days after conditioning (day 8) and culled using the method described previously to ensure robust initial preference. Rats were assigned to a treatment group such that the magnitude of CPP expressed during CPP Test 1 was approximately equal for both treatment groups. Rats were subsequently administered 10 once-daily home cage injections (protocol days 9-18) of baclofen (2mg/kg) or baclofen vehicle (1ml/kg). Three days after the last home cage injection (protocol day 21), rats were tested in a drug-free state to ascertain if preference demonstrated during CPP Test 1 could be disrupted. This three-day period was imposed to allow the sub-chronic baclofen treatment to be cleared from the system prior to testing so we could determine baclofen effects on memory *maintenance*.

Statistical Analysis

CPP was defined as spending significantly more time in the Meth- *vs.* the saline-paired chamber. For Experiment 1, a two-way ANOVA was used (Treatment x Chamber) to determine the effect of treatment on preference expressed during

the CPP Test. A two-way repeated measures ANOVA was employed for Experiments 2 and 3 (Chamber x Test) to evaluate the changes in preference that occurred before and after home cage treatments (i.e., CPP Test 1 *vs.* CPP Test 2). For all experiments, a *post-hoc* Newman-Keuls test was conducted. Similar approaches were used by Stewart and colleagues (Paolone et al., 2009; Botreau et al., 2006). Time spent in the center compartment was not used for CPP statistical analysis but we verified that time spent in the center chamber was not different between treatment groups or during any test within a treatment group (Student's *t*-test or repeated measures ANOVA, respectively). All data are presented as mean ± standard error of the mean (SEM). Statistical outliers, determined as those rats that spent greater than two standard deviations above or below the mean time spent in any chamber, were not included for analysis.

Results

Experiment 1: Early Withdrawal (Short vs. Long-Term Maintenance)

To determine if processes engaged soon after conditioning could be inhibited by baclofen, two once-daily, post-conditioning, home cage injections of baclofen or vehicle were administered on protocol days 6 & 7 (Fig. 9A) and the rats were tested one day later (protocol day 8). Baclofen did not inhibit the maintenance and subsequent expression of Meth-induced CPP in either protocol. A two-way ANOVA revealed a significant effect of Chamber ($F_{(1,54)}$=33.86, p<0.0001) with a non-significant effect of Treatment ($F_{(1,54)}$=0, p=0.996) and Treatment x Chamber interaction ($F_{(1,54)}$=1.83, p=0.182). A *post-hoc* Newman-Keuls test revealed

significantly more time was spent in the Meth-paired chamber compared to the saline-paired chamber for vehicle (Fig. 9B, n=14, $p<0.001$) and baclofen (Fig. 9B, n=15, $p<0.01$) treated rats. Time spent in the middle chamber was not significantly different between treatment groups (Student's *t*-test, $p>0.05$). Thus, two injections of baclofen administered on the days soon after Meth conditioning failed to alter the expression of CPP one day.

To determine if the time between baclofen administration and CPP testing altered the ability of baclofen to disrupt the preference for the Meth-associated context, another group of Meth-conditioned rats, administered baclofen (2mg/kg) or baclofen vehicle (1ml/kg) on days 6 and 7, were tested for the expression of the preference on day 18. Similar to those tested one day after baclofen, these rats successfully expressed CPP (time spent Meth-paired chamber, 1141±46sec *vs.* time spent in the saline-paired chamber, 455±45sec; paired *t*-test, $p<0.0001$). Findings from these two experiments converge to demonstrate that two injections of baclofen given soon after conditioning (i.e., protocol days 6 and 7) did not inhibit the expression of Meth-induced CPP one or 11 days later.

Experiment 2: Protracted withdrawal (Long-Term Maintenance)

In this experiment, CPP Test 1 was incorporated on protocol day 11 (see Fig. 10A) in order to verify that rats successfully developed the preference for the Meth-paired chamber prior to administration of the home cage treatments and then to allow identification of the strongest learners to provide a more rigorous

evaluation of the ability of baclofen to reduce this learning. As a group, the 30 rats tested expressed CPP (time spent Meth-paired chamber, 896±48sec; *vs.* saline-paired chamber, 758±48sec; paired *t*-test, $p<0.05$); however, six out of 30 rats did not increase time spent in the Meth-paired chamber on CPP Test 1 by 10% (180s) more than time spent in the same compartment during the pre-test, thus they were not tested for antagonism by baclofen.

To determine if baclofen administered during a later withdrawal time (protocol days 16 & 17) would disrupt previously acquired place preference when CPP expression was tested one day later (day 18). Vehicle treated rats (Fig 10B, n=9) expressed a preference for the Meth-paired chamber before (protocol day 11) and after the two home cage vehicle injections (protocol day 18). Two-way repeated measures ANOVA revealed a significant effect of Chamber ($F_{(1,16)}=19.832$, $p=0.0004$) as well as a significant Chamber x Test interaction ($F_{(1,16)}=4.528$, $p=0.049$) with no effect of Test ($F_{(1,16)}=0.020$, $p=0.889$). A *post-hoc* Newman-Keuls revealed significantly more time was spent in the Meth-paired chamber compared to the saline-paired chamber for both tests ($p<0.01$). This indicates that the acquired preference demonstrated during CPP Test 1 was not diminished on CPP Test 2 (day 18); therefore, repeated CPP testing, intervening home cage injections, or withdrawal time did not alter subsequent expression of place preference. The baclofen treatments resulted in similar outcomes obtained with vehicle; i.e., preference maintained after two once-daily injections of baclofen (Fig. 10C, n=13). A two-way repeated measures ANOVA

revealed a significant effect of Chamber ($F_{(1,24)}$=17.483, p=0.0003) with no effect of Test ($F_{(1,24)}$=0.072, p=0.790) or Chamber x Test interaction ($F_{(1,24)}$=0.122, p=0.730). A *post-hoc* Newman-Keuls test revealed significant CPP during CPP Test 1 (p<0.01) and CPP Test 2 (p<0.05). For each test, time spent in the middle chamber did not significantly change for either treatment group (Student's *t*-test, p>0.05). Thus, findings from Experiments 1 and 2 indicate that two once-daily injections of baclofen independent of when they are administered (days 6 & 7 *vs*. 16 & 17) or tested (day 8 or 18) failed to alter the expression of CPP induced by a five-day Meth conditioning protocol.

Experiment 3: Sub-Chronic Baclofen Administration & the Long-Term Maintenance of Meth-induced CPP

As a group, the 32 rats tested expressed CPP during Test 1 (time spent Meth-paired chamber, 999±43sec *vs.* saline-paired chamber, 645±44sec; paired *t*-test, p<0.0001); however, four out of 32 did not increase time spent in the Meth-paired chamber on CPP Test 1 by 10% (180s) more than time spent in the same compartment during the pre-test thus they were not used to evaluate antagonism by baclofen.

In this Experiment, a three day period was imposed between the last home cage injection to allow baclofen to be cleared from the system prior to testing for preference, to clearly delineate between baclofen effects on maintenance (tested in a drug-free state) *vs.* baclofen effects on expression (baclofen present

during the CPP test). Post-conditioning administration of the vehicle (days 9-18) did not impact the ability of rats to express a preference for the Meth-paired chamber (Fig. 11B, n=12). Two-way repeated measures ANOVA revealed a significant effect of Chamber ($F_{(1,22)}$=27.298, *p*<0.0.0001) but no effect of Test ($F_{(1,22)}$=0.051, p=0.823) or Chamber x Test interaction ($F_{(1,22)}$=0.103, p=0.751). A *post-hoc* Newman-Keuls test revealed significant preference for both CPP tests (Fig. 11B; p<0.01). This demonstrated that the acquired preference demonstrated during CPP Test 1 was not diminished on CPP Test 2 (day 21); therefore, repeated CPP testing, intervening home cage injections, and withdrawal time did not alter subsequent CPP. In contrast, CPP that was observed during CPP Test 1 was not maintained after 10 days of home-cage baclofen injections (Fig. 11C, n=13). Two-way repeated measures ANOVA revealed a significant effect of Chamber ($F_{(1,24)}$=20.814, p=0.0001) and a Chamber x Test interaction ($F_{(1,24)}$=5.742, p=0.025) but no effect of Test ($F_{(1,24)}$=0.009, p=0.926). A *post-hoc* Newman-Keuls test reveled significant preference for CPP Test 1 (p<0.01) but not for CPP Test 2 (p>0.05). These findings demonstrate that sub-chronic treatments with baclofen can disrupt the maintenance of Meth-induced CPP. The inability of rats to express a preference for the Meth-paired chamber did not reflect motor deficits engendered by the repeated baclofen treatment for activity during CPP Test 2 was statistically indistinguishable between vehicle (n=12) and baclofen (n=13) treated rats. (Horizontal Activity; vehicle, 3281±291 *vs.* baclofen, 3390±193, Student's *t*-test,

p>0.05. Vertical Activity; vehicle, 593±76 *vs.* baclofen, 554±59, Student's *t*-test, p>0.05.)

Discussion

Results from this study revealed that 10 once-daily home cage injections of baclofen inhibited the maintenance of Meth-induced CPP. The critical feature of this effect appeared to be the sub-chronic exposure as opposed to time during which baclofen was administered or when the CPP was tested, for baclofen given during the first two or last two days of this treatment period were ineffective, and place preference was sustained whether CPP testing occurred one or 11 days after the last injection. These findings suggest that persistent adaptations engendered by repeatedly augmenting $GABA_BR$ signaling (i.e., for more than two days) was sufficient to disrupt processes necessary to maintain Meth-induced associative learning.

Psychostimulant-induced brain adaptations are complex, involving multiple neurotransmitter systems and downstream signaling molecules (Nestler, 2004; Nestler, 2001; McDaid et al., 2006b; McDaid et al., 2007). Contributing to the dysregulated brain state during withdrawal from repeated psychostimulant administration is a decrease in the expression of $GABA_BRs$ (Frankowska et al., 2008b) as well as a reduction in functional coupling of the receptor to the G-protein (Zhang et al., 2000; Kushner and Unterwald, 2001). Withdrawal from repeated psychostimulant exposure is a dynamic process with different brain

states occurring in the hours, days, and weeks after terminating psychostimulant administration. For example, functional coupling of the $GABA_BR$ is decreased in the nucleus accumbens and increased in the cortex at 14 days withdrawal from repeated amphetamine, an effect that is not observed at one day withdrawal (Zhang et al., 2000). The time dependent nature of these changes may translate into periods of time when the brain is more vulnerable, or sensitive, to pharmacological interventions. In the current study, two once-daily injections administered during the two days following conditioning (protocol days 6 & 7) or delayed by 10 days (protocol days 16 & 17) were not able to reveal withdrawal time-dependent effects. This indicates that the brain state during early *vs.* more protracted withdrawal does not impact the ability of two once-daily treatments of baclofen to disrupt the maintenance of Meth-induced CPP. Rather, our findings point to the duration of the baclofen treatment being a critical factor in disrupting maintenance previously acquired place preference.

The maintenance of psychostimulant-induced behaviors is relatively unexplored; the current study has demonstrated for the first time that 10 once-daily injections inhibit the maintenance of Meth-induced associative learning. To the best of our knowledge, no literature has demonstrated baclofen effects on memory maintenance; however, memory acquisition and expression are sensitive to pharmacological disruption by baclofen (Nakagawa et al., 1995; McNamara and Skelton, 1996; Castellano et al., 1989; Swartzwelder et al., 1987; Zarrindast et al., 2002; Zarrindast et al., 2004; Levin et al., 2004; Li et al., 2001; Stackman and

Walsh, 1994). A single study by Bartoletti and colleagues has demonstrated that 10 injections of 2mg/kg baclofen inhibit the maintenance of amphetamine-induced motor sensitization (Bartoletti et al., 2004). As a collective, these findings suggest that 10 injections of baclofen target a mechanism that is engaged during Meth-induced CPP (current results) and amphetamine-induced behavioral sensitization (Bartoletti et al., 2004).

Ten once-daily baclofen injections have influenced the expression of these psychostimulant-induced behaviors by a number of mechanisms. Repeated administration of an agonist may result in receptor down-regulation and/or desensitization. A single baclofen administration (5-30mg/kg) has been reported to dose-dependently decrease $GABA_BR$ expression in gerbil brain when measured 2hr after the injection (Park et al., 2004); however, mRNA expression of the $GABA_BR$ is not altered by a tolerance-inducing baclofen administration protocol (2 x daily 5mg/kg, x 7 days) in rat spinal cord (Sands et al., 2003). Chronic baclofen administration (10mg/kg x 21 days) can functionally down-regulate $GABA_BR$ responses in rat spinal cord (Malcangio et al., 1995). Therefore, in the current study we cannot determine if a change in $GABA_BR$ expression may have contributed to the observed behavioral results; however based on previous studies which either blunt or augment neuronal activity it seems unlikely that reducing inhibitory GABAergic tone would inhibit the maintenance of Meth-induced CPP. Other candidate mechanisms may involve downstream consequences of repeated (i.e., > 2 injections) $GABA_BR$ activation.

$GABA_BR$ negatively regulate signal transduction, ion channel function and gene transcription, *via* inwardly rectifying potassium channels (GIRKs), calcium channels, and cAMP (Bettler et al., 2004; Mott and Lewis, 1994; Barthel et al., 1996). Indeed, increasing the dose and/or duration of baclofen administration appears to have a more profound effect on the downstream consequences of $GABA_BR$ activation. No changes in neuropeptide gene expression (e.g., preprodynorphine and preproenkephalin) were observed with a single systemic injection of 2.5mg/kg baclofen; however, a single large systemic dose (10mg/kg) increases c-Fos expression in specific brain regions (van Nieuwenhuijzen et al., 2009) and seven injections (10mg/kg) alter the expression and activation of the transcription factor cAMP response element binding protein (CREB) (Yin et al., 2006). Activation (i.e., phosphorylation) of transcription factors in limbic brain regions is a molecular consequence of psychostimulant administration (McDaid et al., 2006b; Carlezon et al., 2005; Olson et al., 2005; Berke and Hyman, 2000; Chen et al., 2009; Nestler, 2001) and is also involved in learning in memory processes (Wang et al., 2006; Bailey et al., 1996; Berke and Hyman, 2000; Alberini, 2009). The $GABA_BR$ activation pharmacologically antagonizes events leading to changes in the transcription factor CREB (Yin et al., 2006; Lhuillier et al., 2007) and also normalizes downstream consequences of changes in gene transcription including ΔFosB (Lhuillier et al., 2007) and neuropeptide gene expression (Zhou et al., 2004). Thus, changes in the function of transcription factors provides an overlapping mechanism by which baclofen can alter the maintenance of memory and psychostimulant-induced behaviors.

Conclusion

In summary, we have found that pharmacologically augmenting $GABA_BR$ signaling for 10 days inhibited brain mechanisms necessary to maintain and subsequently express Meth-induced associative learning. The results of these experiments provide insight into the role of the $GABA_BR$ in memory processes engaged during the maintenance of Meth-induced CPP and may be of value as an addiction therapy for abstinent, Meth abusing individuals an effect which may be independent of when the treatment is initiated.

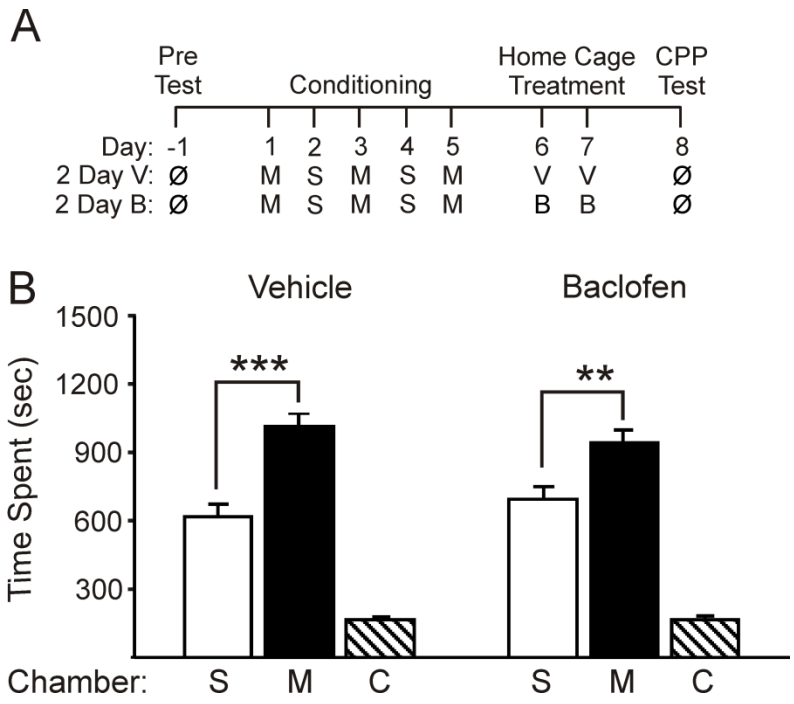

Figure 7. Maintenance of Meth-induced CPP was not altered by two-once daily injections of baclofen administered during the early post-conditioning phase. **A.** Illustration of Experiment 1 treatment protocol. M, methamphetamine (1mg/kg); S, saline (1mg/kg); Ø, no drug; V, baclofen vehicle (0.9% saline, 1ml/kg); B, baclofen (2mg/kg). Data were analyzed with a two-way ANOVA followed by a *post-hoc* Newman-Keuls which compared time spent in the Meth- (filled bar) and saline- (open bar) paired chambers (significance denoted by *** $p < 0.001$ or ** $p < 0.01$). The center compartment (hatched bar) was not included for statistical comparisons. **B.** Rats that received two post-conditioning vehicle injections expressed a preference for the Meth-paired chamber one day later (protocol day 8, left set of bar graphs; n=14). Rats that received two injections of baclofen also expressed CPP (right set of bar graphs; n=15).

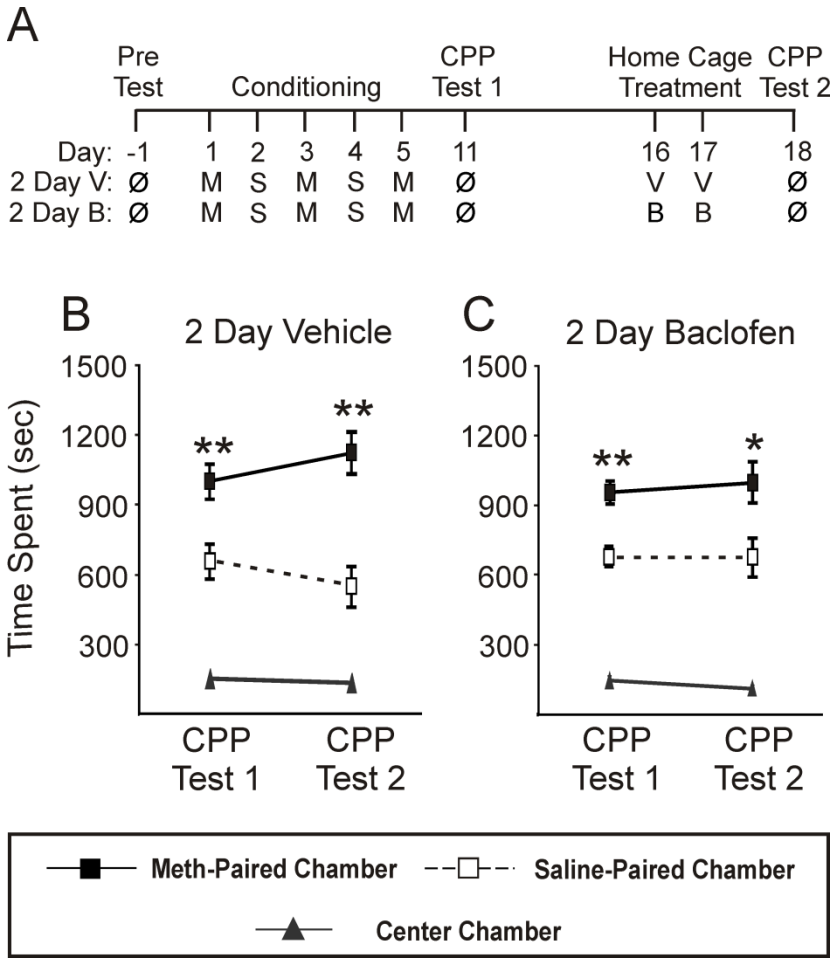

Figure 8. Maintenance of Meth-induced CPP was not inhibited by two once-daily home cage injections of baclofen initiated during the late post-conditioning phase. **A.** Illustration of Experiment 2 treatment protocol. M, methamphetamine (1mg/kg); S, saline (1mg/kg); Ø, no drug; V, baclofen vehicle (0.9% saline, 1ml/kg); B, baclofen (2mg/kg). Data were analyzed with a two-way repeated measures ANOVA followed by a *post-hoc* Newman-Keuls which compared time spent in the Meth- (filled square) and saline- (open square) paired chambers (significance denoted by ** $p<0.01$ or * $p<0.05$). The center compartment (filled triangle) was not included for statistical comparisons. **B.**

Rats assigned to the baclofen vehicle (n=9) expressed CPP on both test days (CPP Test 1 & CPP Test 2). **C.** Rats that received baclofen treatment (n=13) also expressed a preference for the Meth-paired chamber on both test days (CPP Test 1 & CPP Test 2).

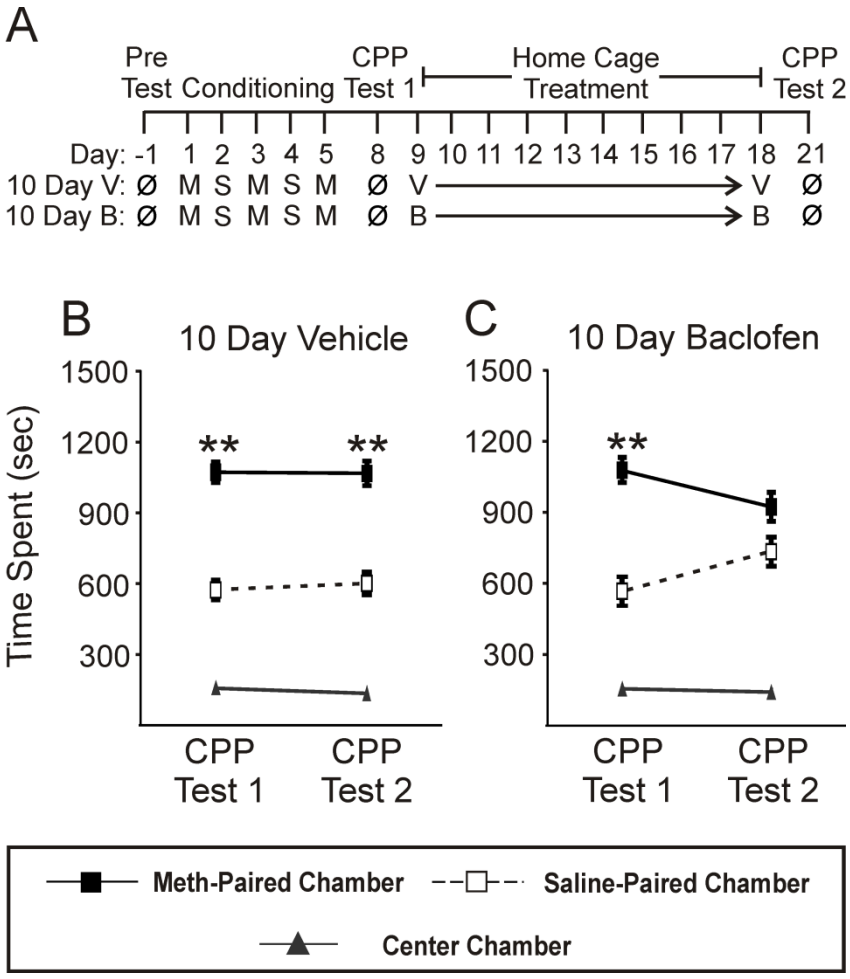

Figure 9. Meth-induced CPP was inhibited by 10 once-daily injections of baclofen. **A.** Illustration of Experiment 3 treatment protocol. M, methamphetamine (1mg/kg); S, saline (1mg/kg); Ø, no drug; V, vehicle (0.9% saline, 1ml/kg); B, baclofen (2mg/kg). Data were analyzed with a two-way repeated measures ANOVA followed by a *post-hoc* Newman-Keuls which compared time spent in the Meth- (filled square) and saline- (open square) paired chambers. (significance denoted by ** $p<0.01$). The center compartment (filled triangle) was not included for statistical comparisons. **B.** Rats assigned to receive baclofen vehicle (n=12) expressed CPP during both CPP tests (CPP Test

1 & CPP Test 2). **C.** Rats that received baclofen treatments (n=13) expressed a preference for the Meth-paired chamber during CPP Test 1 but the preference was no longer evident during CPP Test 2 ($p>0.05$).

CHAPTER V

POST-CONDITIONING ADMINISTRATION OF THE $GABA_B$ RECEPTOR POSTITIVE ALLOSTERIC MODULATOR / Ca^{++} CHANNEL BLOCKER FENDILINE ANTAGONIZES THE MAINTENANCE AND EXPRESSION OF METHAMPHETAMINE-INDUCED CONDITIONED PLACE PREFERENCE

Abstract

The current study evaluated the potential of fendiline, a $GABA_BR$ positive allosteric modulator and L-type calcium channel blocker, to inhibit the maintenance and expression of learned associations between methamphetamine (Meth; the unconditioned stimulus) and a unique environmental context (a conditioned stimulus). Meth (1mg/kg)-induced associative learning was established using a six-day conditioned place preference (CPP) paradigm, and fendiline or its vehicle was administered at various post-conditioning times. The dose selected for fendiline (5mg/kg) did not inhibit motivated motor behavior assessed on the rotarod nor produced rewarding or aversive effects on its own as determined with the CPP/CPA task. In rats demonstrating Meth-induced CPP, two once-daily injections of fendiline did not influence the maintenance of place preference when tested in a drug-free state regardless of the post-conditioning phase or the length of time between terminating the fendiline treatments and the test for preference when tested. In contrast, 10 once-daily fendiline treatments were successful in inhibiting the *maintenance* of the

preference. Following re-conditioning, an injection of fendiline administered immediately prior to the CPP test revealed that *expression* of Meth-induced CPP was inhibited in rats with a treatment history of 10 fendiline injections and rats that received two injections of fendiline corresponding to the last two days of the 10 day treatment. These experiments reveal duration-dependent effects of fendiline on the maintenance of Meth-induced CPP and withdrawal time-after fendiline treatment-dependent effects of fendiline on the expression of Meth-induced CPP. As a reduction in the significance of Meth-associated cues is a means to reduce relapse, fendiline, or other drugs with similar chemical properties may be of value for addiction therapy in abstinent Meth-addicted humans.

Introduction

During repeated psychostimulant administration, associations are made between the rewarding effects of the stimulant and the context in which the drug was administered. After terminating the drug treatments, the brain is hyper-responsive to subsequent re-exposure to psychostimulant-paired cues (Hotsenpiller et al., 2001; Rebec and Sun, 2005; Rebec and Sun, 2005; Hotsenpiller and Wolf, 2002). This hyper-responsivity may be an underlying mechanism contributing to cue-induced drug-craving and seeking in humans (Childress et al., 1999; O'Brien et al., 1998; Childress et al., 1999; Ehrman et al., 1992) and rodents (Crombag et al., 2008). This behavior can be observed in the laboratory using conditioned place preference (CPP) (Childs and deWit H., 2009;

Tzschentke, 2007; Tzschentke, 1998). CPP provides a useful means to explore the potential for pharmacotherapies to reduce cue-elicited drug-craving and –seeking in the abstinent addict. The current study is focused on methamphetamine (Meth). Meth is a potent and highly abused psychostimulant for which no FDA-approved pharmacotherapy is available, therefore; there is a large unmet need to develop an anti-relapse therapy for Meth addiction.

The maladapted brain state that occurs after repeated psychostimulant administration is the consequence of several factors, including a down-regulation of the $GABA_BR$ system (Frankowska et al., 2008b; Kushner and Unterwald, 2001; Zhang et al., 2000) and an up-regulation of L-type calcium channels (Shibasaki et al., 2010; Ford et al., 2009; Nasif et al., 2005a; Nasif et al., 2005b; Hu, 2007). The inhibitory effects of the $GABA_BR$ are mediated by inhibition of high voltage-activated Ca^{++} channels (Mott and Lewis, 1994; Bowery, 1993) thus this provides a unique opportunity for $GABA_BRs$ to blunt the neuronal excitability that occurs as a consequence of the up-regulated Ca^{++} channels. The $GABA_BR$ and the L-type calcium channel have received attention as possible targets for a pharmacotherapy for addiction (Brebner et al., 2002; Xi and Gardner, 2008; Rose and Grant, 2008; Vetulani, 2001). The $GABA_BR$ agonist baclofen inhibits the maintenance and expression of amphetamine-induced behavioral sensitization (Bartoletti et al., 2004; Bartoletti et al., 2005), the expression of Meth-induced CPP (Li et al., 2001), and decreases Meth (Ranaldi and Poeggel, 2002), amphetamine (Brebner et al., 2005) and cocaine self-administration (Filip et al.,

2007; Filip and Frankowska, 2007). A drawback to the practical application of direct acting $GABA_BR$ agonists, such as the baclofen, is sedation and motor impairment (Cryan et al., 2004; Ling et al., 1998; Shoptaw et al., 2003; Heinzerling et al., 2006). Positive allosteric modulators (PAMs) of the $GABA_BR$ selectively augment $GABA_BR$-mediated signaling by acting only where endogenous GABA is already bound to the $GABA_BR$ (Bettler et al., 2004; Urwyler et al., 2001; Gjoni et al., 2006; Urwyler et al., 2005). This action affords PAMs considerable regional and temporal specificity compared to direct acting agonists such as baclofen. Consequently, $GABA_BR$ PAMs present fewer negative side effects than those associated with direct (orthosteric) acting agonists like baclofen. Furthermore, the receptor desensitization and down regulation that occurs with direct agonists is not observed with PAMs (Gjoni and Urwyler, 2008) which is an additional therapeutic advantage as long-term treatment would likely be necessary for an anti-addiction therapy. Like baclofen, $GABA_BR$ PAMs (i.e., CGP7930 and GS39783) reduce the maintenance of Meth-induced CPP (Voigt et al., unpublished data), cocaine self-administration (Smith et al., 2004; Filip et al., 2007) and cocaine- and cue-induced reinstatement of cocaine-seeking (Filip and Frankowska, 2007). Attenuation of stimulant-induced behaviors is also observed with L-type calcium channel blockers which inhibit the expression of nicotine- (Biala, 2003), Meth-, and cocaine-induced CPP (Suzuki et al., 1992), cocaine-induced behavioral sensitization (Martin-Iverson and Reimer, 1994), and attenuates drug-primed reinstatement of nicotine self-administration (Biala and Budzynska, 2008). Collectively, these studies demonstrate that $GABA_BR$ PAMs

and L-type calcium channel blockers modulate stimulant-mediated behaviors. The capacity of these targets to modify the *maintenance and expression* of Meth-induced behaviors was to date, unknown, and thus, is the focus of the current study. We consider the dual actions of fendiline, a $GABA_BR$ PAM (Ong and Kerr, 2005; Ong et al., 2005; Chen et al., 2005) and L-type calcium channel blocker (Tripathi et al., 1993; Nawrath et al., 1998; Bayer and Mannhold, 1987) to be particularly interesting which compelled us to evaluate the ability of fendiline to mitigate previously acquired CPP.

Psychostimulant administration initiates a cascade of down-stream signaling events which change over the course of hours, days, and weeks following the last exposure (Zhang et al., 2000; Zhang et al., 2001; Camp et al., 1997; Ernst and Chang, 2008; Jayaram and Steketee, 2005; McDaid et al., 2006b). For example, functional coupling of the $GABA_BR$ is observed at 14 but not one day withdrawal from repeated amphetamine administration (Zhang et al., 2000). Changes such as these are manifested as different phases of withdrawal which have been observed during the three weeks following the cessation of Meth use (McGregor et al., 2005). The dynamic brain state present after psychostimulant administration presents an opportunity during which the brain may be more sensitive or vulnerable to disruption of mechanisms necessary to maintain and subsequently express psychostimulant-induced behaviors. The current study, evaluated the post-conditioning time and duration-dependent effects of fendiline on the maintenance and expression of Meth-induced CPP.

Materials and Methods

Animals

Male Sprague-Dawley rats (n=118, Harlan, Indianapolis, IN) weighing 225-250g at the start of the study were acclimated to the *vivarium* for at least five days prior to the onset of the experiments. Rats were housed in pairs in a climate-controlled environment on a 12hr light/dark cycle and allowed *ad libitum* access to food and water. Cage mates were given identical pharmacological treatments. Housing facilities are accredited through the Association for Assessment and Accreditation of Laboratory Animal Care, and all experiments were carried out in accordance with the conditions set forth by the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996) and with the approval of the Rush University Medical Center Institutional Animal Care and Use Committee.

Drugs

Conditioning: (+)Methamphetamine HCl (Sigma, St. Louis, MO) was dissolved in 0.9% sterile saline and administrated as 1mg/ml/kg (calculated as the base) and saline was administered as 1ml/kg. Home Cage Treatment: Fendiline [*N*-(3,3-diphenylpropyl)-α-methylbenzylamine] (Sigma, St. Louis, MO) 5mg/ml/kg or fendiline vehicle (25% EtOH) 1ml/kg. Rotarod: Fendiline 5mg/ml/kg; fendiline vehicle 1ml/kg; (+/-)baclofen 4mg/kg/ml (a direct $GABA_BR$ agonist, Sigma, St. Louis, MO); baclofen vehicle (0.9% saline) 1ml/kg; and positive control

pentobarbital 10mg/kg/ml (an agonist of ionotropic $GABA_A$ receptors; Ovation Pharmaceuticals, Inc., Deerfield, IL). All injections were given intraperitoneally (i.p.).

The 5mg/kg dose of fendiline was selected for CPP experiments based on literature demonstrating that this dose provides improved cardiovascular function (e.g., antihypertensive effects) while having a favorable side effect profile in humans (Bayer & Mannhold 1987) and pilot studies conducted in our laboratory (a higher dose of 30mg/kg resulted in adverse side effects in rodents). For example, 4mg/kg baclofen was selected as a $GABA_BR$ positive control for a rotarod motor-control study because it was within the range of doses previously used in laboratory rats to successfully attenuate psychostimulant-induced behaviors including Meth-induced CPP (1.25, 2.5, 5mg/kg, i.p.) (Li et al., 2001) and amphetamine self-administration (1.8, 3.2, 5.6mg/kg, i.p.) (Brebner et al., 2005). As another positive control, we conducted a pilot study to verify that the 10mg/kg pentobarbital dose was below that which is necessary to induce anesthesia or inhibit the righting reflex but was sufficient to induce motor slowing.

Experiment 1: Assessment of motivated motor function

Rotarod assessments were used to ascertain the effects of treatments used in the CPP protocols on motivated motor function requiring a high degree of motor coordination. The rotarod apparatus (San Diego Instruments, San Diego, CA) consisted of four animal lanes (each 11cm wide). The drum (7cm diameter) was

positioned to achieve a 46cm fall height. The rotating drum was accelerated 5-40rpm over 5min. Previously Meth-conditioned rats were trained until all rats met the criterion of remaining on the rotarod for a minimum of 3min. On the test day, a test compound was administered and latency to fall from the apparatus (sec) was measured at the following time points: 0 (immediately prior to injection), 20, 40, 60, 180, 360min, and 24hr after the injection. A repeated measures design was used (all rats were evaluated with each test compound). Rats were "retrained" on the apparatus 24hr prior to each test to ensure that minimum criterion (i.e., had to remain on the rotarod apparatus for a minimum of 3min) was maintained across multiple tests.

Conditioning apparatus

The test room was dimly lit (54-108 lux) with white noise (white noise generator, San Diego Instruments, San Diego, CA) continuously present. The CPP apparatus (AccuScan Instruments, Inc., Columbus, OH) (63cm x 30cm x 30cm) consisted of three chambers divided by Plexiglas sliding doors; two large conditioning chambers (25cm x 30cm x 30cm) were separated by a small center chamber (13cm x 30cm x 30cm). Each chamber had distinct visual and tactile cues (chamber A, vertical lines on walls and an overturned paint dish glued to the center of a randomly patterned floor; chamber B, horizontal lines on walls and a square patterned floor; center chamber, no stripes on walls and a smooth, slightly raised platform floor). Time spent in each chamber and motor activity

was monitored *via* two sets of photobeams (24 in the horizontal plane and 12 vertical in the vertical plane).

Experiment 2: Assessment of fendiline in conditioned place preference/conditioned place aversion

This experiment was designed to test rewarding or aversive properties associated with fendiline. The timeline is illustrated in Fig. 12. The rats were transported from the animal housing room to the adjacent test room at least 30min prior to the start of the experiment. Rats were subjected to a 30min pre-test at least 72hr prior to initiating conditioning to determine unconditioned preference. Pre-test results verified that the box configuration did not engender a bias for either chamber; however individual rats tended to spend more time in one chamber or another other so rats were counterbalanced; half received fendiline in the initially preferred chamber and half in the initially non-preferred chamber. This experimental design was employed because it was unclear if fendiline would produce a preference (CPP) or an aversion (conditioned place aversion, CPA) the counterbalanced design allows for detection of both; accordingly rats which spent more than 75% of the pre-test in one chamber were excluded from further analysis (2 out of 10 rats from the study). Conditioning occurred over 10 days; two conditioning sessions took place each day. Each morning, rats were injected with vehicle (25% EtOH 1ml/kg i.p.) and immediately placed in one CPP chamber for 45min; 4hr later, the other chamber was paired with fendiline (5mg/kg i.p.) for 45min. Three days following the final conditioning

session rats, were given a drug-free CPP test. The three day period between conditioning and the CPP test was imposed to allow for fendiline to be cleared from the system prior to testing for preference. For the 30min CPP test, rats were allowed free access to the entire CPP apparatus and time spent in each chamber and motor activity was monitored.

Experiments 3-5: Methamphetamine-induced conditioned place preference

Rats were pre-tested as described above, and were paired with Meth in the chamber in which they spent the least amount of time during the pre-test. As illustrated in the experimental timelines (Fig. 12B & 12C), conditioning occurred over six days; Meth-conditioned rats were given a Meth injection every other day for three days and a saline injection on the alternate three days. During conditioning, rats were placed into the appropriate chamber of the CPP box immediately after the injection (Meth or saline) for 45min. A 30min, drug-free CPP test was conducted three days after the last conditioning session (day 9). Rats that did not increase time spent in the Meth-paired chamber on CPP Test 1 compared to the same chamber during the pre-test by at least 10% (180s) were excluded from the study. Culling rats based on the strength of learning, as previously employed (Paolone et al., 2009), helps assure that only those rats that *clearly acquired the task* (i.e., made the association between drug and context) were used to determine the potential for $GABA_BR$ ligands to subsequently reduce the acquired preference. Rats were assigned to a treatment group such that the magnitude of preference expressed during CPP Test 1 was approximately equal

for all treatment groups. The effects of fendiline on the maintenance (tested in a drug-free state) and expression (tested immediately after a fendiline injection) were tested in specific protocols described below.

Experiment 3 was designed to ascertain if early post-conditioning treatments of fendiline were sufficient to disrupt the short-term maintenance of previously acquired CPP when measured in a drug-free state. To do so, Meth-conditioned rats were injected in the home cage, once-daily for two days (on protocol days 10 & 11; refer to Fig 12B) with 5mg/kg fendiline or its vehicle. Rats were tested three days after the last home cage treatment using the method described previously. The three day drug-free period was imposed to allow fendiline to be removed from the system prior to testing for preference. *Experiment 4* was designed to determine fendiline effects on the long-term maintenance of Meth-induced CPP when measured in a drug-free state. For this experiment, Meth-conditioned rats received one of the following four post-conditioning treatments to determine the influence of post-conditioning time and treatment duration: (1) 10 days of fendiline vehicle, (2) 10 days of fendiline, (3) two days of fendiline followed by eight days of vehicle, or (4) eight injections of vehicle followed by two days of fendiline. Rats were tested for CPP expression in a drug-free state three days after the last home cage treatment (day 22), as in Experiment 3. We then used these rats to test the effects of fendiline on CPP expression when tested immediately after a fendiline injection, as well as to ascertain if a fendiline history influenced outcomes of this assessment (Experiment 5). To do so, 7-10 days

after CPP Test 2, the rats were 're-conditioned' for four days (protocol days R^1-R^4, Fig 12C) to re-establish preference in all groups and prevent the extinction of the preference that can occur due to repeated testing. Three days after re-conditioning, rats were given a drug-free CPP test (CPP Test 3, protocol day R^7) to verify CPP and the 10% criterion for task acquisition was applied. Three days later, an acute injection of fendiline was administered immediately prior to CPP Test 4 (protocol day R^{10}) and preference was determined as in CPP Tests 1-3. On days when experimental procedures were not conducted (i.e., intervening days are not indicated on the experimental timelines in Fig. 12A, B, & C), rats remained undisturbed in the home cage.

Statistical Analysis

Motor Function: Rotarod assessments were conducted using a repeated measures ANOVA with the within subject factor of time and the between subject factor of treatment followed by a *post-hoc* Newman-Keuls for between group differences at each time point. Motor activity during CPP Test 4 was assessed using a one-way ANOVA followed by a *post-hoc* Newman-Keuls. *Conditioning:* Preference or aversion was defined as spending significantly more time in the drug-treated or vehicle-treated chamber, respectively. This comparison was accomplished by two-way repeated measures ANOVA (factors were chamber and CPP Test) followed by a *post-hoc* Newman-Keuls test for between chamber differences. All data are shown as mean ± SEM. Statistical outliers were determined as those rats with assessments that were greater than two standard

deviations above or below the mean. Using this criterion, 14 rats were removed from the studies for being outliers.

Results

Experiment 1: Effect of fendiline on rotarod performance

The rotarod test was used to determine if fendiline (5mg/kg), or its vehicle (25% EtOH), induced deficits in motivated motor function task (using the natural fear of falling as a motivating factor) that requires a high degree of motor coordination. Baclofen (4mg/kg) and pentobarbital (10mg/kg) were used as positive controls. All rats (n=10) maintained minimum criterion throughout the repeated testing design (refer to methods). Two-way repeated measures ANOVA revealed a significant effect of Time ($F_{(6,270)}$=3.357, p=0.003) as well as a significant Treatment x Time interaction ($F_{(24,270)}$=3.587, p<0.0001) with a non-significant factor of Treatment ($F_{(4,45)}$=1.367, p=0.260). As shown in Fig 13, a *post-hoc* Newman-Keuls test revealed that baclofen (p<0.05) and pentobarbital (p<0.01) administration significantly impaired motivated motor function compared to the saline treatment at 20 and 40min post injection, an effect that was not observed with fendiline or its vehicle (p>0.05). These findings indicate that the 5mg/kg dose used to determine if fendiline could produce CPP or CPA (Experiment 2) and/or alter the maintenance of Meth-induced CPP (Experiments 3-5) was not sufficient to alter even a rigorous motor-function task.

Experiment 2: Assessments of fendiline in conditioned place preference/conditioned place aversion (Refer to Fig. 12A for timeline)

An important consideration for medication development targeted toward chronic therapy for addiction is to determine potential abuse liability or aversive side effects of the putative therapeutic agent. Thus, we evaluated the capacity of fendiline to produce either a preference or an aversion in the conditioning protocol using the 5mg/kg dose used in the rotarod task which did not alter motor function. We designed a conditioning paradigm for fendiline that mimicked the most robust fendiline treatment protocol to be used to disrupt the maintenance of Meth-induced CPP, i.e., 10 once daily injections. Thus, a morning/afternoon conditioning paradigm was employed where saline-pairings were conducted in the morning and fendiline-pairings were conducted in the afternoon. Following 10 days of twice-daily conditioning, rats demonstrated neither a preference nor an aversion for the fendiline-paired chamber (Fig. 14, n=8). That is, a two-way repeated measures ANOVA revealed no significant effects of Chamber ($F_{(1,14)}$=0.009, p=0.928), Test ($F_{(1,14)}$=0.004, p=0.949), or Chamber x Test interaction ($F_{(1,14)}$=0.600, p=0.452). Thus, using the fendiline dose (5mg/kg) identified as having no effects on motivated motor behavior (Fig. 13) or rewarding or aversive subjective effects (Fig. 14) we sought to determine if post-conditioning administration of fendiline inhibited the maintenance and subsequent expression of Meth-induced CPP.

Experiment 3: Effect of fendiline administered during early the post-conditioning phase on short-term maintenance of Meth-induced preference (refer to Fig. 12B for timeline)

Following conditioning with Meth, preference for the Meth-paired chamber was observed before (CPP Test 1) and after (CPP Test 2) two once-daily treatments of vehicle (Fig. 15A, n=8) and fendiline (Fig. 15B, n=9). A two-way repeated measures ANOVA revealed the following. Vehicle-treated rats demonstrated a significant Chamber effect ($F_{(1,14)}$=77.807, p<0.0001) with no effect of Test ($F_{(1,14)}$=0.017, p=0.898) or Chamber x Test interaction ($F_{(1,14)}$=0.121, p=0.733). A *post-hoc* Newman-Keuls test revealed a significant preference during CPP Test 1 and CPP Test 2 (p<0.01). Likewise, fendiline-treated rats demonstrated a significant effect of Chamber ($F_{(1,16)}$=14.746, p=0.001) with no effect for Test ($F_{(1,16)}$=0.120, p=0.733) or a Chamber x Test interaction ($F_{(1,16)}$=1.734, p=0.207). A *post-hoc* Newman-Keuls test revealed a significant preference for the Meth-paired chamber during CPP Test 1 (p<0.01) and during CPP Test 2 (p<0.05). Thus, CPP was expressed independent of treatment history; two days of fendiline administered during the early post-conditioning phase had no impact on the short-term maintenance of Meth-induced CPP.

Experiment 4. Effects of fendiline administration on the long-term maintenance of Meth-induced CPP (Refer to Fig. 12C for timeline)

To ascertain if increasing the number of fendiline treatments would inhibit the maintenance of Meth-induced CPP (tested in a drug-free state), the once-daily

injections of fendiline were increased from two to ten. To verify that Meth-induced CPP persisted for the duration of the study, we determined that post-Meth conditioning administration of ten once-daily *vehicle* injections did not impact the ability of rats to express a preference for the Meth-paired chamber (Fig. 16A, n=11). That is, a two-way repeated measures ANOVA revealed a significant effect of Chamber ($F_{(1,20)}$=63.355, p<0.0001) but a non significant effect of Test ($F_{(1.20)}$=0.165, p=0.689) and Chamber x Test interaction ($F_{(1,20)}$=0.284, p=0.600). This finding indicates the preference exhibited three days after conditioning (CPP Test 1) did not diminish for at least 15 days, due to repeated CPP testing, or with the home cage vehicle treatments. In contrast to these results, in rats administered ten once-daily treatments of fendiline, the preference that was evident during CPP Test 1 was no longer maintained during CPP Test 2 (Fig. 16B, n=12). A two-way repeated measures ANOVA revealed a significant effect of Chamber ($F_{(1,22)}$=11.129, p=0.003) and a significant Chamber x Test interaction ($F_{(1,22)}$=4.800, p=0.039) but no effect of Test ($F_{(1,22)}$=0.035, p=0.853). A *post-hoc* Newman-Keuls test revealed significant CPP for Test 1 ($p<0.01$) but not for CPP Test 2 ($p>0.05$). These results demonstrate that 10 days of fendiline treatment is sufficient to nullify previously established preference.

The positive outcome with ten-days of fendiline led to the question as to whether a critical time during this ten-day period could be identified during which the Meth memories are particularly vulnerable to fendiline-induced disruptions. That is, if

the behavioral antagonism reflected processes that occurred only at the beginning or end of the chronic treatment period. Thus, we tested the effects of two fendiline injections given on days 10 and 11 (2 Day Early Fendiline) or 18 and 19 (2 Day Late Fendiline). Neither 2 Day Early Fendiline (Fig. 16C, n=14) or 2 Day Late Fendiline (Fig. 5D, n=16) influenced the expression of the preference on CPP Test 2. For early fendiline-treated rats, a two-way repeated measures ANOVA revealed a significant effect of Chamber ($F_{(1,26)}$=47.050, p<0.0001) and Chamber x Test interaction ($F_{(1,26)}$=11.056, p=0.003) but no effect of Test ($F_{(1,26)}$=0.003, p=0.956). Two-way repeated measures ANOVA of data collected for the late fendiline-treated rats revealed a significant effect of Chamber ($F_{(1,30)}$=44.607, p<0.0001) with no effect of Test ($F_{(1,30)}$=0.001, p=0.971) or Chamber x Test interaction ($F_{(1,30)}$=0.863, p=0.360). A *post-hoc* Newman-Keuls test revealed a significant preference for the Meth-paired chamber during CPP Test 1 and CPP Test 2 for both treatment groups (p<0.01). Thus, while ten days of once-daily fendiline injections inhibited the maintenance of Meth-induced CPP, this effect did not appear to reflect any particular post-conditioning phase-dependent phenomenon. Thus, it appears that the larger number of treatments is critical for inhibiting the maintenance of Meth-induced CPP.

To verify the conclusions drawn from the rotarod experiment, we also monitored motor activity during the CPP Test 2. The fendiline treatment which terminated three-days prior to CPP Test 2 did not result in any significant changes in motor activity for any treatment group (one-way ANOVA, $F_{(3,52)}$=2.55, p=0.07).

Horizontal activity (horizontal beam breaks); 10 Day Vehicle (n=11) 3649±246, 10 Day Fendiline (n=12) 3373±362, 2 Day Early Fendiline (n=14) 4280±232, 2 Day Late Fendiline (n=16) 4239±190.

Experiment 5: Effects of fendiline administered during the test for expression of Meth-Induced CPP (Refer to Fig. 12C for timeline).

Experiment 5 was designed to ascertain if a 5mg/kg fendiline challenge, administered immediately prior to the CPP test, inhibited CPP expression regardless of the effects fendiline may have had on maintenance. We also set out to determine if fendiline treatment history was an important factor in the effects seen with the fendiline challenge. To accomplish these objectives, all rats used for Experiment 4 were re-conditioned (refer to Fig. 12C) to ensure that CPP was maintained and expressed by all groups (i.e., to re-establish CPP in the 10 Day Fendiline-treated group as well as to assure that extinction of the preference did not occur during repeated CPP testing required for Experiment 4). Thus, following re-conditioning, all rats were subsequently given a drug-free CPP test (CPP Test 3) to verify that the preference was present. The 10% criterion for preference acquisition during CPP Test 3 was applied as described for Experiments 3 and 4. This excluded only 6 rats, and these were distributed approximately equally across treatment groups. Moreover, rats that received the 10 fendiline injections which did not express a preference during CPP Test 2 were not impaired in “re-acquiring” a preference for the Meth-paired chamber compared to other treatment groups. Rats which met the criterion were tested

three days later, immediately after an injection of vehicle or fendiline. Meth-conditioned rats with a vehicle treatment history between CPP Test 1 and CPP Test 2, and who demonstrated preference for the Meth context during CPP Test 3 (i.e., the 10 Day Vehicle group), also showed preference during CPP Test 4 following an acute challenge of vehicle (Fig. 17A, n=9). Two-way repeated measures ANOVA revealed a significant effect of Chamber ($F_{(1,16)}$=18.780, p=0.001) with no effect of Test ($F_{(1,16)}$=0.012, p=0.913) or Chamber x Test interaction ($F_{(1,16)}$=0.113, p=0.741). Indeed, *post-hoc* Newman-Keuls verified significant CPP Test 3 and CPP Test 4 (p<0.01). Similarly, rats in the 2 Day Early Fendiline treatment group expressed preference for the Meth-paired chamber during both drug-free CPP Test 3 and fendiline challenged CPP Test 4 (Fig. 17C, n=12). Two-way repeated measures ANOVA revealed a significant effect of Chamber ($F_{(1,22)}$=14.315, p=0.001) and no effect of Test ($F_{(1,22)}$=0.280, p=0.602) or a Chamber x Test interaction ($F_{(1,22)}$=0.634, p=0.434). A *post-hoc* Newman-Keuls verified the significant preference during both CPP tests (p<0.01). In contrast, rats in the 10 Day Fendiline (Fig. 17B, n=12) or 2 Day Late Fendiline (Fig. 6D, n=14) treatment groups did not express a preference for the Meth-paired chamber after an acute challenge of fendiline. For the 10 Day Fendiline group, a two-way repeated measures ANOVA revealed a significant Chamber x Test interaction ($F_{(1,22)}$=14.483, p=0.001) with non significant effects of Chamber ($F_{(1,22)}$=3.063, p=0.094) or Test ($F_{(1,22)}$=0.011, p=0.916). Likewise, a two-way repeated measures ANOVA of rats in the 2 Day Late Fendiline group demonstrated a significance for the effect of Chamber ($F_{(1,26)}$=7.105, p=0.013)

and Chamber x Test interaction ($F_{(1,36)}$=8.574, p=0.007) but no effect of Test ($F_{(1,26)}$=0.036, p=0.851). A *Post-hoc* Newman-Keuls test revealed significant preference for the drug-free CPP Test 3 (p<0.01) and no preference during the fendiline challenged CPP Test 4 (p>0.05) for both groups. Throughout each CPP test, time spent in the middle chamber did not significantly change in any treatment group throughout any of the experiments. These findings indicate that there may be a critical time that is later in the post-conditioning period, when fendiline causes neuroadaptive alterations that subsequently render an acute fendiline challenge effective in reducing expression of Meth-induced CPP.

As the CPP study indicated that brain adaptations may have occurred following fendiline administration, we also examined motor activity measured during the CPP Test 4 to determine if this behavior also showed changes indicative of neuronal adaptations. A between group comparison, Fendiline injection administered prior to CPP Test 4 significantly altered horizontal motor activity (horizontal beam breaks; one-way ANOVA, p<0.0001). A post-hoc Newman-Keuls revealed that horizontal beam breaks were significantly decreased for all fendiline-treated groups (independent of treatment history) compared to the vehicle treated rats (10 Day and 2 Day Late Fendiline history, p<0.0001; 2 Day Early Fendiline, p<0.001). While all groups had significantly reduced motor activity, it did not impede the capacity of rats to express a preference for the Meth-paired chamber as rats with a treatment history of 2 Day Early Fendiline expressed a preference for the Meth-paired chamber in spite of decreased motor

activity. Illustrated in Fig 17E is horizontal activity but this was representative for all behaviors including total distance and vertical activity.

Discussion

The current study revealed that three conditioning sessions with Meth (alternated with three saline-condition sessions) induced a preference for the Meth-paired chamber that persisted for at least 16 days, and that this preference was not diminished by the home cage injections or repeated CPP testing. The memory of this preference was disrupted by repeated fendiline treatment in a duration-dependent and post-conditioning time-related manner.

To the best of our knowledge, this is the first evaluation of the ability of fendiline to alter behavioral effects of a psychostimulant. Consequently, it was prudent to demonstrate that the dose employed did not alter motivated (i.e., rotarod) motor behavior in Meth-conditioned rats, and that it was not rewarding or aversive. Ten days of fendiline treatment proved to be more important than the post-conditioning phase during which fendiline was administered, as two, once-daily injections of fendiline given either at the early or late post-conditioning phase of the 10 Day protocol failed to diminish the maintenance of Meth-induced CPP. Furthermore, the duration of time between terminating two days of fendiline treatment and the test for preference (compare Experiment 3, three days; Experiment 4, 2 Day Early Fendiline, 11 days) had no effect on the ability of rats to express a preference for the Meth-paired chamber. The findings may reflect

sustained interruptions of mechanisms critical for maintenance and/or adaptations that only occur with repeated treatments (i.e., >2 treatments and does not reflect actions of fendiline administered at the beginning or the end of the 10 day treatment period) of fendiline which serve to weaken the conditioned response memory.

The maintenance of associative memories involves a variety of neurotransmitter systems (e.g., dopamine and glutamate receptors) and concomitant changes in ion channels (e.g., calcium and sodium channels) (Alberini et al., 2006; Bailey et al., 2004; Wang et al., 2006; Vianna et al., 2000). Fendiline, acting as a $GABA_BR$ PAM (Kerr et al., 2002; Ong et al., 2005; Ong and Kerr, 2005) as well as an L-type calcium channel blocker (Bayer and Mannhold, 1987; Nawrath et al., 1998; Tripathi et al., 1993), has dual mechanisms that may serve to alter memory maintenance. Indeed, augmented $GABA_BR$ signaling (*via* decreased cAMP/PKA) (Knight and Bowery, 1996; Malcangio and Bowery, 1993) and calcium channel blockade (*via* calcium calmodulin kinase and ERK) (Rajadhyaksha and Kosofsky, 2005) influence CREB-mediated gene transcription of proteins that are important for psychostimulant-induced and neuronal plasticity as well as learning and memory (Berke and Hyman, 2000; Nestler, 2001; Kelley, 2004). The specific adaptations are dependent on duration of time after the last psychostimulant administration. For example, following repeated Meth administration the activated form of CREB (pCREB) is increased in the cortex at three and not at 14 days withdrawal. In the current study, we

were not able to identify post-conditioning withdrawal time dependent effects of two fendiline injections on the maintenance of Meth-induced CPP when tested in a drug-free state. Future studies focused on molecular and electrophysiological consequences of repeated fendiline are needed to verify the mechanisms that underlie the ability of fendiline to disrupt memory maintenance.

Treatment history is critically important in the ability of an acute fendiline injection to inhibit the expression of Meth-induced CPP. Here, two (2 Day Late Fendiline) and ten fendiline injections both terminating on protocol day 19, but not the 2 day treatment which terminated on protocol day 11, both rendered the acute fendiline challenge effective in blocking preference expression. One explanation is that two once-daily fendiline injections is sufficient to induce an adapted brain state that persists for at least 19 days (2 Day Late Fendiline) but less than 25 days (2 Day Early Fendiline). This adapted brain state is vulnerable to the inhibitory effects of fendiline on the expression of Meth-induced associative learning. The fact that fendiline treatments can produce effects which persist is functionally important, and determining if this reflects pharmacodynamic and/or pharmacokinetic mechanisms is another area that will need to be addressed. The adaptations engendered by two fendiline injections were also maintained after 10 injections which indicate that desensitization and/or down-regulation has not occurred over the additional eight fendiline injections; therefore, this compound may be a viable long-term treatment. Regardless of the underlying mechanisms, it is clear that fendiline treatment history dictated the capacity of a

fendiline challenge to inhibit the expression of Meth-induced CPP, and the time of the treatment, not the number of injections, was the critical factor.

Re-exposure to cues associated with abused substances increases neuronal activity in a region specific manner in humans (Childress et al., 2008; Childress et al., 1999; Kilts et al., 2004; Kilts et al., 2001) and rodents (Brown et al., 1992; Ciccocioppo et al., 2001; Franklin and Druhan, 2000; Zombeck et al., 2008; Rhodes et al., 2005). This activation is also observed with memory recall processes and thus we can infer that blunting neuronal activity may result in an inability to express Meth-induced CPP. Administration of fendiline prior to the CPP test may have blunted the hyper-responsive state that occurs during re-exposure to Meth-associated contextual cues through mechanism such as activation of inwardly rectifying potassium channels ($GABA_BR$ mediated GIRK activation) and inhibition of L-type calcium channels. Indeed, if neuronal activity is blunted by augmenting GABA receptor signaling (*via* administration of gamma vinyl GABA), the expression of Meth-primed reinstatement of CPP is blunted (DeMarco et al., 2009). However, the treatment history dependent effects suggest that prior fendiline-induced adaptations must have occurred in order for the acute fendiline successfully inhibit the expression of the behavior.

An alternative explanation is that the later post-conditioning time (protocol days 18 & 19) is vulnerable to fendiline-induced effects whereas the early post-conditioning time (protocol days 10 & 11) are not. Withdrawal is a dynamic

process with different brain states occurring in the days and weeks following psychostimulant administration. For example, functional coupling of the $GABA_BR$ is decreased in the nucleus accumbens and increased in the cortex at 14 days withdrawal from repeated amphetamine, an effect that is not observed at one day withdrawal (Zhang et al., 2000). Similarly, cocaine-induced sensitization induced subunit-specific and withdrawal-time dependent effects on L-type calcium channel expression and distribution in the cortex; the increase was more profound at 21 days than at three days withdrawal (Ford et al., 2009). Thus, fendiline administered during the different withdrawal time may have different effects on brain function and this factor may have contributed to the effects of treatment history on fendiline inhibition of the expression of Meth-induced CPP.

The goal of this study was to evaluate the ability of fendiline, an L-type Ca^{++} channel blocker and $GABA_BR$ PAM, to disrupt mnemonic processes necessary to maintain and subsequently express the learned associations established during CPP. The results suggest that fendiline successfully disrupted the learned associations between the drug and the cues which is of value as an anti-addiction therapy; both long-term and recent fendiline treatment appears to provide a protective effect in reducing the salience of drug-associated cues and/or the maintenance of the associative memory. Fendiline is clinically available in Europe as a coronary vasodilator and therefore may be repurposed and rapidly translated into the clinic as an anti-addiction therapy.

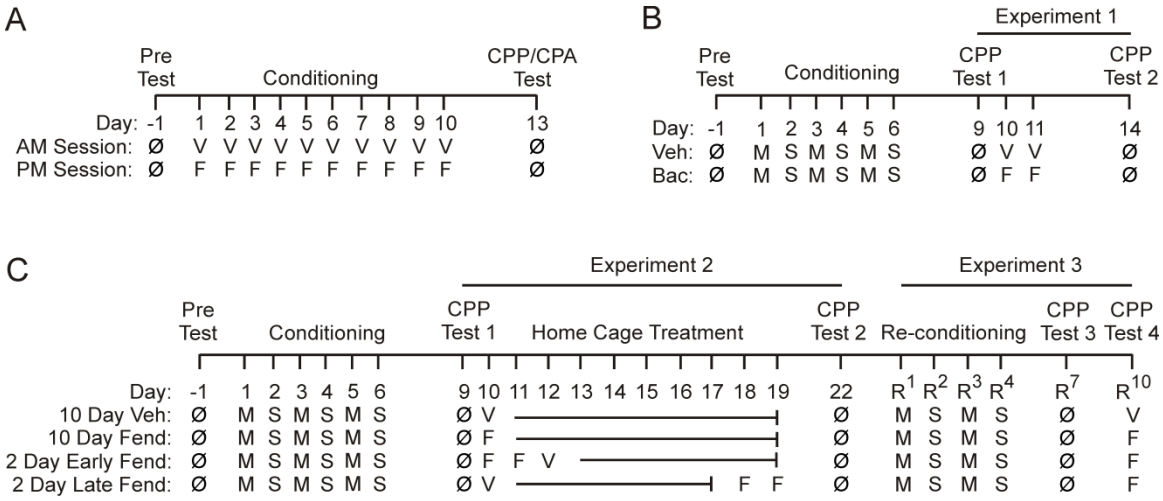

Figure 10. Illustration of treatment protocols. **A.** Fendiline conditioned place preference/conditioned place aversion. For the pre-test (day -1), drug-free rats were allowed to explore the apparatus for 30min; these data were used to counterbalance rats based on unconditioned preference. Conditioning occurred twice daily for 10 days; fendiline vehicle was paired with one chamber in the morning and fendiline was paired in the opposite chamber in the afternoon. Three days after the last conditioning session (day 13), drug-free rats were tested for rewarding or aversive subjective effects of fendiline. **B & C.** Rats were pre-tested as described previously, and assigned to receive Meth in the chamber in which the least amount of time was spent during the pre-test. Conditioning occurred for six days; Meth was paired with one chamber on days 1, 3, & 5, and saline-paired in the opposite chamber on days 2, 4, & 6. Three days later (day 9), rats were tested to confirm the development of CPP (CPP Test 1). Rats were then assigned to one of the following treatment groups. **B.** Experiment 1

evaluated the effects of two home cage injections (days 10 & 11) of fendiline or its vehicle on the maintenance of Meth-induced CPP. Three days after the home cage treatments (day 14), drug-free rats were tested for the expression of Meth-induced CPP (CPP Test 2). **C.** Experiment 2 evaluated the effect of fendiline on the maintenance of Meth-induced CPP by administering 10 once-daily home cage injections (days 10-19): 10 injections of vehicle (10 Day Veh), 10 injections of fendiline (10 Day Fend), two injections of fendiline followed by eight injections of vehicle (2 Day Early Fend), or eight injections of vehicle followed by two injections of fendiline (2 Day Late Fend). Rats were subsequently tested in a drug-free state three days after the last home cage injection (CPP Test 2, day 22). Experiment 3 evaluated the effects of an acute fendiline injection on the expression of Meth-induced CPP. Rats were re-conditioned to re-establish CPP and prevent extinction of the behavior that can occur due to repeated testing. CPP Test 3 was used to verify that rats expressed CPP. Three days later, rats were given an injection of fendiline and immediately tested for the expression of Meth-induced CPP (CPP Test 4). Ø, no drug; V or Veh, vehicle (1ml/kg); F or Fend, fendiline (5mg/kg), M, Meth (1mg/kg); S, saline (1ml/kg).

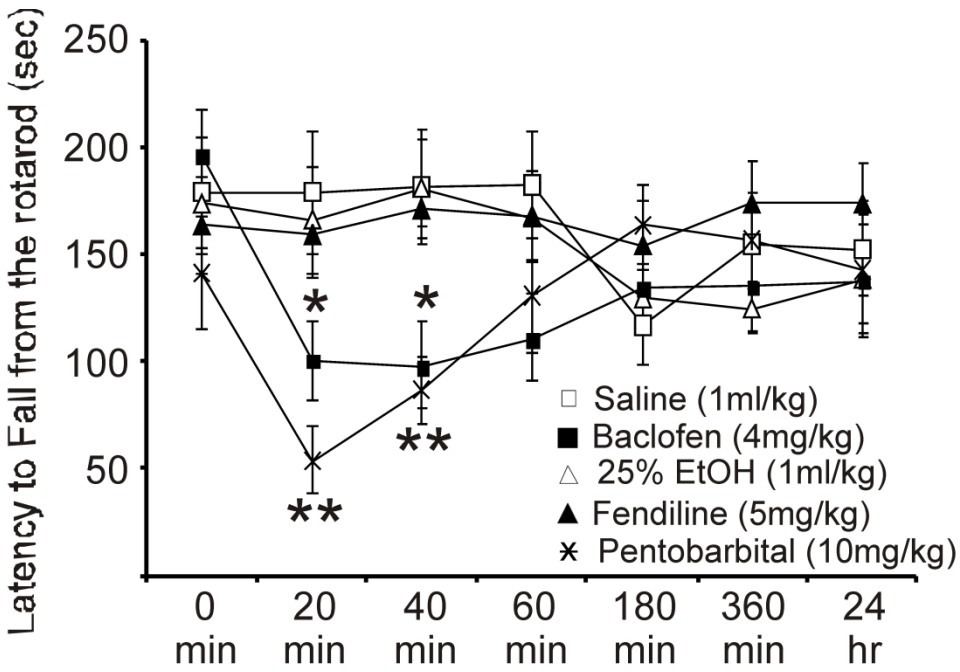

Figure 11. Motivated motor behavior assessed on the rotarod was not inhibited by 5mg/kg fendiline. Rats were trained on the rotarod to meet minimum criterion for inclusion in the study; a repeated measures design was employed such that each rat (n=10) was tested with each ligand or it's vehicle (i.e., saline, fendiline (5mg/kg), fendiline vehicle (25% EtOH, 1ml/kg), baclofen (4mg/kg), and pentobarbital (10mg/kg). Latency to fall from the rotating drum (which was accelerated from 5-40rpm over 5min) was recorded at the following times 0 (immediately prior to injection), 20, 60, 60, 180, 360min, and 24hr. Two way repeated measures ANOVA followed by a *post-hoc* Newman-Keuls revealed that the positive controls, baclofen ($p<0.05$, filled square) and pentobarbital ($p<0.01$, star), significantly impaired motivated motor function compared to saline treated

rats (open square) at 20 and 40min post injection; an effect that was not observed for fendiline ($p>0.05$, filled triangle) or fendiline vehicle rats ($p>0.05$, open triangle).

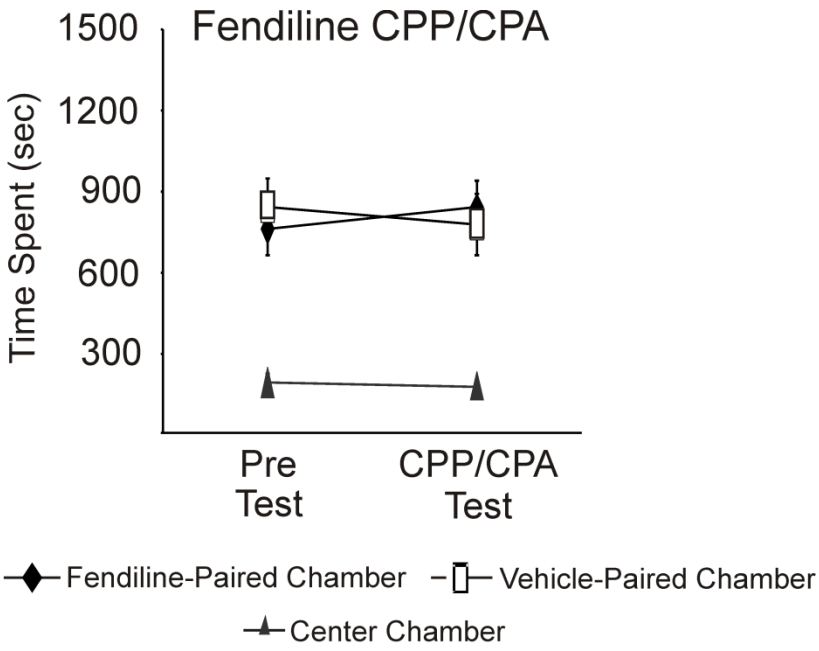

Figure 12. Fendiline (5mg/kg) was neither rewarding nor aversive. Time spent in each chamber was recorded before (Pre-Test) and after 10 days of fendiline (5mg/kg) conditioning (CPP/CPA test). Two-way repeated measures ANOVA followed by a *post-hoc* Newman-Keuls revealed no significant changes between time spent in each chamber during the Pre-Test and the test for preference (n=8, $p>0.05$) (center chamber was not included in the statistical analysis). Filled diamonds, time spent in the fendiline-paired chamber; open square, time spent in the fendiline vehicle-paired chamber; filled triangle, time spent in the center chamber.

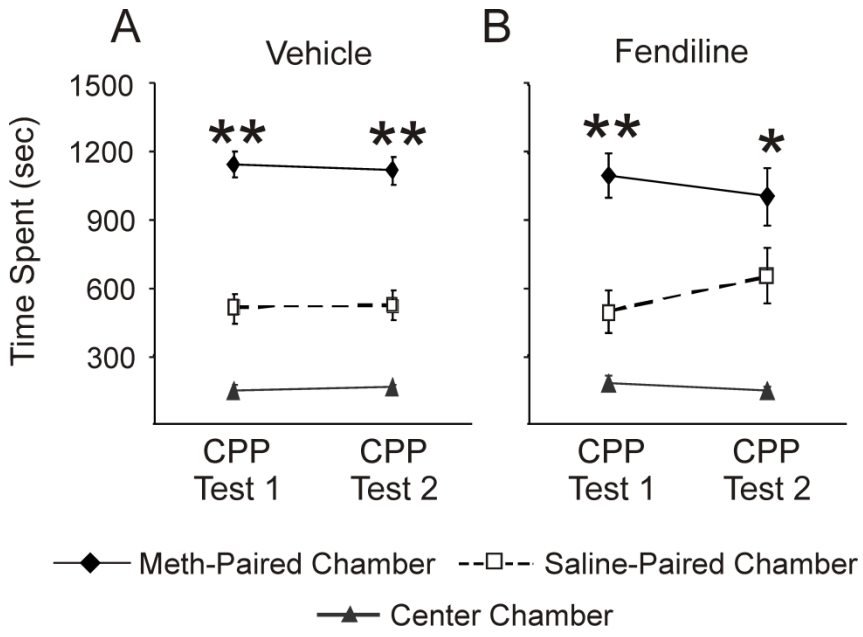

Figure 13. Fendiline administered during the early post-conditioning phase did not inhibit short-term memory maintenance. After six days of conditioning a CPP test was conducted to verify the development of CPP (CPP Test 1); home cage injections were then administered and a drug-free test was subsequently conducted (CPP Test 2). **A.** Rats administered two fendiline vehicle injections (25% EtOH, n=8) expressed CPP on both test days (CPP Test 1 & 2). **B.** Rats that received two once-daily fendiline injections (4mg/kg, n=9) also expressed a preference for the Meth-paired chamber during both tests. Two-way repeated measures ANOVA followed by a *post-hoc* Newman-Keuls test was used to determine between chamber differences (center chamber not included in the statistical analysis), ** $p<0.01$, * $p<0.05$. Filled diamond, time spent in the Meth-paired chamber; open square, time spent in the saline-paired chamber; filled

triangle, time spent in the center chamber.

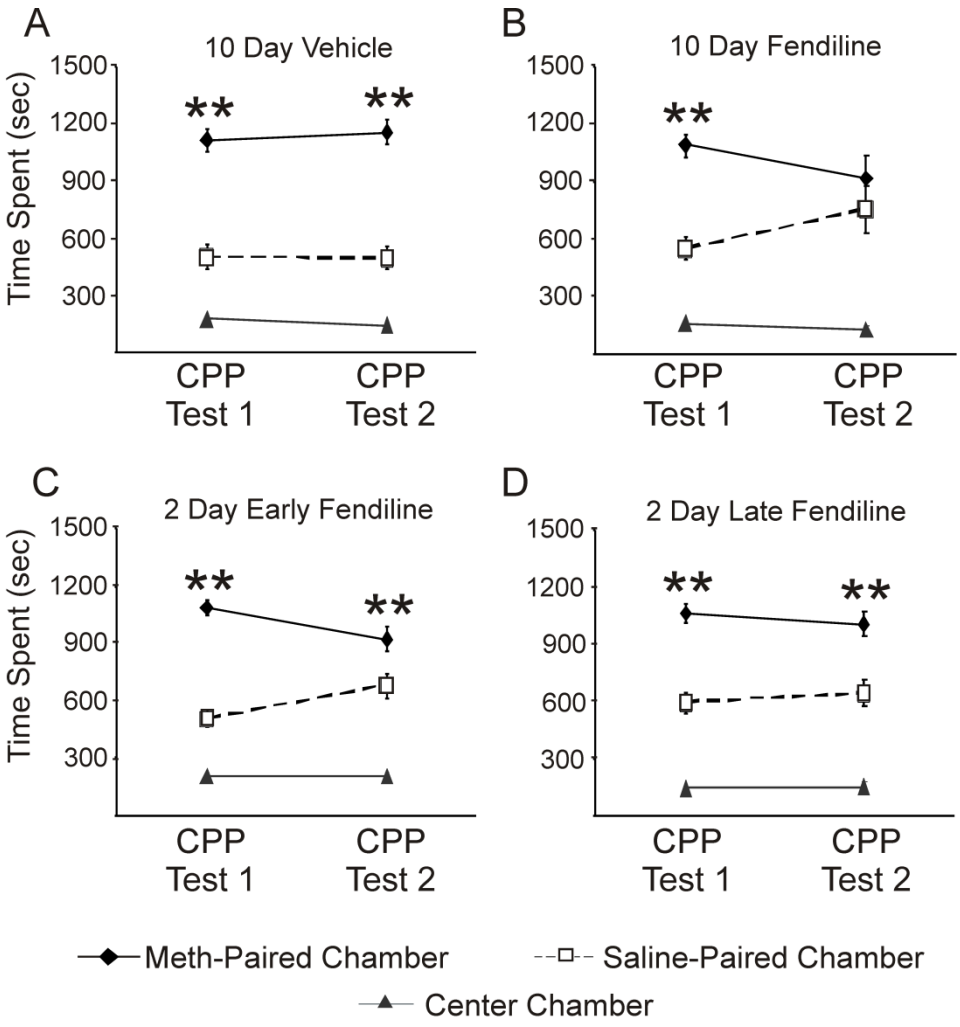

Figure 14. The maintenance of Meth-induced CPP was inhibited by 10 injections but not two post-conditioning injections of fendiline. After six days of conditioning a CPP test was conducted to verify the development of CPP (CPP Test 1); home cage injections were then administered and a drug-free test was subsequently conducted (CPP Test 2). **A.** Rats administered 10 fendiline vehicle injections (25% EtOH, n=11) expressed CPP on both test days (CPP Test 1 & 2).

B. Rats administered 10 once daily fendiline injections (n=12) did not express a preference for the Meth-paired chamber on CPP Test 2. Rats administered 2 once-daily injections of fendiline corresponding to the first two days (**C**, n=14) or the last two days (**D**, n=16) of the 10 day treatment expressed CPP during both CPP Test 1 and CPP Test 2. Two-way repeated measures ANOVA followed by a p*ost-hoc* Newman-Keuls test was used to determine between chamber differences (center chamber not included for statistical analysis), ** $p<0.01$. Filled diamonds, time spent in the Meth-paired chamber; open squares, time spent in the saline-paired chamber; filled triangles, time spent in the center chamber.

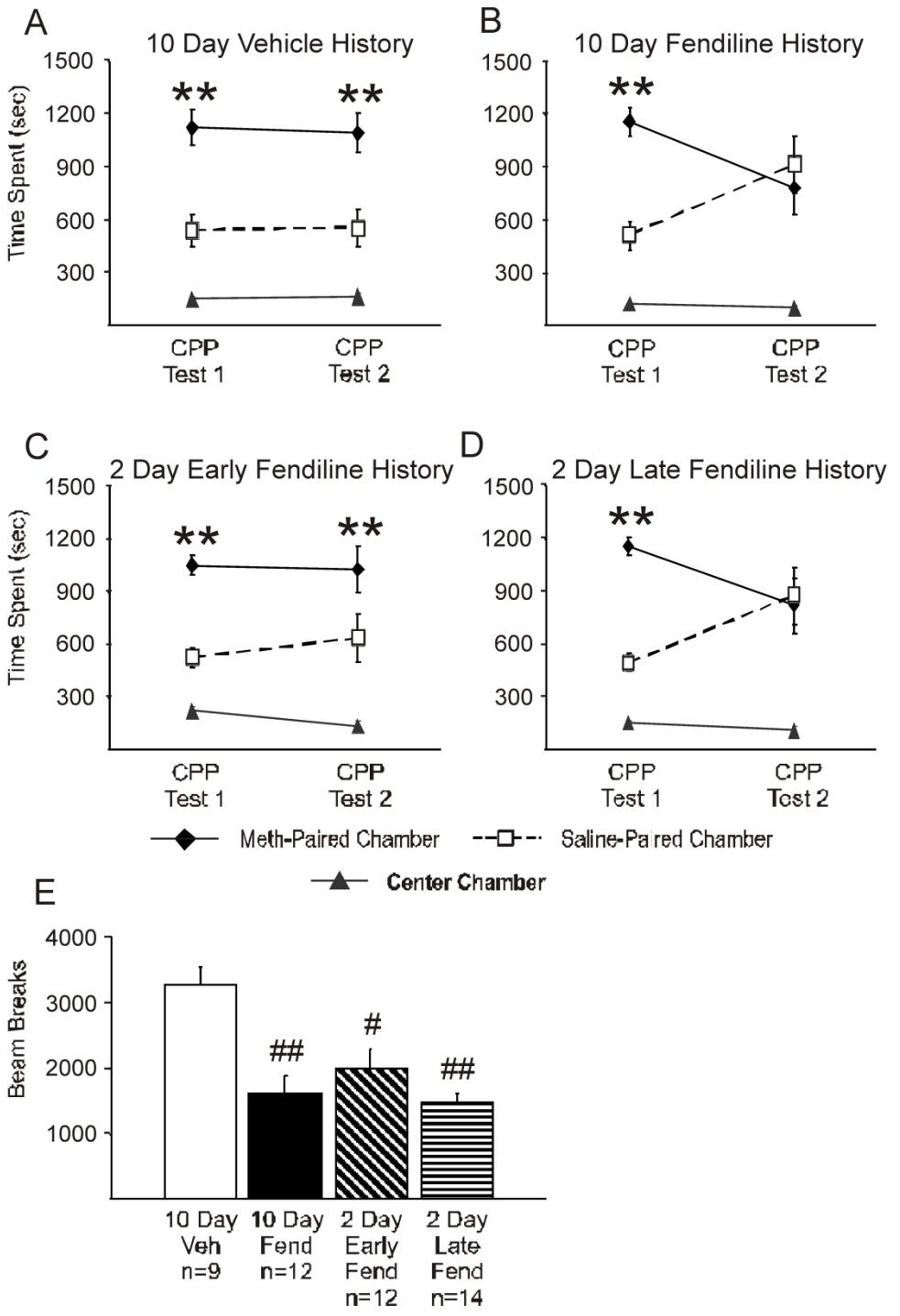

Figure 15. The expression of Meth-induced CPP was inhibited by a fendiline injection administered immediately prior to the CPP test only in rats with a treatment history of 10 injections and two injections corresponding to the last two injections of the 10 day treatment. Rats were re-conditioned to prevent the extinction that can occur after repeated testing and re-establish CPP in the 10

Day Fendiline treated group. CPP Test 3 was conducted drug-free state to verify the expression of CPP. Three days later rats were tested for the expression of Meth-induced CPP after an acute injection of fendiline (5mg/kg) or fendiline vehicle (25% EtOH, 1ml/kg). **A.** Rats with a treatment history of 10 fendiline vehicle injections (n=9) expressed CPP during the drug-free test and after a fendiline vehicle injection (CPP Test 3 & 4). **C.** Likewise, rats with a treatment history of two once-daily fendiline injections corresponding to the first two days of the 10 day treatment expressed CPP during both tests (n=12). In contrast, rats with a treatment history of 10 once daily fendiline injections (**B**, n=12) as well as rats with a treatment history of two once-daily injections of fendiline corresponding to the last two days of the 10 day treatment (**D**, n=14) expressed CPP during the drug-free test (CPP Test 3) but not after the acute fendiline injection (CPP Test 4). Two-way repeated measures ANOVA followed by a p*ost-hoc* Newman-Keuls test was used to determine between chamber differences (center chamber not included for statistical analysis), ** $p<0.01$. Filled diamonds, time spent in the Meth-paired chamber; open squares, time spent in the saline-paired chamber; filled triangles, time spent in the center chamber. **E.** Horizontal Activity assessed during CPP Test 4 revealed that fendiline injection, independent of treatment history, significantly decreased motor activity. One-way ANOVA followed by post-hoc Newman-Keuls, ## $p<0.001$, # $p<0.01$.

CHAPTER VI

ADMINISTRATION OF $GABA_B$ RECEPTOR POSITIVE ALLOSTERIC MODULATORS GS39783 AND CGP7930 BUT NOT BACLOFEN INHIBIT THE MAINTENANCE OF PREVIOUSLY ESTABLIHSED METHAMPHETAMINE-INDUCED CONDITIONED PLACE PREFERENCE

Abstract

Little is known about the role of $GABA_BR$ in the maintenance of memories associated with abused substances. The current study determined if baclofen, and/or $GABA_BR$ positive allosteric modulators, GS39783 and CGP7930, could negate previously established conditioned place preference (CPP) induced by methamphetamine. Post-conditioning, home-cage treatments with GS39783 or CGP7930 antagonized the expression of methamphetamine-induced CPP but baclofen did not. These data indicate that selectively augmenting $GABA_BR$ signaling in areas where GABA is endogenously released after repeated methamphetamine administration may reduce the maintenance and/or the salience of drug-associated cues.

Introduction

Cues associated with the use of abused substances can activate limbic brain regions (Childress et al., 1999; Childress et al., 2008) and elicit drug-craving and

drug-seeking behavior (O'Brien et al., 1992; Ehrman et al., 1992). This powerful, long-lasting associative learning process that occurs in addicts is modeled in rodents using conditioned place preference (CPP) with high face, construct, and predictive validity (Childs and deWit H., 2009; Tzschentke, 1998; Tzschentke, 2007). The current study evaluated CPP induced by the psychostimulant, methamphetamine (Meth).

There is no FDA-approved pharmacotherapy for psychostimulant addiction. However, literature suggests that the $GABA_BR$ system is a viable target (Brebner et al., 2002; Xi and Gardner, 2008). Administration of the $GABA_BR$ agonist baclofen inhibits the *development* and *expression* of CPP induced by Meth (Li et al., 2001) as well as the *development* and *expression* of amphetamine-induced motor sensitization (Bartoletti et al., 2004; Bartoletti et al., 2005). Baclofen also decreases several aspects of Meth (Ranaldi and Poeggel, 2002), amphetamine (Brebner et al., 2005) and cocaine self-administration (Filip et al., 2007) in rodents and cocaine-primed reinstatement of cocaine-seeking in baboons (Weerts et al., 2007). These data demonstrate that baclofen prevents the development of psychostimulant-induced behaviors and subsequent stimulant-seeking. Moreover, imaging studies indicate that baclofen blunts the limbic activation associated with visual drug cues in drug-addicted humans (Brebner et al., 2002). Clinical studies also demonstrate the efficacy of baclofen to reduce cocaine craving (Ling et al., 1998) and reduce cocaine (Shoptaw et al., 2003) and Meth use (Heinzerling et al., 2006). While the use of $GABA_BR$ agonists as a

therapy for addiction has considerable support (Xi and Gardner, 2008; Rose and Grant, 2008), the side effects associated with agonist administration (e.g., sedation and motor impairment) is a drawback to their practical application (Cryan et al., 2004; Ling et al., 1998; Shoptaw et al., 2003; Heinzerling et al., 2006; Jacobson and Cryan, 2005). Positive allosteric modulators (PAMs) of the $GABA_BR$ provide an alternative means to augment GABAergic signaling. $GABA_BR$ PAMs do not directly activate receptors; instead they increase the efficacy of endogenous GABA (Bettler et al., 2004; Urwyler et al., 2001; Gjoni et al., 2006; Urwyler et al., 2005). While $GABA_BRs$ are located throughout the brain, expression levels differ greatly among regions with high expression levels found in brain regions important for reward and motivated behavior (e.g., ventral tegmental area, substantial nigra, and the thalamus) (Margeta-Mitrovic et al., 1999). The differential expression of $GABA_BRs$ and the temporal and regional specificity of GABA release afford PAMs considerable therapeutic discretion compared to $GABA_BR$ agonists.

Recent reports reveal the ability of $GABA_BR$ PAMs to reduce reward-mediated behaviors. The PAM CGP7930 reduces cocaine self-administration (Smith et al., 2004; Filip et al., 2007) and both cocaine- and cue-induced reinstatement of cocaine-seeking (Filip and Frankowska, 2007). The PAM GS39783 blunts locomotion induced by acute cocaine, blocks the development of cocaine-induced motor sensitization, and normalizes molecular adaptations resulting from repeated cocaine administration (Lhuillier et al., 2007). These studies

demonstrate the capacity of $GABA_BR$ PAMs to modulate cocaine-mediated effects; yet, the capacity of PAMs to modify Meth-induced behaviors is unknown.

Administration of GABAergic ligands during the development and expression phases of reward-mediated behaviors provides insight into the role of the $GABA_BR$ system in these behaviors; however, there are limited therapeutic applications for these treatment protocols. A more relevant approach is to administer a potential therapy *after* the brain and behavioral adaptations have taken place. Using this treatment strategy, baclofen successfully inhibits the expression of previously established amphetamine-induced motor sensitization (Bartoletti et al., 2004). We have revealed that a similar strategy with the atypical antidepressant mirtazapine which inhibits the expression of Meth-induced CPP (Herrold et al., 2009) and Meth-induced motor and cellular sensitization (McDaid et al., 2007). These studies illustrate the feasibility of post-conditioning treatments to nullify previously established Meth-induced behavioral effects. The current study used this approach to determine if baclofen and/or PAMs would inhibit the expression of Meth-induced CPP when administered *after* the development of the behavior.

The GABA system undergoes many temporal and region specific adaptations following psychostimulant administration including changes in GABA turnover and receptor-mediated function. These adaptations are influenced by neuronal phenotype, number of drug exposures, and withdrawal duration. During early

withdrawal (<10 days) the $GABA_BR$ system is down-regulated which includes decreased receptor expression (Frankowska et al., 2008b; Frankowska et al., 2008a), uncoupling of the $GABA_BR$ from the G-protein (Kushner and Unterwald, 2001), and increased extracellular GABA concentrations (Jayaram and Steketee, 2005; Jayaram and Steketee, 2005; Bustamante et al., 2002). In the current study, the $GABA_BR$ ligands baclofen, GS39783, and CGP7930 were administered during the early withdrawal phase (days 5 and 6) and we hypothesized that augmenting $GABA_BR$ signaling during this phase would reduce the salience of Meth-associated cues.

Materials and Methods

Animals

Male Sprague-Dawley rats (n=73, Harlan, Indianapolis, IN) weighing 250-300g at the start of the study were acclimated to the *vivarium* for at least one week prior to the onset of the experiments. Rats were housed in pairs in a climate-controlled environment on a 12 hr light/dark cycle and allowed *ad libitum* access to food and water. Cage mates were given identical pharmacological treatments. Housing facilities at Rush University are accredited through the Association for Assessment and Accreditation of Laboratory Animal Care, and all experiments were carried out in accordance with the conditions set forth by the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996) and with the approval of the local Institutional Animal Care and Use Committee.

Drugs

Conditioning: (+)Methamphetamine HCl (Sigma-Aldrich, St. Louis, MO) was dissolved in 0.9% sterile saline and administrated as 1mg/ml/kg. We have demonstrated that this dose (as opposed to, e.g., 0.1mg/kg) reliably produces an enduring CPP (unpublished data). Saline was administered as 1ml/kg. Home cage injections: baclofen (2mg/kg; Sigma, St Louis, MO), baclofen vehicle (0.9% saline; 1ml/kg), GS39783 and CGP7930 (30mg/kg; gift from Novartis Pharma AG, Basel, Switzerland), PAM vehicles (10% propylene glycol in sterile water; 1ml/kg). All injections were given intraperitoneally (i.p.).

Doses of GABAergic ligands were selected based on the following behavioral endpoints in laboratory rats. The dose of 30mg/kg GS39783 and CGP7930 successfully reduces cocaine self administration when administered 10 min prior to the session (Smith et al., 2004) (3, 10, 30mg/kg i.p.). The dose of 2mg/kg baclofen was selected because it was within the range of doses found to successfully i) attenuate the *development* and *expression* of Meth-induced CPP when administered 30min prior to each daily conditioning session or CPP test, respectively (doses tested were 1.25, 2.5, 5mg/kg, i.p.) (Li et al., 2001), ii) inhibit the *reinstatement* of nicotine-induced CPP and self-administration when administered 5min prior to testing (0.612, 1.25, 2.5mg/kg, i.p.) (Fattore et al., 2009), iii) reduce cocaine self-administration when administered 10min prior to the session (2.5mg/kg, i.p.) (Smith et al., 2004), iv) reduce amphetamine self-

administration break point when administered 30min prior to testing (doses tested were 1.8, 3.2, 5.6mg/kg, i.p.) (Brebner et al., 2005), and v) inhibit the *maintenance* of amphetamine-induced motor sensitization (2mg/kg, i.p.) (Bartoletti et al., 2004). In addition, we determined that motivated motor behavior assessed on the rotarod was inhibited by 4mg/kg baclofen (20 and 40min collapsed results were as follows; latency to fall 209±24sec (n=5), *vs*. 355±29sec for vehicle (n=11); Student's *t*-test, $p<0.01$). Additionally, while 3mg/kg baclofen produced an impairment in spontaneous motor activity, 2mg/kg baclofen resulted in no difference from vehicle (horizontal activity, photobeam breaks over 75min post injection for vehicle (n=7), 2422±189; baclofen 2mg/kg (n=6), 1728±388; baclofen 3mg/kg (n=6), 1067±258; ANOVA p=0.012, *post-hoc* Dunnett's).

Apparatus

The apparatus used to monitor activity consisted of three chambers divided by opaque Plexiglas sliding doors (AccuScan Instruments, Inc., Columbus, OH); two large conditioning chambers (25cm x 30cm x 30cm) separated by a small center chamber (13cm x 30cm x 30cm). Each chamber had distinct visual (horizontal or vertical stripes or a solid color wall) and tactile cues (textured floors). Motor activity and time spent in each chamber was monitored *via* two sets of photobeams (24 horizontal and 12 vertical).

Conditioned Place Preference

The behavioral testing room was dimly lit (54-108lux) with white noise continuously present. The rats were transported from the *vivarium* to the test room (located across the hall, in the same animal facility suite) at least 30min prior to the start of the experiment. The protocol consisted of four phases (refer to Figure 18): pre-test, conditioning, home-cage intervening treatments, and a CPP test. A 30min pre-test, verified that the box configuration did not result in a significant group preference for either chamber (time spent left: 789±42s, time spent right: 883±42s, p=0.265). Individual rats tended to spend unequal amounts of time in each chamber; thus, for conditioning, rats were administered Meth in the chamber in which they spent the least amount of time during the pre-test. Conditioning occurred over 6 days. During the conditioning phase, rats were given a Meth injection (1mg/kg, i.p.) every other day (days 1, 3, and 5) and immediately placed into the appropriate chamber for 45min. On the alternate days (2, 4, and 6), rats were given a saline injection (1ml/kg, i.p.) and immediately placed into the opposite chamber for 45min. In half the rats, the order of Meth or saline pairing occurring first was switched such that saline was administered on days 1, 3, and 5 and Meth on days 2, 4, & 6. A *drug-free* CPP test was performed on day 9, (termed CPP Test 1) to confirm that the preference developed. This was accomplished by placing rats into the center chamber and the sliding doors were immediately removed allowing free access to the entire CPP box. The test session lasted 30min and time spent in each chamber was determined. There were no differences between those rats which received the

Meth or the saline pairing first thus they were pooled for the analysis. Rats that did not increase time spent in the Meth-paired chamber on CPP Test 1 compared to the same chamber during the pre-test by at least 10% (180s) were excluded from the study. This culling procedure, as previously shown by others (Paolone et al., 2009) helps assure that only those rats that *clearly acquired the task* were used to determine the potential for $GABA_BR$ ligands to subsequently reduce the acquired preference. Based on the results generated during CPP Test 1, rats were assigned to one of the following once-daily home cage treatments (days 10 & 11) such that the expression of the preference was approximately equal across all treatment groups which included: vehicle, baclofen, GS39783, or CGP7930. Selection of the early withdrawal time treatment (days 10 & 11) was based on literature demonstrating that the $GABA_BR$ system is down-regulated during early withdrawal times (Frankowska et al., 2008b; Kushner and Unterwald, 2001) and therefore might benefit from augmented $GABA_BR$ signaling. While the pharmacokinetics of GS39783 and CGP7930 are unknown, orally administered baclofen (5mg/kg) elicits centrally mediated events including hypothermia (Cryan et al., 2004) within one hour after baclofen administration suggesting that the onset of action is relatively short. Plasma and brain concentrations of baclofen are reported to be relatively consistent for at least 180min after a single bolus i.v. injection of baclofen in the rat (50mg/kg) (Deguchi et al., 1995) which aligns with enduring effects of GABAergic ligands on drug-induced behaviors. Baclofen (2.5mg/kg), GS39783 (3mg/kg), and CGP7930 (30mg/kg) significantly reduce cocaine self administration for at least 10 hours, indicating that these ligands may

actively alter brain processes for several hours after a single administration (Smith et al., 2004). Therefore, in the current study the administration of ligands once-daily for two days likely altered GABAergic signaling for a sustained period of time. Three days after the home cage treatments (day 14), rats were tested for the expression of CPP in a drug-free state (termed CPP Test 2). This period aided in clearing the $GABA_BR$ compounds from the rat prior to CPP testing to avoid any potential influences of residual drug on the *expression* of CPP. Thus, this approach allowed us to more confidently interpret the effects of $GABA_BR$ ligands in terms of memory *maintenance*.

Statistical Analysis

CPP was defined as spending significantly more time in the Meth-paired *vs.* saline-paired chamber. This was accomplished by two-way repeated measures ANOVA (factors were Chamber and CPP Test) followed by *post-hoc* Newman-Keuls for between chamber differences for each test. All data are shown as mean ± SEM. Statistical outliers were determined as those rats that spent greater than two standard deviations above or below the mean time spent in any chamber.

Results

The 6 day conditioning protocol resulted in a significant preference for the Meth-paired chamber. As a group, the 73 rats tested expressed CPP on Test 1 (day 9) (time spent Meth-paired chamber, 970±33sec; time spent saline-paired chamber,

675±32sec; paired *t*-test: p<0.0001); however, 13 rats were not tested for antagonism by $GABA_BR$ ligands because time spent in the Meth-paired chamber on CPP Test 1 was not least 10% (180s) more than time spent in the same compartment during the pre-test. This culling procedure helped assure that only those rats that *clearly acquired the task* were used to determine the potential for $GABA_BR$ ligands to subsequently reduce the acquired preference (see Materials & Methods).

Post-conditioning administration of the vehicle solutions did not impact the ability of drug-free rats to express a preference for the Meth-paired chamber (Figure 19, Baclofen Vehicle (n=9): Chamber, $F_{(1,16)}$=23.850, p=0.0002; Test, $F_{(1.16)}$=0.0001, p=0.994; Chamber x Test Interaction, $F_{(1,16)}$=2.572, p=0.128. Figure 20, PAM Vehicle (n=14): Chamber, $F_{(1,26)}$=18.759, p=0.0002; Test, $F_{(1,26)}$=0.065, p=0.801; Chamber x Test Interaction, $F_{(1,26)}$=2.05, p=0.164). This demonstrated that the acquired preference demonstrated three days after conditioning did not diminish for at least five days, due to repeated CPP testing, or with the intervening home cage injections.

Rats administered baclofen as an intervening treatment maintained CPP (Figure 19, Baclofen (n=8): Chamber, $F_{(1,14)}$=25.326, p=0.0002; Test, $F_{(1,14)}$=0.055, p=0.818; Chamber x Test Interaction, $F_{(1,14)}$=6.167, p=0.026). In contrast, administration of the $GABA_BR$ PAMS, GS39783 and CGP7930 nullified previously established preference; i.e., the preference for the Meth-paired

chamber observed on CPP Test 1 was no longer evident on CPP Test 2 (Figure 3. GS39783 (n=11): Chamber, $F_{(1,20)}$=11.141, p=0.003; Test, $F_{(1,20)}$=0.0002, p=0.989; Chamber x Test Interaction, $F_{(1,20)}$=6.947, p=0.016. CGP7930 (n=9): Chamber, $F_{(1,16)}$=3.506, p=0.080; Test, $F_{(1,16)}$=0.002, p=0.965; Chamber x Test Interaction, $F_{(1,16)}$=9.108, p=0.008.)

Throughout each test, (Pre-Test, CPP Test 1, CPP Test 2) time spent in the middle chamber did not significantly change in any treatment group (Figures 19 & 20).

These data reveal the unique ability of post-conditioning injections of the $GABA_BR$ PAMS (administered while the rats remained in the home cage) to diminish the expression of the previously established preference.

Discussion

This study revealed that $GABA_BR$ PAMs administered to rats in the neutral environment of the home cage was sufficient to nullify the previously expressed preference for the Meth-paired chamber. This outcome was not obtained with the direct $GABA_BR$ agonist, baclofen. At the dose selected, GS39783 and CGP7930 are thought to act only at $GABA_BRs$ that are occupied by endogenous GABA (Gjoni et al., 2006; Urwyler et al., 2001). Therefore, these findings indicate that augmenting the efficacy of occupied $GABA_BRs$ during early phases

of withdrawal is sufficient to disrupt the maintenance of the acquired salience for the Meth-associated context.

There are numerous studies demonstrating the efficacy of baclofen, GS39783, and CGP7930 to modulate the development and expression of behaviors induced by psychostimulants when administered *during* conditioning, or within 30min prior to testing (Li et al., 2001; Bartoletti et al., 2005; Filip et al., 2007; Filip and Frankowska, 2007; Weerts et al., 2007; Smith et al., 2004; Lhuillier et al., 2007; Brebner et al., 2005; Fattore et al., 2009). However, to date only Bartoletti and colleagues (Bartoletti et al., 2004) have demonstrated efficacy of a $GABA_BR$ ligand to modify the *maintenance* of a previously established psychostimulant-induced behavior. These authors report that the maintenance of amphetamine-induced motor sensitization is blunted when 10 administrations of 2mg/kg baclofen were initiated 36 days after the behavior developed and terminated 20 days prior to the amphetamine challenge. Our research efforts are expanding this literature by evaluating $GABA_BR$ influences on the maintenance of place preference memory induced by Meth. The inhibitory effects previously observed for baclofen on the maintenance of *amphetamine-induced motor sensitization* were not observed in the current study for the maintenance of *Meth-induced CPP* when 2mg/kg baclofen treatments were initiated 4 days after Meth, and given once-daily for two days after conditioning. This discrepancy may be due to several factors including different behavioral endpoints (motor sensitization *vs.* CPP), different psychostimulant (amphetamine *vs.* Meth), different treatment

duration, and/or different withdrawal time for initiating the baclofen treatment (36 days *vs.* 4 days). Thus, the possibility still remains that a different treatment regimen might afford a viable window of opportunity by which baclofen may be able to interrupt the maintenance of place preference memories for Meth.

The PAMs GS39783 and CGP7930 successfully inhibited the maintenance of previously established CPP. As PAMs act by positively modulating GABA-occupied receptors, this suggests that during the early withdrawal period, regions that endogenously release GABA have a sufficient number of functional $GABA_BRs$ to allow PAMs to inhibit the maintenance of place preference memory. This conclusion is consistent with the recent demonstration that enhancing GABA levels (*via* reducing its metabolism with *gamma* vinyl GABA, an irreversible inhibitor of GABA transaminase), inhibits the expression of Meth-primed reinstatement of CPP (administered 2.5hr prior to the Meth challenge) (DeMarco et al., 2009). Thus, it may be that augmenting $GABA_BR$ signaling in brain regions where GABA is tonically released is particularly important for disrupting memory maintenance.

Several laboratories report *region specific* changes in GABA turnover, $GABA_BR$ expression, and receptor-mediated function during the early days following repeated psychostimulant exposure. Examples of increased GABAergic tone occurring within the first seven days of withdrawal include increased extracellular GABA levels in the medial prefrontal cortex after repeated cocaine administration

(Jayaram and Steketee, 2005) and enhanced substantia nigra and striatum K^+-evoked GABA release after repeated Meth administration (Bustamante et al., 2002). These observations may be the consequence of the brain attempting to maintain homeostasis (i.e., blunt the neuronal hyper-excitability associated with psychostimulant use). Possibly contributing to the hyper-excitability associated with psychostimulant administration, $GABA_BRs$ are reduced throughout the brain of rats after cocaine self-administration (Frankowska et al., 2008b) and in the ventral tegmental area a decrease in the functional coupling of $GABA_BRs$ is observed (Kushner and Unterwald, 2001). Changes such as these may contribute to the hyper-excitable state of the brain observed during psychostimulant withdrawal (Hu, 2007). Behavioral observations made the current study suggest that the "new brain state' induced by Meth conditioning includes GABA-occupied $GABA_BRs$ in regions critical for the maintenance of Meth-associated memories that are selectively enhanced by PAMs. The negative findings for baclofen then suggest that activating $GABA_BRs$ throughout the brain (regardless of endogenous GABAergic tone) overrides the selective augmentation of occupied $GABA_BRs$ achieved by administration of the PAMs.

The current study focused on the short-term maintenance of Meth-associated memories, a phase of mnemonic processing that is not widely studied with regard to $GABA_BR$ influences. In contrast, there is ample literature demonstrating that administration of baclofen alters other mnemonic processes including acquisition (Nakagawa et al., 1995; McNamara and Skelton, 1996),

consolidation (Castellano et al., 1989; Swartzwelder et al., 1987; Castellano et al., 1989; Zarrindast et al., 2002; Zarrindast et al., 2004), and expression (Stackman and Walsh, 1994; Stackman and Walsh, 1994; Levin et al., 2004; Li et al., 2001) of spatial and/or associative learning tasks. We determined that a dose of baclofen that was subthreshold to impairing motor function was not sufficient to inhibit the maintenance of CPP when given as two once-daily injections. Therefore, while moderate doses of baclofen effectively inhibit memory acquisition, consolidation, and expression it does not appear to be effective to inhibit memory maintenance during the first few days of withdrawal. Phase-dependent effects also observed with the PAM GS39783 which was shown to inhibit the acquisition but not the expression of nicotine-induced CPP (Mombereau et al., 2007). The current work extended this literature by demonstrating that selective positive modulation of activated $GABA_BRs$ by GS39783 and CGP7930 can also inhibit the early phase maintenance of drug-associated memories.

In summary, we have identified two structurally distinct $GABA_BR$ PAMs, GS39783 and CGP7930, which inhibit the expression of Meth-induced CPP when administered during the early withdrawal phase. This information allows us to infer that $GABA_BR$ expressing neurons in regions that have endogenous GABAergic tone are critical for the maintenance of the associative memory that forms during Meth-induced CPP. Thus, $GABA_BR$ PAMs may be a viable

therapeutic alternative to direct acting $GABA_BR$ agonists for the treatment of psychostimulant addiction.

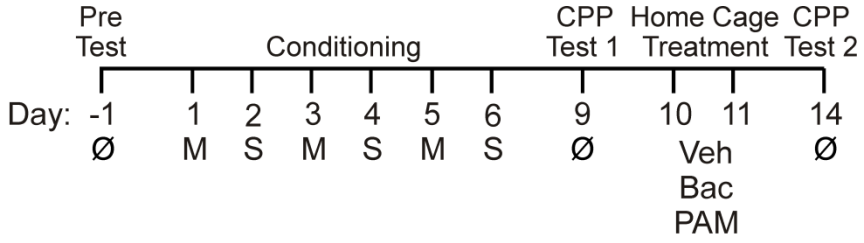

Figure 16. Illustration of the treatment protocol. A drug-free pre-test was conducted and rats were assigned to receive Meth (1mg/kg, i.p.) in the initially non-preferred chamber. Conditioning occurred on days 1–6. A drug-free CPP test was conducted on day 9 to verify the development of CPP (CPP Test 1). Vehicle, baclofen, or a PAM was administered in the home cage on days 10 & 11. A final drug-free CPP test (CPP Test 2) was conducted on day 14 to determine the capacity of GABAergic ligands to influence the expression of Meth-induced CPP. M, methamphetamine (1mg/kg), S, saline (1mg/kg), Veh, vehicle (1ml/kg), Bac, baclofen (2mg/kg), PAM, positive allosteric modulator: GS39783 (30mg/kg), CGP7930 (30mg/kg)

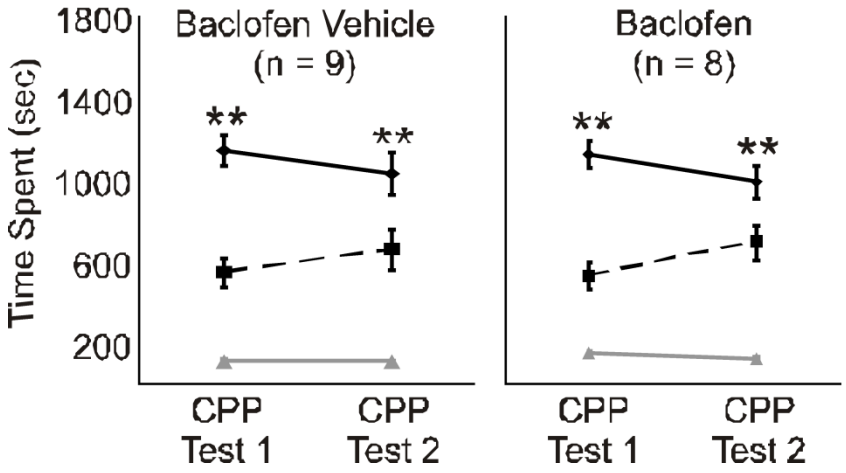

Figure 17. Meth-induced CPP was not inhibited by post-conditioning administration of baclofen. Rats assigned to the baclofen vehicle (0.9% saline) group (n=9) expressed CPP on both test days (CPP Test 1 & 2). Rats that received baclofen treatment (2mg/kg; n=8) also expressed a preference for the Meth-paired chamber on both test days. *Post-hoc* Newman-Keuls test was used to determine between chamber differences (center chamber not included for statistical analysis), ** $p<0.01$. Solid line, time spent in the Meth-paired chamber; dashed line, time spent in the saline-paired chamber; grey line, time spent in the center chamber

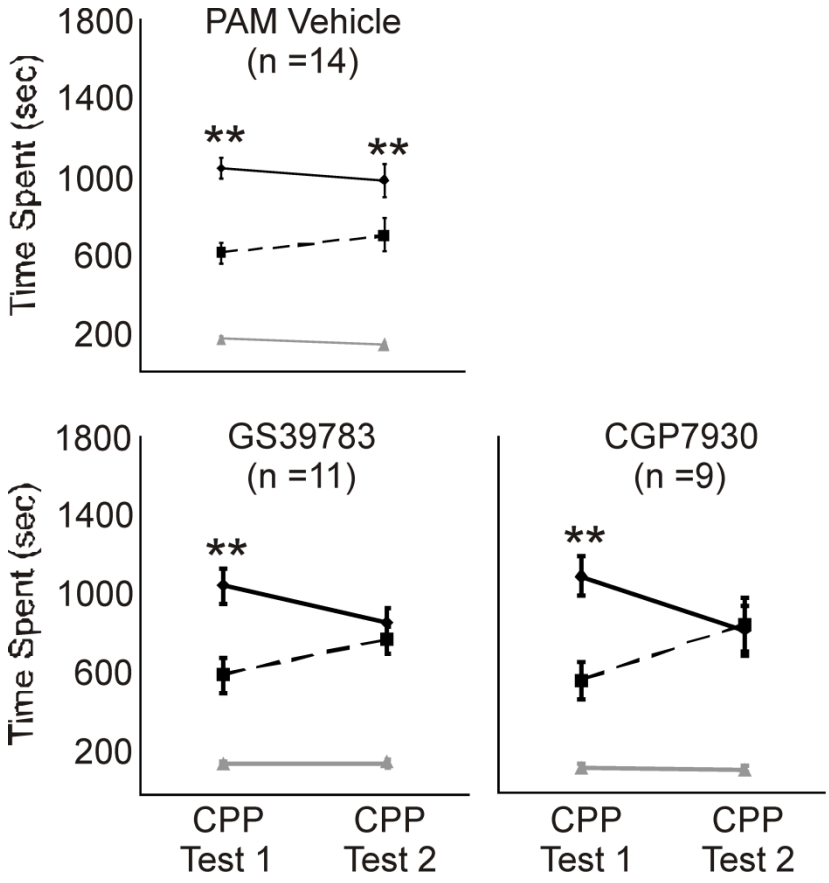

Figure 18. Meth-induced CPP is inhibited by post-conditioning administration of the PAMs GS39783 and CGP7930. Rats assigned to the PAM vehicle (10% propylene glycol) group (n=14) expressed CPP on both test days (CPP Test 1 &2). Rats administered GS39783 (n=11) or CGP7930 (n=9) as an intervening treatment did not express a preference for the Meth-paired chamber on CPP Test 2. *Post-hoc* Newman-Keuls test was used to determine between chamber differences (center chamber not included for statistical analysis), ** $p<0.01$. Solid line, time spent in the Meth-paired chamber; dashed line, time spent in the saline-paired chamber; grey line, time spent in the center chamber

CHAPTER VII

POST-CONDITIONING ADMINISTRATION OF BACLOFEN INTO THE MEDIAL DORSAL THALAMUS INHIBTS METHAMPHETAMINE-INDUCED CONDITIONED PLACE PREFERENCE

Abstract

The medial dorsal nucleus of the thalamus (MDT) is connected to brain regions important in the regulation of psychostimulant-induced behaviors and mnemonic processes. However, the role of the MDT in the maintenance of psychostimulant-induced associative learning is unclear. The MDT expresses moderate to high levels of $GABA_BR$; which modulate psychostimulant-induced neurotransmitter release and downstream molecular adaptations. The present study evaluated the role of $GABA_BRs$ in the MDT in the maintenance of methamphetamine (Meth)-induced associative learning using conditioned place preference (CPP). After the development of Meth (1mg/kg)-induced CPP, rats were administered two once-daily injections of baclofen (0.5nmol/0.5µl/side) or vehicle into the MDT, or the overlying hippocampus (HC) which served as a site control. The following day, place preference was determined for drug-free rats by comparing time spent in the Meth-paired chamber during the CPP test to the same chamber during the pre-test. Intra-MDT administration of vehicle and intra-HC administration of either the vehicle or baclofen did not impact the ability of rats to demonstrate significant CPP. In contrast, rats that received intra-MDT

injections of baclofen did not show CPP. These results implicate $GABA_BRs$ in the MDT as regulators of short term processes that allow for subsequent expression of Meth-induced CPP.

Introduction

When psychostimulants are administered in a unique context or environment, that context may become associated with the positive attributes of the stimulant. Once this drug-context association is made, re-exposure to the context, in the absence of the drug, can increase in neuronal activity in limbic brain regions (Childress et al., 1999; Childress et al., 2008; Rhodes et al., 2005; Brown et al., 1992; Zombeck et al., 2008; Franklin and Druhan, 2000; Ciccocioppo et al., 2001) and induce drug-craving, drug-seeking during cue re-exposure which can lead to relapse to drug use (Ehrman et al., 1992; Hartz et al., 2001; O'Brien et al., 1992). The enhanced salience of drug-associated cues can be studied in the laboratory with conditioned place preference (CPP) paradigms (Childs and deWit H., 2009; Tzschentke, 1998; Tzschentke, 2007). CPP measures the tendency to spend more time in an environmental context previously coupled with the rewarding effects of an unconditioned stimulus. Methamphetamine (Meth) is a potent and highly abused psychostimulant with no FDA-approved pharmacotherapies available. Drug-associated cues can hinder abstinence in addicts; therefore, understanding the brain circuits and transmitter systems that are involved in responsiveness to these cues might help identify targets for relapse reduction pharmacotherapy.

Though largely ignored by the addiction field, the MDT is connected with regions known to regulate drug-induced behaviors and mnemonic processes (Kalivas et al., 1999; Kalivas et al., 2001). Through glutamatergic (Pirot et al., 1994; Kuroda et al., 1995; Kuroda et al., 1998; Giguere and Goldman-Rakic, 1988; Pirot et al., 1995) and GABAergic (Churchill et al., 1996b; Groenewegen, 1988; Mogenson et al., 1987; Zahm et al., 1996) connections, the MDT modulates the medial prefrontal cortex (mPFC) (Ferron et al., 1984) and ventral pallidum (VP) (Churchill et al., 1996b; Churchill et al., 1996a; Churchill and Kalivas, 1999), respectively. Given the involvement of the mPFC in associative learning it is not surprising that neuronal activity in the MDT is correlated with associative learning tasks (Oyoshi et al., 1996); lesions of the MDT disrupt the acquisition of sucrose-induced associative learning (McAlonan et al., 1993), blockade of *mu*-opioid receptors in the MDT inhibits the acquisition of morphine-induced associative learning (Guo et al., 2008b), and baclofen administration into the MDT inhibits working memory (e.g., short-term memory) (Floresco et al., 1999; Romanides et al., 1999). Based on these observations, we sought to determine the yet unexplored role of the MDT in the maintenance of Meth-induced associative learning.

Systemic administration of the $GABA_BR$ agonist baclofen inhibits the development and expression of Meth-induced behaviors including CPP (Li et al., 2001) and self-administration (Ranaldi and Poeggel, 2002). $GABA_BRs$ are

located throughout the brain (Margeta-Mitrovic et al., 1999; Charles et al., 2003); however, moderate to high expression levels are observed in the thalamus, including the MDT (Margeta-Mitrovic et al., 1999; Charles et al., 2003). Thus, effects of systemically administered baclofen likely reflect, at least in part, activation of $GABA_BRs$ in the MDT. Indeed, activating $GABA_BRs$ within the MDT, *via* intra-cerebral injections of $GABA_BR$ agonists, disrupts working memory (Floresco et al., 1999; Romanides et al., 1999) and $GABA_BR$ expression within the MDT is significantly decreased following cocaine self-administration (Frankowska et al., 2008b; Frankowska et al., 2008a). The current project was designed to expand this literature to ascertain if activating MDT $GABA_BRs$ can reduce the maintenance of place preference memory that was induced by Meth.

Materials and Methods

Animals

Forty male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 250-275g at the start of the study were acclimated to the *vivarium* (a climate-controlled environment on a 12hr light/dark cycle), for at least one week prior to the onset of the experiments. Rats were housed in pairs and allowed *ad libitum* access to food and water. Cage mates were given identical pharmacological treatments. The rat housing room was in the same animal facility suite as, and adjacent to, the behavioral testing room. Loyola University Medical Center and Rush University Medical Center housing facilities are accredited through the Association for Assessment and Accreditation of Laboratory Animal Care, and all

experiments were carried out in accordance with the conditions set forth by the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996) and with the approval of the Loyola University Medical Center and Rush University Medical Center Institutional Animal Care and Use Committee.

Drugs

(+)Methamphetamine HCl (Sigma, St. Louis, MO) was dissolved in 0.9% sterile saline and the dose, 1mg/ml/kg, was calculated as the salt and administered intraperitoneally (i.p.). Vehicle (0.9% saline; 0.5µl/side) or baclofen (0.5nmol/0.5µl/side; Sigma, St Louis, MO) were administered *via* intracerebral (i.c.) injections. The dose of baclofen selected was because it is within the range of MDT-injected doses found to be behaviorally relevant to inhibit working memory (doses used were 0.03-0.3nmol) (Romanides et al., 1999) and influence motor activity (doses used were 0.003-1.0nmol) (Churchill et al., 1996a) .

Surgical Procedures for intracerebral implantation of guide cannula

To allow direct delivery of vehicle or baclofen into the MDT of awake, behavior rats, the rats were surgically fitted with guide cannulae overlying the MDT prior to initiating the study. To do so, 41 rats were allowed one week of acclimation to the *vivarium*, and then were anesthetized with pentobarbital (50mg/kg, i.p., Ovation Pharmaceuticals, Inc., Deerfield, IL) and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). After the skull was exposed, guide cannulae

(28 gauge, 1.2mm wide, 11.4mm long) were lowered into the brain 1.2mm posterior to bregma, +/- 0.6mm off the midline, and 4.4mm below dura (Pellegrino et al., 1979) as has been used previously (Churchill et al., 1996a). For placement controls, guide cannulae were implanted into the hippocampus (HC) which is dorsal to the MDT that was 2.4mm below dura (2mm dorsal to the MDT). Stainless steel mounting screws and dental acrylic were used to fix the cannulae to the skull. The skin incision was closed with sutures. Each guide cannula was fitted with dummy cannula (33 gauge, which did not extend below the tip of the implanted guide cannula), and was changed daily to prevent obstruction of the cannula and to acclimate the rats to having the cannula manipulated. At least five days of recovery were allowed between the surgery and the initiation of the experiment.

Behavioral Evaluations

The behavioral test room was dimly lit (54-108lux) with white noise (San Diego Instruments, San Diego, CA) continuously present. The small animal activity box used for CPP (AccuScan Instruments, Inc., Columbus, OH) was 63cm x 30cm x 30cm and consisted of three chambers divided by Plexiglas sliding doors; two large conditioning chambers (25cm x 30cm x 30cm) separated by a small center chamber (13cm x 30cm x 30cm). Each chamber had distinct visual and tactile cues. Chamber A had vertical stripes on the walls and a textured floor with an overturned paint dish glued to the center, the opposite, chamber B, had horizontal stripes and a textured floor with a flat rectangle glued to the center,

and the center chamber had solid color walls and a smooth, slightly raised platform floor. Time spent in each chamber and motor activity was monitored *via* two sets of photobeams (24 in the horizontal plane and 12 vertical).

The rats were transported from the animal housing room to the adjacent test room at least 30min prior to the start of the experiment for habituation. Rats were subjected to a 30min pre-test (refer to the timeline in Fig.21A) which verified that there was not a significant preference for either chamber (data presented in Results); however, individual rats tended to spend more time in one chamber compared to the other. Thus, for conditioning, rats were administered Meth (1mg/kg) in the chamber in which they spent the least amount of time during the pre-test. Conditioning occurred over five days; Meth was paired with one of the chambers on days 1, 3, & 5 and a saline (1ml/kg) was paired in the opposite chamber on days 2 & 4. Pairing occurred immediately after each injection and lasted for 45min. During the withdrawal period after conditioning, baclofen or vehicle was administered in the home cage, once-daily for two days (days 6 & 7). One day later (day 8), rats were tested for expression of CPP in a drug-free state. To do so, the rats were placed into a small neutral, center chamber and the sliding doors were immediately removed allowing the rats free access to the entire CPP box. The test session lasted 30min and time spent in each chamber was determined.

Intracerebral Microinjection Procedure

For the intracerebral injections (which occurred in the housing room), dummy cannulae were removed and injectors which extended 1.0mm below the guide cannulae were inserted. The injectors were connected to a Hamilton syringe (Reno, NV) *via* polyethylene tubing, and the syringes were held in a CMA/100 microinjection pump (Carnegie Medicine, Stockholm, Sweden). Baclofen (0.5nmol/0.5µl/side, dose based on (Churchill et al., 1996a; Romanides et al., 1999)) or vehicle (0.5µl/side) was administered to behaving, unrestrained rats at a rate of 0.1µl/min for 5min for a total volume of 0.5µl/side. Injectors were left in place for 1min to allow for diffusion of the drug away from the injector tip after which time the injectors were removed, dummy injectors were replaced, and rats returned to the home cage.

Histology

After the completion of behavioral testing, pontamine sky blue (Sigma, St. Louis, MO) was injected to allow for visualization of injector tip placement. The brains were removed, fresh frozen and cut in 50µm sections. Sections were mounted on subbed slides and stained with pyronin-Y or cresyl violet. The intracerebral infusion site (marked by blue dot or lesion) was agreed on by two treatment-blind observers using light microscopy and reconstructed onto a rat stereotaxic brain map (Paxinos and Watson, 1998) (Fig. 22)

Statistics

A two-way repeated measures ANOVA (treatment x test) was employed to determine shifts in chamber preference across time (pre-test *vs.* CPP test). P*ost-hoc* Newman-Keuls was used to identify between test differences. All data are presented as mean ± SEM. Statistical outliers were determined as those rats that spent greater than two standard deviations above or below the mean time spent in any chamber during the pre-test or CPP Test.

Results

Results of the 30min pre-test demonstrated that, before conditioning, there was a significant group preference for chamber A (n=41, time spent in chamber A, 1025±45sec *vs.* chamber B 643±44sec; paired *t*-test, $p<0.001$, center chamber time was 133±8sec). Based on pre-test results, rats were assigned to receive Meth in the chamber in which the least amount of time was spent during the pre-test such that time spent in the chamber to be paired with Meth was approximately equal across all treatment groups. Rats were assigned to one of the following Meth-conditioned treatment groups: HC vehicle, HC baclofen, MDT vehicle, or MDT baclofen.

Figure 22 shows the injection sites within the MDT and HC for the intracerebral injections administered during the post-conditioning period; out of the total of 41 rats used for the study one was excluded due to the cannula blockage and four for placement outside of the MDT and HC. Rats receiving HC injections demonstrated significant CPP independent of treatment history (i.e., vehicle or

baclofen) (Fig. 23A), a two-way repeated measures ANOVA revealed a significant effect of Test ($F_{(1,11)}$=22.068, p=0.001) and no effect of Treatment ($F_{(1,11)}$=0.467, p=0.508) or Treatment x Test interaction ($F_{(1,11)}$=1.274, p=0.283). A *Post-hoc* Newman-Keuls test showed that time spent in the Meth-paired chamber during the CPP test (day 8) was significantly increased from time spent in the same chamber during the pre-test (day -1) for both treatment groups (HC vehicle, n=8; HC baclofen, n=5; p<0.05). In contrast, treatment history significantly impacted CPP outcomes for MDT injected rats (Fig. 23B), a two-way repeated measures ANOVA revealed a significant effect of Test ($F_{(1,21)}$=26.822, p<0.0001) and a Treatment x Test interaction ($F_{(1,21)}$=4.614, p=0.044) but no effect of Treatment ($F_{(1,21)}$=1.327, p=0.262). A *Post-hoc* Newman-Keuls test revealed that time spent in the Meth-paired chamber during the CPP test (day 8) was significantly increased from time spent in the same chamber during the pre-test (day -1) for the MDT vehicle treated rats (n=13, p<0.01) but not for those rats which received MDT baclofen (n=10, p>0.05).

Discussion

Repeated pairing of Meth with a unique context results in robust associations between Meth (unconditioned stimulus) and the context in which it is administered (conditioned stimulus) (Tzschentke, 1998; Tzschentke, 2007). In the current study, preference for the Meth-paired chamber was not disrupted by

intra-cerebral vehicle injections into the MDT or HC indicating that the injection procedure did not impact the preference for the Meth context. Baclofen administered into the MDT, and not the overlying HC, inhibited the expression of Meth-induced CPP when tested in a drug-free state 24hr after the last intracerebral injection. These findings demonstrate that pharmacological augmentation of $GABA_BR$ signaling in the MDT disrupted the processes necessary to maintain and subsequently express a preference for the Meth-paired chamber.

Prior work has demonstrated that the $GABA_BR$ agonist, baclofen, modifies the development (administered immediately after training) and expression (administered as a pretreatment prior to behavioral testing) of mnemonic processes including spatial memory (Levin et al., 2004; McNamara and Skelton, 1996; Nakagawa et al., 1995) and fear conditioning (Castellano et al., 1989; Swartzwelder et al., 1987; Zarrindast et al., 2004). Baclofen also antagonizes the expression of conditioned behaviors; baclofen administered as a pre-treatment prior to testing for the expression of Meth-induced CPP (Li et al., 2001) or amphetamine-induced conditioned motor sensitization (Hotsenpiller and Wolf, 2003) inhibits the expression of these behaviors. The current study demonstrated that intra-MDT administration of baclofen in Meth-conditioned rats resulted in a reduction of CPP drug-free rats one day after the last MDT treatment an effect which was not observed with two once-daily systemic injections of 2mg/kg baclofen (Chapter VI) indicating a selective influence of the

MDT on the maintenance of Meth-induced CPP rather than globally activating $GABA_B$Rs *via* systemic baclofen administration. The behavioral effects of locally injected baclofen can be antagonized by co-administration of $GABA_B$R antagonists (i.e., CGP35348 or saclofen) (Zarrindast et al., 2002; Churchill et al., 1996a) indicating the involvement of the $GABA_B$R in the observed behavioral phenomenon; however, future studies with a $GABA_B$R antagonist will be necessary to validate the involvement of $GABA_B$Rs in the observed behavioral effects.

The MDT expresses moderate to high levels of $GABA_B$Rs (Margeta-Mitrovic et al., 1999; Charles et al., 2003); therefore, it is possible that augmented $GABA_B$R signaling within the MDT may be responsible, at least in part, for mediating the effects of the systemically administered ligands. The MDT is important for learning and memory processes; neuronal activity in the MDT is enhanced during the recall of associative tasks (Oyoshi et al., 1996), lesions of the MDT inhibit the acquisition of sucrose-induced associative learning (McAlonan et al., 1993) as well as working memory (Floresco et al., 1999), morphine-induced associative learning is disrupted by blockade of μ-opioid receptors in the MDT (Guo et al., 2008b), and baclofen administered into the MDT disrupts working memory (Romanides et al., 1999). We have now demonstrated that the maintenance of associative memories is regulated by the MDT. The current study demonstrated that intra-MDT administration of baclofen in Meth-conditioned rats inhibited the maintenance of CPP when drug-free rats were tested one day after the last MDT

treatment. As this outcome was not obtained when the overlying HC was exposed to the same treatment this helped verify that the effect of baclofen reflected actions within the MDT, and not *via* back diffusion of baclofen up the cannulae track to more dorsal structures. Although the HC has long been implicated in learning and memory processes (Jarrard, 1993); the current study found that augmenting $GABA_BR$ signaling in the HC 24 and 48hr after conditioning had no effect on memory maintenance. When baclofen was administered into the HC immediately after passive avoidance training subsequent expression of the behavior was inhibited, this observation is consistent with the involvement of the HC in early memory mnemonic events (<24hr) (Arolfo et al., 1998; Bourtchouladze et al., 1998; Packard and Teather, 1997). Taken together, these studies suggest that augmenting $GABA_BR$ signaling within the MDT, but not the HC, during the early phases after Meth-conditioning is sufficient to weaken the maintenance of Meth-induced associative learning.

The MDT is anatomically positioned to influence a number of brain regions that are known to influence drug-induced behaviors and mnemonic processes; one such region is the medial prefrontal cortex (mPFC) which receives dense glutamatergic projections from the MDT (Kuroda et al., 1995; Churchill et al., 1996b; Kuroda et al., 1998; Pirot et al., 1994; Groenewegen, 1988). Glutamate in the mPFC is a critical regulator of memory processes (Wang et al., 2006) and neuronal activity in the mPFC is increased by electrical stimulation of the MDT

(Ferron et al., 1984; Bubser et al., 1998). Thus a decrease in MDT (e.g., due to administration of baclofen as in the current study) activity might be expected to reduce glutamate release in the mPFC and blunt neuronal activity. This effect may account for inhibiting working memory (Romanides et al., 1999) and disrupting the maintenance of Meth-induced associative memory in the current study.

In conclusion, these results implicate $GABA_BRs$ in the MDT as critical regulators of processes that are necessary to maintain and subsequently express Meth-induced CPP.

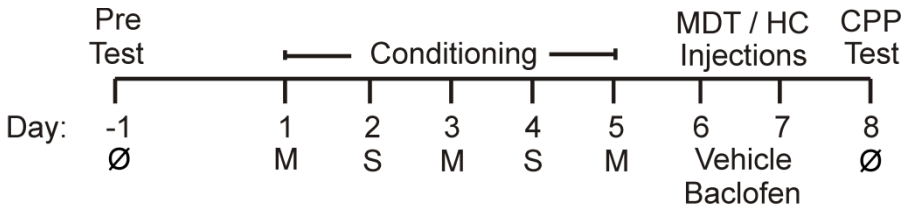

Figure 19. Illustration of treatment the protocol. For the pre-test (day -1), drug-free rats were allowed to explore the entire CPP box for 30min. Conditioning occurred for five days; Meth was paired with one chamber on days 1, 3, & 5, and the saline was paired with the opposite chamber on days 2 & 4). Baclofen (0.5nmol/0.5µl/side) or its vehicle (0.9% saline, 0.5µl/side) was administered on days 6 and 7. One day later (day 8), rats were tested for the expression of Meth-induced CPP in a drug-free state. M, methamphetamine (1mg/kg); S, saline (1mg/kg); Ø, no drug.

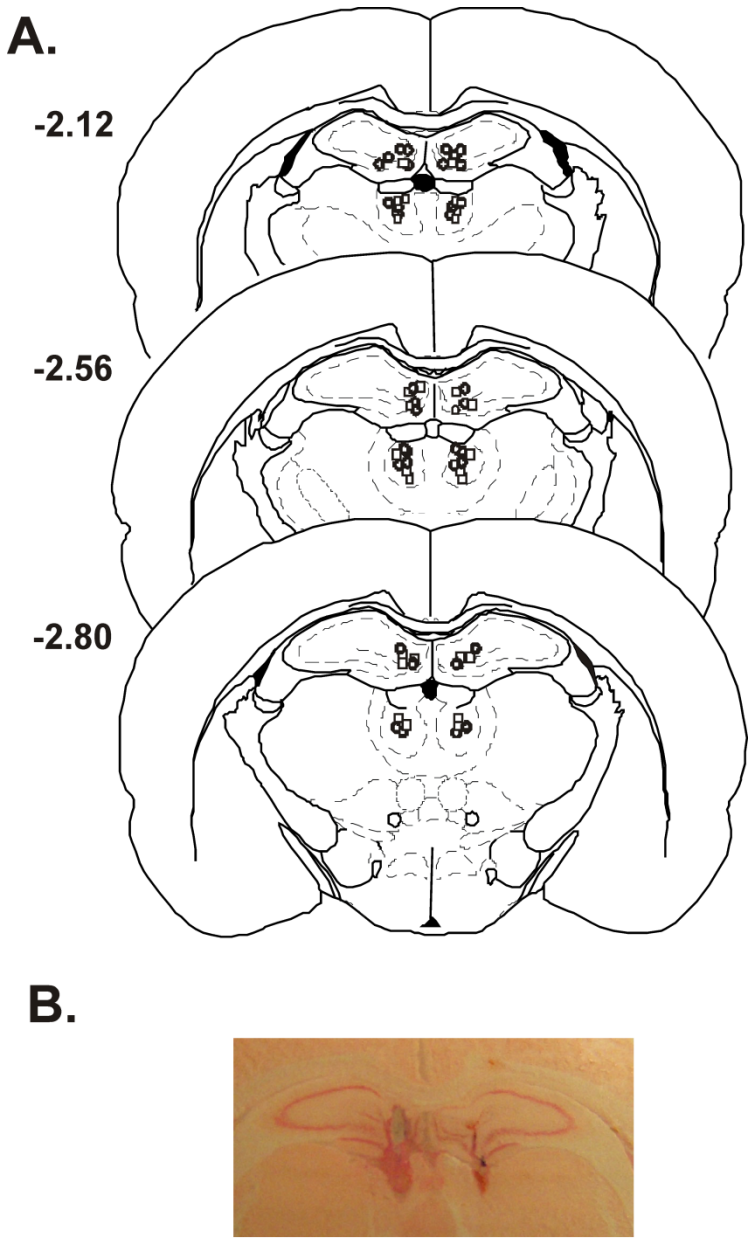

Figure 20. Brain map Illustrating location of intra-cerebral injection sites. A. Illustration of the injector tip location for all intra-cerebral sites tested. Stereotaxic maps were adapted from the atlas of Paxinos and Watson (Paxinos and Watson, 1998). The numbers refer to the coronal level of the section, in mm relative to bregma. B. Representative photomicrographs of Nissl-stained coronal brain sections showing injector tips into the medial dorsal thalamus.

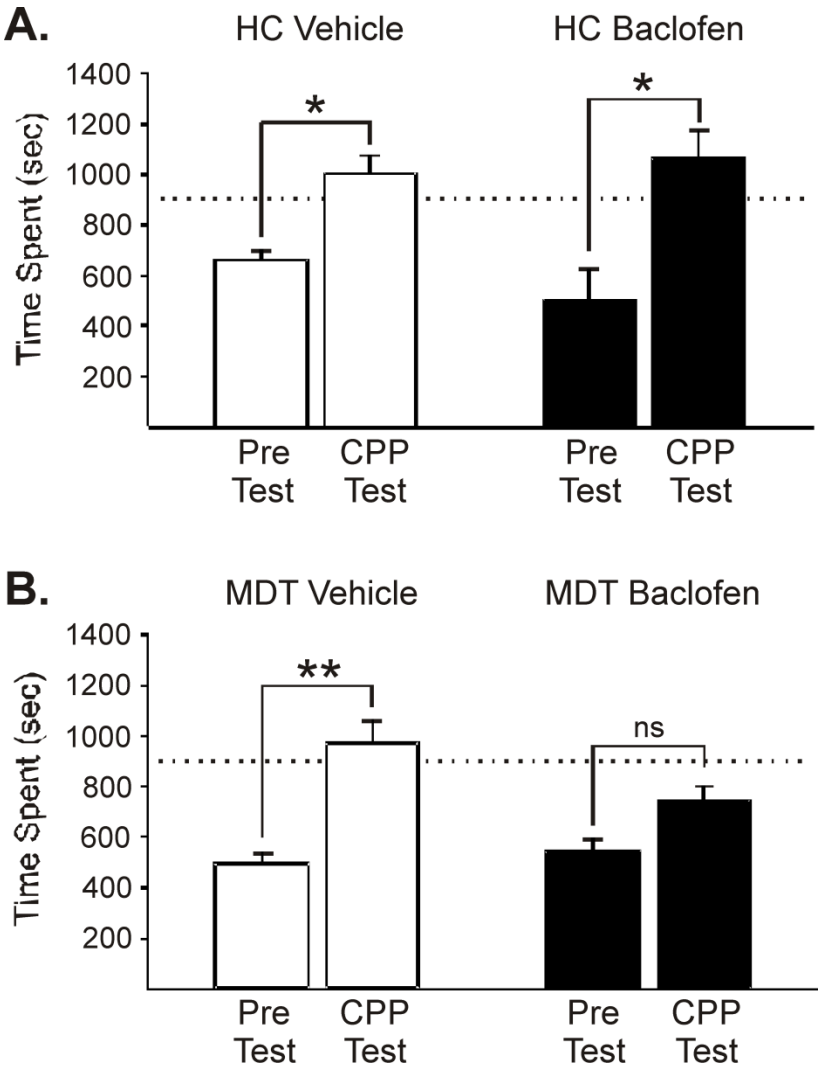

Figure 21. The maintenance of Meth-induced CPP was inhibited by two-once daily injections of baclofen into the medial dorsal thalamus (MDT). Data were analyzed with a two-way repeated measures ANOVA followed by a *post-hoc* Newman-Keuls test to identify between test (pre-test *vs.* CPP test) differences. *Post-hoc* test values are illustrated, * $p<0.05$, ** $p<0.01$, ns, not significant. The dashed line indicates 900sec; 50% of the total CPP test time (1800sec). **B.** Intra-hippocampus (HC) injections of vehicle (open bars, n=8, $p<0.05$) or baclofen (filled bars, n=5, $p<0.05$) did not impact the ability of rats to demonstrate

CPP. **C.** Rats that received intra-MDT injections of vehicle demonstrate a significant CPP (open bars, n=13, $p<0.01$), whereas those rats which received baclofen did not (filled bars, n=10, $p>0.05$).

CHAPTER VIII

ANALYSIS OF $GABA_B$ RECEPTOR EXPRESSION AND DISTRIBUTION USING THE CROSS-LINKING TECHNIQUE IN RAT BRAIN FOLLOWING REPEATED METHAMPHETAMINE ADMINISTRATION

Abstract

Repeated psychostimulant administration creates persistent molecular, cellular, and circuit adaptations which are manifested as altered neuronal function and behavior. These adaptations are the consequence of adaptations in a number of neurotransmitter systems including the inhibitory $GABA_BR$ system; however, little is known about the $GABA_BR$ at a time when psychostimulant-induced behaviors are expressed. To provide an *ex vivo* snap-shot of an *in vivo* brain state we applied the bis(sulfosuccinimidyl)suberate (BS^3) cross-linking technique to assess $GABA_BR$ expression and distribution, the first time that such an evaluation has been conducted with a metabotropic receptor in psychostimulant-treated brain tissue. Using this technique, we evaluated $GABA_BR$ expression and distribution within brain regions involved in psychostimulant-induced behaviors at a time when methamphetamine (Meth)-induced conditioned place preference (CPP, Experiment 1) and motor sensitization (MS, Experiment 2) were expressed. For CPP, we used a six-day conditioning protocol (Meth-paired on days 1, 3, & 5 and saline-paired on days 2, 4, & 6) which was sufficient to induce CPP and MS. Brain tissue was harvested one day after rats

demonstrated Meth-induced CPP (day 10). In spite of the robust behavioral effects, we observed no significant changes in the expression or distribution of the $GABA_BR$ in the medial prefrontal cortex (mPFC), nucleus accumbens (NAc), ventral pallidum, or medial dorsal thalamus. Likewise, 14 days after three once-daily Meth injections, at a time when rats expressed Meth-induced MS, $GABA_BR$ expression or distribution was unaltered in the mPFC and NAc. Thus, we conclude that in the expression of Meth-induced CPP and MS in the current studies was not dependent on a change in the expression and or distribution of the $GABA_BR$ in limbic brain regions evaluated. However, future studies evaluating downstream mediators of the $GABA_BR$ and/or receptor function will be of value to determine if there is a down-regulation of the $GABA_BR$ system contributing to the psychostimulant-induced behaviors such as CPP and motor sensitization. These studies also suggest that the BS^3 cross-linking technique will likely be of value to assess a wide variety of surface proteins.

Introduction

Psychostimulants, including methamphetamine (Meth), induce long-lasting changes in neuronal function (Chen et al., 2009; Nestler, 2001; McDaid et al., 2007). These adaptations are highly dynamic and paradigm-specific, with different brain states occurring during the hours, days, and weeks following psychostimulant administration (Kalivas and Hu, 2006). While the basal brain state is highly variable, it is clear that brain regions/neurons that are withdrawn from repeated exposure to psychostimulants are hyper-responsive to re-

exposure to cues which are associated with drug-administration (Brown et al., 1992; Zombeck et al., 2008; Franklin and Druhan, 2000; Ciccocioppo et al., 2001; Rhodes et al., 2005; Childress et al., 1999; Childress et al., 2008; Lin et al., 2007) as well as subsequent psychostimulant administration (Robinson et al., 1988; Kazahaya et al., 1989; Nishikawa et al., 1983; McDaid et al., 2006b; McDaid et al., 2007). This hyper-excitable brain state can be observed behaviorally in the laboratory using conditioned place preference (CPP) and motor sensitization (MS). These behaviors model different aspects of psychostimulant-induced brain adaptations but both behaviors indicate persistent alterations in neuronal function that persist long-after psychostimulant administration.

The $GABA_BR$ modulates neuronal excitability, signal transduction, and protein expression; thus, changes in the $GABA_BR$ system will significantly affect brain function and resulting behavior. Effects on neuronal excitability are mediated by G-protein dependent activation of inwardly rectifying K^+ channels and inhibition of high voltage activated Ca^{++} channels (Mott and Lewis, 1994; Bowery, 1993); the $GABA_BR$ regulates neuronal activity and the release of a neurotransmitters including dopamine (Gong et al., 1998; Kalivas et al., 1993; Santiago et al., 1993a; Santiago et al., 1993c; Smolders et al., 1995). Indeed, pharmacological augmentation of $GABA_BR$ signaling blunts stimulus-evoked release of dopamine (Fadda et al., 2003) and glutamate (Hotsenpiller and Wolf, 2003). In addition, $GABA_BRs$ also mediate effects on protein expression and signal transduction *via*

a G-protein dependent mechanism (e.g., cAMP) to regulate the activity of the transcriptional regulator cAMP response element binding protein (CREB). Thus, a down-regulation in $GABA_BR$ system function may contribute to neuronal hyper-responsivity observed after repeated psychostimulant administration. Indeed, the $GABA_BR$ system is down-regulated after robust psychostimulant administration protocols including decreased $GABA_BR$ expression following a minimum of six days of stable cocaine self-administration (average of 15-18mg/kg/day) (Frankowska et al., 2008b; Frankowska et al., 2008a) and functional coupling of the receptor to the $G_{i/o}$ G-protein is reduced after chronic cocaine (Kushner and Unterwald, 2001) and after a sensitizing regimen of amphetamine (Zhang et al., 2000). Furthermore, these effects are withdrawal time dependent; which makes it of value to determine withdrawal time dependent effects on the $GABA_BR$ system which may contribute to the development, maintenance, and/or expression of Meth-induced behaviors. Thus, the current study sought to determine the expression and distribution of the $GABA_BR$ at a time when Meth-induced CPP and MS are expressed.

Trafficking of $GABA_BRs$ is a dynamic process that can profoundly influence stimulant-medicated behaviors and learning and memory. Several methods have been used to determine the cellular distribution of $GABA_BRs$ including biotinylation (Fairfax et al., 2004), α-bungarotoxin tag (Wilkins et al., 2008), and florescent protein tags (Fairfax et al., 2004). However, each of these techniques has aspects which make them less than ideal. The cross-linking technique used

in the current study, pioneered by Boudreau and Wolf (Boudreau and Wolf, 2005) to evaluate the surface expression of AMPA receptor subunits, provides an *ex vivo* snapshot of an *in vivo* brain state and is an efficient method to determine the distribution and expression of surface proteins. The membrane impermeable cross-linking agent bis(sulfosuccinimidyl)suberate (BS^3) binds to extracellular basic residues (*via* an amide bond) of proteins inserted into the membrane resulting in a high molecular weight aggregate that is easily differentiated from the intracellular protein by using an antibody for protein detection targeted to the carboxy-terminal region of the receptor. In order for the cross-linking technique to work there must be an adequate number of extracellular basic resides for BS^3 to bind to in the extracellular domain. The $GABA_BR$ is part of the Class C family of receptors which has a very large extracellular domain (Brauner-Osborne et al., 2007) making it amenable to the cross-linking procedure. Using this technique, we were able to successfully evaluate the expression and distribution of $GABA_BRs$ in brain regions critical for regulating psychostimulant-induced behaviors (i.e., medial prefrontal cortex, mPFC; nucleus accumbens, NAc; ventral pallidum, VP; and medial dorsal thalamus, MDT) at a time when Meth-induced CPP and MS are demonstrated. These experiments demonstrate, for the first time, that the cross-linking procedure can be used to assess expression and cellular distribution of a metabotropic receptor in brain tissue. Applying this technique, these experiments were designed to assess behavior- (CPP *vs.* MS) and/or withdrawal-time (4 or 14 days after the last Meth administration) dependent effects on the $GABA_BR$.

Materials and Methods

Animals

A total of 70 male Sprague-Dawley rats weighing between 250-275g at the start of the study were acclimated to the *vivarium* for at least one week prior to the onset of the experiment. Rats were housed in pairs in a climate-controlled environment on a 12hr light/dark cycle and allowed *ad libitum* access to food and water. Cage mates were given identical treatments. Housing facilities were accredited through the Association for Assessment and Accreditation of Laboratory Animal Care, and all experiments were carried out in accordance with the conditions set forth by the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996) and with the approval of the Loyola University Medical Center and Rush University Medical Center Institutional Animal Care and Use Committee.

Drugs

(+)Methamphetamine HCl (Meth; Sigma, St. Louis, MO) was dissolved in 0.9% sterile saline and the dose, 1mg/ml/kg, was calculated as the base (Experiment 1) or as the salt (Experiment 2). Meth and Meth vehicle (0.9% saline) were given at a volume of 1ml/kg *via* subcutaneous (s.c.) or intraperitoneal (i.p.) injection, as indicated.

Conditioned Place Preference Apparatus

The test room was dimly lit (54-108lux) with white noise (white noise generator, San Diego Instruments, San Diego, CA) continuously present. The CPP apparatus (63cm x 30cm x 30cm) consisted of three chambers divided by Plexiglas sliding doors (AccuScan Instruments, Inc., Columbus, OH); two large conditioning chambers (25cm x 30cm x 30cm) were separated by a small center chamber (13cm x 30cm x 30cm). Each chamber had distinct visual and tactile cues. Chamber A, vertical stripes on walls and an overturned paint dish glued to the center of a patterned floor; Chamber B, horizontal stripes on walls and a grid floor; Center chamber, solid color walls and a smooth, slightly raised platform floor. Time spent in each chamber and motor activity was monitored *via* two sets of photobeams (24 horizontal and 12 vertical).

Behavioral Experiment 1: Repeated Meth administration, four days post-conditioning

The rats were transported from the animal housing room to the adjacent test room at least 30min prior to the start of the experiment. The behavioral paradigm consisted of three phases: pre-test, conditioning, and CPP test (Fig. 24A). Rats were given a 15min pre-test to determine, initial, unconditioned chamber bias (time spent Chamber A, 348±26s *vs*. time spent Chamber B, 459±24s, Student's *t*-test, p=0.034). Rats were assigned to one of two treatment groups (i.e., Meth or saline conditioned) such that unconditioned preference was approximately equal for each group. For conditioning, rats were counterbalanced wherein half of the rats were assigned to receive the day 1 pairing in the chamber in which

they spent the greatest amount of time during the pre-test and the other half were assigned to receive the day 1 pairing in the chamber in which they spent the least amount of time during the pre-test. Rats were subsequently conditioned for six days; Meth (1mg/kg, as the base, i.p.) administered on days 1, 3, & 5 and saline on the alternate days (termed Meth conditioned group; Meth Grp) or saline was administered on days 1-6 (saline conditioned group; Sal Grp). During conditioning, rats were injected (Meth or saline) and were immediately placed into the appropriate chamber of the CPP box for 45min; motor activity was assessed during this time (illustrated is vertical time which represent sensitized motor activity induced by psychostimulants including Meth, Figs. 24B & C). The rats were evaluated for chamber preference three days after the last conditioning session (day 9). To do so, rats were placed into the center chamber and sliding doors immediately removed allowing free access to the entire CPP box. The test session lasted 15min and time spent in each chamber was evaluated to determine preference. Brain tissue was harvested one day after the CPP test as described below.

Behavioral Experiment 2: Repeated Meth administration, 14 days post-administration

The rats were transported from the animal housing room to the behavior room at least 30min prior to the start of the experiment. The behavioral paradigm consisted of three phases: repeated treatment, withdrawal, and motor sensitization test (Fig. 25A). For the repeated treatment (days 1-3), rats were

administered a once-daily injection of Meth (1mg/kg, as the salt, s.c.) or vehicle (1ml/kg) and placed into a chamber of the CPP box (which was used to measure motor activity and not used for conditioning) and motor activity was monitored for 60min. During the 14 day withdrawal period, rats remained in the home cage undisturbed. To ascertain if motor sensitization was expressed on day 17, rats were given an acute challenge of Meth (1mg/kg, as the salt, s.c.) immediately prior to being placed into the CPP box for 60min. Vertical activity reliably represents sensitized motor activity induced by psychostimulants including Meth, illustrated are vertical beam breaks and vertical time. Brain tissue was harvested (described below) from a separate group of rats on the same day as the behavioral sensitization test (day 17).

Biochemical assessments

Tissue Preparation

The surface receptor cross-linking method was adapted from that of Boudreau and Wolf (Boudreau and Wolf, 2005). Rat mPFC, VP, NAc, and MDT tissues were harvested one day after the CPP test (Fig. 3). After being dissected, each brain region was chopped into 400μm slices with a McIlwain tissue chopper (Mickle Laboratory Engineering Co. LTD, Goose Green, UK) and immediately placed into artificial cerebrospinal fluid (aCSF) with 2mM Bis(sulfosuccinimidyl)suberate (BS^3; Thermo Scientific, Rockford, IL). The cross-linking reaction took place for 30min with gentle agitation at 4°C and the reaction was terminated by administration of 100mM glycine (10min at 4°C). The tissue

pellet was separated from the supernatant by centrifugation at 14,000rpm for 2min (4^{o}C); supernatant was removed and the pellet was re-suspended in lysis buffer containing: 25mM HEPES, 500mM NaCl, 2mM EDTA, 20mM NaF, 0.1% Nonidet P-40, 1mM DTT, 1mM PMSF, protease inhibitor cocktail (Calbiochem, La Jolla, CA), phosphatase inhibitor cocktail I (Sigma-Aldrich), and phosphatase inhibitor cocktail II (Sigma-Aldrich). Cellular membranes were disrupted by sonication. Tissues were spun at 14,000rpm for 2min at 4°C and subsequently aliquoted and frozen at -80°C for future use. Protein concentrations were determined *via* the Bradford method (Bradford, 1976).

Non-cross-linked samples (Non CL, Fig. 27A) were hand dissected and immediately fast-frozen on dry ice then kept at -80°C until tissue preparation. For tissue preparation, brain regions were Dounce homogenized and sonicated in homogenation/lysis buffer (25mM HEPES, 1mM EGTA, 1mM EDTA, 100nM okadaic acid, 1mM sodium orthovanadate, 100µM PMSF, 10µg/ml of pepstatin, leupeptin & aprotinin in cocktail form). Protein concentrations were determined *via* the Bradford method (Bradford, 1976) and samples were prepared with sample buffer (NuPAGE LDS Sample Buffer, Invitrogen, Carlsbad, CA) and reducing agent (NuPAGE Sample Reducing Agent, Invitrogen), aliquoted and frozen until use at -80°C.

Immunoblotting

Tissue homogenate samples for cross-linking experiments were prepared with in 1:1 dilution of Laemmli sample buffer (Bio-Rad, Hercules, CA) with β-mercaptoethanol (volume determined based on protein assay result) and subsequently heated to 100°C for 3-5min. Protein (20µg) was loaded into a 4-15% Tris-HCl linear gradient gel (Bio-Rad) and electrophoresed at 200V for 45min. The gels were then transferred to a Hy-Bond PVDF membrane (GE Healthcare Limited, Buckinghamshire, UK) for 1.5hr at a current of 1.25 amperes. Nonspecific binding was blocked by incubation of the membrane with TBST (0.05% Tween 20, Sigma-Aldrich), 1% normal donkey ($GABA_BR1$-R20) or 1% normal goat ($GABA_BR1$-R300) serum for at least 1hr. Membranes were then incubated with $GABA_BR1$-R20 (1:300 in TBST, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), $GABA_BR1$-R300 (1:500 in TBST, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or actin (1:20,000 in TBST and 5% non-fat dry milk, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) primary antibody overnight at 4°C with gentle agitation. Membranes were washed repeatedly with TBST and then incubated with the appropriate HRP-conjugated secondary antibody for $GABA_BR1$-R20 (donkey anti-goat 1:50,000 in TBST; Jackson ImmunoResearch, West Grove, PA), $GABA_BR1$-R300 (goat anti-rabbit 1:15,000 in TBST; Jackson ImmunoResearch, West Grove, PA), or actin (goat anti-rabbit 1:20,000 in TBST and 5% non-fat dry milk; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Membranes were subsequently washed with TBST, TBS, and distilled water. Chemiluminescent substrate (SuperSignal West Pico, Thermo Scientific, Rockford, IL) was applied to the membrane to visualize protein of interest by light

sensitive film (HyBlot CL, Denville Scientific, Inc., Metuchen, NJ). Optical density was determined *via* densitometric analysis with Un-Scan-It Software (Silk Software,Inc., Orem, UT).

Preabsorption Assay

Immunoblotting was conducted as stated above with the following adaptations. The PVDF membrane was cut in half. One half was incubated with antibody plus the blocking peptide (pre-incubated for 1hr at room temperature) and the other half was processed as described previously; membranes were incubated overnight at 4°C. The $GABA_BR$ blocking peptide (1:60 in 1xTBST; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) specific to the epitope of the anti-$GABA_BR1$-R20 goat immunoaffinity purified IgG primary antibody (1:300 in 1xTBST; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Immunoblots were subsequently washed, incubated with the appropriate secondary antibody and developed as described previously.

Quantification and Data Summary

Optical density of the high molecular weight aggregate (>400kD) corresponding to receptors located at the neuronal surface (S), the intracellular components (I) which were located at the expected molecular weights ($GABA_{B1a}R$ ~130kD, $GABA_{B1b}R$ ~100kD), and actin (A, ~43kD) were determined. Using these data the following were calculated: 1) the ratio of surface to intracellular protein (S/I

ratio), 2) total surface protein (S/A), 3) total intracellular protein (I/A), and 4) total protein ((S+I)/A).

Statistics

All data are represented as mean ± SEM. Statistical outliers were determined as more than two standard deviations above or below the mean for any data set.

Behavior

Conditioned place preference: Significant preference was achieved when a significantly greater amount of time was spent in the Meth-paired compared to the saline-paired chamber during the CPP test as assessed with a paired *t*-test (Bonferroni adjustment set significance at $p<0.025$). Development of motor sensitization: A within group comparison of vertical activity between the first and the last Meth treatment (Paired *t*-test, $p<0.05$). Expression of motor sensitization: A between groups analysis of vertical activity following a Meth challenge (saline *vs.* Meth) (Student's *t*-test, significance set at $p<0.05$).

Immunoblotting

The surface to intracellular ratio (S/I ratio), total protein, surface (S), and intracellular (I) values were normalized to the average saline within each gel and multiplied by 100 to determine optical density as a % Average Saline. Student's *t*-tests (significance set at $p<0.05$) were used to determine differences between

$GABA_BR$ distribution and expression in Meth and saline treatment groups for S/I ratio, total protein, S, and I.

Results

Experiment 1: Repeated Meth administration, four days post-conditioning (Refer to Fig. 24A for conditioning protocol)

Meth-conditioned rats demonstrated a significant preference for the Meth-paired chamber (n=16, p=0.0004; Fig. 24C) while saline-conditioned rats did not show preference for either chamber (n=16, p=0.304, Fig. 24B). The conditioning procedure was also sufficient to induce motor sensitization; vertical time (p=0.040) was significantly greater on day 5 compared to first conditioning day (day 1) which was representative of other vertical parameters as well. Saline conditioned rats demonstrated a significant decrease in vertical time ($p<0.001$) when comparing the first and last conditioning days which is consistent with habituation to the environment and the conditioning procedure. Together these data indicate that the Meth administration protocol induced brain adaptations which were sufficient to induce Meth-induced associative learning and MS; thus to determine the status of the $GABA_BR$ at a time when the behavior was expressed brain tissue was harvested one day after the CPP test. No differences in $GABA_BR$ expression or distribution were observed in any brain region comparing Meth-conditioned rats which expressed a preference for the Meth-paired chamber when compared to saline-conditioned rats (representative

blots in Fig. 27C; Table 1); this was true when evaluating the $GABA_{B1a}R$ (130kD) and $GABA_{B1b}R$ (100kD) isoforms separately (data not shown) or when included together (100+130kD). Meth conditioning did not alter $GABA_BR$ surface/intracellular ratio, total protein, surface, or intracellular in the mPFC, NAc, VP, or MDT (Table 1). Furthermore, the magnitude of the preference was not correlated with biochemistry outcomes.

Experiment 2: Repeated Meth administration, 14 days post-administration (Refer to Figure 25A for repeated treatment protocol) (Behavioral data, collected by Amanda Mickiewicz, Ph.D.)

These data revealed that three once-daily treatments of 1mg/kg Meth (administered as the salt, s.c.) was sufficient to induce brain changes that manifested themselves as the development (Figs. 25B & C) and expression (Fig. 25D) of Meth-induced MS. Vertical parameters (which reliably represent the profile of psychostimulant motor sensitization in rodents) including vertical time (time spent in the vertical position) which was significantly increased during the repeated treatment period (day 1 *vs.* day 3) for rats which received repeated Meth (Fig. 25C, n=8, paired *t*-test, p=0.005) but not for those repeatedly administered saline (Fig. 25B, n=8, paired t-test, p=0.527). In addition, significant between group differences were observed on day 17 following a Meth challenge injection; with the Meth repeated treatment rats spending more time in the vertical position (Student's *t*-test, p=0.018). Analysis of tissue collected on

day 17 revealed no differences in $GABA_BR$ expression or distribution in the mPFC or NAc when comparing rats repeatedly treated with saline or Meth (representative blots in Fig. 27D; Table 2); this was true when evaluating the $GABA_{B1a}R$ (130kD) and $GABA_{B1b}R$ (100kD) and 130kD isoforms separately (data not shown) or when included together (100+130kD). Repeated Meth administration sufficient to induce behavioral sensitization did not alter $GABA_BR$ expression or distribution (i.e., surface/intracellular ratio, total protein, surface, or intracellular) in the mPFC or NAc (Table 2).

Cross-linking of the $GABA_B$ receptor

The $GABA_BR$ was identified as the first receptor to function as an obligate heterodimer (Kaupmann et al., 1998; White et al., 1998; Jones et al., 1998; Kuner et al., 1999); the receptor is comprised of a ligand-binding $GABA_{B1}R$ (Kaupmann et al., 1998) (which has two isoforms ($GABA_{B1a}R$ & $GABA_{B1b}R$) which differ from one another by the presence of a sushi domain repeat) (Kaupmann et al., 1997) and a $GABA_{B2}R$ which couples to the G-protein ($G_{i/o}$) (Bettler et al., 2004; Pin et al., 2004). In addition, the dimerization is critical for the trafficking of the functional receptor to the neuronal surface; the binding of the $GABA_{B2}R$ masks the endoplasmic reticulum retention signal located on the $GABA_{B1}R$ (Couve et al., 1998; Kaupmann et al., 1997). For the current study, evaluation of the $GABA_{B1}R$ would be expected to mirror changes that also occurred for the $GABA_{B2}R$; thus, it is appropriate to evaluate the $GABA_{B1}R$ in the current study.

Using the cross-linking technique we have been able to differentiate between the high molecular weight aggregate corresponding to receptor inserted into the neuronal membrane bound to BS^3 (>400kD) and the intracellular protein which ran at the expected molecular weight (~130 & 100kD) in the same tissue sample. This is illustrated in Fig. 27A which demonstrates the difference between Western blot results obtained from cross-linked (CL) and non-cross-linked (Non CL) samples. The high molecular aggregate is not present in the Non CL tissue. The ability to cross-linked receptor proteins is not ubiquitous, the inwardly rectifying potassium channel (GIRK, a major downstream mediator of the $GABA_BR$) could not be cross-linked due to a relatively small extracellular domain, and resulting lack of basic amino acid residues for BS^3 to bind to (data not shown). Using antibodies targeting toward the intracellular portion of the receptor we were able to reliably assess expression and distribution of the $GABA_BR$. We then validated that the antibodies used were specific for the $GABA_BR$ using a blocking peptide which inhibited all antibody binding (illustrated in Fig. 27B). Results obtained in this assay revealed small inter-sample variability making the results highly reproducible.

Discussion

In the current study, the cross-linking technique revealed no changes in the expression or distribution of the $GABA_BR$ at a time when Meth-induced CPP and MS are expressed. This result conflicts with the decreased $GABA_BR$-selective radioligand binding observed throughout the brain, including regions evaluated in the current study (i.e., cortex, NAc, MDT) of rats repeatedly self-administered cocaine (Frankowska et al., 2008b; Frankowska et al., 2008a); however, the amount of cocaine that was administered (15-18mg/day for more than 6 days) far exceeded the Meth administered (1mg/kg/day for 3 days) in the current study. Our possibility to account for the divergent results between our study and the previous self-administration study, a more robust psychostimulant administration paradigm might be required to obtain significant changes in $GABA_BR$ expression and/or distribution. Our findings are in agreement with an earlier study which demonstrated that during a time when amphetamine-induced behavioral sensitization was expressed (14 days after 5mg/kg x 5 days) $GABA_BR$ density was unchanged although there was a change in the functional coupling of the receptor to the G-protein (Zhang et al., 2000). This is not the first report to demonstrate that $GABA_BR$ expression does not always correlate with $GABA_BR$ function (Enna and Bowery, 2004). Based on these data, we may conclude that adaptations in $GABA_BR$ expression and distribution are not necessary for the expression of psychostimulant-induced behaviors or an alternative conclusion may be that there was a functional down-regulation of the $GABA_BR$ system which was not detected in these assays. Our laboratory has previously demonstrated that the inhibitory effects of GABA are diminished in the VP following a

sensitizing regimen of Meth (McDaid et al., 2005) which may reflect a change in the functional coupling of the $GABA_BR$ to the G-protein or one of the downstream mediators of the $GABA_BR$ including the expression or function of GIRKs and/or Ca^{++} channels (Bettler et al., 2004; Mott and Lewis, 1994; Barthel et al., 1996). Taken together psychostimulant-induced behaviors which appear to not involve alterations in $GABA_BR$ expression or distribution may be the consequence of a *functional* down-regulation of the $GABA_BR$ system. Further analysis will be required to validate this possibility.

These findings indicate that one day after the expression of Meth-induced CPP the expression and distribution of the $GABA_BR$ are not altered in rats that learned to associate the rewarding properties of Meth with the contextual cues of the chamber. Likewise, 14 days after repeated Meth administration, at a time when Meth-induced behavioral sensitization is expressed, the expression and distribution of the $GABA_BR$ were unaltered. While we were not able to identify changes in $GABA_BR$ expression or distribution at a time when Meth-induced CPP or MS were expressed, this report describes a re-purposing of a technique to assess changes in cellular distribution of a metabotropic receptor for the first time. This technique allows for a reliable and efficient means to assess expression and distribution of surface proteins which may be applied to a wide variety of proteins inserted into the neuronal surface.

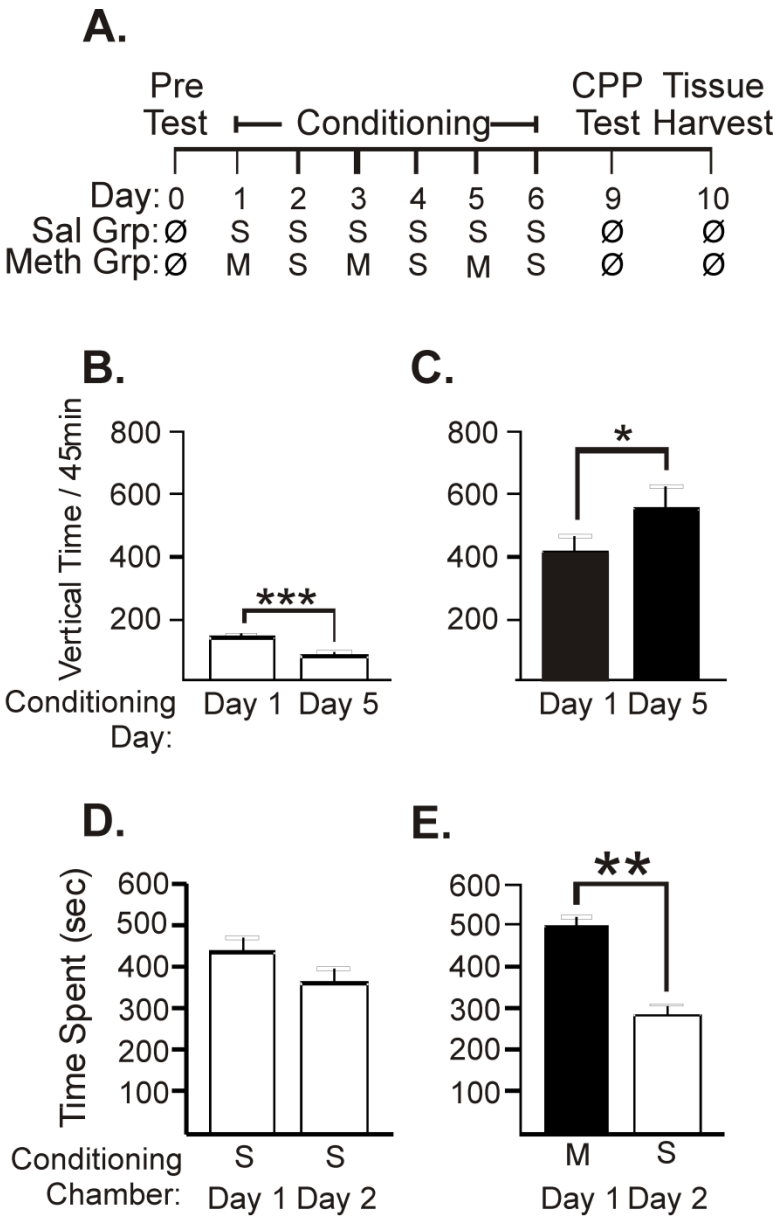

Figure 22. A six-day conditioning protocol was sufficient to induce methamphetamine-induced conditioned place preference and behavioral sensitization. **A.** Illustration of treatment protocol. A drug-free Pre-test was conducted (day 0) and rats were counterbalanced (half were given the day 1 treatment in chamber in which rats spent the least amount of time during the Pre-

test and the other half in the chamber in which the greatest amount of time was spent during the Pre-test). Conditioning occurred on days 1–6; saline conditioned group was administered saline on days 1-6 (n=16) and the Meth conditioned group was administered Meth on days 1, 3, & 5 and saline on the alternate days (n=16). A drug-free CPP test was conducted on day 9 to verify the development of CPP (CPP Test 1) and tissue was collected from drug-free rats one day later (day 10). Sal Grp, saline conditioned group; Meth Grp, methamphetamine conditioned group; M, methamphetamine (1mg/kg, as the base); S, saline (1mg/kg); Ø, no drug. During conditioning, the saline group exhibited a significant decrease in time spent in the vertical position (s) (day 1 *vs*. day 5) consistent with habituation (**B**; Paired t-test, $p<0.001$) whereas the Meth group had a significant increase in time spent in the vertical position (seconds) when comparing the first to the last Meth injection (day 1 *vs*. day 5) demonstrating the development of behavioral sensitization (**C**; Paired t-test, $p<0.05$). The drug-free CPP test conducted on day 9 revealed that saline conditioned rats had no preference for either chamber (**D**; Paired t-test, $p>0.05$) while the Meth-conditioned rats demonstrated a significant preference for the chamber paired with Meth (**E**; Paired *t*-test, $p<0.01$). S, saline-paired chamber; M, methamphetamine-paired chamber.

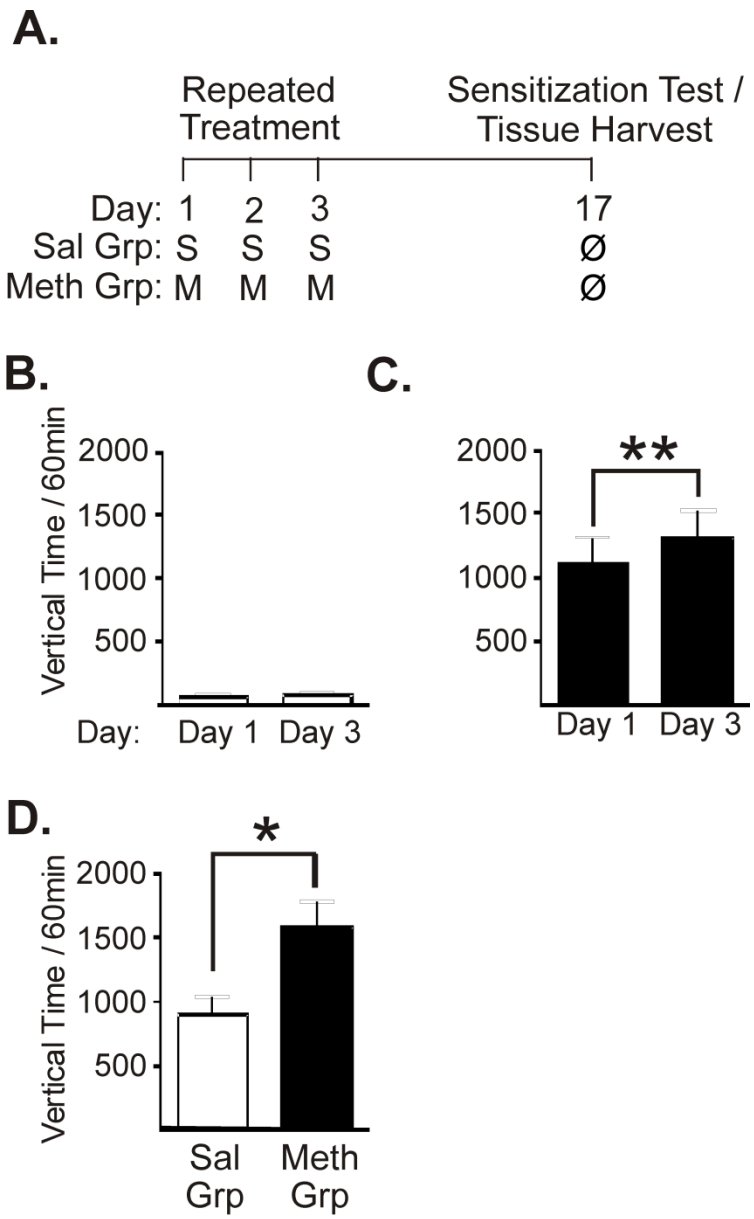

Figure 23. Three once-daily injections of methamphetamine induced behavioral sensitization which was expressed at 14 days withdrawal. **A.** Illustration of treatment protocol. Three once-daily injections of saline or vehicle were administered (day 1-3) and 14 days later (day 17) rats were either given an acute challenge or tissue was harvested from drug-free rats. Sal Grp, saline repeated

treatment group; Meth Grp, methamphetamine repeated treatment group; M, methamphetamine (1mg/kg, as the salt); S, saline (1mg/kg); Ø, no drug. During repeated treatment, the saline-treated rats did not exhibit a significant change in vertical time (seconds spent in the vertical position) (day 1 *vs*. day 3) (**B**; Paired t-test, $p>0.05$) whereas the Meth group had a significant increase in time spent in the vertical time when comparing the first to the last Meth injection (day 1 *vs*. day 3) demonstrating the development of behavioral sensitization (**C**; Paired *t*-test, $p<0.01$). On day 17, rats with a treatment history of Meth (n=20, filled bars) had significantly higher motor activity than rats repeatedly administered saline (n=18, open bars) over the 60min post Meth injection time period. **D.** Vertical time; Student's *t*-test, $p<0.05$.

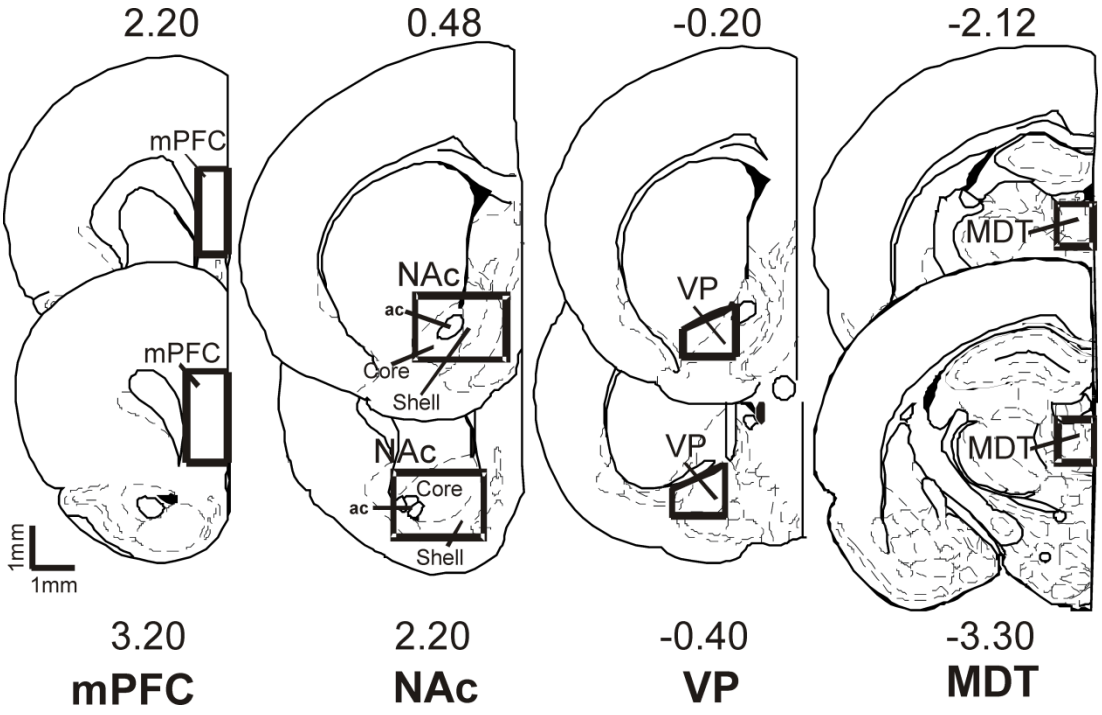

Figure 24. Anatomical illustration of the brain regions dissected for immunoblotting. Sections were redrawn from Paxinos and Watson (1998) and the numbers illustrate the distance in millimeters from Bregma. mPFC, medial prefrontal cortex; NAc, nucleus accumbens; VP, ventral pallidum; MDT, medial dorsal thalamus.

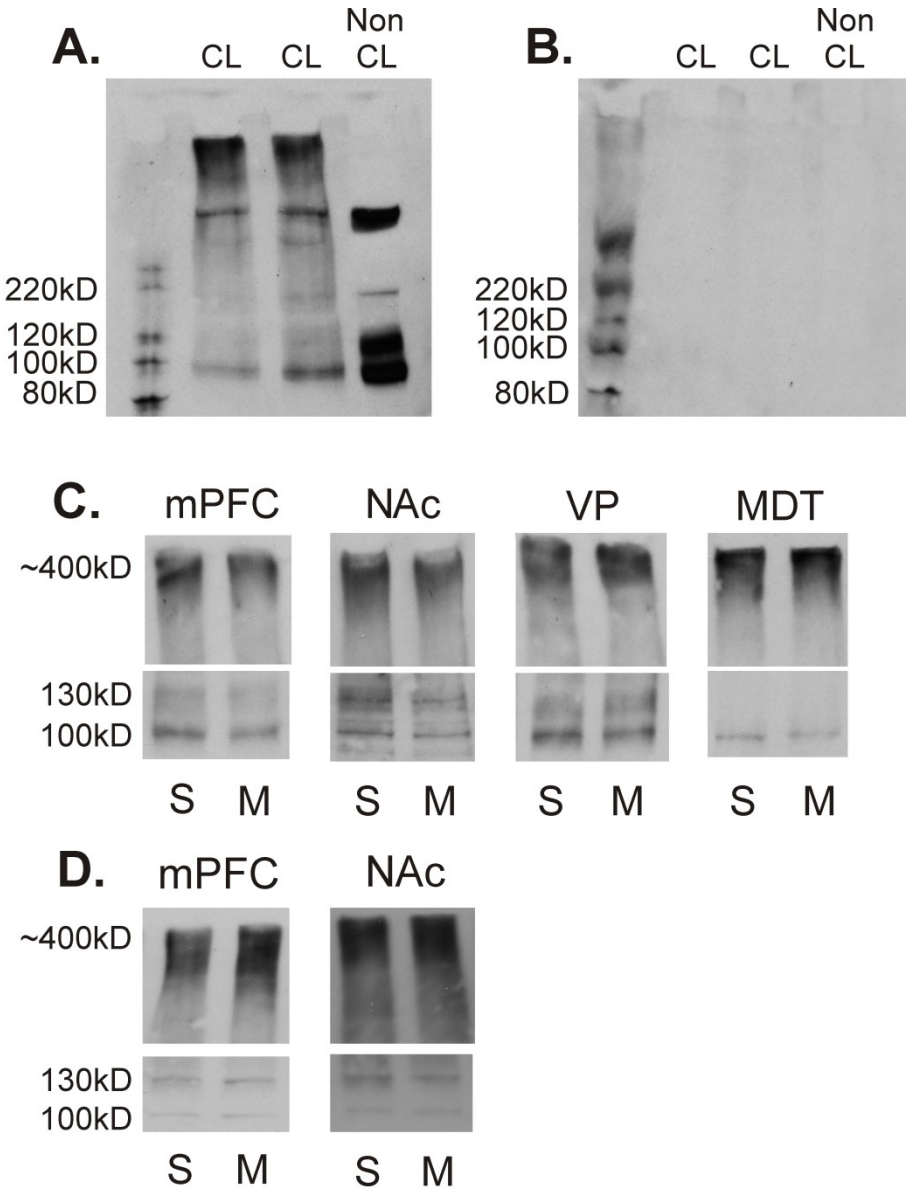

Figure 25. Validation of the cross-linking technique and $GABA_B$ receptor antibody specificity. **A.** Immunoblotting of cross-linked (CL) and non-cross-linked (Non CL) mPFC tissues demonstrated a high molecular weight aggregate (~400kD) which corresponds to BS^3 bound $GABA_BR$ inserted into the neuronal membrane in the CL tissue which is not present in the Non CL tissue. Bands corresponding to receptors contained in the intracellular compartment were found

at the expected molecular weights of the two isoforms, $GABA_{B1a}R$ (~130kD) and $GABA_{B1b}R$ (~100kD) in CL and Non CL samples. A band corresponding to the dimerized receptor ($GABA_{B1}R$ & $GABA_{B2}R$) was also observed in CL and Non CL samples. **B.** Pre-incubation of the antibody with blocking peptide inhibited all antibody binding, indicating antibody specificity. Representative blots from CL tissue used for quantification in behavioral studies. **C.** Experiment 1 ($GABA_BR1$-R20), tissue was collected one day after the test for chamber preference in which Meth (1mg/kg, as the base, i.p.) conditioned rats demonstrate a preference for the Meth-paired chamber and saline (1ml/kg) conditioned rats did not. **D.** Experiment 2 ($GABA_BR1$-R300), tissue was collected 14 days after three once daily treatments of saline (1ml/kg) or Meth (1mg/kg, as the salt, s.c.). mPFC, medial prefrontal cortex; NAc, nucleus accumbens; VP, ventral pallidum; MDT, medial prefrontal cortex; M, methamphetamine treated rats; S, saline treated rats.

Table 1. There were no significant changes in $GABA_B$ receptor expression or distribution after Meth conditioning (Experiment 1).			
	Saline Conditioned	Meth Conditioned	Student's *t*-test
Surface/Intracellular Ratio: S/I			
mPFC	100±6, n=15	115±6, n=15	p=0.077
NAc	100±4, n=11	96±6, n=12	p=0.583
VP	100±3, n=9	110±6, n=11	p=0.202
MDT	100±5, n=14	98±8, n=13	p=0.804
Total Protein: (S+I)/A			
mPFC	100±3, n=12	94±5, n=13	p=0.338
NAc	100±4, n=7	101±7, n=7	p=0.930
VP	100±4, n=10	107±6, n=11	p=0.353
MDT	100±3, n=13	94±3, n=10	p=0.182
Surface: S/A			
mPFC	100±4, n=13	104±7, n=15	p=0.636
NAc	100±3, n=7	105±9, n=7	p=0.625
VP	100±4, n=11	102±8, n=9	p=0.859
MDT	100±2, n=14	100±6, n=13	p=0.944
Intracellular: I/A			
mPFC	100±4, n=14	89±5, n=15	p=0.100
NAC	100±8, n=8	103±8, n=7	p=0.824
VP	100±3, n=10	109±6, n=7	p=0.139
MDT	100±4, n=14	96±4, n=13	p=0.543

Table 2. There were no significant changes in $GABA_B$ receptor expression or distribution 14 days after repeated Meth administration (Experiment 2).			
	Saline Repeated Treatment	Meth Repeated Treatment	Student's *t*-test
Surface/Intracellular Ratio: S/I			
mPFC	100±5, n=8	98±6, n=9	p=0.839
NAc	100±8, n=7	92±7, n=5	p=0.529
Total Protein: (S+I)/A			
mPFC	100±9, n=9	92±6, n=8	p=0.459
NAc	100±5, n=10	101±3, n=9	p=0.839
Surface: S/A			
mPFC	100±6, n=7	101±6, n=9	p=0.955
NAc	100±4, n=11	98±3, n=10	p=0.653
Intracellular: I/A			
mPFC	100±7, n=7	106±10, n=8	p=0.619
NAc	100±5, p=12	105±6, n=10	p=0.465

CHAPTER IX

BACLOFEN FACILITATES THE EXTINCTION OF METHAMPHETAMINE-INDUCED CONDITIONED PLACE PREFERENCE IN RATS

Abstract

Exposure to cues associated with abused drugs can trigger drug-craving, seeking and relapse in the abstinent addict. The powerful, long-lasting association between the rewarding effects of a drug and contextual cues associated with drug administration can be studied in laboratory rats using conditioned place preference (CPP). Extinction therapy is based on the concept that repeated exposure to drug-associated cues in the absence of the drug reduces the significance of cues and the likelihood of cue-induced relapse. Extinction processes engage multiple brain systems that are modulated by GABA and baclofen, the $GABA_BR$ agonist, which has been shown to facilitate the extinction of morphine-induced CPP in mice. The current study extended this work by determining if baclofen could enhance the extinction of methamphetamine (Meth)-induced CPP in rats. Meth-induced CPP was established using a six day conditioning protocol. Rats were subsequently administered baclofen (2mg/kg i.p. or its vehicle) in conjunction with six once-daily extinction training sessions followed by a CPP test (collectively termed an extinction training cycle). Our results demonstrate that CPP persisted for at least four extinction cycles in vehicle-treated rats. In contrast, the expression of Meth-

induced CPP was completely inhibited following the first extinction training cycle in which baclofen was administered immediately after each daily extinction training session. These data indicate that Meth-induced CPP was resistant to extinction, but extinction training combined with baclofen administration rapidly extinguished the preference for the Meth-paired chamber. These findings converge with the prior demonstration of baclofen facilitating the extinction of morphine-induced CPP indicating that $GABA_BR$ actions are independent of the primary (unconditioned) stimulus (i.e., the opiate or the stimulant) and likely reflect mechanisms engaged by extinction learning processes *per se*. Thus, baclofen administered in conjunction with extinction training may be of value for addiction therapy in abstinent addicted humans regardless of the class of drug being abused.

Introduction

Methamphetamine (Meth) is a highly abused psychostimulant. Even after long periods of abstinence, cues associated with the rewarding properties of psychostimulants can elicit drug-craving and seeking (O'Brien et al., 1992; Ehrman et al., 1992; Hartz et al., 2001). Thus, relapse to drug use remains a major challenge for psychostimulant-addicted individuals. These responses are attributed at least in part to the robust associative learning that occurs between contextual cues (conditioned stimulus) and the rewarding effects of abused substances (unconditioned stimulus) as well as the enduring nature of the drug-context memory. This long-lasting association can be demonstrated in the

laboratory with conditioned place preference (CPP) (Childs and deWit H., 2009; O'Brien et al., 1998; Tzschentke, 1998; Tzschentke, 2007). Unwanted associative memories can be disrupted by purposefully employing extinction learning procedures. For example, extinction procedures can reduce anxiety associated with post traumatic stress disorder in humans (McCleery and Harvey, 2004; Brunet et al., 2008). However, extinction of reward-related memories is not particularly efficacious in reducing relapse in abstinent drug-dependent humans (Conklin and Tiffany, 2002) or in rodent models of addiction (Crombag and Shaham, 2002; Di Ciano P. and Everitt, 2004); but there is evidence that combining extinction therapy with a pharmacotherapy may reduce cue-elicited relapse in humans (O'Brien et al., 1990) and mice (Heinrichs et al., 2010).

Currently there are no FDA-approved pharmacotherapies for Meth addiction; however, the $GABA_BR$ has received considerable attention as a potential pharmacotherapeutic target (Xi and Gardner, 2008; Rose and Grant, 2008; Brebner et al., 2002). $GABA_BRs$ negatively regulate neurotransmitter systems important for reward-mediated behaviors and mnemonic processes, including glutamate (Yamada et al., 1999; Lei and McBain, 2003; Porter and Nieves, 2004; Harte and O'Connor, 2005) and dopamine (Santiago et al., 1993c; Santiago et al., 1993b; Smolders et al., 1995; Westerink et al., 1996). Cues associated with drug reward activate limbic brain regions in drug-addicted humans (Childress et al., 1999; Childress et al., 2008) and rodents (Brown et al., 1992; Zombeck et al., 2008; Franklin and Druhan, 2000; Ciccocioppo et al., 2001; Rhodes et al., 2005).

This activation is attributed to the hyper-responsiveness of glutamatergic (Bell et al., 2000; Hotsenpiller et al., 2001) and dopaminergic (Lin et al., 2007) systems. Imaging studies indicate that baclofen, a $GABA_BR$ agonist, blunts the limbic activation associated with visual drug cues in drug-addicted humans (Brebner et al., 2002). Baclofen also inhibits the expression of many psychostimulant-induced behaviors in rodents including CPP (Li et al., 2001), conditioned motor sensitization (Hotsenpiller and Wolf, 2003), motor sensitization (Bartoletti et al., 2004; Frankowska et al., 2009; Lhuillier et al., 2007), and self-administration (Ranaldi and Poeggel, 2002; Brebner et al., 2005; Campbell et al., 1999; Filip et al., 2007; Roberts and Andrews, 1997; Smith et al., 2004; Weerts et al., 2007). It is clear that baclofen can alter learning and memory process. Particularly relevant to the current study is the recent demonstration that baclofen administered immediately after extinction training facilitated the extinction of morphine-induced CPP (Heinrichs et al., 2010). As baclofen modulates neurotransmitter systems that are critical for the expression of psychostimulant-induced behaviors as well as the extinction of opiate-induced CPP, we sought to determine the efficacy of baclofen to facilitate the extinction of Meth-induced associative learning. This experimental endeavor also determined if the baclofen effect seen with morphine-induced CPP generalized to the psychostimulant Meth.

Materials and Methods

Animals

Forty male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 250-300g at the start of the study were acclimated to the local *vivarium* (a climate-controlled environment on a 12hr light/dark cycle) for at least one week prior to the onset of the experiments. Rats were housed in pairs and allowed *ad libitum* access to food and water. Cage mates were given identical pharmacological treatments. Rush University Medical Center housing facilities are accredited through the Association for Assessment and Accreditation of Laboratory Animal Care, and all experiments were carried out in accordance with the conditions set forth by the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996) and with the approval of the Rush University Medical Center Institutional Animal Care and Use Committee.

Drugs

(+)Methamphetamine HCl (Sigma, St. Louis, MO) was dissolved in 0.9% sterile saline, and the dose, 1mg/ml/kg, was administered as the base. Baclofen was also dissolved in 0.9% saline and administered at a dose of 2mg/ml/kg (Sigma, St Louis, MO). Saline vehicle injections were administered as 1ml/kg. All injections were given intraperitoneally (i.p.).

The baclofen dose of 2mg/kg is within the range of doses used in laboratory rats to successfully attenuate psychostimulant-induced behaviors, including Meth-induced CPP (1.25, 2.5, 5mg/kg, i.p.) (Li et al., 2001), cocaine self-administration (2.5mg/kg, i.p.) (Smith et al., 2004), break point of amphetamine self-

administration (1.8, 3.2, 5.6mg/kg, i.p.) (Brebner et al., 2005), and amphetamine-induced motor sensitization (2mg/kg, i.p.) (Bartoletti et al., 2004). In addition, in pilot studies, we determined that motivated motor behavior assessed on the rotarod (San Diego Instruments, San Diego, CA) was inhibited by 4mg/kg baclofen and spontaneous motor activity was decreased by 3mg/kg baclofen but not by 2mg/kg (unpublished data).

Apparatus for Assessing Behavior

The test room was dimly lit (54-108lux) with white noise (San Diego Instruments, San Diego, CA) continuously present. The CPP apparatus (63cm x 30cm x 30cm) consisted of three chambers divided by Plexiglas sliding doors (AccuScan Instruments, Inc., Columbus, OH); two large conditioning chambers (25cm x 30cm x 30cm) separated by a small center chamber (13cm x 30cm x 30cm). Each chamber had distinct, yet neutral, visual and tactile cues. Chamber A had white vertical stripes and chamber B had white horizontal stripes on walls. These chambers were randomly fitted with floors that were either a textured surface with an overturned paint dish glued to the center or different textured floor with a smooth, flat rectangle glued to the center. The center chamber had white walls and a smooth, slightly raised platform floor. Time spent in each chamber and motor activity was monitored *via* two sets of photobeams (24 in the horizontal plane and 12 vertical).

Conditioned Place Preference

The rats were transported from the animal housing room to the adjacent test room at least 30min prior to the start of the experiment for habituation. Rats were subjected to a 30min pre-test (refer to the timeline in Fig.28A) which verified that there was not a significant preference for either chamber (data presented in Results); however, individual rats tended to spend more time in one chamber compared to the other. Thus, for conditioning, rats were administered Meth (1mg/kg) in the chamber in which they spent the least amount of time during the pre-test. Conditioning occurred over six days; Meth was paired with a unique context (i.e., chamber) on days 1, 3, & 5 and a saline (1ml/kg) was paired with a different context on days 2, 4, & 6. Pairing occurred immediately after each injection and lasted for 45min. In order to confirm that the preference developed, a drug-free CPP test was conducted on day 9 (termed CPP Test 1; Fig. 28A). For this test, rats were placed into the center chamber and the sliding doors were immediately removed allowing free access to the entire CPP box. The test session lasted 30min and time spent in each chamber was determined. Rats that did not increase time spent in the Meth-paired chamber on CPP Test 1 compared to the same chamber during the pre-test by at least 10% (180s) were excluded from the studies. This culling procedure, as previously shown by others (Paolone et al., 2009) helps assure that only those rats who clearly acquired the task were used to determine the potential of the $GABA_BR$ agonist baclofen to facilitate the extinction of CPP. Rats were assigned to either the baclofen or baclofen vehicle treatment group such that the magnitude of the preference during CPP Test 1 was approximately equal between the two groups. Each

extinction cycle consisted of six consecutive once-daily forced extinction sessions (45min) followed by a CPP test three days later (referred to as the "Extinction Test", Ext Test; Fig. 28A). The once-daily forced extinction sessions consisted of pairing a saline (1ml/kg) treatment with each chamber for 45min (termed "Pre-Training Injection"; Fig. 28A), alternating between the previously Meth- or saline-paired chamber (as done during conditioning), a commonly used approach for forced extinction training (Mueller and Stewart, 2000; Heinrichs et al., 2010; Schroeder and Packard, 2004). Immediately after each daily extinction session, baclofen vehicle (1ml/kg) or baclofen (2mg/kg) was administered (termed "Post-Training Injection"; Fig. 28A) and rats were returned to the home cage. Four extinction cycles (each including six once-daily forced extinction sessions and the Ext test) were conducted (Fig. 28A).

Statistical Analysis

A two-way repeated measures ANOVA was employed using the within group factor of chamber and the repeated measure of test. P*ost-hoc* Newman-Keuls was used to identify between chamber differences; significant preference was achieved when time spent in the Meth-paired chamber was significantly greater than time spent in the saline-paired chamber for any CPP/Ext Test. This approach has been used by Stewart and colleagues for similar evaluations (Paolone et al., 2009; Botreau et al., 2006). All data are presented as mean ± SEM. Statistical outliers were determined as those rats that spent greater than

two standard deviations above or below the mean time spent in any chamber during any of the CPP or Ext tests.

Results

Results of the 30min pre-test demonstrated that, before conditioning, rats spent approximately equal amount of time in each chamber (time spent in chamber A, 868±64s *vs.* chamber B 810±63s; paired *t*-test, *p*=0.652 center chamber time was 122±9s; n=40). After conditioning (CPP Test 1, day 9), rats expressed a significant preference for the Meth-paired chamber compared to the saline-paired chamber (time spent in the Meth-paired chamber, 958±41s *vs*. time spent saline chamber 672±43s; paired *t*-test, p=0.0003; center chamber time was 170±8sec; n=40). Of these rats, 28 met the learning criteria detailed in the methods, and these rats with a strong preference for the Meth-chamber (i.e., robust learners) were subsequently assigned to receive either baclofen or baclofen vehicle in order to assess the ability of baclofen to facilitate the extinction of Meth-induced CPP. As detailed in the methods, rats that were outliers for any of the tests (CPP Test-Ext Test 4) were excluded (n=7) thus a total of 10 and 11 rats were included in the baclofen vehicle and baclofen groups, respectively.

Significant preference was expressed on CPP Test 1 and it persisted through four extinction cycles when vehicle was administered in conjunction with extinction training (n=10, Fig. 28B). A two-way repeated measures ANOVA revealed a significant effect of Chamber ($F_{(1,18)}$=15.966, *p*=0.001) but no effect of

Test ($F_{(4,72)}$=0.022, *p*=0.999) and no Chamber x Test interaction ($F_{(4,72)}$=1.011, *p*=0.407). A *post-hoc* Newman-Keuls test revealed significant CPP (significantly greater amount of time spent in the Meth-paired than the saline-paired chamber) for the CPP test and all subsequent extinction tests (Fig. 1B; p<0.05 or p<0.01). In contrast, baclofen administered immediately after each daily extinction session during extinction cycle 1 nullified preference for the Meth-paired chamber during all subsequent preference tests (n=11; Fig. 28C, Ext Test 1-4). A two-way repeated measures ANOVA revealed a significant Chamber x Test interaction ($F_{(4,80)}$=2.799, *p*=0.031) with no effect of Chamber ($F_{(1,20)}$=2.950, *p*=0.101) or Test ($F_{(4,80)}$=0.057, *p*=0.994). A *post-hoc* Newman-Keuls test revealed significant preference for the Meth-paired chamber compared to the saline-paired chamber only during CPP Test 1 (p<0.01). Although time spent in the center chamber was not included in the statistical analyses reported above, we verified that time spent in the center chamber did not significantly change for either treatment group during any of the preference tests (CPP Test-Ext 4) (one-way ANOVA; vehicle treated rats $F_{(4,45)}$=1.300, p=0.284, baclofen treated rats $F_{(4,50)}$=1.185, p=0.329).

Motor activity was monitored during each of the preference tests (CPP and Ext Tests). Examination of these data revealed no significant between group differences during any test for horizontal or vertical activity (Student's *t*-test, p>0.05; Table 1).

Discussion

The Meth-induced preference observed in the current study was highly resistant to extinction; repeated re-exposure to the chambers over four extinction cycles did not extinguish the Meth-induced preference in vehicle treated rats. In contrast, baclofen administered immediately after chamber re-exposure inhibited the preference for the Meth-paired chamber after the first extinction cycle (Extinction Test 1). This inhibitory effect was produced even in rats which demonstrated robust preference during CPP Test 1 (see culling procedure in Methods). Motor activity was not significantly altered by repeated baclofen treatment; thus, the place preference results do not reflect a change in the capacity of rats to successfully execute the task. These findings indicate that augmenting $GABA_BR$ signaling facilitates the extinction of Meth-induced CPP.

These results corroborate the recent publication by Heinrichs and colleagues which demonstrates that baclofen facilitates the extinction of morphine-induced CPP in mice (Heinrichs et al., 2010). This suggests that baclofen is working *via* an overlapping mechanism(s) engaged during the extinction of morphine- and Meth-induced CPP. During extinction training, rats were re-exposed to contextual cues (i.e., the conditioning chambers) that were previously associated with Meth- as well as those paired with saline. Re-exposure to cues associated with Meth (Rhodes et al., 2005; Chiang et al., 2009) and morphine (Guo et al., 2008a; Schroeder et al., 2003; Schroeder and Kelley, 2002; Schroeder et al., 2000) increases neuronal activity (e.g., amygdala and orbitofrontal cortex). This may reflect hyper-responsive glutamatergic (Bell et al., 2000; Hotsenpiller et al.,

2001) neurotransmission. Indeed, blocking glutamatergic AMPA receptors inhibits the increase in Fos expression that occurs during re-exposure to cocaine cues in limbic and cortical brain regions (Zavala et al., 2007). In the current study, blunting the cue-induced brain hyperactivity immediately after re-exposure to the Meth-paired context is a credible candidate mechanism by which baclofen facilitates the extinction of Meth-induced CPP with the same argument holding true for the extinction of opiate-induced CPP. If true, this indicates that the persistence of drug-induced associative memories is dependent on hyper-responsive glutamate neurotransmission in the time period after re-exposure to contextual conditioning cues which influences protein expression and neuronal structure.

Extinction of a memory is believed to involve new learning; the new memory becomes stronger than the previously established memory resulting in a different conditioned response (Quirk and Mueller, 2008). For example, memory consolidation can be facilitated by augmenting glutamatergic neurotransmission (Ungerer et al., 1998); the extinction of cocaine-induced CPP is enhanced by administering a mGluR5 positive allosteric modulator prior to extinction training (Gass et al., 2009) or a NMDA receptor agonist immediately after extinction training (Botreau et al., 2006). In the current study, baclofen was administered immediately after each daily extinction session (i.e., after re-exposure to the saline- or Meth-paired chamber), thus the extinction enhancing effects of baclofen may be due to enhanced consolidation of the extinction memory likely

involving mechanisms including PKA and CREB. Although not typically thought of as memory enhancing, the $GABA_BR$ agonist baclofen has been reported to improve passive avoidance learning when administered immediately after training (Georgiev et al., 1988; Saha et al., 1993) and recognition memory deficits induced by repeated Meth administration when administered prior to training (Arai et al., 2009). $GABA_BRs$ blunt glutamatergic (Yamada et al., 1999; Lei and McBain, 2003; Porter and Nieves, 2004) and dopaminergic (Santiago et al., 1993c; Santiago et al., 1993b; Smolders et al., 1995; Westerink et al., 1996) neurotransmission and this effect would serve to inhibit rather than facilitate memory consolidation. Antagonism of NMDA glutamate receptors impairs the extinction of fear conditioning (Liu et al., 2009) as well as the extinction of cocaine self-administration (Feltenstein and See, 2007) and similar impairing effects on the extinction of fear conditioning are observed with dopamine receptor antagonisms (Holtzman-Assif et al., 2010; Hikind and Maroun, 2008). Therefore, while baclofen has been reported to have positive influences on memory consolidation, the negative regulation of glutamate and dopamine does not account for the extinction facilitating effects observed in the current study.

Extinction of a previously established memory can occur when a memory is recalled (which makes the memory labile and sensitive to disruption) but not successfully reconsolidated (McGaugh, 2000; Tronson and Taylor, 2007; Taylor et al., 2009; Bevilaqua et al., 2008). Inhibiting reconsolidation as a practical means to reduce cue-induced cocaine seeking has been successfully

demonstrated (Lee et al., 2006; Lee et al., 2005). $GABA_BR$-induced decrease in glutamatergic neurotransmission (Yamada et al., 1999; Lei and McBain, 2003; Porter and Nieves, 2004; Harte and O'Connor, 2005) should serve to blunt memory reconsolidation as administration of an NMDA receptor antagonist inhibits reconsolidation of cocaine-induced CPP memory (Brown et al., 2008; Itzhak, 2008). Dopamine receptor antagonism has been reported to have both no effect on the extinction of cocaine CPP (Yim et al., 2009) but has been reported to facilitate the extinction of conditioned fear (an effect that may be attributed to the disruption of re-consolidation mechanisms) (Ponnusamy et al., 2005). In the current protocol, we cannot determine if baclofen enhanced extinction learning or inhibited memory re-consolidation, a task made more difficult by the fact that these processes have many overlapping mechanisms (Alberini, 2005), these avenues need further exploration to determine how baclofen facilitated the extinction of Meth-induced CPP.

Baclofen effects on memory maintenance, independent of re-exposure to the conditioning cues, should also be considered for the current behavioral outcomes. To date only Bartoletti and colleagues (Bartoletti et al., 2004) have demonstrated efficacy of baclofen to modify the *maintenance* of a previously established psychostimulant-induced behavior. These authors reported that the maintenance of amphetamine-induced motor sensitization is diminished by 10 home cage administrations of 2mg/kg baclofen. In the current study, baclofen may have inhibited memory maintenance independent of re-exposure to the

conditioning cues. However, while we have observed that the maintenance of Meth-induced CPP was not inhibited with two once-daily baclofen treatments (2mg/kg) (Chapter VI) the maintenance of the behavior can be inhibited by two once-daily injections of negative allosteric modulators of the metabotropic glutamate receptor subtype 5 (mGluR5). Likewise, 10 administrations of baclofen in the home cage inhibited the maintenance of Meth-induced CPP (Chapter IV); however, we contend that the inhibitory effects of baclofen on the maintenance of Meth-induced CPP may be more therapeutically efficacious by combining baclofen treatment with extinction training.

In summary, we have found that baclofen administered in conjunction with extinction training resulted in rapid and complete extinction of Meth-induced CPP. While the mechanism is unclear this exciting study provides insight into the role of the $GABA_BR$ in memory processes engaged after re-exposure to salient drug-associated contextual cues and may be of value as an addiction therapy for abstinent, Meth-addicted individuals.

Table 3. Motor activity recorded during testing revealed no significant differences between vehicle- (n=10) and baclofen- (n=11) treated rats.

	CPP Test 1	Ext Test 1	Ext Test 2	Ext Test 3	Ext Test 4
Horizontal Activity					
Vehicle	4242±226	3306±115	3098±198	3296±356	2876±152
Baclofen	4003±259	3291±252	3239±263	2899±201	2773±126
Vertical Activity					
Vehicle	746±51	528±44	519±49	446±33	406±32
Baclofen	757±82	569±62	527±60	380±32	398±26

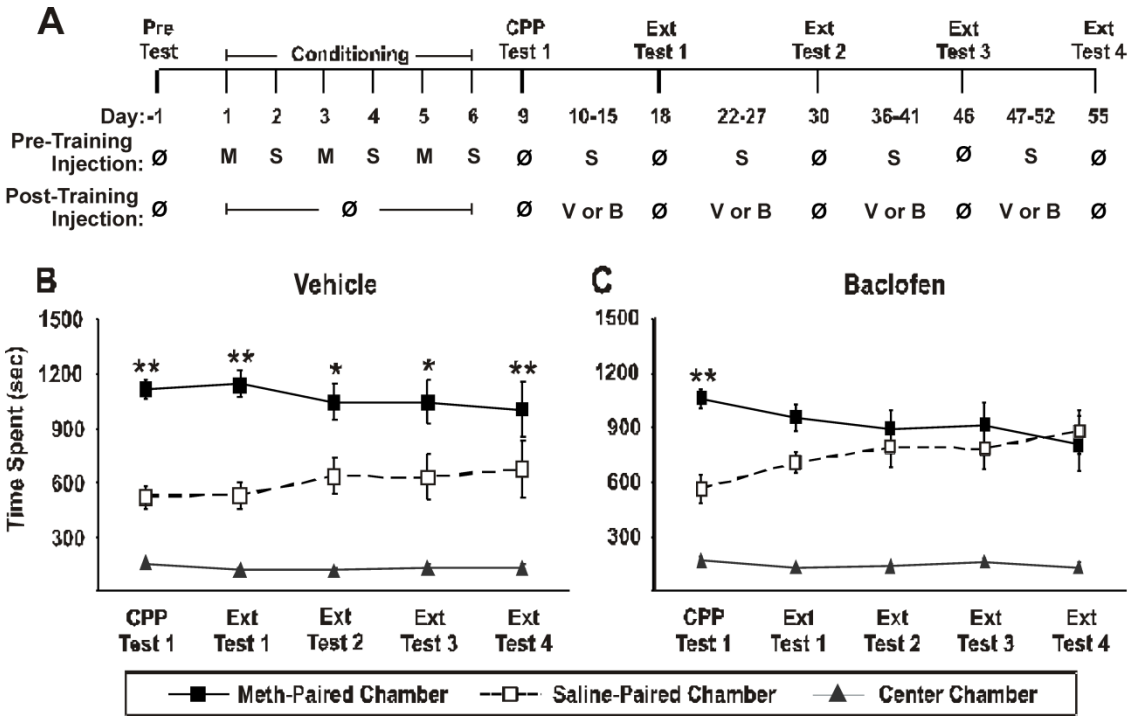

Figure 26. Extinction of Meth-induced CPP was facilitated by baclofen. A. Illustration of treatment protocol. A drug-free pre-test was conducted and rats were assigned to receive Meth in the chamber in which the least amount of time was spent during the pre-test. Conditioning occurred on days 1–6. A drug-free CPP test was conducted on day 9 to verify the development of CPP. Daily extinction sessions consisted of a saline injection prior to being placed into either the previously saline- or Meth-paired chamber. Immediately following each extinction training session, baclofen vehicle or baclofen was administered before the rats were returned to the home cage. Each six-day extinction cycle was followed by a drug-free test for preference three-days after the last extinction session (denoted Ext Test 1-4, on days 18, 30, 46 and 55). M, methamphetamine (1mg/kg); S, saline (1mg/kg); Veh, vehicle (0.9% saline,

1ml/kg); Bac, baclofen (2mg/kg); Ø, no drug. B. Meth-induced CPP established by 6 days of conditioning and subsequently expressed during CPP Test 1 was not altered by post-conditioning administration of vehicle; significant CPP was expressed on CPP Test 1 through Ext Test 4 (Vehicle, n=10). C**.** CPP Test 1 validated that rats expressed a preference prior to initiating extinction training. The preference evident during CPP Test 1 was not present after the first extinction cycle with baclofen (Baclofen, n=11). *Post-hoc* Newman-Keuls was used to determine between chamber differences ** $p<0.01$. Solid line, time spent in the Meth-paired chamber; dashed line, time spent in the saline-paired chamber; grey line, time spent in the center chamber.

CHAPTER X

GENERAL DISCUSSION

Relapse to drug use is a major problem for abstinent drug-addicted individuals; cues associated with drug use can precipitate drug-craving, -seeking, and –relapse (Childress et al., 1993; Ehrman et al., 1992; O'Brien et al., 1998). Thus, a mechanism to functionally 'uncouple' the cue from the response (i.e., drug-craving and –seeking) may reduce the propensity of individuals to relapse to drug use. Towards that end, this dissertation project explored the role of the $GABA_BR$ in the maintenance of Meth-induced CPP. The maintenance of CPP, compared to the development and expression phases, is a relatively unexplored area which has significant clinical applications, for it is those individuals who are already addicted who require treatment and will struggle with the consequences of being re-exposed to drug-associated cues. Thus, disrupting processes necessary to maintain the previously acquired drug-cue association may be of value as an anti-relapse therapy. This dissertation project was developed to assess if augmenting $GABA_BR$ signaling was sufficient to alter brain circuits necessary to maintain and subsequently expresses Meth-induced CPP. The collective results from this project demonstrate that the maintenance of Meth-induced learned associations are sensitive to disruption by augmenting $GABA_BR$ signaling. $GABA_BRs$ regulate the development and expression of drug-induced behaviors

(e.g., CPP and MS) as well as learning and memory; however, the influence of these receptors in the maintenance of drug-induced behaviors is largely unexplored. To date, only Bartoletti and colleagues have demonstrated that 10 once-daily injections of baclofen administered after the development of amphetamine- (Bartoletti et al., 2005) or morphine- (Bartoletti et al., 2007) induced motor sensitization inhibits the subsequent expression of the behavior and Heinrichs and colleagues have demonstrated that baclofen facilitates the extinction of morphine-induced CPP (Heinrichs et al., 2010). Findings from the current project extend these previous results by revealing that i) 10 once-daily systemic injections of baclofen or fendiline, ii) two once-daily systemic injections of GS39783 and CGP7930, iii) two once-daily intra-MDT injections of baclofen administered in the context of the home cage altered brain processes necessary to maintain Meth-induced CPP, and iv) six systemic injections of baclofen administered in conjunction with extinction training accelerated the disruption of the preference. $GABA_BR$ activation influences neuronal function *via* several different mechanisms which may disrupt Meth-induced CPP; below, I have overviewed the most plausible theories that help define these mechanisms and which dovetail into the findings from the current project.

Repeated administration of psychostimulants has significant effects on neuronal excitability. Studies overwhelmingly support the notion that the brain is hyper-excitable during re-exposure to drug-associated cues. This hyper-excitability was measured as an increase in i) Fos expression (Brown et al., 1992;

Ciccocioppo et al., 2001; Franklin and Druhan, 2000; Rhodes et al., 2005; Zombeck et al., 2008; Miller and Marshall, 2005), ii) neuronal firing (Rebec and Sun, 2005) iii) metabolic activity (Childress et al., 2008; Childress et al., 1999), and iv) extrasynaptic concentrations of neurotransmitters glutamate (Bell et al., 2000; Hotsenpiller et al., 2001; Hotsenpiller and Wolf, 2002) and dopamine (Lin et al., 2007). These studies consistently demonstrate neuronal hyper-excitability within the amygdala, thalamus, and PFC (especially the anterior cingulate and the orbitofrontal cortices) when the psychostimulant withdrawn individual is re-exposed to drug-associated stimuli. Both the amygdala and the thalamus send glutamatergic projections to the PFC; therefore, the PFC may be a site of convergence for multiple brain circuits which respond to drug-associated stimuli. Thus, restoring the inhibitory/excitatory balance in the PFC (which plays an important role in learning/memory processes as well as executive function) and associated circuits may reduce the maintenance and subsequent expression of Meth-induced CPP. Outcomes obtained in Chapter VIII indicate that any putative adaptations did not involve the level of expression or cellular distribution of the $GABA_BR$, in several limbic regions at times when Meth-induced behaviors (which presumably involve neuronal hyper-excitability) were expressed (i.e., cue-induced hyper-responsivity (CPP) and drug-induced hyper-excitability (MS)). Thus, it does not appear that changes in $GABA_BR$ expression and/or distribution are responsible for rats to demonstrate Meth-induced behaviors, and that down-regulation of some other component of the $GABA_BR$ system may have occurred. For example, decreased G-protein expression is observed after chronic cocaine

(Nestler et al., 1990) and functional coupling of the $GABA_BR$ to the G-protein after a sensitizing regimen of amphetamine (Zhang et al., 2000) or chronic cocaine (Kushner and Unterwald, 2001). It might be that overt changes in the expression of the $GABA_BR$ only occur with robust psychostimulant exposure (> 10 days, 15-18mg/kg i.v. cocaine/day) as was observed previously (Frankowska et al., 2008b; Frankowska et al., 2008a) and not with the more modest Meth-treatment protocols which induced CPP and MS (1mg/kg x 3 days). Although no changes were observed in the current project, a previous report has demonstrated that $GABA_BR$ expression does not predict function of the $GABA_BR$ system (Enna and Bowery, 2004). Determination of such changes in $GABA_BR$ system function at a time when Meth-induced behaviors are observed will require further study. Independent of the status of the $GABA_BR$ system, this project has demonstrated that augmenting $GABA_BR$ signaling inhibited the maintenance of Meth-induced CPP.

Activating $GABA_BRs$ inhibits Ca^{++} influx *via* high voltage activated Ca^{++} channels (Gong et al., 1998; Kalivas, 1993; Santiago et al., 1993c; Santiago et al., 1993b; Smolders et al., 1995; Westerink et al., 1996; Harte and O'Connor, 2005; Lei and McBain, 2003; Porter and Nieves, 2004; Yamada et al., 1999; Bonanno et al., 1997; Huston et al., 1990; Amico et al., 1995; Cardozo and Bean, 1995; Mintz and Bean, 1993; Scholz and Miller, 1991; Takahashi et al., 1998). This effect would oppose the enhanced excitatory response to drug-stimuli (i.e., subsequent drug exposure or re-exposure to cues) caused by repeated psychostimulant

administration which increases the expression of L-type Ca^{++} channels (Shibasaki et al., 2010; Ford et al., 2009; Nasif et al., 2005a; Nasif et al., 2005b; Hu, 2007). Whether attenuated inhibition or augmented excitation underlies maintenance, increasing $GABA_BR$ signaling effectively disrupted mechanisms necessary to maintain and subsequently express Meth-induced CPP.

Baclofen administered in conjunction with extinction training rapidly disrupted the Meth-induced preference beyond that achieved with extinction training alone (Chapter IX). This experimental protocol highlights events which occur during re-exposure to Meth-associated cues; it suggests that blunting neuronal activity and/or down-stream signaling events at a time when neuronal activity was increased (i.e., during re-exposure to cues) rapidly disrupted the Meth-induced preference. $GABA_BRs$ directly influence neurotransmitter release *via* inhibition of high-voltage activated Ca^{++} channels (Gong et al., 1998; Kalivas, 1993; Santiago et al., 1993c; Santiago et al., 1993b; Smolders et al., 1995; Westerink et al., 1996; Harte and O'Connor, 2005; Lei and McBain, 2003; Porter and Nieves, 2004; Yamada et al., 1999; Bonanno et al., 1997; Huston et al., 1990; Amico et al., 1995; Cardozo and Bean, 1995; Mintz and Bean, 1993; Scholz and Miller, 1991; Takahashi et al., 1998), neuronal excitability *via* activation of GIRKs (Tabata et al., 2005; Luscher et al., 1997; Nicoll, 2004; Chen and Johnston, 2005; Takigawa and Alzheimer, 1999), and down-stream molecular events *via* inhibition of cAMP and CREB (Bettler et al., 2004; Bowery et al., 2002; Bowery, 1993). Thus, the $GABA_BR$ has multiple mechanisms that may underlie the

observed behavioral outcomes. $GABA_BR$-mediated reduction in excitatory neurotransmitter release, is akin to blunting neuronal excitation *via* pharmacological antagonism of glutamate receptors; a well documented means to impair mnemonic processes (i.e., consolidation and reconsolidation) (Liu et al., 2009; Feltenstein and See, 2007; Brown et al., 2008; Itzhak, 2008; Holtzman-Assif et al., 2010; Hikind and Maroun, 2008; Ponnusamy et al., 2005). In addition, $GABA_BR$-mediated reduction in cAMP (Bettler et al., 2004) may reduce the activity of the transcription factor CREB (Barthel et al., 1996; Bettler et al., 2004; Mott and Lewis, 1994; Lhuillier et al., 2007; Yin et al., 2006). Indeed, augmenting $GABA_BR$ signaling at the same time as psychostimulant administration pharmacologically antagonizes events leading to psychostimulant-induced changes in the transcription factor CREB (Yin et al., 2006; Lhuillier et al., 2007) and also inhibits downstream consequences of changes in gene transcription including ΔFosB (Lhuillier et al., 2007) and neuropeptide gene expression (Zhou et al., 2004). This transcriptional regulation by the $GABA_BR$ may also reduce the expression of proteins that regulate mnemonic processes, e.g., NMDA receptors which are known to be regulated by CREB (Mayr and Montminy, 2001; McClung and Nestler, 2003). In addition, $GABA_BR$ mediated inhibition PKA (*via* cAMP) would be expected to negatively impact memory processes as direct inhibition of PKA impairs the maintenance of spatial, fear conditioned, and recognition memory (Abel and Nguyen, 2008; Wang et al., 2006) as well as memory consolidation (Bourtchouladze et al., 1998; Quevedo et al., 2004; Vianna et al., 2000; Isiegas et al., 2006) and reconsolidation (Micheau

and Riedel, 1999) when administered immediately before or after training/testing/re-exposure to cues. $GABA_BR$ G-protein independent mechanisms, where transcription factors bind directly to the receptor, may also be involved (e.g., CREB2) (White et al., 2000; Nehring et al., 2000; Vernon et al., 2001). Thus, there are multiple avenues by which augmenting $GABA_BR$ signaling can disrupt the cue-activated brain state which may account for the behavioral inhibition observed when baclofen was administered in conjunction with extinction training.

In contrast to the cue-activated brain produced during re-exposure to drug-associated cues (e.g., during extinction training) (Brown et al., 1992; Ciccocioppo et al., 2001; Franklin and Druhan, 2000; Rhodes et al., 2005; Zombeck et al., 2008; Miller and Marshall, 2005; Rebec and Sun, 2005; Bell et al., 2000; Hotsenpiller et al., 2001; Hotsenpiller and Wolf, 2002; Lin et al., 2007), the remaining studies primarily evaluated the effects of baclofen given without re-exposure to conditioning cues. Thus, administration of PAMs, which are highly dependent on extracellular GABA concentrations, may function quite differently in a cue-activated brain *vs.* a 'non-activated' brain. Although these brain states are quite different, results from this project demonstrated that augmented $GABA_BR$ signaling during the maintenance period is also sufficient to disrupt processes necessary to maintain Meth-induced CPP. This effect may be the consequence of the highly dynamic brain state that exists during the days and weeks following psychostimulant administration and learning with different kinases, transcription

factors, and proteins being expressed and activated in a time dependent manner (Lonze and Ginty, 2002; Selcher et al., 2002; Wang et al., 2006; Bailey et al., 1996; Nestler, 2001; Chen et al., 2009; Berke and Hyman, 2000; Carlezon et al., 2005; McDaid et al., 2006b; Ford et al., 2009; Alberini, 2009). Based on results obtained with the PAMs, regions which are critical for the maintenance of Meth-induced CPP express $GABA_BRs$ and have endogenous GABAergic tone.

While it is difficult to predict what brain regions systemic administration of baclofen, fendiline, GS39783, or CGP7930 might most influence; guided several criterion we chose to explore the MDT as a critical mediator of the effects of systemically administered ligands. The MDT i) expresses moderate to high levels of $GABA_BRs$ (Margeta-Mitrovic et al., 1999; Charles et al., 2003), ii) is tonically regulated by endogenous GABA (Churchill et al., 1996a), iii) sends a glutamatergic projection to the PFC which is known to regulate drug-induced behaviors and mnemonic processes (in fact the orbitofrontal cortex is defined almost entirely as a projection region of the MDT) (Goldman-Rakic, 1995; Kuroda et al., 1995; Krettek and Price, 1977; Sesack et al., 1989) which alters PFC neuronal activity (Bubser et al., 1998; Pirot et al., 1994; Ferron et al., 1984; Gigg et al., 1992), and iv) studies which demonstrate an involvement of the MDT in drug-induced behaviors (Churchill and Kalivas, 1999) and mnemonic processes (Romanides et al., 1999; Floresco et al., 1999; McAlonan et al., 1993; Kalivas et al., 1999; Kalivas et al., 2001; Oyoshi et al., 1996). As this anatomical connectivity would predict, augmenting $GABA_BR$ signaling in the MDT, which

should reduce glutamate release in the cortex (Bubser et al., 1998; Pirot et al., 1994; Ferron et al., 1984; Gigg et al., 1992), was sufficient to disrupt processes necessary to maintain Meth-induced CPP (Chapter VII). Glutamate in the PFC is a critical regulator of mnemonic processes (Wang et al., 2006). Administration of baclofen into other regions which provide significant glutamatergic input to the PFC would be expected to have similar behavioral outcomes. The HC provides glutamatergic input to the PFC and intra-HC baclofen inhibits passive avoidance memory retention (Zarrindast et al., 2002) as well as the acquisition of spatial memories (Arolfo et al., 1998). In contrast to these results, baclofen injected into the HC in the current study did not inhibit the maintenance of Meth-induced CPP (Chapter VII). Thus, intra-HC baclofen does influence mnemonic processes, but this effect may be a task-specific (i.e., associative learning *vs*. spatial or passive avoidance learning), phase specific (i.e., acquisition *vs.* maintenance), or dose-specific. It is interesting that the HC, which does provide glutamate to the PFC, was not identified by Rhodes and colleagues as a region which is engaged during re-exposure to Meth-associated cues in a Meth-CPP protocol (Rhodes et al., 2005). Future studies evaluating the amygdala which does provide a glutamatergic input and is activated during cue re-exposure would be of value in determining the neurocircuitry underlying the maintenance of Meth-induced CPP.

Our results suggest that brain region-selective augmentation of $GABA_BR$ signaling disrupts Meth-induced CPP more rapidly than non-selective activation of $GABA_BRs$ throughout the brain. This conclusion is drawn from the observation

that two once-daily systemic injections of the selective PAMs GS39783 and CGP7930 (Chapter VI), and local injection of baclofen into the MDT (Chapter VII), inhibited the maintenance of Meth-induced CPP, whereas two administrations of baclofen (Chapters IV & VI) or the PAM/L-type Ca^{++} channel blocker fendiline did not (Chapter V). Thus, global activation of $GABA_BRs$ by baclofen or the global inhibition of L-type Ca^{++} channels by fendiline may negate the effects of selectively augmenting $GABA_BRs$ in areas where GABA is endogenously released. That is, the $GABA_BR$ influence on neuronal activity in particular brain regions appears to have been overridden by global inhibition throughout the brain. This effect was independent of the post-conditioning time during which the two once-daily injections of baclofen and fendiline were administered (i.e., early post-conditioning time *vs.* a later post-conditioning time). Although the brain is highly dynamic during the days and weeks following repeated psychostimulant administration (Camp et al., 1997; Ernst and Chang, 2008; Jayaram and Steketee, 2005; McDaid et al., 2006b; McGregor et al., 2005; Zhang et al., 2000; Zhang et al., 2001; Giorgetti et al., 2002; Ford et al., 2009) and memory acquisition (Abel and Kandel, 1998; Alberini, 2009; Bailey et al., 1996), we did not observe time-dependent effects of baclofen or fendiline on the maintenance of Meth-induced CPP. Rather, the inhibitory effects of baclofen and fendiline appeared to be dependent on the number of treatments (i.e., protocol duration) and not on the post-conditioning withdrawal time during which the ligands were administered.

These findings indicate that persistent adaptations engendered by repeated $GABA_BR$ activation and/or Ca^{++} channel inhibition are needed to disrupt the place memory. Alterations in neuronal function resulting from two fendiline injections were not sufficiently robust to inhibit maintenance and subsequent expression of Meth-induced CPP when rats were tested in a drug-free state whereas drug-free inhibition of the preference was observable after 10 injections. Reports underscore that such adaptations are long lasting; e.g., 10 once-daily injections of baclofen inhibits the expression of amphetamine- and morphine-induced motor sensitization when evaluated 28 (Bartoletti et al., 2004) and 30 (Bartoletti et al., 2007) days after the last administration, respectively. Such persistent effects may reflect long term changes in gene expression which do not occur with two injections. Evidence for this effect can be found in Chapter V, two once-daily injections of fendiline rendered the brain vulnerable to an acute injection of fendiline; however, this effect was only observed for those rats which received fendiline during the late post-conditioning phase and not when fendiline was administered during the early post-conditioning phase. This indicates that the adaptations engendered by two injections of fendiline were not sufficiently robust (i.e., they are not observable in drug-free rats) nor did they persist (i.e., note behavioral differences between two injections during the early *vs.* late post-conditioning phase).

It should be noted that we interpret these effects in terms of 'maintenance of previously acquired associations' but the behavioral outcomes also might reflect

changes in motivation (Ma et al., 2010). Future studies evaluating the maintenance of other learned behaviors (e.g., Morris water maze, fear conditioning, novel object recognition) *vs.* tasks that more directly assess motivation (e.g., assess break point) are necessary to conclusively determine the mechanism underlying the observed behavioral effects. This will be especially important in light of the observation that in a progressive ratio experiment, baclofen (3.2mg/kg, i.p.) reduces cocaine self-administration break point when given 30min prior to the test while having no effect on food self-administration (Brebner et al., 2000). Thus, it will be informative to conduct a similar test with $GABA_BR$ agonists and/or PAMs administered during the post-acquisition phase on a drug-free test for break point.

While many of the exact underlying mechanisms are conjecture it is clear that the $GABA_BR$ is an exciting and highly therapeutically relevant target for an anti-addiction therapy. Results from this project suggest that not only do PAMs have an improved side effect profile over the agonist baclofen but also that they may afford significant therapeutic advantage wherein the selective PAMs GS39783 and CGP7930 were able to disrupt the maintenance of CPP with fewer administrations than baclofen or the non-selective PAM fendiline. Thus, it will certainly be of value to explore the use of the selective $GABA_BR$ PAMs GS39783 and CGP7930 as anti-addiction therapies.

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VITA

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