EVIDENCE FOR THE ROLE OF THE DOPAMINE D_3 RECEPTOR IN MEDIATING METHAMPHETAMINE ADDICTION

by

AMANDA E. HIGLEY

B.S., Jamestown College, 2003
M.S., Kansas State University, 2005

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Psychology
College of Arts and Sciences

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2009

Abstract

Methamphetamine is a potent psychomotor stimulant and a major drug of abuse. There is currently no effective medication available for treatment for methamphetamine addiction. The present study investigated the role of the dopamine D_3 receptor on IV methamphetamine self-administration and its effect on methamphetamine induced neurochemical changes. Acute administration of the putative D_3 receptor antagonists PG-01037 (10, 30 mg/kg, ip) and SB-277011A (12, 24, mg/kg, ip) significantly decreased the break-point for methamphetamine self-administration under a progressive-ratio (PR) schedule by 45 - 70%. Furthermore, both drugs dose dependently attenuated methamphetamine -triggered reinstatement of drug-seeking behavior in the reinstatement model of relapse. As with other drugs of abuse, the rewarding effects of methamphetamine are believed to be mediated by elevated levels of extracellular dopamine in the mesocorticolimbic system. The present study utilized *in vivo* microdialysis to examine the neurochemical mechanisms modulating the rewarding effects of methamphetamine actions evident in the various animal models of addiction. In the nucleus accumbens and ventral pallidum, acute methamphetamine (1.0 mg/kg, i.p.,) increased extracellular dopamine by 800 - 900% and decreased GABA by 60 – 65 % in the nucleus accumbens and ventral pallidum. Pretreatment with SB-277011A (12, 24 mg/kg) potentiated the methamphetamine induced dopamine increase but attenuated the methamphetamine-induced GABA decrease. Take together these data suggest that D_3 selective antagonists' pharmacotherapeutic potential in the treatment of methamphetamine addiction may involve a GABAergic mechanism.

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Acknowledgements

I would like to express my sincere appreciation to my major professor and mentor, Dr. Stephen W. Kiefer for his guidance and unconditional support throughout my graduate career. Also, I'd like to thank my committee members Drs Jerome Frieman, Mary Cain, Mark Weiss and Anna Whitfield for you assistance, direction, and cooperation. I am also grateful to Dr Eliot Gardner and NIDA IRP for the exceptional training experience I received at NIDA. Lastly, I would like to acknowledge my friends and family for your unconditional support and confidence in me. You raise me up! Thank you.

Dedication

This dissertation is dedicated to my family. To my mom and dad for teaching me to never give up on my dreams, for Todd, Cam & Ginny for giving me fight and for Baba, you started this process a long time ago and I'll spend the rest of my life making you proud. I am grateful to you all for your love and support, and remember… "Be careful where you set your goals for that is where you may end up."

CHAPTER 1 - Dopamine D_3 Receptors and Methamphetamine Addiction

Methamphetamine is a profoundly addictive drug that detrimentally affects health, family, businesses, and the environment in much of the United States. Methamphetamine related emergency department admissions increased 420% between 1992 and 2002 (U.S. Office of Applied Studies, 2004). One Midwestern state estimates that methamphetamine is the culprit in 90% of all newborns prenatally exposed to drugs (Lucas, Schlussman, Ho, McEwen, & Kreek, 1997). In 2004, 8% of treatment facility admissions were for the abuse of psychostimulants, and 99% of those psychostimulant admissions were for methamphetamine abuse (U.S. Office of Applied Studies, 2005). Patients with methamphetamine as their primary drug of choice are now the predominant population in public funded treatment systems in most states (Rose & Grant, 2008). Methamphetamine use continues to evolve as part of a growing epidemic. Internationally, it is estimated that more than 40 million people abuse methamphetamine (versus 15 million abusers of cocaine and less than 10 million abusers of opiates) (Meredith, Jaffe, Ang-Lee, & Saxon, 2005). Historically, its use had been rooted in the Western parts of the United States but recent trends show a highlighted emergence of methamphetamine within major East coast cities. Methamphetamine's outreach has far exceeded its geographic specificity and the U.S. Attorney General now considers methamphetamine the most dangerous drug in America (Jefferson, 2005).

The past decade has witnessed alarmingly widespread increases in methamphetamine use across all demographics and its debilitating effects transcend age,

gender, and sexual orientation. Adolescents report using methamphetamine to party or stay up all night; executives, to focus and be more productive; athletes, to enhance physical performance; the urban gay population to enhance sex; and women report using it to lose weight or cope with difficult emotions. As such, methamphetamine abuse is on the rise among those with eating disorders (Matsumoto, 2000, Gettig, Grady & Nowosadzka, 2006). Reports from the 2005 National Survey on Drug Use and Health indicated that 5.2% of the non-institutionalized, civilian, U.S. population, or 12.4 million Americans, report having tried methamphetamine at least once in their lifetime (US Office of Applied Studies, 2005). The 2*007 Monitoring the Future Survey*, a national survey of 8th, 10th, and 12th graders, indicates that 7.6% of students report having used methamphetamine in their lifetime (Johnston et al., 2008). These numbers show a staggering increase from the 1994 estimate of 0.6% (or 3.8 million Americans). Additionally, recent reports estimate a two-fold increase in the number of people who have illicitly used methamphetamine only because it is less expensive than cocaine (Crampton, Mishra & Zerfas, 2008).

The methamphetamine problem has become pandemic and there currently is no medication available to treat methamphetamine abuse. Ingestion of methamphetamine can cause a variety of cardiovascular problems, including rapid heart rate, irregular heartbeat, increased blood pressure, and irreversible, stroke-producing damage to small blood vessels in the brain (Gettig et al., 2006). Hyperthermia and convulsions occur with methamphetamine overdose, and without immediate treatment, can result in death. Chronic methamphetamine abuse can result in inflammation of the heart lining and, among users who inject the drug, damaged blood vessels and skin abscesses. Heavy users

also show progressive social and occupational deterioration. Additionally, methamphetamine's continuous stimulation of the nervous system has been shown to induce negative psychological states that include anxiety, confusion, insomnia, aggression, depression, paranoia, suicide ideation, and a number of additional psychotic features. These symptoms may persist for months or years after use has ceased and stress has been shown to precipitate spontaneous reoccurrence of methamphetamine psychosis (i.e., sensations of insects creeping under the skin) in former methamphetamine abusers (Perdue, Hagan, Thiede, & Valleroy, 2003).

Recent rodent and primate research data has confirmed methamphetamine's toxological repercussions, adding that they may persist for months or years following only a few exposures to methamphetamine (Cadet, Jayanthi, & Deng, 2003; Rose & Grant, 2008). Nordahl, Salo and Leamon (2003) found significant biochemical alterations in the brains of rats within three days of a single exposure to methamphetamine, which included reduced dopamine transporter function, and increased dopaminergic nerve terminal damage. Prolonged use of methamphetamine is associated with depletion of pre-synaptic monoamine reserves, down-regulation of neurotransmitter receptors and transporters, and neurotoxicity/ excitotoxicity due to the metabolic by-products of dopamine, serotonin, and prolonged release of glutamate (Davidson, Gow, Lee & Ellinwood, 2001; Nordahl et al., 2003). Moreover, these neurotoxic effects occur preferentially in the destruction of dopamine synaptic terminals rather than total cell loss (Halkitis, Zade, Shrem, & Marmor, 2004; Meredith, et al., 2005). Monkeys exposed to methamphetamine continued to have depleted dopamine store three years post exposure (Seiden, 1991) and dopamine transporter levels were still reduced 11 months post

methamphetamine exposure. The damage to the brain caused by methamphetamine use is comparable to damage caused by Alzheimer's disease, stroke, and epilepsy.

Methamphetamine is a highly lipophilic cationic molecule that readily crosses the blood brain barrier and has potent actions on the sympathetic and central nervous systems (Beebe & Walley, 1995). Though, classified as a psychostimulant and structurally similar to amphetamine and dopamine, methamphetamine effects are more pronounced. Amphetamine's stimulant effects last just a few minutes, due in part to its short duration of action, and its quick and almost complete metabolism within the body. Conversely, methamphetamine's stimulant effects last for hours, it has a 10-fold longer duration of action than cocaine, and no efficient mechanism exists for its metabolism. Following oral administration, peak methamphetamine concentrations occur within 2.6 – 3.6 hours with a mean elimination half-life of 10.1 hours (range 6.4 – 15 hours) (Rose & Grant, 2008; Schepers Oyler, Joseph et al., 2003;). Moreover, 15% of Caucasians are deficient in the only enzyme that helps metabolize methamphetamine (cytochrome P450-2D6) rendering them ultrasensitive to the effects of methamphetamine (Cho & Melega, 2002) and leaving a large percentage unchanged in the body (Glittenberg & Anderson, 1999). Unlike cocaine, which elevates extracellular dopamine by blocking the plasma membrane transporters, which in turn inhibit dopamine reuptake, methamphetamine attacks several neural systems. For example, methamphetamine induces its effects by blocking monoamine transporter activity, redistributing catecholamines from synaptic vesicles, inhibiting dopamine transporter expression on the cell surface, inhibiting monoamine oxidase, and by stimulating tyrosine hydroxylase activity (Barr, Panenka, MacEwan, et al., 2006; Sulzer, Sonders, Poulsen, & Galli, 2005;). Additionally, methamphetamine

inhibits both the plasmalemmal monoamine transporter and the intraneuronal vesicular transporter; this combined inhibition is likely responsible for the neurotoxic concentrations of monoamines within the nerve terminal levels (Carta, Gerfen & Steiner, 2000; Kitamura, Wee, Specio, Koob & Pulvirenti, 2006) and for the rapid escalation from methamphetamine abuse to addiction.

Pathophysiology of Addiction: The Dopaminergic System

Drug addiction is a chronic, relapsing disorder characterized by reduced pleasure from natural rewards, and compulsive drug-seeking despite severe negative consequences (Kalivas & Volkow, 2005, Kelley, 2004; Nestler, 1992; Wise, 1996a). The development of drug addiction occurs in a chronological sequence, beginning with the acute reinforcing effects of the drug, and in some individuals, progresses to chronic habitual use and dependence (Leshner & Koob 1999; Wise 1996b). Neuropharmacologic studies indicate that addictive behavior is driving the negative emotional state stemming from dysregulated neurochemical mechanisms in specific brain circuits (i.e., CRF, dopamine, GABA, glutamate, opioids, and serotonin) that reside in the reward pathway. As such, addiction and its components seemingly develop through maladaptive learning processes where associations between the rewarding interoceptive aspects of the drug and environmental cues associated with the drug overtake previous rewarding stimuli and behavior in such a way that addicts have a difficult time stopping drug use and experience high rates of relapse following periods of abstinence. This maladaptive learning process may be mediated by long-lasting (possibly permanent) alterations in the brain structure and neurocircuitry (Kalivas & Volkow, 2005; Kelley, 2004; Nestler, 1992; Wise, 1996b). Koob et al., (2004) understood these neuroadaptive processes to be in

direct opposition to the acute reinforcing properties of the addictive drug leading to impairment in the mechanisms that mediate "normal" positive reinforcement and thus the emergence of effective changes such as anxiety, dysphoria, and depression in the drug's absence. It is this combination of lowered function of neurotransmitters involved in the positive/reinforcing properties of drugs and the recruitment of the reward pathway which alter the hedonic set point and can lead to the compulsive drug –seeking and drug-taking behavior that is characteristic of methamphetamine addiction. What is more, recent reports indicate low levels of striatal dopamine in the autopsied brains of recreational methamphetamine users. Even recreational use can cause depletion of neurotransmission and it is likely that these low dopamine levels explain some of the unpleasant withdrawal symptoms.

Dopaminergic System

The mesocorticolimbic system, which originates with the dopamine neurons in the ventral tegmental area (VTA) and projects into the nucleus accumbens (NAc), prefrontal cortex (PFC) and amygdala has been implicated in the hedonic and reinforcing effects of drugs of abuse (Kalivas & Volkow, 2005; Pierce & Kumaresan, 2006; Wise, 1996a, 1996b, 2005). The involvement of these regions in rewarding effects was first reported by Olds and Milner (1954) who demonstrated that animals will self administer electrical-stimulation in these brain areas. A common property of all drugs of abuse is their activation of the ascending dopaminergic neurons in the VTA that project to the nucleus accumbens and elevate dopamine levels (Di Chiara & Imperato, 1988; Koob, 1992) As with other drugs of abuse, the rewarding effects of methamphetamine are also

believed to be mediated by elevating extracellular dopamine in the NAc (McCann & Ricaurte, 2004; Riddle, Fleckenstein & Hanson, 2006).

Drugs of abuse (i.e., cocaine) often increase extracellular dopamine by binding to the dopamine transporter (DAT) protein (Hyman, Malenka & Nestler, 2006). DAT is responsible for terminating dopamine synaptic transmission by binding to dopamine and transporting it out of the synaptic cleft and back into the presynaptic terminal. By binding to DAT, psychostimulants prevent the removal of dopamine, thereby increasing the effect of released dopamine by both prolonging its lifetime in the synaptic cleft and permitting dopamine to diffuse more effectively between synapses (Kalivas, 2007). Further support for the dopamine hypothesis is that the pharmacological and behavioral effects of psychostimulants can be blocked by lesions of dopamine terminals with 6-hydroxydopamine (Nestler, 1992) or by pharmacological blockade of dopamine receptors in the NAc (Bressan & Crippa, 2005; Gardner, 2000). Based on this proposed mechanism of reward, and the ubiquitous enhancement of dopamine as a result of addictive drug intake, development of new medications for the treatment of drug addiction has focused on manipulation of dopamine transmission or dopamine receptors in the reward circuitry of the brain.

Dopamine exerts its actions via two families of dopamine receptors classified by their pharmacological profile, function, and homology. The dopamine D_1-like receptor family includes D_1 and D_5 receptors (Girault & Greengard, 2004) and the D_2-like family which includes the D_2, D_3 and D_4 (Vallone et al., 2000). Stimulation of the D_1 like family leads to activation of adenylylcyclase and the cyclic adenosine monophosphate (cAMP)-cAMP-dependent protein kinase (PKA) cascade (Stoof & Kebabian, 1981).

They are found primarily in the cortex and hippocampus and also in the caudate and nucleus accumbens (NAc). The D_2-like family (D_2, D_3 and D_4) are coupled to inhibitory G proteins such that stimulation inhibits adenylyl cyclase and in turn the cAMP-PKA cascade. D_2- like receptors are located throughout the brain with slightly higher densities in the caudate, putamen, and NAc. D_3 is found primarily in limbic regions with moderate to high concentrations in the Isles of Calleja, NAc, olfactory tubercle and basolateral amygdala (BLA) (Levant, 1997; Vallone et al., 2000).

Previous research has implicated both D_1 and D_2 receptors are involved in drug reward and addiction (Cabib, Castellano, Cestair, Filibeck & Puglisi-Allegra, 1991; Platt, Rowlett & Spealman, 2002; Rothman & Glowa, 1995). In preclinical studies, D_1 or D_2 receptor antagonists inhibit cocaine's rewarding effects, as assessed by drug self-administration, conditioned place preference, and brain stimulation reward experiments. Such effects, however, are mediated at doses that also inhibit natural reward (food, sucrose), locomotor behaviors, and/or produce dysphorogenic effects (Gorelick, Gardner & Xi, 2004; Platt et al., 2002; Wise, 2006). Moreover, cocaine self-administration is not abolished in the D_1 receptor knockout mouse (Xu, 1998) and D_2 receptor knockout-mice actually self-administer at a higher rate and lower dose than their wild type counterparts (Caine, Negus, Mello et al., 2002). Clinical findings for D_1 antagonists are mixed; Haney et al., (2001) report that some individuals experience no effect of D_1 antagonists on their cocaine craving, while others experience both an increase in the rate of cocaine self-administration and convey more positive subjective effects of cocaine (Haney, Ward, Folton & Fischman, 2001). Alternatively, clinical trials utilizing D_2 antagonists universally report that D_2 receptor blockade causes extreme dysphoria, depressive

symptoms, and motor side effects in the human population (Bressan & Crippa, 2005; Verhoeff et al., 2003). Collectively, these data support the hypothesis that other dopamine receptor subtypes are involved in mediating drug intake.

Since its identification in 1990 (Sokoloff, Giros, Martres, Bouthenet & Schwartz, 1990) the D_3 receptor has received much attention in the anti-addiction field in part due to its distinct features suggesting a functional role in mediating the rewarding effects of addictive drugs. D_3 receptors have the highest affinity to endogenous dopamine of all known dopamine receptors suggesting its predominant role in the normal function of the mesolimbic dopamine system (Diaz, Pilon, Le Foll, et al., 2002; Levant, 1998; Sokoloff et al., 1990; 1992; Stanwood, Artymyshyn, Kung, et al., 2000). Unlike D_1 and D_2 receptors, D_3 receptors are expressed preferentially in granule cells of the islands of Calleja and in the medium-sized spiny neurons of the mesolimbic dopamine system (Drago, Padungchaichot, Accili, & Fuchs, 1998; Gorwood et al., 1995; Gurevich & Joyce, 1999; Le Foll, Schwartz & Sokoloff, 2000; Shafer & Levant, 1998). Furthermore, in situ hybridization shows that D_3 receptor mRNA is present at low levels throughout the brain but is most abundant in the ventral striatum, NAc, dentate gyrus, and islands of Calleja, structures deemed critical in the motivation of drug taking behavior (Gurevich & Joyce, 1999; Sokoloff et al., 2001; 2006). This unique and restricted anatomical localization suggests an important role of D_3 receptors in drug reward and addiction (Heidbreder, Gardner, Xi, et al., 2005; Le Foll, Goldberg & Sokoloff, 2005; Newman, Grundt & Nader, 2005; Pilla, Perachon, Sautel, et al., 1999; Sokoloff et al., 2001; 2006; Xi & Gardner, 2007).

The density of D_3 receptors are elevated one-to-threefold in the NAc and ventromedial subregions of the caudate-putamen in the brains of cocaine overdose fatalities (Mash & Staley, 1999; Staley & Mash, 1996). Likewise, Segal, Moraes and Mash (1997) reported a six fold increase in the D_3 receptor mRNA and binding in victims of cocaine overdose. Neisewander et al., (2004) found elevated D_3 mRNA in the rat NAc shell following a single exposure to cocaine and, interestingly, termination of cocaine self-administration result in gradual increases in D_3 binding in the NAc. Le Foll et al. (2005) found selective increases in D_3 expression with no changes in D_1 or D_2 expression in rats exposed to cocaine or nicotine. Moreover, this D_3 over-expression was more pronounced in rats receiving repeated nicotine in a distinct environment versus their home cage, suggesting D_3 hyper-responsiveness to drug and drug-associated environmental stimuli in the addicted brain. Growing evidence demonstrates that pharmacological blockade of D_3 receptors implicate the central D_3 role in drug-induced reward, drug-taking, and cue-, drug-, and stress-induced reinstatement of drug-seeking behavior (for a more comprehensive review see Le Foll et al., 2005). These data support the potential use of D_3 receptor antagonists in clinical treatment of drug addiction.

Elucidating the role of D_3 in drug addiction has been hampered due to a lack of pharmacological tools showing considerable selectivity for D_3 over D_2 receptors. Until 2000, when SB-277011A was introduced, most compounds used in animal models of drug addiction have a 10- to 30-fold selectivity for D_3 over D_2 receptors in vivo. SB-277011A, is a potent, competitive, D_3 receptor antagonist with high affinity for human (pKi = 8.40) and rat (pKi = 7.97) dopamine D_3 receptors. SB-277011A has 120 and 80 fold functional D_3/D_2 selectivity for human and rat and lacks D_2 or D_3 agonist activity. It

is 100-fold selective over 66 other receptors, enzymes and ion channels and has an elimination half life of 2 hrs in the rat (Reavill et al, 2000). Recent data confirmed SB277011A's selectivity profile by screening 16 additional receptors, 16 ion channels, and 64 kinases (see Heidbreder et al., 2005 for review). Thus, SB-277011A represents a useful tool with which to probe dopamine D_3 receptor function.

SB-277011A blocked both the acquisition and expression of cocaine-induced conditioned place preference, suggesting that D_3 receptor specifically was involved in hedonic actions of cocaine-taking (Vorel, Ashby, Paul, et al., 2002). Utilizing the intracranial self stimulation paradigm, Di Ciano et al. (2003) demonstrated that SB-277011A (3.0 mg/kg, i.p.) blocked the cocaine-induced enhancement of brain stimulation reward while having no effect on brain stimulation reward thresholds by itself at this or higher doses. Spiller et al. (2008) demonstrated that 12 mg/kg SB-277011A blocked methamphetamine-induced enhancement of brain stimulation reward using the intracranial self-stimulation paradigm.

Gilbert et al. (2005) examined the effects of SB-277011A on cocaine self-administration under both fixed-ratio (FR) and progressive-ratio (PR) schedules of reinforcement in rats. Systemic administration of SB-277011A (3–24 mg/kg) did not significantly alter cocaine self-administration under an FR-1 schedule. However, pretreatment with SB-277011A (24 mg/kg i.p.) produced a significant decrease in cocaine self-administration when the schedule of reinforcement was increased from an FR1 to FR10 schedule and dose-dependently attenuated PR breakpoint levels for cocaine. SB-277011A also inhibits drug and stress-triggered reinstatement of cocaine seeking behavior. Xi et al. (2004) investigated the effect of stress on relapse to cocaine seeking in

rats using a stress-induced reinstatement paradigm which exposed rats to intermittent foot shock (0.5 mA; 0.5 s on; mean off period of 40 s) prior to self-administration testing. Results indicated that 12 mg/kg SB-277011A attenuated stress-induced relapse to cocaine seeking behavior. These data suggest that SB-277011A is effective in antagonizing actions produced by psychostimulants in rats (see a comprehensive review by Heidbreder et al., 2005).

Recent studies have shown that highly selective D_3 receptor antagonists block both the acquisition and expression of cocaine and heroin-induced conditioned place preference (Ashby et al., 2003; Vorel et al., 2002), inhibit alcohol consumption and reinstatement (Heidbreder et al., 2007; Thanos et al., 2005), attenuate cocaine-self-administration under a PR schedule (Gilbert et al, 2005; Xi et al., 2004, 2005, 2006), and inhibit drug-, cue-, and stress- induced reinstatement to cocaine seeking (Di Ciano and Everitt, 2003; Gilbert et al., 2005; Vorel et al., 2002; Xi et al 2004). Although the efficacy of D_3 receptor antagonists in attenuating the addictive potential of cocaine may be well established, the effectiveness of D_3 antagonists against the addictive potential of methamphetamine has been grossly neglected. The present study, for the first time, characterizes the effectiveness of SB-277011A and PG01037 (a novel D_3 receptor antagonist) in several preclinical animal models of methamphetamine reward and addiction.

SB-277011A is the most well characterized D_3 receptor antagonist to date, however, due to poor bioavailability (~2%) and a very short half-life (< 20 min) in primates (Austin et al., 2001; Remington and Kapur, 2001) GlaxoSmithKline Pharmaceuticals has halted further clinical development. Nevertheless, SB-277011A

remains an important research tool to probe the role of D_3 receptors in animal models of addiction. PG01037 is a novel, highly selective, D_3 receptor antagonist (Grundt et al., 2005, 2007). *In vitro* binding studies reveal that PG01037 has high affinity for the D_3 receptor ($K_i(D_3) = 0.7 \pm 0.1$ nM) and 133-fold selectivity over D_2 receptors in HEK 293 cells transfected with human D_3 and D_2 receptors (Grundt et al., 2005) and a 2.6 hr eliminataion half life in rats (Personal Communication with Dr. Amy Newman, NIDA/IRP). Pharmacological MRI studies have shown that PG01037 readily enters the brain and is localized in D_3 receptor rich brain regions such as the nucleus accumbens and islands of Calleja, without significant localization in the caudate putamen, a brain region rich with D_2 receptors (Grundt, et al., 2007). PG01037 has a similar regional activation pattern to SB-277011A (Schwartz et al. 2004), significantly higher binding affinity for the D_3 receptor and has been shown both *in vitro* (Grundt et al., 2005, 2007) and *in vivo* (Collins et al., 2005, Higley et al., 2008) to function as a selective D_3 antagonist.

The present study observed the effects of SB-277011A and PG01037 on the acute rewarding effects of methamphetamine using a low effort-high reward (FR2) schedule of intravenous methamphetamine self-administration. Experiment 1b used a progressive ratio (PR) schedule of reinforcement to examine the effect of D_3 antagonists on methamphetamine's reinforcing efficacy. The PR paradigm (Richardson & Roberts, 1996; Roberts, Loh & Vickers, 1989) imposes incrementally increasing work demand to receive a single "hit" of reinforcer until the animal "gives up" and stops responding. This failure to meet the response requirement is termed the PR "break-point" and is an index of the reinforcers appetitive value. The reinstatement paradigm (Shaham et al., 2003;

Shalev et al., 2002;) is an animal model developed to evaluate the high rates of relapse, which is a prominent characteristic of drug addiction. Abstinent addicts report three distinct triggers that cause relapse to drug seeking: 1) re-exposure to the drug previously used, 2) re-exposure to environmental stimuli (sights, sounds, smells) previously associated with the drug-taking behavior, and/or 3) elevated levels of stress. The reinstatement paradigm mimics the human condition by exposing "abstinent" animals to triggers previously associated with drug use and was used to investigate the role of D_3 on drug- and cue-induced relapse (Experiment 1c). Additionally, the neurochemical mechanisms underlying methamphetamine's rewarding effects were assessed (Experiment 2) via *in vivo* microdialysis techniques which allowed us to analyze alterations in reward pathway DA, GABA, and glutamate levels following exposure to methamphetamine.

CHAPTER 2 - Method

Experiment 1: Methamphetamine Self-Administration

Subjects

For all experiments, male Long-Evans rats (Charles River Laboratories, Raleigh, NC, USA), experimentally naive and initially weighing 250 – 300 g were utilized. Rats were housed individually in a climate controlled animal colony room on a reversed light-dark cycle (lights on at 1900 hr, lights off at 700 hr) with *ad libitum* access to food and water. All animals were maintained in a facility fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, and all experimental

procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, 1996) and were approved by the Animal Care and Use Committee of the National Institute on Drug Abuse of the United States National Institute of Health.

Surgery

All animals were prepared for experimentation by surgical catheterization of the right external jugular vein. The right jugular vein was exposed by blunt dissection and the catheter inserted into the vein and sutured into place. The catheter was then passed subcutaneously to the skull top and made to exit into a connector (a modified 22 gauge cannula; Plastics One, Roanoke, VA) which was mounted to the skull with jewelers screws and cranioplastic acrylic. The venous catheters were constructed of microrenathane (Braintree Scientific Inc., Braintree, MA, USA), and catheterization was performed under sodium pentobarbital anesthesia (65 mg/kg, i.p.) with standard aseptic surgical techniques. To prevent clogging, catheters were flushed daily with a gentamiacin-heparin-saline solution (30 IU/ml heparin; ICN Biochemicals, Cleveland, OH, USA), and a cannula cap was placed over the opening of the connector during the recovery period and at all times when the animal was not in a self-administration session.

Apparatus

All self-administration experiments were conducted in standard MED Associate (Georgia, VT, USA) operant response test chambers (32 × 25 × 33 cm). Each test chamber had one active and one inactive lever, located 6.5 cm above the floor. Depression of the active lever resulted in activation of the infusion pump; depression of

the inactive lever was recorded but had no consequence. A cue light and speaker were located 12 cm above the active lever. To aid acquisition and maintenance of drug self-administration behavior, each drug infusion was always paired with a conditioned cue-light and cue-sound (tone). The house light remained illuminated for the duration of the test session.

General Drug Self-Administration Training

Following recovery from surgery (5 – 7 days) animals were given the opportunity to self-administer intravenous (IV) methamphetamine (0.05 mg/kg/infusion) in daily three-hour sessions. During experimental sessions, each rat's catheter was connected to a microprocessor controlled infusion pump via tubing encased in a protective metal spring, from the head-mounted connector to the top of the experimental chamber. Training sessions began with the insertion of the operant retractable lever into the chamber and illumination of a 15 watt house-light that remained illuminated for the session's duration. Each depression of the retractable, active lever triggered one IV methamphetamine (0.05 mg/kg) infusion, in a volume of 0.08 ml delivered over 4.6 seconds. A white cue light located above the lever was illuminated, and a cue tone was emitted for the duration of the infusion. Further responses on the active lever during the infusions were recorded and resulted in additional infusions. A fixed ratio 1 (FR1) schedule of reinforcement was used for three to five days to facilitate acquisition of self-administration behavior. After this initial training, animals were switched to an FR2 schedule of reinforcement, such that two lever presses resulted in one IV infusion of methamphetamine (0.05 mg/kg/infusion). This dose of methamphetamine was chosen based on a pilot study conducted in the present lab indicating that rats trained with 0.05 mg/kg/infusion display rapid and reliable

acquisition of self-administration (Higley et al., 2007). Additional analysis revealed that 0.05 mg/kg/infusions methamphetamine lies within the range of the descending limb of the methamphetamine dose-response curve for self-administration where stable and reliable dose-dependent effects are observed (Xi et al., 2005). To avoid overdose during the self-administration period, each animal was limited to a maximum of 50 methamphetamine infusions per 3 hr session. Total self-administration training lasted 10 – 14 days. At the end of each daily session, the intravenous catheters were flushed with gentamiacin-heparin-saline solution and animals were returned to their individual chamber in the colony room.

Experiment 1a: Fixed Ratio 2 Methamphetamine Self-Administration

Twenty-four, male, Long-Evans rats were tested under a FR2 reinforcement schedule to investigate the role of D_3 antagonism on the acute rewarding properties of methamphetamine. Animals remained on the FR2 schedule for the methamphetamine until the following criteria for responding were met: 1.) a minimum of 20 presses on the active operandi per test session, 2.) less than 10% variability in inter-response interval, 3.) less than 10% variability in number of infusions taken, and 4.) less than 10% variability in number of presses on the active operandi for at least three consecutive days. After stable methamphetamine self-administration had been established (7 – 10 days), rats were divided into groups based on D_3 antagonist treatment condition: Those receiving SB-277011A (0, 6, 12, 24 mg/kg, i.p.) and those receiving PG01037 (0, 3, 10, 30, mg/kg, i.p.). On the test day, each subject received one dose of their respective treatment (either SB-277011A or PG01037) 30 min prior to the three-hour self-

administration test session. Data collection and all other conditions were identical to daily training sessions.

Normal, daily FR2 sessions resumed the following day and continued until stable baseline levels were achieved. Once the aforementioned criteria for baseline responding were met, animals received a different dose of the same D_3 antagonist with which they were previously tested. This same procedure continued until each animal was tested with all three doses plus vehicle. The order of testing for the various doses of either SB-277011A or PG01037 was counterbalanced according to a Latin square design.

Experiment 1b: Progressive Ratio Methamphetamine Self-Administration.

Ninety-six, experimentally naive, male, Long-Evans rats were used to investigate the role of D_3 antagonism on the reinforcing efficacy of methamphetamine using a progressive ratio (PR) paradigm. The PR paradigm is an effective tool for studying the reinforcing properties of drugs because it incrementally elevates the work effort required to achieve drug reward. Initially, animals were trained to self-administer methamphetamine under FR1 and FR2 reinforcement schedules before training on a PR schedule. The present study utilized the following exponential equation, in which the reinforcement number is a natural logarithmic function of the ratio value: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, 328, 402, 492, and 603. The break-point is operationally defined as a failure to complete the work effort ratio necessary to receive a reinforcer within 60 minutes from previous methamphetamine infusion. Said another way, it is the maximum reinforcing ratio achieved in a given

session. Animals quickly learned to administer methamphetamine on this PR schedule and displayed regular, controlled responding within several days.

Animals underwent continuous daily sessions of methamphetamine self-administration under PR reinforcement until stable break-points were achieved. Break-points were considered stable when the day-to-day variability in break-points fell within 1-2 ratio increments for three consecutive days. Once stable, animals were randomly assigned to one of eight subgroups (n = 12 each) based on the ensuing D_3 antagonist treatment: SB-277011A (0, 6, 12, 24 mg/kg; i.p.) or PG01037 (0, 3, 10, 30, mg/kg; i.p.). As it is relatively difficult to re-achieve basal break-point levels after each drug test, each animal received only two treatments; either vehicle or one dose of D_3 antagonist, delivered 30 min prior to initiation of PR self-administration sessions. Aside from this pretreatment, all other conditions were identical to training. Break-point values were calculated based on the number of earned infusions and active lever presses. Following the test session, animals were returned to their home cages and, on the next day, resumed normal PR sessions until the aforementioned criteria for stable baseline responding were met (approximately 7 – 10 days). Once stable baseline levels of responding were met, rats received their second and final treatment (vehicle or their respective D_3 antagonist) 30 min prior to their PR session and completed the test session in the manner previously described. The order of treatment (vehicle or D_3 antagonist) was counterbalanced.

Experiment 1c: D3 Receptor Antagonism and Relapse to Drug Seeking

Ninety-six, Long-Evans male rats, experimentally naïve at the study's start were used to examine the effect of pretreatment with SB-277011A or PG01037 on

reinstatement to drug seeking. The surgery, apparatus, and general procedure to establish stable FR2 methamphetamine self-administration were identical to that outlined above.

After stable responding for methamphetamine was established (approximately 14 days), rats underwent two weeks of daily extinction trials, during which methamphetamine was replaced by saline, and the methamphetamine-associated tone and cue light were off. Thus, active lever presses led only to a saline infusion. Daily 3-hour extinction sessions continued for each animal until the animal made less than 10 lever presses per 3-hour session for at least three consecutive days. After meeting extinction criteria, animals were randomly assigned to the drug-induced relapse to methamphetamine seeking group (n = 48) or the cue-induced reinstatement group (n = 48) for reinstatement testing.

Methamphetamine-Induced Relapse to Drug Seeking

On the reinstatement test day, 48 rats were randomly assigned to receive one of four treatment doses of SB-277011A (0, 6, 12, or 24 mg/kg, i.p) followed 30 minutes later by a priming injection of methamphetamine (1.0 mg/kg, i.p). The reinstatement test initiated immediately following the methamphetamine injection. Active and inactive lever presses were recorded but never resulted in the presentation the conditioned cues (light and tone) or in a methamphetamine infusions. Consequently, responses made on the active lever resulted in only a saline infusion.

Cue-Induced Relapse to Drug Seeking

The remaining 48, Long-Evans, male rats were randomly subdivided into four treatment groups for the exploration of PG01037 effects on cue-induced relapse to drug

seeking behavior. On the cue-induced reinstatement test day, rats were administered one dose of PG01037 (0, 3, 10, or 30 mg/kg, i.p.) 30 minutes prior to the start of the 3 hr reinstatement test. The cue-induced reinstatement test was initiated by two non-contingent presentations of the cue light and tone previously associated with a methamphetamine infusions All other conditions were identical to the extinction session in that responses on the active lever only resulted in a saline infusion.

Experiment 1d: Behavioral Effects of PG01037

PG01037 Self-Administration Maintenance

The initial self-administration training was identical to that outlined above for FR2 methamphetamine. Once stable responding for methamphetamine was met, rats were randomly assigned to one of three replacement conditions (n = 9 each) in which 1.) Methamphetamine (0.05 mg/kg/infusion) was available for self-administration in the following days during their standard 3-hr test session; 2.) Methamphetamine was replaced by PG01037 (0.066 mg/kg/infusion); 3.) Methamphetamine was replaced by physiological sterile saline (0.08 ml/infusion). As it may take several days to support self-administration for a novel reinforcer, the replacement test was conducted for five consecutive days. Sessions lasted three hours each.

Sucrose Self-Administration

To investigate the effect of PG01037 on natural reward, 24, male, Long-Evans rats were trained to lever press for 5% sucrose (100 mg sucrose dissolved into 2000 ml distilled water; sucrose obtained from Sigma Chemical Co., Saint Louis, MO). The

procedures for oral sucrose self-administration were identical to the procedures for methamphetamine self-administration except for the following: 1) No surgery was performed on the animals in the sucrose experiment; 2) Active lever presses led to delivery of 0.1 ml of 5% sucrose solution into a liquid food tray located on the operant chamber wall.

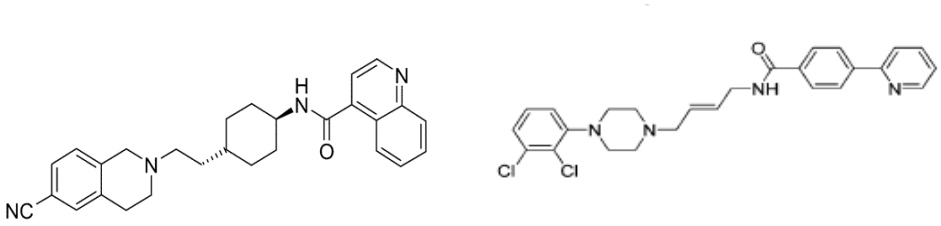
Half of the animals (n = 12) were trained and tested on an FR2 schedule of sucrose reinforcement, and half (n = 12) were trained and tested on a PR schedule; in both cases, a repeated measures design was utilized. Once the previously mentioned criteria for stable responding were met, animals were randomly assigned to receive one dose of PG01037 (0, 3, 10, or 30 mg/kg, i.p.) 30 minutes prior to the start of their regular self-administration session. Aside from the pretreatment with PG01037, all other conditions were identical to training. At the end of the test session, animals were returned to their home cage and resumed regular self-administration sessions the following day until stable baseline responding was regained. This procedure was repeated until each animal was tested with each dose of PG01037 and vehicle. The order of testing for the various doses was counterbalanced according to a Latin square design.

Drugs

(+/-) Methamphetamine (Sigma-Aldrich Corporation, St Louis, MO, USA) was dissolved in sterile physiological saline. SB-277011A (trans-N-[4-[2-(6-cyano-1,2,3,4-tetrahydroisoquinolin-2-yl)ethyl]cyclohexyl]-4-quinolinecarboxamide) was synthesized at MegaPharma Kft (Budapest, Hungary) and PG01037 ((E)-N-(4-(4-(2,3-dichlorophenyl)piperazin-1-yl)but-2-enyl)-4-(-2-yl)benzamide was synthesized by Dr.

Peter Grundt in the Medicinal Chemistry Section, Intramural Research Program, NIDA (Baltimore, MD) using procedures previously reported (Grundt et al., 2005). Both SB-277011A and PG01037 (see Figure 1) were dissolved in 0.5% Tween-80 (Sigma-RBI, St. Louis, MO). The 0.5% Tween-80 alone was used as vehicle for systemic injections.

Figure 1 Chemical Structure of SB-277011A and PG01037



Statistical analyses

All self-administration data are presented as mean (± *SEM*) number of active lever responses or infusions. Progressive ratio data is presented as percent change from stable baseline responding. Separate One Way analyses of variance (ANOVA) were used to evaluate the effect of SB-277011A or PG01037 on methamphetamine self-administration and reinstatement to methamphetamine seeking. Individual group comparisons were carried out using pre-planned Bonferroni *t* – tests. The minimum acceptable statistical significance was set at a probability level of $p < 0.05$ for all tests.

CHAPTER 3 - Results

Effect of D3 antagonism IV methamphetamine Self-Administration under an FR2 reinforcement schedule (Experiment 1a)

Treatment with SB-277011A (0, 6, 12, 24 mg/kg, i.p.) administered 30 minutes prior to the initiation of FR2 intravenous methamphetamine self-administration (0.05 mg/kg/infusion) had no significant effect on methamphetamine self-administration behavior compared to vehicle treated animals (see Figure 2A). A One Way repeated measures ANOVA revealed no significant treatment effect of SB-277011A on the number of active lever presses for IV methamphetamine ($F(3, 33) = 1.46$, p = NS) under FR2 reinforcement conditions. Likewise, PG01037 (0, 3, 10, or 30, mg/kg, i.p.) administered 30 min prior to self-administration sessions for IV methamphetamine had no statistically significant effect on self-administration behavior (see Figure 2B). A one-way ANOVA with repeated measures over PG01037 dose range revealed no statistically significant differences between any dose of PG01037 on methamphetamine self-administration under FR2 reinforcement conditions ($F(3, 33) = 0.56$; p = NS). Similarly, there were no differences in the inactive lever presses between treatments. Figure 3 illustrates an individual representative record for an animal receiving pretreatment with either vehicle or 30 mg/kg PG01037 Each vertical line represents an earned methamphetamine infusion. On average, animals earned approximately 25 infusions per hour (range 23.86 ± 2.7 to 27.23 ± 0.91) regardless of treatment condition.

Figure 2 SB277011A and PG01037 on FR2 Self Administration

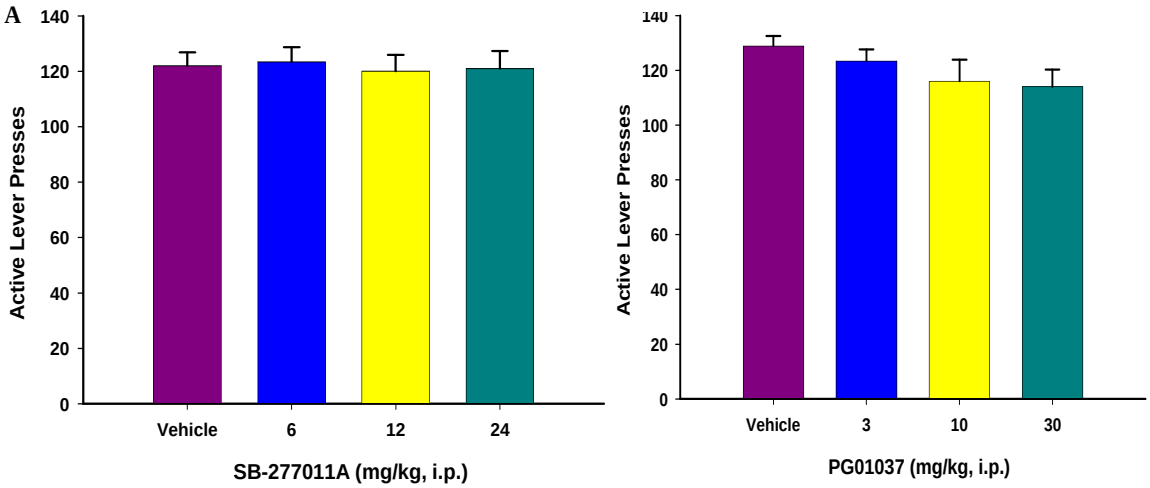
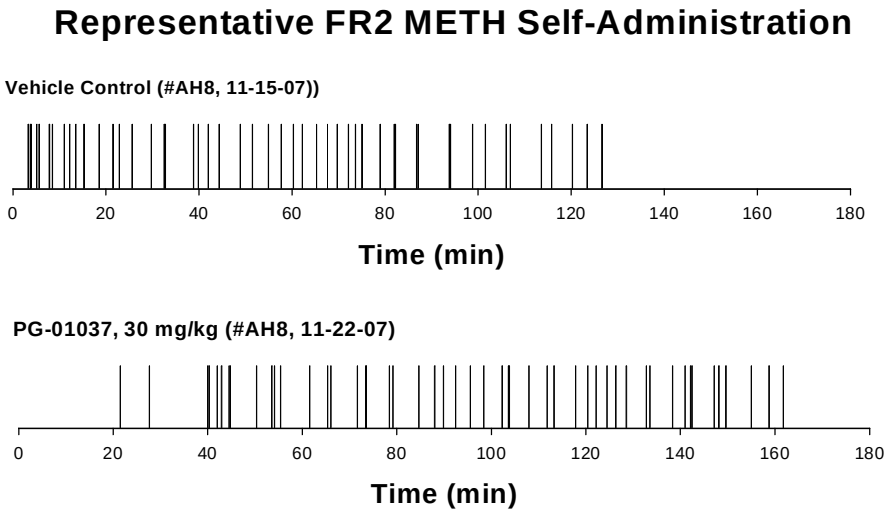


Figure 3 Representative Record FR2 Self Administration

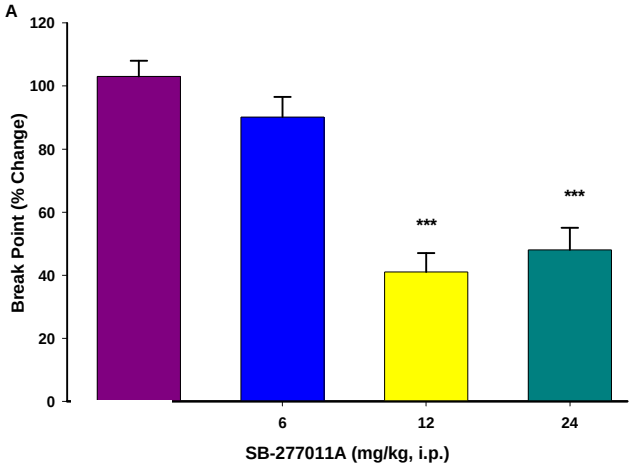


Effect of D3 antagonists on PR IV methamphetamine self-administration (Experiment 1b)

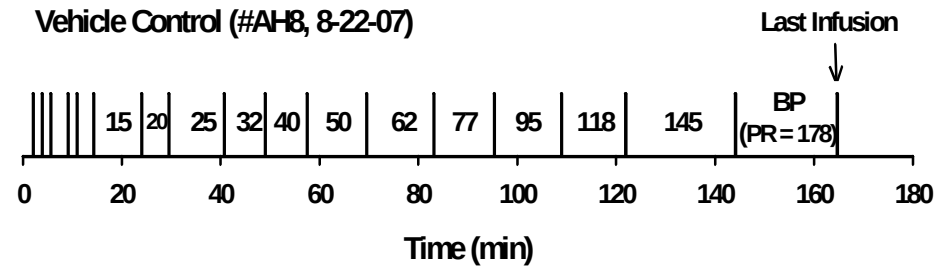
Figure 4A depicts the percent change in PR break-point for methamphetamine self-administration produced by 0, 6, 12, or 24 mg/kg SB-277011A pretreatment on test day compared to PR break-point for methamphetamine self-administration after vehicle pretreatment on test day. Treatment with SB-277011A significantly lowered the break-point for methamphetamine self-administration behavior reinforced under a PR schedule. A One Way ANOVA revealed a statistically significant treatment effect such that pretreatment with SB-277011A significantly reduced PR break-point values for methamphetamine self-administration ($F(3,44) = 8.27$, $p < 0.001$, $\eta 2 = 0.71$). Individual group comparisons using Bonferroni post hoc analysis revealed a statistically significant difference between PR break-point for methamphetamine self-administration following 12 mg/kg SB-277011A ($t = 4.39$, $p < .001$; n = 12) and 24 mg/kg SB-277011A ($t = 3.34$, $p < 0.01$; n = 12) versus pretreatment with vehicle. Figure 4B shows a representative record of an individual animal's active lever pressing pattern for IV methamphetamine under a PR schedule following pretreatment with vehicle and 12 mg/kg SB-277011A. Each vertical line indicates a methamphetamine infusion (0.05 mg/kg per infusion). The number between the vertical lines indicates the number of lever presses (PR ratio) required for a subsequent methamphetamine infusion with the last number indicating the PR break-point. Figure 4B is representative illustration of one rat's reduction in PR Breakpoint from 178 after pretreatment with vehicle (0.5% Tween80, i.p.; upper trace) to a break-point of 50 following SB-277011A pretreatment (12 mg/kg i.p., lower trace). Pretreatment with PG01037 had similar effects as SB-277011A on PR break-point for

methamphetamine (see Figure 5). A one-way ANOVA revealed a statistically significant main effect of pretreatment with PG01037 on PR break-point values for methamphetamine ($F(3, 36) = 26.56$, $p < 0.001$, $\eta^2 = 0.65$). Individual group comparisons using the Bonferroni post hoc analysis revealed that rats treated with 10 mg/kg PG01037 ($t = 6.14$, $p < 0.001$, n = 12) or 30 mg/kg PG01037 ($t = 6.31$, $p < 0.001$, n = 12) had significantly lower PR breakpoint values for methamphetamine than rats treated with 3 mg/kg PG01037 (n = 12) or vehicle (n = 12). There were no statistically significant differences between rats treated with 10 mg/kg PG01037 or 30 mg/kg PG01037. Figure 5A illustrates the dose dependent decrease in PR break-point following systemic administration of PG01037. Figure 5B shows a representative response record for methamphetamine self-administration under PR reinforcement conditions. It illustrates a PR break-point value of 268 in an animal receiving pretreatment with vehicle; pretreatment with 10 mg/kg PG01037, however, resulted in a drastically lower break-point value of 50 (lower trace). Regardless of condition or treatment, there were no significant effects on inactive lever presses.

Figure 4 SB277011A on PR Break-Point for Methamphetamine



B



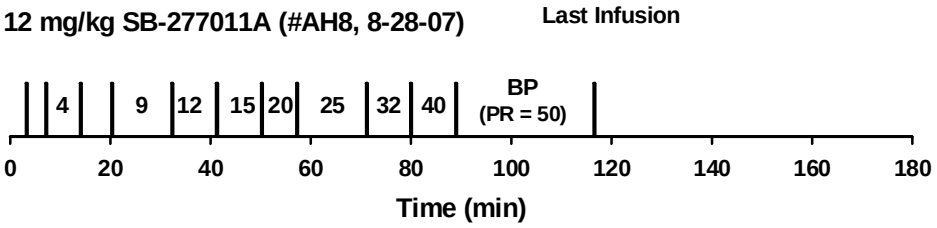
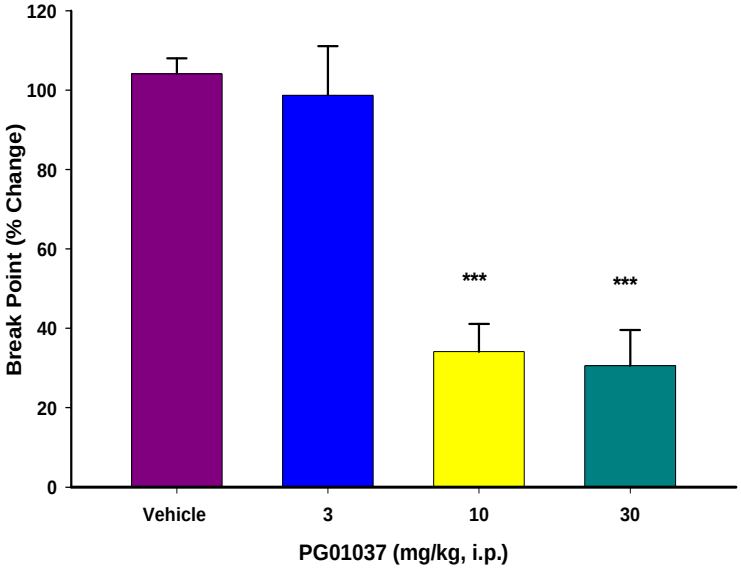


Figure 5 PG01037 on PR Break-Point for Methamphetamine

A



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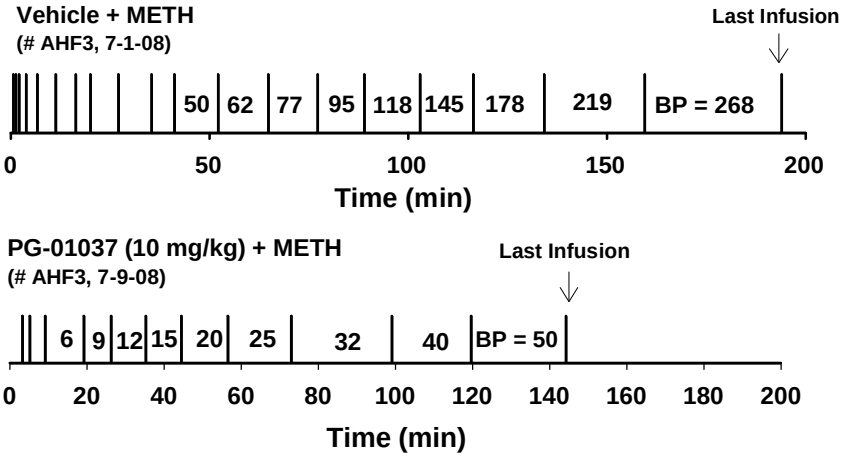


Figure 6 SB277011A on Methamphetamine Induced Relapse to Drug Seeking

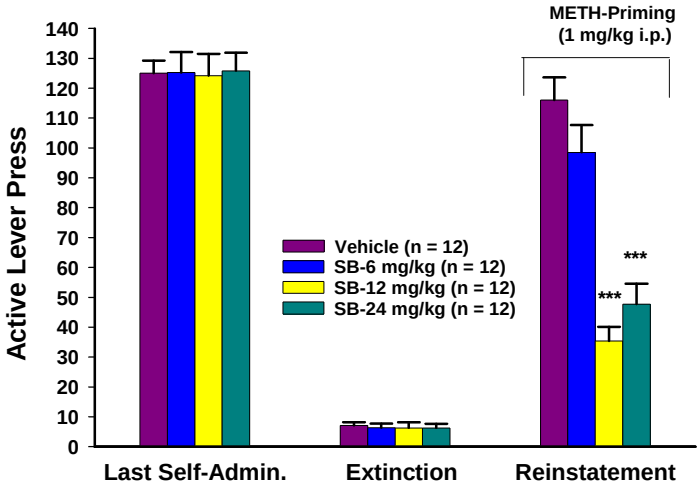


Figure 7 PG01036 on Cue-Induced Relapse to Drug Seeking

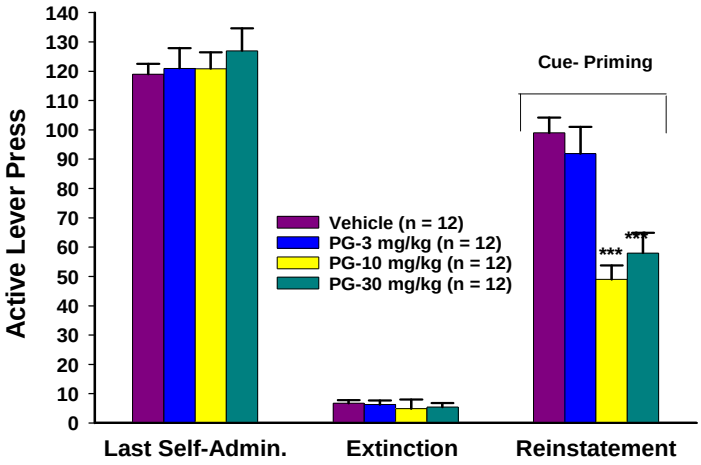


Figure 8 Ability of PG01037 to Maintain Self-Administration

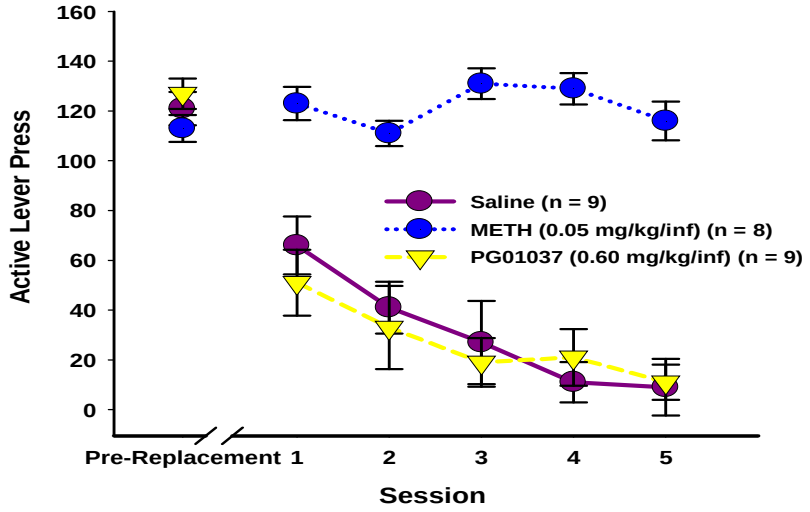
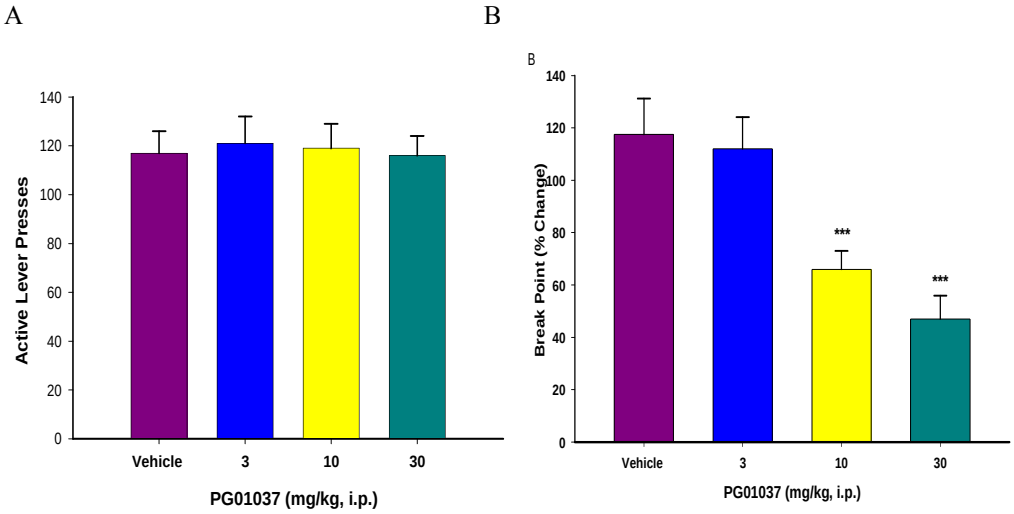


Figure 9 PG01037 on Sucrose Self-Administration



CHAPTER 4 - Introduction to Brain Mechanisms Modulating Drug Addiction

In vivo microdialysis provides a means for understanding the neurochemical changes in different brain regions that occur before and after drug exposure. This

technique requires a semi-permeable membrane to be stereotaxically implanted into discrete brain regions so that later dialysis buffer (which is similar to cerebral spinal fluid in ionic composition) can be perfused through the membrane. Substances in the brain that are in low concentration in the dialysis (including all small molecule neurotransmitters and some peptides), will diffuse into the dialysis membrane and flow through another tube to a vial for collection. The contents of the dialysis samples can then be determined using high pressure liquid chromatography (HPLC) coupled to a fluorescent and electrochemical detector.

Classically, drug addiction research has focused on dopaminergic systems because all known drugs of abuse and natural rewards have been shown to enhance dopamine release in the NAc (despite having diverse mechanisms of action). Research during the past decade has shown that repeated cocaine and methamphetamine administration cause a number of alterations in dopamine and glutamate transmission in the NAc that may be linked to addiction (Carta et al., 2000; Nestler, 1992; Vanderschuren & Kalivas, 2000). The NAc receives major glutamatergic input from the limbic system (Heimer et al., 1997) and has two main outputs, which are GABAergic projections to the ventral pallidum (VP) and the VTA. Meanwhile, dopaminergic neurons in the VTA innervate nearly every nucleus in the limbic system including the prefrontal cortex, dorsal striatum, NAc, and VP (Berendse et al., 1992; Hyman et al., 2006).

Changes in dopaminergic transmission play a critical role in modulating the flow of information through the limbic circuit comprising these interconnected nuclei (Kalivas

& Nakamura, 1999). As previously noted, the final result of psychostimulant intake is to increase extracellular levels of dopamine. Increased dopamine transmission in the NAc in particular, appears to play a critical role in the maintenance of psychostimulant self-administration behavior. Moreover, *in vivo* microdialysis experiments demonstrate that extracellular levels of dopamine in the NAc of rats and monkeys are increased during cocaine or methamphetamine self-administration session and that during post-session extinction, dopamine levels rapidly return to baseline levels (Czoty et al., 2000; Di Ciano et al., 1995). This decrease in NAc dopamine between injections may trigger responding for cocaine or methamphetamine self-administration, presumably to maintain extracellular dopamine above a threshold level (Ranaldi et al., 1999; Koob, 2002).

Basal levels of dopamine in the NAc may also change over the course of withdrawal, Parsons et al. (1991) found that basal dopamine in the NAc was significantly reduced after 10 days abstinence from chronic cocaine. Chen et al. (1996) also found that a cocaine challenge enhanced NAc dopamine release after 7 days withdrawal from chronic cocaine compared to drug naïve controls, but that this enhancement only occurred in response to systemically administered cocaine, not when administered via reverse dialysis directly into the NAc, indicating that the effect of cocaine challenge on dopamine efflux in the NAc requires the activity of other brain regions. In addition to elevating dopamine levels in the NAc, research indicates elevated levels of NAc glutamate (Neisewander et al., 2004; Pierce et al., 1996) and decreased levels of NAc GABA (Tang et al., 2005). Moreover, activation of NAc glutamate receptors reinstates cocaine seeking, whereas blockade of glutamate receptors prevents cocaine-primed

relapse (Cornish et al., 1999). Likewise, preclinical models of cocaine relapse reveal that decreased GABA release in the ventral pallidum is associated with cocaine-seeking (Tang et al., 2005). Thus, cocaine-induced changes in glutamate or GABA may also be involved in psychostimulant intake. Experiment 2 used *in vivo* microdialysis to elucidate methamphetamine's effects on DA, GABA and glutamate in the NAc and VP and to determine whether the effects observed with SB-277011A on behaviour are mediated by acting on local levels of DA, GABA or glutamate in the NAc or VP, two neuroanatomical regions involved in reward.

CHAPTER 5 - In Vivo Microdialysis Method

Subjects

Male, Long-Evans rats (Charles River Laboratories, Raleigh, NC, USA), experimentally naive at the start of the experiment and initially weighing 250 – 300 g were utilized for all manipulations. Rats were housed individually in a climate controlled animal colony room on a reversed light-dark cycle (lights on at 1900 hr, lights off at 700 hr) and received access to food and water ad libitum. All animals were maintained in a facility fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, and all experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, 1996) and were approved by the Animal Care and Use Committee of the National Institute on Drug Abuse of the United States National Institute of Health.

Surgery

Rats were surgically implanted with bilateral guide cannula (20 gauge, 14 mm length; Plastics One, Roanoke, VA) into the NAc (N = 78) or VP (N = 83) under sodium pentobarbital anesthesia (65 mg/kg, i.p.) using standard aseptic surgical techniques. According to Paxinos and Watson (1998), the coordinates used for guide cannula placement into the nucleus accumbens were 1.7 mm anterior to Bregma, 2.0 mm lateral to midline and 4.0 mm ventral to the skull surface using a 6 degree approach angle. The stereotaxic coordinates for guide cannula placement into the VP were 0.26 mm posterior to Bregma, 3.2 mm lateral to midline and 6.0 mm ventral to the skull surface using a 6 degree approach angle. The guide cannula was mounted to the top of the skull with 4 stainless steel 36ehaviou screws (Small Parts Inc., Miami Lake, FL) and cranioplastic acrylic. In vivo microdialysis experiments began 5 – 7 days after animals recovered from surgery.

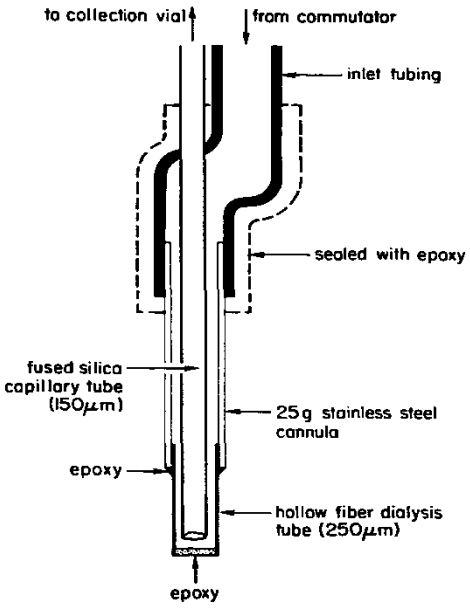
Procedure

Microdialysis probes (see Figure 10) were constructed as previously described (see Xi et al., 2003; Robinson & Whishaw, 1988). The active operational length of the semipermeable microdialysis membrane (Spectra hollow cellulose fiber, MWCO 5000, Spectrum Medical Industries, Houston, TX, USA) was 1.0 – 1.5 mm, and the probe diameter was approximately 100 μm. The probes, which are porous enough to allow the passage of neurotransmitter molecules and metabolites through its wall, were inserted through implanted guide cannula into the NAC or VP 12-14 hours prior to the start of the experiment to minimize tissue trauma effects. Microdialysis buffer (5 mM glucose, 2.5 mM KCl, 140 mM NaCl, 1.4 mM CaCl2, 1.2 mM MgCl2, 0.15% phosphate buffered saline, pH 7.4) was perfused, via a glass syringe pump (Bioanalytical Systems, Inc., West

Lafayette, IN) through the probes at flow rate of 0.10 µl/min overnight. The flow rate was increased to 2.0 µl/min for at least 2 hrs prior to the start of testing and dialysis buffer (2.0 µl/min) was continuously infused via reverse dialysis for the experiment's duration. In vivo dialysis samples were collected every 20 minutes into vials containing 10 µl of 0.5 M perchloric acid to prevent degradation of neurotransmitter.

Baseline samples were collected for 60 minutes prior to the systemic (i.p.) administration of either SB-277011A (12 or 24 mg/kg), methamphetamine (0.20, 1.0 mg/kg), or vehicle (0.5% Tween-80), depending on treatment condition. To measure the neurochemical alterations induced by methamphetamine animals were injected with vehicle, 0.20 mg/kg or 1.0 mg/kg methamphetamine and dialysis samples were collected for 3 hours. Similarly, a separate group of animals was injected with SB-277011A (12, 24 mg/kg, i.p) to elucidate the effect of D3 blockade of DA, GABA and glutamate. Finally, to measure the effect of SB-277011A on methamphetamine-induced neurochemical alterations, additional rats were randomly assigned to receive SB-277011A (12 or 24 mg/kg, i.p.) or vehicle followed, 20 minutes later, by a systemic injection of 1 mg/kg methamphetamine. The 1 mg/kg (rather than 0.2 mg/kg) methamphetamine dose was chosen as it was closer to the cumulative methamphetamine dose animals obtained in the PR self-administration experiment (~ 0.90 mg/kg). Brain samples were collected for 5 hours following methamphetamine administration. All collected samples were immediately frozen at -80°C until analyzed.

Figure 10 Schematic Drawing of Microdialysis Probe



Quantification of Dopamine.

Microdialysate DA was measured using high-performance liquid chromatography (HPLC) coupled with electrochemical detection (ESA Biosciences Inc., Chelmsford, MA). The mobile phase contained 4.76 mM citric acid, 150 mM Na2HPO4, 3 mM sodium dodecyl 38ehaviou, 50 mM EDTA, 10% methanol, and 15% acetylnitrile, pH 5.6 and the mobile phase flow rate was 0.9 ml/min. DA and its metabolites were separated using an ESA Biosciences MD-150 H 3.2 mm reversed phase column, and were oxidized/reduced using an ESA Biosciences Coulochem® III electrochemical detector. Three electrodes were used: a preinjection port guard cell (+0.25 V) to oxidize the mobile phase, an oxidation analytical electrode (E1, -0.1V), and a reduction analytical electrode (E2, 0.2 V). The areas under the curve (AUC) of the peaks of DA and its metabolites were measured using the EZChrom EliteTM chromatography data analysis system (ESA

Biosciences, Inc.). The values of DA, DOPAC and/or HVA were normalized to the internal standard dihydroxybenzylamine and quantified with an external standard curve (1-1000 fM). The minimum detection limit for DA and the measured metabolites was 1-10 fmol.

Quantification of GABA and Glutamate

The concentration of glutamate and GABA in the dialysis samples was determined using HPLC with flourometric detection. The mobile phase consisted of 18% acetylnitrile (v/v), 100 mM Na2HPO4, 0.1 mM EDTA, pH 6.04. A reversed-phase column (VELOSER RP-18, 10 cm × 3 μm ODS, BAS Inc., West Lafayette, IN) was used to separate the amino acids, and precolumn derivatization of amino acids with o-phthalaldehyde using an ESA Model 542 autosampler (Chelmsford, MA). Glutamate and GABA were detected by a fluorescence spectrophotometer (LINEAR FLOUR LC 530, from ESA Inc.). Two sets of different excitation wavelengths (Exλ) and emission wavelengths (Emλ) were used simultaneously to measure glutamate (Exλ, 314 nm; Emλ, 394 nm) and GABA (Exλ, 336 nm; Emλ, 420 nm) levels from the same samples. The area under the curve of glutamate or GABA peak was measured with EZChrom Elite for ESA Chromatography Data System. Glutamate or GABA values were quantified with an external standard curve. The limits of detection for glutamate and GABA were 1-2 pM and 0.1-1 pM, respectively.

Histology and Probe Verification

Immediately following the microdialysis manipulations, animals were given an overdose of pentobarbital (150 mg/kg, i.p.) and perfused transcardially with 0.9% saline

followed by 10% formalin solution. Brains were removed and post fixed in 10% formalin for at least 1 week to ensure thorough fixation. The tissue was blocked and coronal sections (100 μm thick) were made by vibratome through the area of microdialysis probe implantation and sections were stained with cresyl violet. Anatomical placement was verified by visual microscopic examination and the rat brain atlas (Paxinos & Watson, 1998).

Drugs

(+/-) Methamphetamine (Sigma-Aldrich Corporation, St Louis, MO, USA) was dissolved in sterile physiological saline. SB-277011A (trans-N-[4-[2-(6-cyano-1,2,3,4-tetrahydroisoquinolin-2-yl)ethyl]cyclohexyl]-4-quinolinecarboxamide) was synthesized at MegaPharma Kft. (Budapest, Hungary) and was dissolved into solution using 0.5% Tween-80 (Sigma-RBI, St. Louis, MO). The 0.5% Tween-80 alone served as vehicle for systemic i.p. injections. Chemicals used for HPLC were purchased from Sigma-Aldrich Corporations (St. Louis, MO).

Data Analyses

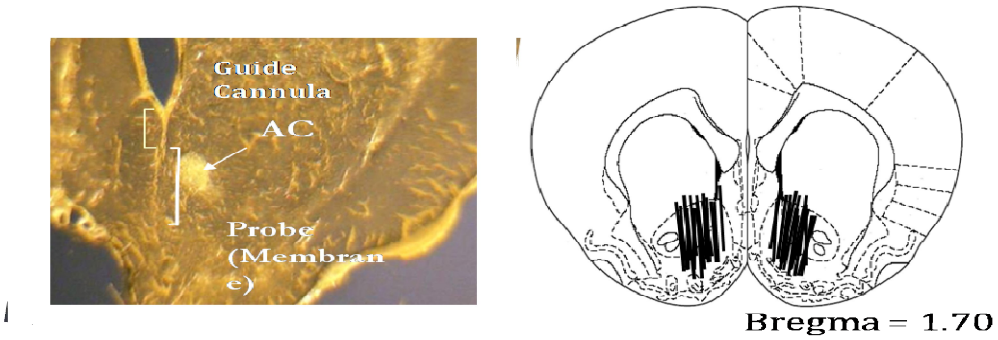
Microdialysis data are presented as means (± S.E.M.). Two Way (treatment vs. Time) ANOVA with repeated measures on time was used to analyze the data reflecting the time courses of the neurochemical changes after drug administration. One Way ANOVA with repeated measures over time was used to analyze the effect of D_3 antagonist-induced changes in neurotransmitters following acute administration of methamphetamine. Post hoc individual group comparisons were carried out using the Bonferroni statistical procedure. When data are expressed as percent of the baseline

values, the mean concentration of the three samples preceding the drug infusion or the behavioural test was defined as 100%.

CHAPTER 6 - Results: *In Vivo* Microdialysis

Figure 11 depicts microdialysis probe locations, demonstrating that the dialysis membranes were located within the nucleus accumbens core and shell. Figure 11a shows a representative microdialysis probe position in rat brain, demonstrating that the active membrane portion, below the non-membrane (stainless steel) portion of the probe, was located in the NAc. Figure 11b illustrates the allover locations of microdialysis probes in the NAc. The active membranes of the dialysis probes were located within both the core and shell, but closer to the core compartment of the NAc. There was no obvious difference in the placement of the dialysis probes across the different experimental groups of rats.

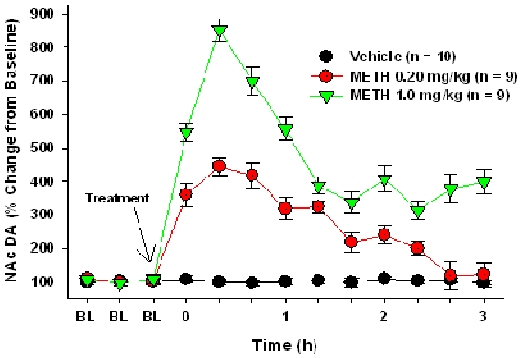
Figure 11 Histological Verification of Microdialysis Probe Placement Within the NAc

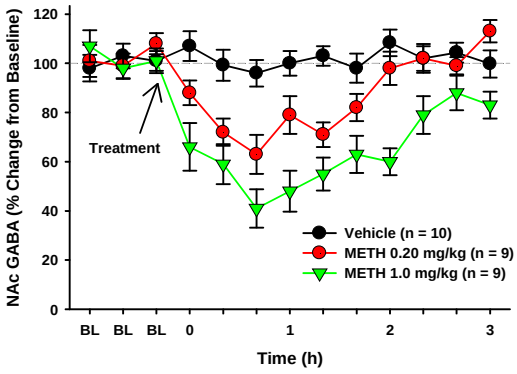


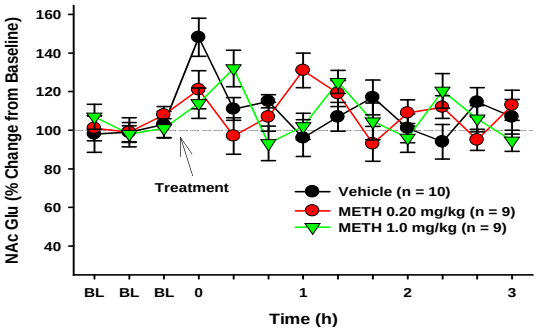
Effect of methamphetamine in the NAc

Figure 12A illustrates the robust, dose dependent elevation of extracellular NAc DA following acute treatment with methamphetamine (0.20, 1.0 mg/kg, i.p.). Peak levels of extracellular NAc DA (~ 900% of baseline) were recorded 40 minutes post injection and gradually attenuated to 250% of baseline. A two way repeated measures ANOVA revealed a statistically significant main effect of treatment ($F(2, 25) = 5.72$, $p < 0.001$), time ($F(17, 374) = 13.65$, $p < 0.001$), and treatment × time interaction ($F(36, 374) = 7.75$, $p < 0.001$). Individual group comparisons using Bonferroni correction revealed a statistically significant increase in DA after administration of 0.2 mg/kg ($t = 4.04$, $p < 0.05$) or 1.0 mg/kg ($t = 9.87$, $p < 0.001$), but not after vehicle ($t = 0.42$, $p = NS$). Similarly, two way ANOVA with repeated measures over time for the GABA data shown in Figure 12B also indicates statistically significant main effects of treatment ($F(2,25) = 5.29$, $p < 0.01$), time ($F(17, 374) = 12.67$, $p < 0.001$), and treatment × time interaction ($F(51, 374) = 3.69$, $p < 0.001$). Post ANOVA individual group comparisons revealed a statistically significant decrease in GABA following 0.2 mg/kg ($t = 2.43$, $p < 0.05$), or 1.0 mg/kg ($t = 5.05$, $p < 0.05$) methamphetamine, but not after vehicle ($t = 1.13$, $p = NS$) administration. Glutamate levels in the NAc, however, were unaffected by systemic administration of methamphetamine (see Figure 12C), with either dose. A two way repeated measures ANOVA indicated no statistically significant effects in treatment ($F(2, 25) = 1.89$, p = NS), time ($F(17, 374) = 1.39$, $p < 0.05$), or treatment × time interaction ($F(51, 374) = 1.28$, p = NS).

Figure 12 Effect of Methamphetamine on Nucleus Accumbens Dopamine, GABA and Glutamate







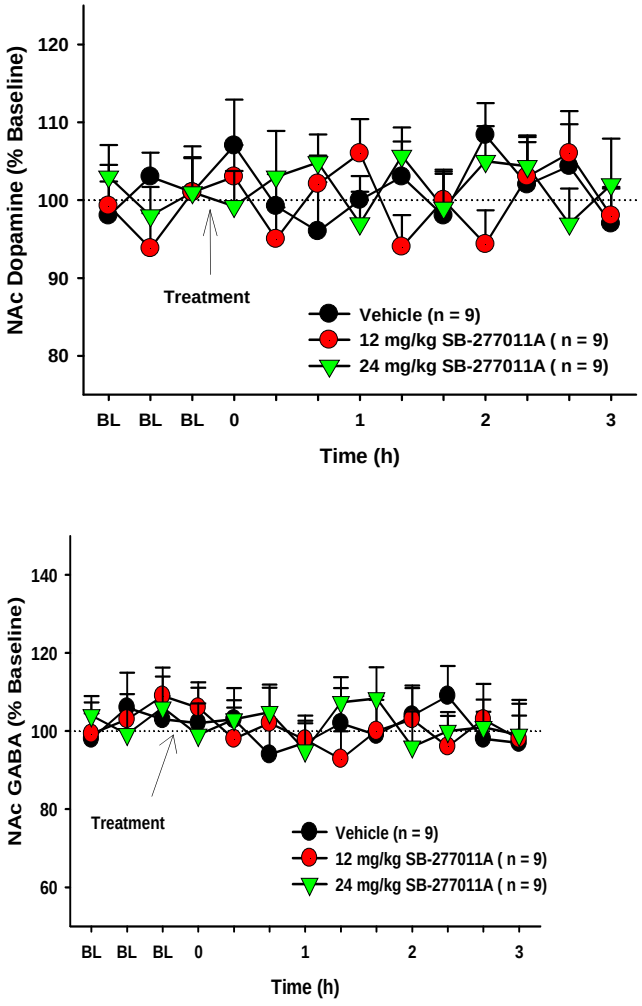
SB-277011A on extracellular NAc DA, GABA, and Glutamate

Administered alone, SB-277011A (12, 24 mg/kg) had no effect on extracellular levels of DA, GABA or glutamate in the NAc at any time point (see Figure 13A-C, respectively). Conversely, pretreatment with SB-277011A potentiated methamphetamine-induced increases in NAc extracellular DA (see Figure 14). A two way repeated measures ANOVA indicated a statistically significant main effect of treatment ($F(2, 20) = 7.37$, $p = < .001$), time ($F(18,436) = 11.72$, $p < 0.01$), and treatment × time interaction ($F(36, 436) = 9.28$, $p < .001$). As seen in Figure 14, methamphetamine (1 mg/kg) significantly increased extracellular NAc DA by 836.72% ± 26.97% from baseline and that pretreatment with SB-277011A (12, 24 mg/kg) potentiated the amplitude and duration of that methamphetamine-enhanced DA. Independent comparisons with Bonferroni correction revealed that pretreatment with 12 mg/kg SB-277011A significantly potentiated extracellular DA to 1145% ($SEM = ± 31.83\%$) of baseline at 40 min post methamphetamine when compared with vehicle treated animals ($t = 9.91$, $p < 0.001$). Moreover, this enhancement of DA following pretreatment with 12 mg/kg or 24 mg/kg SB-277011A remained elevated (> 250% over vehicle) for the duration of the 5 h session.

Figure 15b illustrates the effect of SB-277011A on methamphetamine induced inhibition of GABA in the NAc. Acute methamphetamine (1.0 mg/kg, i.p.) resulted in a 60% GABA decrease in the NAc, an effect that was unaltered by pretreatment with SB-277011A. Two way ANOVA with repeated measures over time revealed a statistically

significant main effect of time ($F(18, 436) = 9.47$, $p < 0.05$) and a significant treatment × time interaction ($F(36, 436) = 3.77$, $p < .05$). There was no significant main effect of treatment on extracellular GABA levels ($F(2, 20) = 0.97$, p = NS. Post-ANOVA individual group comparisons indicated pretreatment with 12 mg/kg SB-277011A significantly inhibited methamphetamine-induced decreases in GABA ($t = 5.95$, $p < 0.05$) 4 hr 20 min – 6 hr post methamphetamine when compared with vehicle or 24 mg/kg SB-277011A. There were no significant effects of methamphetamine or SB277011A treatment on extracellular levels of NAc glutamate (see Figure 15c).

Figure 13 SB-277011A on NAc DA, GABA, Glutamate



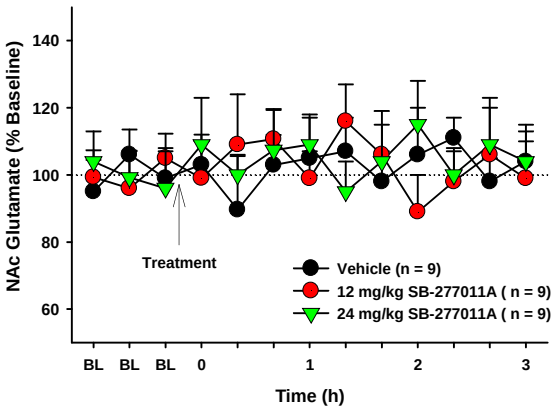

NAc Glutamate (% Baseline)
140
120
100
80
60
BL
BL
BL
0
1
2
3
Time (h)
Treatment
Vehicle (n = 9)
12 mg/kg SB-277011A (n = 9)
24 mg/kg SB-277011A (n = 9)

Figure 14 SB277011A Potentiates Methamphetamine induced DA increase

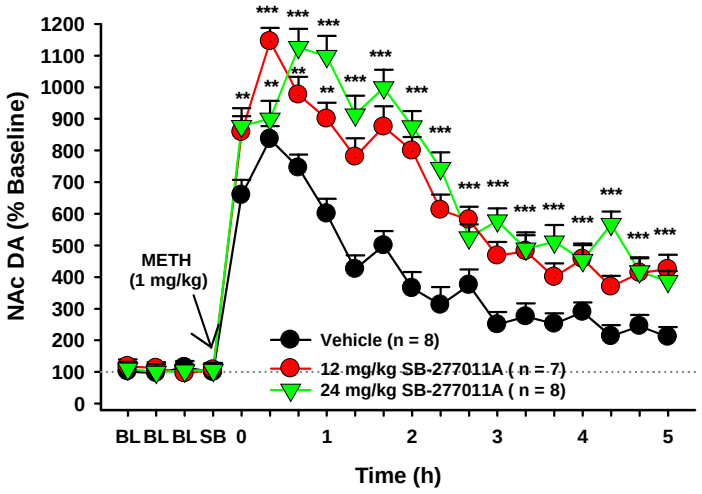


Figure 15 SB-277011A on Methamphetamine Induced GABA decrease

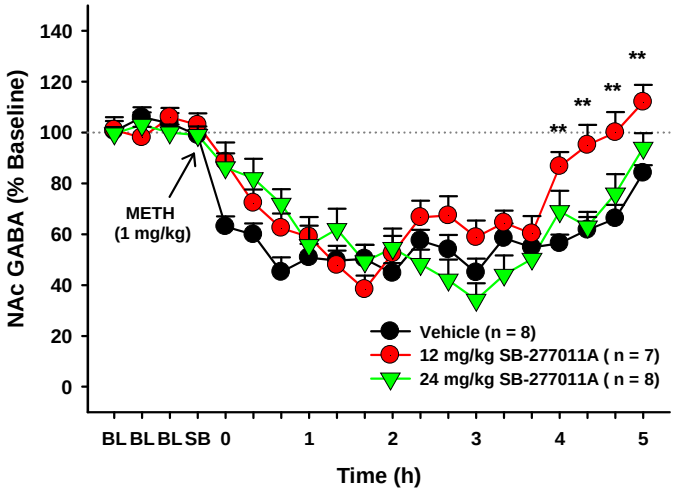
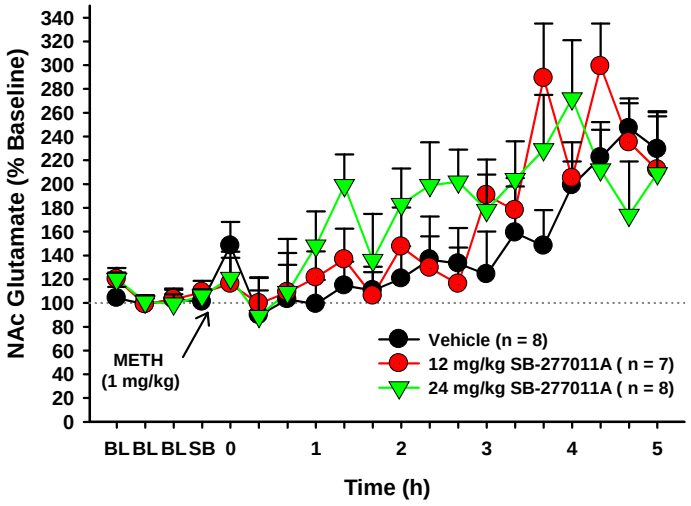


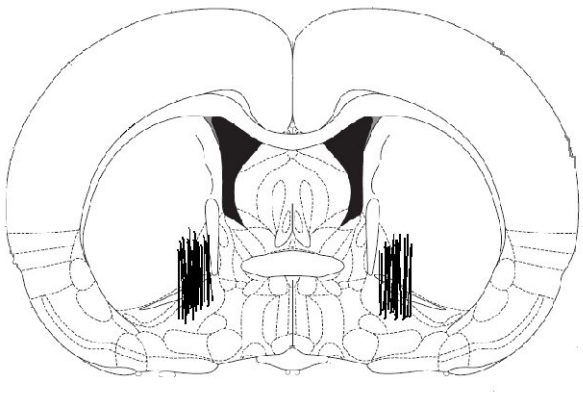
Figure 16 SB277011A has no effect on Glutamate



Methamphetamine on extracellular VP DA, GABA, and Glutamate

Figure 17 depicts the allover locations of the microdialysis probes in the ventral pallidum. In all cases, the active membranes of the dialysis probes were located within the VP.

Figure 17 Schematic Representation of VP Microdialysis Probes

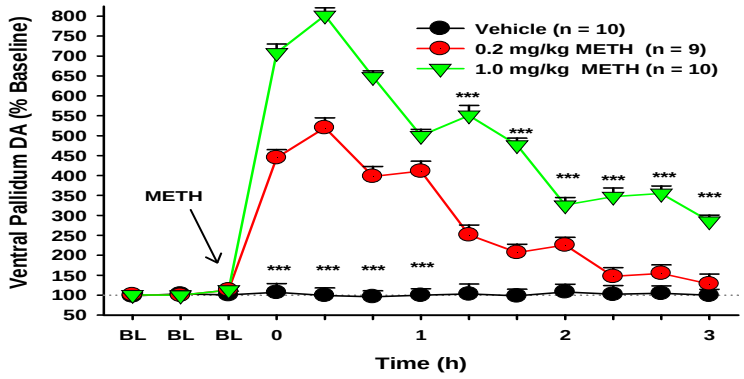


Bregma = 0.24

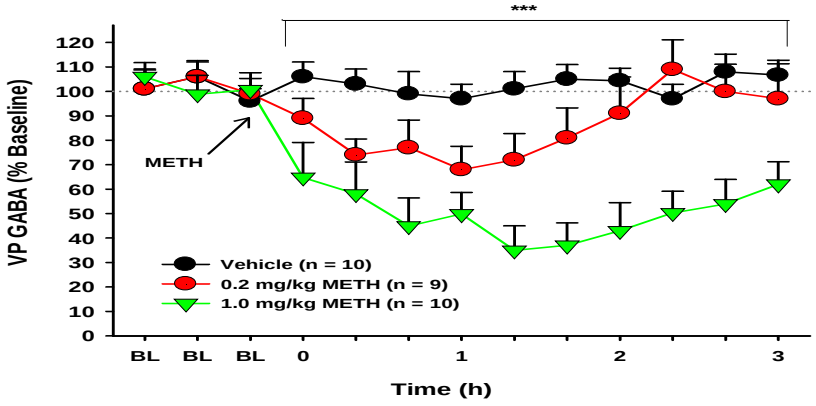
Figure 18A illustrates the robust, dose dependent elevation of extracellular VP DA following acute treatment with methamphetamine (0.20, 1.0 mg/kg, i.p.). Peak levels of extracellular NAc DA (~800% of baseline) were recorded 20 minutes post injection of 1 mg/kg methamphetamine and gradually attenuated to baseline. A Two Way repeated measures ANOVA revealed a statistically significant main effect of treatment ($F(2, 25) = 21.72$, $p < 0.001$), time ($F(17, 374) = 16.65$, $p < 0.001$), and treatment × time interaction ($F(54, 374) = 31.75$, $p < 0.001$). Individual group comparisons using the Bonferroni correction revealed a statistically significant increase in DA after administration of 0.2 mg/kg ($t = 9.04$, $p < 0.001$) or 1.0 mg/kg ($t = 13.87$, $p < 0.001$), but not after vehicle ($t = 0.22$, p = NS). Similarly, Figure 18B illustrates the significant (40 – 60%) inhibition of VP GABA following acute administration of 0.2 or 1.0 mg/kg methamphetamine. Two-way ANOVA with repeated measures over time indicate a statistically significant treatment main effect ($F(2,25) = 5.29$, $p < 0.01$), time main effect (F (17, 374) = 16.44, $p < 0.001$), and treatment × time interaction ($F(54, 374) = 17.94$, $p < 0.001$). Post ANOVA individual group comparisons revealed a statistically significant decrease in GABA following 0.2 mg/kg ($t = 4.43$, $p < 0.05$), or 1 mg/kg ($t = 9.05$, $p < 0.01$) methamphetamine, but not after vehicle ($t = 0.98$, $p = NS$) administration. Systemic methamphetamine had no consistent effects on extracellular levels of glutamate (Figure 18C). A two way ANOVA revealed no statistically significant effects of treatment ($F(2, 25) = 1.67$, p = NS), time ($F(18, 436) = 2.10$, p = NS), or treatment by time interaction ($F(42, 436) = 2.91$, p = NS).

Figure 18 Methamphetamine on VP DA, GABA, and Glutamate

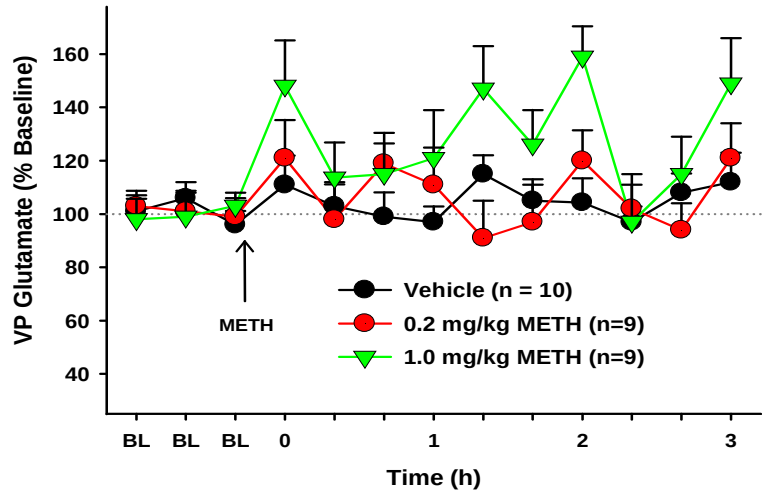
A



B



C



Effect of SB-277011A on extracellular VP DA, GABA and Glutamate

Figure 19 exhibits the effects of SB-277011A administration on VP extracellular DA (Panel A), GABA (Panel B), and glutamate (Panel C). Similar to the observations within the NAc, SB-277011A (12, 24 mg/kg) had no statistically significant effects on extracellular levels of VP DA ($F(2, 24) = 1.88$, p = NS), GABA ($F(2, 24) = 1.13$, p = NS) or glutamate ($F(2, 24) = 1.73$, p = NS). However, as a pretreatment to 1 mg/kg methamphetamine, SB-277011A (12, 24 mg/kg) resulted in a significant increase in the duration of DA enhancement.

Figure 19 SB-277011A on VP DA, GABA, Glutamate

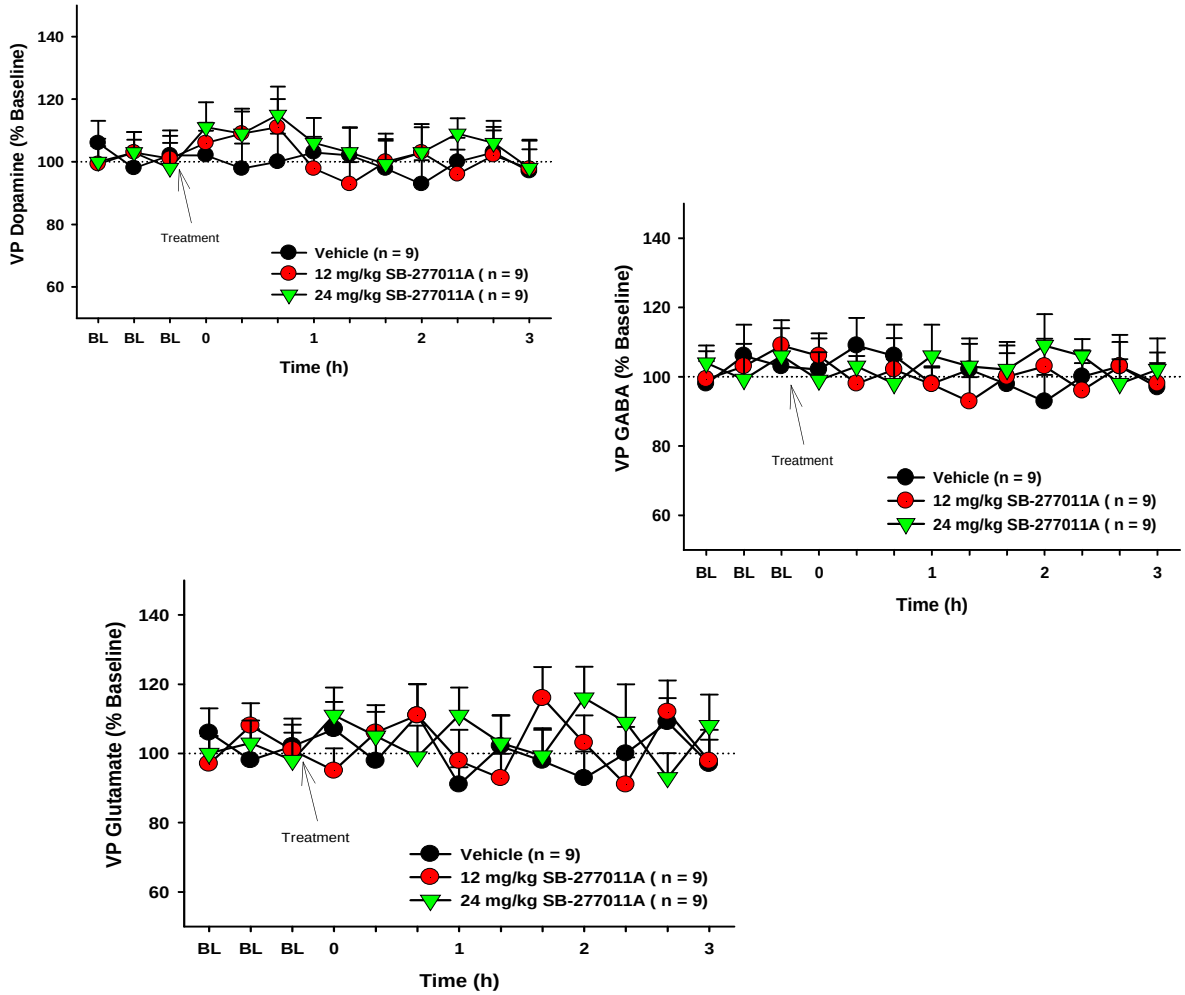


Figure 20 illustrates that, when pretreated with vehicle, methamphetamine (1 mg/kg) produced a quick, short term increase in VP DA of 802.72% ± 19.97% (greater than baseline) while animals pretreated with 12 or 24 mg/kg SB-277011A displayed similar changes in magnitude, the effects lasted considerably longer (140 vs. 20 min) . A two way repeated measures ANOVA indicated a statistically significant main effect of treatment ($F(2, 24) = 22.37$, $p < .001$), time ($F(21, 436) = 21.72$, $p < 0.001$), and treatment × time interaction ($F(42, 436) = 14.28$, $p < .01$) of SB277011A on extracellular DA in the VP. Independent comparisons with Bonferroni correction reveal that pretreatment with 12 mg/kg ($t = 9.91$, $p < 0.001$) or 24 mg/kg SB-277011A ($t = 6.91$, $p < 0.001$) significantly potentiated extracellular DA when compared with vehicle treated animals. Moreover, this enhancement of DA following pretreatment with 12 mg/kg or 24 mg/kg SB-277011A remained elevated (200% – 350% over vehicle) for 180 minutes post methamphetamine injection. There were no significant differences between 12 and 24 mg/kg SB-277011A.

Figure 21 illustrates the effect of SB-277011A on methamphetamine-induced inhibition of GABA in the VP. Acute methamphetamine (1.0 mg/kg, i.p.) resulted in a 65% decrease in VP GABA, an effect that was attenuated by pretreatment with SB-277011A. Two way ANOVA with repeated measures over time revealed a statistically significant main effect of treatment ($F(2, 24) = 18.99$, $p < .001$), time ($F(18, 436) = 9.47$, $p < 0.001$), and a treatment × time interaction ($F(36, 436) = 3.77$, $p < .001$). Post ANOVA individual group comparisons indicated pretreatment with 12 mg/kg ($t = 6.95$, $p < 0.001$) and 24 mg/kg SB-277011A ($t = 3.62$, $p < 0.01$) significantly attenuated methamphetamine-induced decreases in GABA (maximally by 25%) when compared to

vehicle treated animals. Moreover, 1 hr 40 min post methamphetamine treatment GABA levels had completely returned to baseline in animals receiving 12 mg/kg SB-277011A pretreatment, an effect not observed in animals receiving vehicle or 24 mg/kg SB-277011A pretreatment. Figure 22 illustrates the effects of SB-277011A pretreatment and methamphetamine on extracellular levels of VP glutamate. There were no significant differences in glutamate levels by pretreatment with 12 mg/kg, 24 mg/kg SB-277011A, or Vehicle, $F(2, 24) = 1.97$, p = NS.

Figure 20 SB277011A Potentiates Meth-Induced DA increase in VP

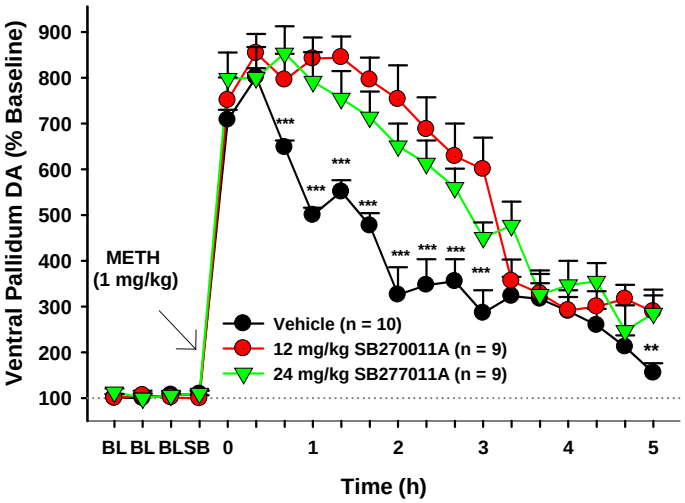


Figure 21 SB-277011A attenuates METH-Induced GABA decreasee in VP

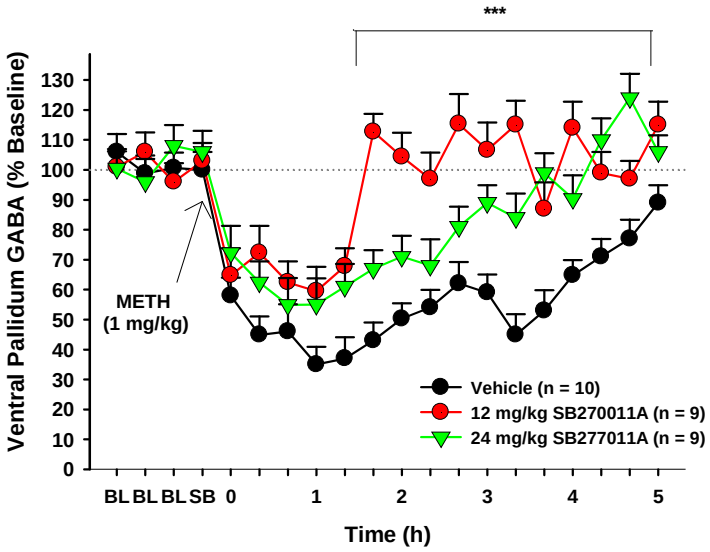
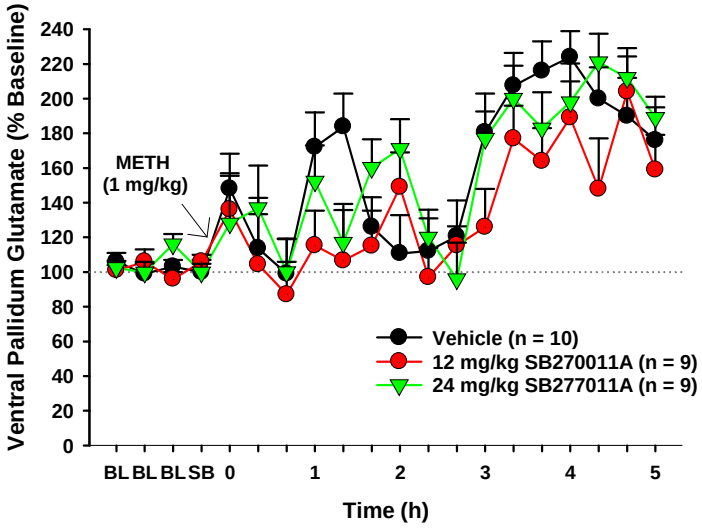


Figure 22 SB-277011A on VP Glutamate



CHAPTER 7 - Discussion

The present study, for the first time, demonstrated that systemic administration of the selective DA D_3 antagonists SB-277011A and PG01037 effectively attenuated the addictive-like properties of methamphetamine. Although pretreatment with SB-277011A

or PG01037 did not alter IV methamphetamine self-administration reinforced on an FR2 schedule, the same doses produced a significant and dose dependent drop in PR break-point levels for IV methamphetamine. Likewise, SB-277011A and PG01037 significantly inhibited reinstatement to methamphetamine seeking behavior induced by drug cues or environmental cues previously associated with methamphetamine intake. These data support the utility of pharmacologic blockade of D_3 receptors as anti-addiction, anti-craving tools.

Intravenous drug self-administration is the most commonly used animal model to evaluate a drug's rewarding effects (Gardner, 2000). Over twenty psychoactive drugs that are abused by humans have also been found to act as reinforcers in rats, thus supporting the hypothesis that drug self-administration in animals may be a reliable predictor of abuse liability in humans. A major focus of preclinical research on drug self-administration has been to examine behavioral and pharmacological variables that modify this behavior. Consequently, different reinforcement contingencies have led to variants of the core self-administration model, most commonly the FR and PR reinforcement schedule. In low fixed-ratio schedules of reinforcement, the response requirements for each drug infusion are set at a fixed number. Within a range of drug doses that maintain stable responding, animals will typically increase their response rate as the unit dose is decreased, but will reduce their rate of self-administration when the unit dose is increased. Low FR schedules of reinforcement are useful for exploring patterns of rate of drug intake, but are less appropriate to assess changes in the reinforcing efficacy of drugs of abuse. The present study found that systemic administration of SB-277011A (0, 6, 12, 24 mg/kg, i.p.) or PG01037 (0, 3, 10, 30 mg/kg, i.p.) had no effect on stable maintenance

of methamphetamine self-administration under an FR2 reinforcement schedule in rats. This finding is consistent with previous studies demonstrating that neither BP-897 nor SB-277011A affects continuous cocaine or nicotine self-administration under low FR schedules (Di Ciano & Everitt, 2003; Gál & Gyertyán, 2003; Vorel et al., 2002; Xi et al., 2005; 2006)

Conversely, the same doses of SB-277011A and PG01037 significantly lowered the break-point for methamphetamine self-administration under PR reinforcement conditions, an effect that lasted less than 24 hrs. During PR schedules of reinforcement rats must complete increasing FR response requirements to obtain a reinforcer (i.e., the drug). The essential feature of the PR schedule is that the response requirement continues to increase until responding ceases altogether and the reinforcer is no longer attained. Thus, the PR break-point is an index of the relative strength of a reinforcer independent of response rate; as such, one can assume that a shift in PR break-point produced by a pharmacological agent indicates that the latter decreases the reinforcing value of the drug. Under this PR reinforcement schedule, both SB-277011A and PG01037 produced a significant decrease of the PR break-point for methamphetamine self-administration. This finding suggests a decreased motivation to work for the next infusion, ultimately resulting in less total drug consumption. These data are congruent with previous experiments which report that highly selective D_3 antagonists inhibit cocaine, alcohol, and nicotine self-administration under PR or high FR conditions, but not under low FR conditions (Andreoli, et al., 2003; Di Ciano et al., 2003; Gilbert et al., 2003; Gal & Gyertyan, 2003; Pilla et al., 1999; Vorel et al., 2002; Xi et al., 2005, 2006). There are several possible explanations for the differential effects of SB-277011A and PG0137 on

FR versus PR methamphetamine self-administration. First, animals may compensate for the D_3 antagonism by increasing their drug intake or their self-administration rate under FR2 conditions. This however is unlikely, as no such compensatory increase in methamphetamine intake or in the individual methamphetamine self-administration patterns was observed. Secondly, the FR2 schedule demands less work to obtain much higher cumulative methamphetamine reward. The total methamphetamine intake during FR2 methamphetamine self-administration averaged 2.5 mg methamphetamine, considerably higher than the average total intake of 0.90 mg methamphetamine during PR self-administration. This high dose of methamphetamine may block the effectiveness of SB-277011A or PG01037 on methamphetamine's actions by inhibiting the antagonists from binding to D_3 receptors or activating other dopamine receptors. This dopamine hypothesis may also explain why the PR self-administration reinforcement schedule is more sensitive than the FR reinforcement in evaluating the changes or extent of a drug's rewarding efficacy (Richardson & Roberts, 1996; Rowlett, 2000). On the other hand, one must also consider the relative insensitivity of low FR reinforcement schedules to detect changes in the reinforcing efficacy of a reward. Many have argued that FR reinforcement schedules simply evaluate the fact of reinforcement rather than the degree of reinforcing efficacy (Arnold & Roberts, 1997; Gardner, 2000; Roberts, Loh & Vickers, 1989; Wise & Gardner, 2004). The PR paradigm, however, is highly sensitive to dose-response functions that reflect a given drug's reinforcing efficacy (Arnold & Roberts, 1997; French et al., 1995; Roberts et al., 1989; Stafford Le Sage & Glowa, 1998). Additionally, if one considers that high effort and high motivation are required to obtain a highly rewarding stimulus, then the PR paradigm succinctly and quantifiably assesses incentive

motivation to self-administer the addictive drug (Richardson & Roberts 1996; Rowlett, 2000; Stafford et al., 1998;). As such, the present finding that D_3 antagonists significantly inhibit methamphetamine self-administration under PR reinforcement conditions suggests an important role for D_3 receptors role in mediating the reinforcing efficacy of methamphetamine and thereby its motivational salience.

Craving-driven relapse to illicit drug use is a core feature of drug addiction (Mendelson & Mello, 1996; O'Brien, 1997). In humans, relapse to drug use can be triggered by administration of the drug, by exposure to stimuli previously associated with drug use, or by exposure to various stressors (Jaffe et al., 1989; O'Brien et al., 1992; Sinha, 2001). Relapse to drug use can similarly be modeled in the laboratory using the reinstatement to relapse paradigm (Shaham et al., 2003; Shalev et al., 2002). In this case, previously extinguished drug-seeking behavior can be elicited by re-exposing the animal to an addictive drug, drug-associated environmental cues, or stressor (Stewart, 2000; Shalev et al., 2002; Di Ciano & Everitt, 2003). The reinstatement paradigm is commonly used to study neurobiological mechanisms underlying drug craving and relapse (Bossert et al., 2005). The present study demonstrated that presenting methamphetamine or cues previously associated with its intake reliably reinstated methamphetamine-seeking behavior in rats as measured by the recovery of extinguished, drug-paired responding.

Using the drug-triggered reinstatement model, the present study found that a single non-contingent injection of 1 mg/kg i.p. methamphetamine produced robust reinstatement of extinguished operant lever pressing previously reinforced by intravenous methamphetamine infusions. Pretreatment with SB-277011A (12, 24 mg/kg i.p.) significantly attenuated methamphetamine-triggered reinstatement of methamphetamine-

seeking behavior. Likewise, the effects of PG01037 on reinstatement of methamphetamine-seeking behavior induced by a single, non-contingent presentation of methamphetamine-associated cues (light-tone) were observed. PG01037 (10, 30 mg/kg, i.p.) dose-dependently inhibited cue-induced reinstatement of methamphetamine-seeking behavior in rats after 10-14 days of extinction from previous methamphetamine self-administration. These findings are consistent with previous research with SB-277011A and BP-897 on reinstatement drug-, cue- , stress- induced reinstatement to cocaine seeking (Gilbert et al., 2005; Xi et al., 2005; 2006) or alcohol-triggered reinstatement of alcohol seeking (Vengeliene et al., 2007), and nicotine-triggered reinstatement (Andreoli et al., 2003).

Importantly, pretreatment with SB-277011A or PG01037 dose-dependently decreased reinstatement of methamphetamine-seeking behavior without affecting inactive lever responding. The presentation of methamphetamine and its associated cues resulted in rapid responding only on the active lever. The fact that animals responded preferentially on the active lever is an important one as it indicates drug seeking rather than a general elevated level of arousal. Moreover, regardless of D_3 treatment condition, there were no changes in responses on the inactive lever, suggesting D_3 receptor antagonism's inhibition of responding was specific to drug and cue induced reinstatement rather than lethargy or impaired locomotor effects These data are consistent with those produced by D_3-selective or D_3-preferring receptor antagonists in experimental animals (Gilbert et al., 2005; Cervo et al., 2003; Self et al, 1996; Khroyan et al, 2000) and suggest PG01037 may have potential in attenuating drug craving and relapse to drug use in humans.

The observed reduction in PR breakpoint, drug- and cue-induced methamphetamine seeking behavior by SB-277011A and PG01037 is unlikely the result of impaired motor function as both drugs failed to alter spontaneous locomotion. Previous research investigating SB-27011A and PG01037 have found no significant effects on locomotion in squirrel monkeys (Newman et al., 2005) nor does it alter spontaneous or stimulant induced locomotion in rats (Reavill et al., 2000; Vorel et al 2002) . They also had no effect on active lever presses under FR2 reinforcement schedules for methamphetamine self administration behavior nor was inactive lever responding altered in any of the operant behavioral experiments conducted PG01037, however, may affect natural reward as pretreatment with 10 or 30 mg/kg inhibited oral sucrose self-administration under PR schedules of reinforcement but did not affect responding under FR2 schedules. Given that 5% sucrose solution is a much weaker reinforcer than methamphetamine, it is possible that the observed reduction in PR breakpoint is due to the low reward pay-off. Under PR conditions, total sucrose intake ranged from 0.6 – 0.8 mg (*SEM* = ± 0.2), which is considerably lower than the average total intake of 5.0 – 7.5 mg (*SEM* = ± 2.3) of sucrose under FR2 conditions. PG01037 reduced the breakpoint level for both sucrose and methamphetamine suggesting that PG01037 may act on the general reward system rather than having specificity to drugs of abuse. It is important to note that, regardless of the reinforcer, PG01037's effects were specific to the reinforcing efficacy (PR) rather than the acute rewarding properties of the stimulus (FR2). Further studies investigating other natural reward (i.e., food-taking or sexual behaviors) are required to elucidate these mechanisms of action.

In vivo microdialysis was conducted to clarify the effects of SB-277011A on basal and methamphetamine-induced alterations in extracellular DA, GABA, and glutamate in the NAc and VP, neuroanatomical areas deemed critical in the motivation of drug taking behavior. Consistent with previous research (Dillon et al., 2008; Xi & Gardner, 2007), the present study found that systemic administration of SB-277011A (12, 24 mg/kg i.p.) had no effect on basal levels of NAc DA, GABA or glutamate. Systemic administration of methamphetamine (0.20, 1.0 mg/kg) however, dose-dependently increased NAc DA by 400-800%; while the same doses resulted in a 40-60 % decrease in NAc GABA. There were no significant or stable alterations in NAc glutamate. Pretreatment with SB-277011A (12, 24 mg/kg, i.p.), 20 minutes prior to a systemic injection of 1 mg/kg methamphetamine significantly potentiated and prolonged the methamphetamine enhanced DA increase by 200%; an effect that lasted the 5 hr duration of testing. Pretreatment with SB-277011A (12, 24 mg/kg, i.p.) had no significant effect on methamphetamine induced alterations in GABA, however, pretreatment with 12 mg/kg SB-277011A resulted in a return to baseline GABA levels at 4 hrs post methamphetamine injection, which was significantly faster than animals pretreated with vehicle or 24 mg/kg SB-277011A.

Similar to the results obtained in the NAc, the present study found no effects of SB-277011A (12, 24 mg/kg i.p.) on basal levels of VP DA, GABA, or glutamate. Likewise, systemic administrations of methamphetamine (0.20, 1.0 mg/kg) dose-dependently increased VP DA by 500-800%; the same doses resulted in a 35-70 % decrease in VP GABA. There were no significant alterations in VP glutamate. Pretreatment with SB-277011A (12, 24 mg/kg, i.p.) resulted in a 200 – 500% potentiation

of 1 mg/kg methamphetamine-enhanced DA; this effect occurred 40 minutes post-methamphetamine injection and lasted until 3 hours post-injection. SB-277011A (12 mg/kg, i.p.) reversed methamphetamine-induced GABA inhibition, completely returning GABA levels to baseline at 1 hour and 40 minutes after methamphetamine injection.

The mechanisms underlying such differential effects on basal and methamphetamine-enhanced DA are unclear. The effect could be related to different DA tone on presynaptic D_3 receptors in the presence or absence of methamphetamine (or other stimulation), such that DA tone is low in the absence of methamphetamine, but increased after methamphetamine administration. Therefore, blockade of D_3 receptors leads to an enhancement of methamphetamine-induced increases in extracellular DA. Given that DA D_3 receptors are distributed on both presynaptic and postsynaptic cells (see review by Joyce & Millan, 2005; Sokoloff et al., 2006), such an increase in DA produced by either SB-277011A or methamphetamine may attenuate D_3 antagonists anti-methamphetamine actions by competing with SB-277011A's binding to D_3 receptors and/or by activation of other DA receptors. In contrast, blockade of presynaptic D_3 receptors may potentiate DA release via a disinhibition mechanism, which then subsequently attenuates the therapeutic effects of D_3 antagonists by competitively binding to D_3 receptors and/or activation of other DA receptor subtypes. This DA enhancement hypothesis not only explains the different effects on basal verses drug induced dopamine levels, but may also explain why D_3 antagonists selectively inhibit intravenous methamphetamine self-administration under PR, but not FR, reinforcement schedules. That is, the cumulative doses of methamphetamine under PR conditions are significantly

lower than FR conditions thereby minimizing the likelihood of competitive binding under such conditions.

Whatever the underlying mechanism, the present study demonstrates, for the first time, that blockade of D_3 receptors inhibits the addictive-like behaviors produced by methamphetamine. In summary, it was demonstrated that although acute intraperitoneal administration of D_3 antagonists failed to alter methamphetamine self-administration under an FR2 (low effort-high reward) schedule of reinforcement, they significantly and dose dependently lowered the break-point for methamphetamine and sucrose self-administration under a PR schedule of reinforcement. Additionally, methamphetamine and its associated cues evoked robust reinstatement of extinguished methamphetamine-seeking behavior, an effect that was inhibited by pretreatment with SB-277011A or PG01037. The inter-model consistency of the present findings strengthen the conclusion that D_3 blockade inhibits methamphetamine's rewarding effects and supports the further exploration and potential development of selective D_3 antagonists as medications to treat psychostimulant addiction. At the same time caution along these lines is warranted. The seeming face validity of these animal models of addiction has not been shown to have predictive validity for human addicts (especially in regards to relapse).

Future studies using the technique of microinjection are required to determine the precise loci of D_3 antagonism in the brain as they relate to drug seeking and drug taking behavior. The present study found that PG01037 significantly decreased responding for sucrose on a PR schedule, suggesting perhaps that PG01037 may have the unwanted side effect of disrupting natural reward. Future studies using alternative paradigms of natural

reward (i.e., the opportunity to mate with a sexually receptive female) are required to better delineate these systems effects.

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