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MODULATION OF COCAINE-LIKE BEHAVIOURAL ACTIVITY BY SEROTONIN UPTAKE INHIBITION RELATIVE TO THE EFFECTS OF THE NOVEL AND SELECTIVE DOPAMINE TRANSPORTER INHIBITOR, D-84

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GENERAL INTRODUCTION

Cocaine Dependence

Psycho-stimulant abuse and addiction is a serious medical, economic and health problem for which there are no effective pharmacotherapies. Cocaine is one of the most commonly abused psychostimulants. The acute rewarding effects produced upon administration of cocaine, followed by cravings and relapse after a period of abstinence, are the hallmark features of addiction to cocaine. Despite the worldwide abuse and almost a decade worth of research into potential treatments, an effective treatment medication remains elusive (Gorelick, Gardner, & Xi, 2004)

Cocaine is a crystalline tropane alkaloid obtained from the leaves of the *Erythroxylon coca* plant. Pharmacologically, cocaine has many prominent actions. It is a local anesthetic, it increases alertness and reduces fatigue, increases motor activity and, importantly, it is a powerful positive reinforcer. Cocaine has been used for years as a recreational stimulant and for its local anesthetic properties. Today, cocaine is considered to be one of the most addictive substances known and epidemiological studies show that cocaine addiction is a markedly persistent problem in the US. Nearly six million Americans (age 12 or older) used cocaine in 2001 (SAMHSA 2003) and the Office of National Drug Control Policy estimates that approximately three million individuals chronically use cocaine. Cocaine dependence is known to develop over a period of time, and becomes a chronic, relapsing disorder characterized by compulsive drug-seeking and drug use despite the negative consequences associated with it (Dackis & O'Brien, 2001). Cocaine is known to elevate mood, induce euphoria, reduce fatigue and increase task performance. It is these positive reinforcing

effects, coupled with the severity of the psychological withdrawal side effects that is responsible for the high rate of relapse seen in addicts attempting to break the addiction.

Mesolimbic DA hypothesis of psycho-stimulant reward

It is thought that cocaine produces its strong reinforcing effects by binding to and blocking the dopamine (DA) uptake transporter (DAT) particularly in the mesolimbic DA reward pathway (Ritz, Lamb, Goldberg, & Kuhar, 1987). Blockade of the transporter inhibits the reuptake of DA into the presynaptic nerve terminals and, therefore, increases its concentration in the synapse, leading to the positive reinforcing effects associated with cocaine (Koe, 1976).

The mesolimbic DA system is critical in mediating the rewarding properties of cocaine (Koob & Swerdlow, 1988; Self & Nestler, 1995; Wise, 2005) and, hence, plays a large role in cocaine addiction. The pathway originates in the ventral tegmental area (VTA) in the midbrain and projects to the nucleus accumbens (NAc), prefrontal cortex (PFc) and amygdala in the forebrain. Increased DA neurotransmission, particularly in the mesolimbic DA reward pathway (Di Chiara, 1995), and indirect activation of DA receptors (Spealman, Bergman, Madras, Kamien, & Melia, 1992), has been shown to play an important role in the positive reinforcing effects of cocaine that are entwined with the euphoria associated with cocaine's abuse (Koob & Swerdlow, 1988; Wise, 2005).

There is an abundance of evidence supporting the role of this DA system in the rewarding properties of many drugs of abuse, starting with the fact that cocaine and other abused drugs have been shown to increase extracellular DA in the NAc (Self DW 1995, Wise

RA2005). It is also widely known that cocaine is readily self-administered by rodents and non-human primates and that this effect can be attenuated through the use of high dose DA receptor antagonists (Gardner, 2000).

Pharmacological treatments for cocaine abuse

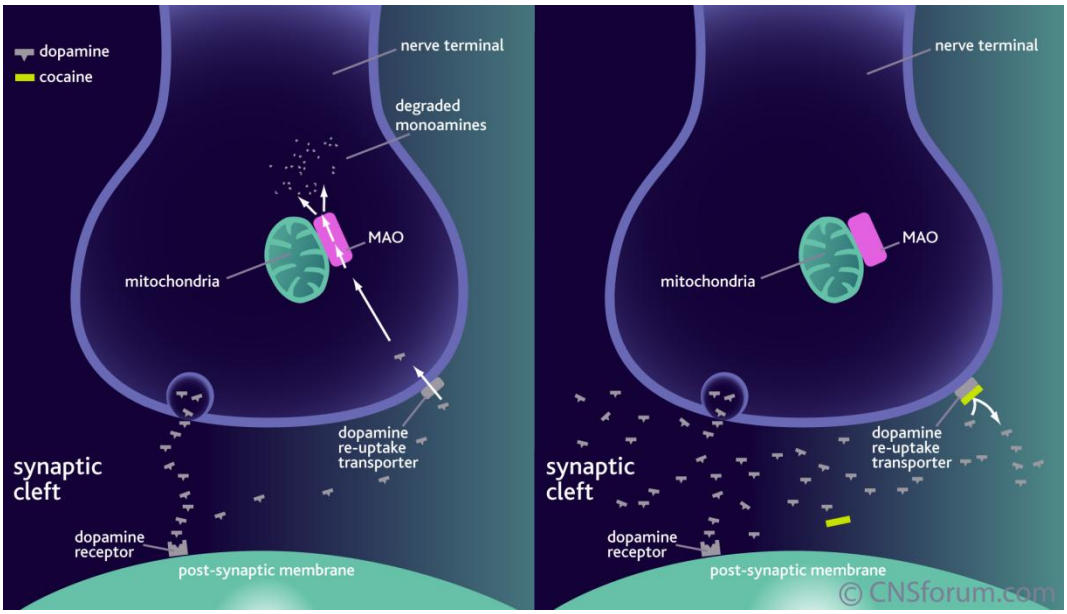
Since it is thought that cocaine produces its strong reinforcing effects through action within the DA reward pathway, it seems reasonable that one cocaine treatment strategy would involve manipulating this DA reward pathway. There are two prominent pharmacological approaches to manipulate this pathway; either by using agents that antagonize the brain DA receptors or by agents that promote their activation, directly or indirectly. However, despite several decades of research into cocaine dependence, an effective treatment strategy remains elusive. A number of approaches have been explored, including DA receptor antagonism (de Lima, de Oliveira Soares, Reisser, & Farrell, 2002), anti-depressants and anti-convulsants (Gorelick, et al., 2004), and more recently a cocaine vaccination to prevent entry of cocaine into the brain (Martell, Mitchell, Poling, Gonsai, & Kosten, 2005). However, of all the potential treatment strategies investigated, DAT agonist substitution appears to have generated the greatest interest to date.

DAT Agonist Substitution as a Potential Treatment Strategy

Given the success of methadone replacement treatment for heroin addiction and the nicotine patch for the cessation of smoking, it seems plausible that a similar strategy may work for cocaine addiction. Cocaine produces its reinforcing effects by binding to and blocking the DAT (Ritz, et al., 1987). This is understood to cause an increase in the extra cellular DA levels, leading to an increased DA receptor activation (Fig 1). It is also thought

that the faster an abused drug enters the brain, the faster extracellular DA is increased and the higher the abuse potential of the drug (Kimmel, O'Connor, Carroll, & Howell, 2007). Therefore, an effective replacement therapy could potentially be a DAT inhibitor, to produce some cocaine-like effects, but with a slower onset of action to convey a lower abuse potential than cocaine itself. Producing such a compound is proving to be difficult, as achieving a balance between possessing some cocaine-like effects but with lower reinforcing efficacy and with adequate safety has yet to be achieved.

Figure 1: Diagrammatic representation of DAT functioning normally (left hand panel) and in the presence of cocaine (right hand panel) (CNSforum.com)



DAT Inhibitors as Potential Treatments for Cocaine Dependence

A number of compounds have been investigated as potential agonist replacement therapies. These include bupropion, benztropines, tropanes, mazindol and substituted piperazines. Some of these compounds have demonstrated cocaine-like behavioural profiles in animal models, and some have shown potential to act as "agonist" therapies, however none have proven 100% effective as a replacement therapy, either due to low efficacy in clinical studies, or because of their toxic effects, or because of a high abuse potential of their own (Gorelick, et al., 2004).

Bupropion is an atypical anti-depressant and smoking cessation aid that is known to inhibit dopamine and noradrenaline reuptake with relatively little inhibitory action at the SERT (Richelson & Pfenning, 1984). Bupropion has high uptake inhibition at the DAT, and since this activity is known to be responsible for the high abuse liability of cocaine-like psychostimulants, it is not surprising that pre-clinical studies have shown bupropion to be self-administered in monkeys and rats at levels similar to cocaine (Bergman, Madras, Johnson, & Spealman, 1989; Tella, Ladenheim, & Cadet, 1997). Although bupropion has been marketed as an anti-depressant and a smoking cessation aid for years, it has not had a significant incidence of abuse (Nieuwstraten & Dolovich, 2001; Wilkes, 2008). The dis-correlation of the animal laboratory studies and the actual abuse of bupropion suggests that results from pre-clinical studies are not always clinically predictive. This suggests that DAT inhibitors, whilst sometimes conveying cocaine-like effects, may not necessarily always possess abuse liability. This could, in part, be due to the route of administration. An early study showed that peak concentrations of bupropion in brain tissue of rats and mice after p.o administration of 10 mg/kg were approximately 10% of concentrations measured after i.v administration of the same dose (Butz, Welch, & Findlay, 1982). However, despite the

cocaine-like, preclinical profile of bupropion, it did not prove to be a successful replacement therapy for cocaine dependence it was thought it could be (Oliveto et al., 2001).

Research to find a DAT inhibitor, without abuse potential, to act as a potential therapy for cocaine dependence has continued beyond bupropion. Benztropine possesses a tropane ring, a predominant feature of the cocaine molecule and is a potent DAT inhibitor (Horn AS, 1971). However, in animal models of drug abuse, benztropine did not demonstrate cocaine-like behaviour despite having a high affinity for the DAT (J. L. Katz, Newman, & Izenwasser, 1997; Newman, Allen, Izenwasser, & Katz, 1994). In fact, benztropine itself is an approved medication for the treatment of Parkinson Disease, attributable to its DAT inhibition, but does not appear to possess the abuse liability of cocaine. Benztropine was studied in a Phase II clinical trial to determine its efficacy in affecting stimulated craving to cocaine cues, although there are no published results of this study (ClinicalTrials.gov identifier NCT00000333.) More recent studies with benztropine analogs have shown that these newer compounds have cocaine-like behaviour but with an apparent lower abuse liability than cocaine itself (Ferragud et al., 2009; Hiranita, Soto, Newman, & Katz, 2009). N-substituted benztropine analogs were studied in rats to characterize their addictive-like properties and their effects on cocaine self-administration (Ferragud, et al., 2009). Results in these open field tests and drug self-administration assays showed that the N-substituted benztropine analog, AHN-1055, produced robust and sustained locomotor activity and also enhance cocaine-induced locomotor stimulation. AHN-1055 showed weak reinforcing efficacy in progressive ratio studies and did not promote cue- induced relapse. AHN-1055 also produced a dose dependent decrease in cocaine intake in rats with a history of cocaine self-administration (Ferragud, et al., 2009). Another recent study investigating the reinforcing effects of benztropine analogs and their effects on cocaine self-administration showed that

benztropine and its newer analogs are less effective than cocaine in producing behavioural effects that are predictive of abuse liability and therefore may be suitable candidates as potential cocaine dependence medications (Hiranita, et al., 2009).

Tropane analogs were among the first DAT inhibitor compounds that were investigated as cocaine replacement therapies. RTI-113 and PTT both have a high selectivity for DAT verses SERT and NET and both were shown to decrease cocaine self-administration in non-human primates (Negus, Mello, Kimmel, Howell, & Carroll, 2008). However, both compounds also decreased food maintained responding at similar doses required to decrease cocaine self administration. These non-specific, behaviourally toxic effects may hinder their potential to be developed as replacement therapies for cocaine (Howell, Czoty, Kuhar, & Carrol, 2000; Nader, Grant, Davies, Mach, & Childers, 1997). Further studies with phenyltropane analogues have shown that high affinity DAT inhibitors with a slower rate of onset and longer duration of action decreased cocaine intake and also maintained less cocaine self administration across a range of conditions (Lile et al., 2002).

RTI-336 (3-beta-(4-chlorophenyl)-2betas-[3-(4'-methlyphenyl)isoxazol-5-yl]tropane) is a novel DAT inhibitor that has a slower onset and longer duration of action than cocaine (F. I. Carroll, Howard, Howell, Fox, & Kuhar, 2006; Kimmel, et al., 2007) and is more selective for the DAT relative to the SERT and NET (Carroll FI et al., 2006). RTI-336 dose dependently attenuated cocaine self-administration in both rats and non-human primates but only at very high doses (Howell, Carroll, Votaw, Goodman, & Kimmel, 2007). RTI-336 was also shown to be reliably self-administered and also generalized to cocaine's discriminative stimulus (Howell, et al., 2007). This study provided evidence to suggest that RTI-336 may have its own abuse potential although it did maintain lower rates of self-administration when compared to cocaine suggesting it may have a lower abuse potential than cocaine itself. RTI-

336 is a promising candidate and has been tested in a Phase 1 clinical trial in healthy male subjects (www.clinicaltrials.gov/ct/show/NCT00808119) although no results have been published.

Mazindol is an anorexic compound that has also been used to label the cocaine binding site on the dopamine transporter (Ritz, et al., 1987). It has been shown to inhibit reuptake of DA, 5-HT and NET but does so without showing the robust reinforcing effects seen with cocaine (Bergman, et al., 1989; Wilson & Schuster, 1976). Mansbach et al (1993) showed that mazindol generalized to cocaine in discrimination studies and also reduced self-administration of cocaine, albeit at doses that also reduced food maintained behavior (Mansbach & Balster, 1993) . A further study showed that mazindol had positive reinforcing properties in rhesus monkeys trained to self-administer cocaine (Wilson and Schuster 1976). Given these properties mazindol was considered to be a suitable candidate as a cocaine replacement therapy, however, a double-blind placebo-controlled study showed that patients treated with mazindol versus placebo showed no response differences (Stine, Krystal, Kosten, & Charney, 1995). Further studies were suggested with higher doses of mazindol but there were concerns about safety with using higher doses and therefore were not pursued (Preston, Sullivan, Berger, & Bigelow, 1993).

GBR-12909 is a 1,4-dialkylpiperazine and was among the first compounds to be characterized as a selective and high affinity DAT inhibitor (van der Zee, Koger, Gootjes, & Hespe, 1980), being several fold less potent at 5-HT and NE transporters (Andersen, 1987; Hirate & Kuribara, 1991; Preti, 2000) and much research has characterized its behavioural profile (Rothman & Glowa, 1995). It was determined to have a slower onset and longer duration of action than cocaine (Kelley & Lang, 1989; Rothman et al., 1992; Rothman et al., 1991), in part due to its slower dissociation and higher affinity at the DAT (Reith, Sershen, &

Lajtha, 1981). It is the most extensively studied piperazine derivative in terms of its potential as a cocaine substitute. GBR-12909 has been shown to increase locomotor activity and inhibit cocaine self-administration in rats and non-human primates (Schenk, 2002; Tella, 1995). GBR-12909 generalizes to cocaine in discrimination studies (Holtzman, 2001), is also self-administered in rats and non-human primates, and shows similar break points to cocaine in progressive ratio studies (Roberts, 1993). These latter results suggest that GBR-12909 may have its own inherent abuse potential. GBR-12909 has also been shown to attenuate the cocaine-induced increase in extracellular dopamine in rat nucleus accumbens (Baumann et al., 1994). GBR-12909 is one of the few DAT inhibitors that has been tested in clinical trials as a potential agonist therapy for treating cocaine dependence. Unfortunately the trial was halted after Phase I due to cardiac toxicity (Vocci & Elkashef, 2005) thought to be cause by an enhanced QT interval (Herman BH et al., 2005).

Rationale for Study

Given the available research, and data that shows that cocaine's abuse related effects are attributable to its inhibitory actions at the DAT (Ritz et al., 1987), it has been determined that a potential treatment for cocaine dependence could involve the use of a DAT inhibitor as a replacement therapy. However, despite an abundance of research, finding or developing a DAT inhibitor with enough DAT inhibition to convey some cocaine-like behavior without the high abuse potential of cocaine has proven difficult. Clearly there is more research needed in order to help in this drug development area. As previously mentioned, the greatest issue with cocaine dependence treatments is to produce a DAT inhibitor that mimics some cocaine-like effects, but with limited abuse liability. Given the previously discussed results, plus the observation that a high affinity DAT inhibitor, like buproprion, has low abuse liability,

further research into high affinity, selective DAT inhibitors may prove productive for developing a treatment for cocaine dependence. Although there is a large body of data showing results of studies with numerous DAT inhibitors, to date very little information is available about the effects of a *selective*, high affinity DAT inhibitor. It is thought that investigating the effects of a very selective, high affinity DAT inhibitor could illuminate what effects are minimally essential for producing drugs with profiles consistent with those thought ideal for a cocaine replacement therapeutic, however, until recently no such tool has been available. To this end, the first section of this dissertation was to characterize the behavioural effects of a novel, selective DAT inhibitor, D-84, as part of a larger drug development project.

BEHAVIOURAL CHARACTERISATION OF A HIGHLY SELECTIVE DAT INHIBITOR

Introduction

As stated earlier, cocaine abuse and addiction is a persistent, worldwide, socioeconomic and medical problem and an estimated 1.5 million people are addicted to cocaine (SAMHSA 2000). Studies have shown that cocaine inhibits the uptake of serotonin, dopamine and noradrenaline (Reith, Meisler, Sershen, & Lajtha, 1986; Taylor & Ho, 1978), however, the preponderance of its abuse-related effects are likely attributable to its inhibition of the DAT (Ritz et al., 1987).

It has been hypothesized that a selective DAT inhibitor may provide an effective therapy for cocaine dependent individuals, and to this end DAT inhibitors have been investigated as potential replacement therapies for cocaine abuse (Bergman, et al., 1989; F. I. Carroll, Howard, et al., 2006; Spealman, Madras, & Bergman, 1989). These drugs possess several effects thought required for a cocaine replacement pharmacotherapeutic, namely an ability to occasion the cocaine discriminative stimulus, the ability to reduce cocaine self-administration, and to have a long duration of activity, however, they also have some undesirable effects (Birmingham, Nader, Grant, Davies, & Nader, 1998; Nader, et al., 1997; Wojnicki & Glowa, 1996). It is unknown whether a drug with greater selectivity for the DAT would have an improved profile. D-84 is an optically active three-hydroxy substituted piperidine derivative of GBR-12935 (Ghorai et al., 2003), and is, to date, one of the most selective DAT inhibitors available (NIDA 31254 Broad spectrum analysis). The aim of the present study was to evaluate the behavioural effects of D-84 in cocaine discrimination and self-administration assays, and to determine the effects of D-84 on cocaine self-

administration in rats, in order to further evaluate its likely usefulness as a replacement therapy for cocaine dependency. Drug discrimination and self-administration are assays that demonstrate the subjective and positive reinforcing effects of compounds, respectively (Balster, 1991b; Schuster & Johanson, 1974; Thompson & Unna, 1977). An ideal replacement therapy should likely possess a certain level of cocaine-like activity but with limited abuse potential. A successful treatment should likely have a slower onset but longer duration of action compared to cocaine, would partially generalize in discrimination studies and would be self-administered but at lower maximal levels when compared with cocaine. Partial to full generalization may predict that the compound is able to produce similar subject effects as cocaine, which consequentially may palliate cravings of the patient. Similarly, to promote patient compliance a compound would likely need to be self-administered but ideally with fewer efficacies (abuse liability) compared to cocaine.

Hypothesis

Evidence has shown that cocaine produces its abuse related effects through inhibition of the DAT (Ritz, et al., 1987). Many non-selective, high affinity DAT inhibitors have been investigated for their potential to be used as replacement therapies, but to date none have proven totally satisfactory (Gorelick, et al., 2004). These non-selective, high affinity DAT inhibitors generalize to cocaine in discrimination studies and are self-administered. Based on these observations, and on the knowledge that D-84 is a highly selective DAT inhibitor, I predict that D-84 will at least partially generalise to cocaine in discrimination studies and will be self-administered above vehicle control levels, thereby satisfying minimal features essential as a potential replacement therapy for cocaine dependence.

Materials and Methods

Subjects

Adult, male, experimentally-naive Long-Evans hooded rats (Harlan Sprague-Dawley, Indianapolis, IN) were used. Subjects were individually housed in an American Association of Animal Laboratory Care-accredited facility and given *ad libitum* food and water. For self-administration studies, free feeding weights were obtained, and food (Harlan Teklad, Madison, Wisconsin) was subsequently restricted to 15 g a day until rats achieved a target weight of approximately 320 g. The rats were maintained at this target weight throughout the study by adjustments in post-session feedings. For discrimination studies, rats were initially free fed, and then maintained at 85% of their free feeding weights. Post session feeding was adjusted to maintain these weights throughout the study.

Studies were approved by the Institutional Animal Care and Use Committee of the Virginia Commonwealth University and conformed with NIH Guidelines for Care and Use of Laboratory Animals.

Drug Discrimination Apparatus and Procedure

Rat discrimination studies were conducted in two-lever operant conditioning chambers (Med-Associates Inc., St. Albans, VT) equipped with a house light and food dispenser that delivered 45 mg food pellets (Research Diets, Noyes Precision Pellets, New Brunswick, NJ). Scheduling of pellet deliveries and collection of data were accomplished by a microcomputer and associated interface (Med-Associates Inc., St. Albans, VT, MED-PC® IV.

Drug discrimination training occurred during daily (M-F) 15-min experimental sessions. The rats were initially trained to press one of two levers under a fixed-ratio 1 (FR 1) schedule of reinforcement in which each lever press resulted in a pellet delivery. The response requirement was gradually increased to FR 10. During the next few sessions the rats were reinforced only for pressing the alternate lever until they pressed reliably under FR 10 scheduling conditions, after which drug discrimination training commenced. Rats were injected with 10 mg/kg cocaine or saline vehicle i.p., 10 min prior to the start of the session. For each rat, one lever was designated correct following drug administration and the other as correct following saline administration. The lever upon which the rats initially acquired the lever press response was designated as the saline-appropriate lever. All responses on the inappropriate lever were recorded, but had no programmed consequences. The lever on which the rats were initially trained and on which they acquired the lever-press response was designated as the vehicle appropriate lever. Alternation of cocaine and saline injections proceeded according to a two monthly cycle (Month #1: CSSCS, SCCSC, SCSCS, CSCSC; Month #2: SCCSS, CSCSC, CSSCC, SCSCS; in which C=cocaine S=saline). Lever pressing produced pellet delivery only on the injection-appropriate lever for that day. Incorrect presses reset the response requirement on the correct lever. Substitution tests began once a rat met the following criteria: 1) the first completed fixed ratio (FFR) occurred on the lever designated correct on at least eight of ten consecutive sessions; and 2) at least 80% of the total responses were emitted on the correct lever during those eight sessions. After these initial training criteria were met, testing could occur twice a week on Tuesdays and Fridays, provided that the rats completed the FFR on the correct lever during the most recent training drug and saline sessions; otherwise, a training day was administered. Test sessions were identical to training sessions except completion of the FR10 contingencies on either lever resulted in

pellet delivery. Dose-response curves were collected first with cocaine (1-30 mg/kg) before substitution tests with D-84 (1-42 mg/kg) were conducted.

Self-administration Apparatus and Procedure

Self-administration tests were conducted in operant conditioning chambers housed inside individual, isolated and ventilated boxes (Med-Associates Inc., St. Albans, VT). The front wall of each chamber was equipped with two retractable levers with a white stimulus light above each lever. A 5-w house light and Sonalert® tone generator were located on the rear wall of the chamber.

Infusion Assembly System

Catheters were constructed from polyurethane tubing (Access Technologies, Skokie, IL; 0.044" O.D. X 0.025" I.D.). The proximal 3.2 cm of the catheter was tapered by stretching following immersion in hot sesame oil. The catheters were prepared with a retaining cuff approximately 3 cm from the proximal end of the catheter. A second larger retaining cuff was positioned approximately 3.4 cm from the proximal end of the catheter. Mid-scapula cannula/connectors were obtained from Plastics One (Roanoke, VA). The cannula/connectors consisted of a threaded plastic post through which passed an "L" shaped section of 22 gauge stainless steel needle tubing. The lower surface of the plastic post was affixed to a 2 cm diameter disc of Dacron mesh. During sessions the exposed threaded portion of the infusion cannula was connected to an infusion tether consisting of a 35 cm length of 0.40 mm i.d. polypropylene tubing encased within a 30 cm stainless steel spring to

prevent damage. The upper portion of the 0.40 polypropylene tubing was connected to a fluid swivel (Lomir Biomedical, Inc, Quebec, Canada) that was, in turn, attached via 0.40 polypropylene tubing to the infusion syringe.

Surgical Procedure

Following acclimation to the laboratory environment, indwelling venous catheters were implanted into the right external jugular vein. Surgical anesthesia was induced with a combination of 50 mg/kg ketamine (KetaThesia, Butler Animal Health Supply, Dublin, OH) and 8.7 mg/kg xylazine (X-Ject E, Butler Animal Health Supply, Dublin, OH). Rats were additionally administered 8 mg/kg oral enrofloxacin (Baytril, Bio-Serv, Frenchtown, NJ) for three days postsurgery. The ventral neck area and back of the rat were shaved and wiped with povidoneiodine,7.5% (Betadine, Purdue Products L.P., Stamford, CT) and isopropyl alcohol. The rat was placed ventral side down on the surgical table and a 3 cm incision was made 1 cm lateral from mid-scapula. A second 0.5 cm incision was then made mid-scapula. The rat was then placed dorsal side down on the operating table and a 2.5 cm incision was made longitudinally through the skin above the jugular area. The underlying fascia was bluntly dissected and the right external jugular vein isolated and ligated. A small cut was made into the vein using an iris scissors and the catheter was introduced into the vein and inserted up to the level of the larger retaining cuff. The vein encircling the catheter between the two cuffs was then tied with silk suture. A second suture was then used to anchor the catheter to surrounding fascia. The distal end of the catheter was passed subcutaneously and attached to the cannula/connector that was then inserted subcutaneously through the larger incision. The upper post portion of the connector/cannula exited through the smaller mid-scapula incision. All incisions were then sprayed with a gentamicin sulfate/betamethasone valerate topical antibiotic (Betagen, Med- Pharmex, Inc., Pomona, CA) and the incisions were closed with

Michel wound clips. Rats were allowed to recover from surgery for at least 5 days before self-administration training began. Periodically throughout training, methohexital (1.5 mg/kg) or ketamine (5 mg/kg) (KetaThesia, Butler Animal Health Supply, Dublin, OH) was infused through the catheters to determine patency as inferred when immediate anesthesia was induced. Between sessions the catheters were flushed and filled with 0.1 ml of a 25% glycerol (Acros, New Jersey)/75% sterile saline locking solution containing: 250 units/ml heparin (Abraxis Pharmaceutical Products, Schaumburg, IL) and 250 mg/ml ticarcillin/9 mg/ml clavulanic acid (Timentin, GlaxoSmithKline, Research Triangle Park, NC). If during the experiment a catheter was determined to be inpatent, the left external jugular was then catheterized and the rat was returned to testing.

Pre-treatment Tests with D-84 in Rats Self-administering Cocaine

Cocaine self-administration training sessions were 2 h in duration and were conducted 5 days a week (M-F), unless testing had begun, and then sessions were run continuously until testing was completed. Initially, each response (fixed ratio 1, FR1) on the right-side lever resulted in delivery of a 0.5-mg/kg cocaine infusion (0.18 ml/6 sec). For the duration of the infusion, the tone sounded and the stimulus lights above both levers flashed at 3 Hz. Active (right-side) lever presses during the infusions as well as all inactive (left-side) lever presses were recorded but were without scheduled consequences.

Pre-treatment testing began once rats had met the following criteria: no increasing or decreasing trends in infusion numbers for 3 consecutive sessions had occurred, and the animals received at least 15 infusions during each session. Once these criteria were met, daily injections of vehicle were administered i.p. immediately prior to each session in order to habituate the rats to pre-session injections.

After training criteria were met, a cocaine self-administration dose-effect curve was

obtained. Initially, saline was substituted for cocaine for at least three consecutive sessions. When the number of saline infusions during the third or later session of its substitution was < 50% the number of infusions during the most recent session of cocaine administration, the rats were returned to 0.5 mg/kg/inf cocaine self-administration. When the number of infusions of the training dose of cocaine obtained during a session was greater than the number of saline infusions on the last of its substitution, a new dose of cocaine could then be substituted. Each dose of cocaine was substituted for one day. Between substitutions the rats were returned to 0.5 mg/kg/inf cocaine self-administration for at least one session and until the number of infusions exceeded the number obtained on the last day of saline substitution. On each cocaine dose test day either vehicle or a dose of a pretreatment test drug was given before test session start. Pretreatment tests with vehicle preceded those with test compounds. Pre-treatment's with cocaine (10 mg/kg) and D-84 (9.6, 17.1 and 30.4 mg/kg) were tested across the self-administration dose-effect curve of cocaine. Self-administered doses of cocaine tested included 0.01, 0.1, 0.5 and 1 mg/kg/inf. These self-administration doses were used based on historical studies performed in the laboratory that the current experiment were conducted in. The 10 mg/kg cocaine pre-treatment dose was selected based on the training dose of cocaine for the discrimination studies, and the pre-treatment doses of D-84 were selected based on ED50 values of D-84 and doses one half log unit higher and lower determined during a previous mouse cocaine discrimination study (Ghorai et al 2003).

Self-administration of D-84

Once pretreatment studies had been completed, other rats were trained to self-administer 0.5 mg/kg/inf cocaine in an identical fashion as described for rats used in the pretreatment study. When neither increasing nor decreasing trends in infusion numbers for 3

consecutive sessions had occurred, and the rats had obtained at least 15 infusions during each session, cue changes (stimulus light flashes above levers and Sonalert activations) during infusions were discontinued. Discontinuation of cue changes were initiated to permit rapid extinction of lever pressing during substitutions with doses of D-84. After three sessions of 0.5 mg/kg/infuse cocaine self-administration occurred during which neither increasing nor decreasing trends in infusion numbers occurred, saline was substituted as the available infusate until the number of infusions obtained during a session was less than 50% the mean of the most recent three cocaine sessions. Rats were then returned to 0.5 mg/kg/cocaine availability without cues until trends in infusion numbers did not occur across three consecutive sessions. Vehicle, and increasing doses of D-84 (0.1, 0.3, 0.56 and 1.0 mg/kg/infusion) were then substituted for three consecutive sessions each. Following tests with D-84, the rats were returned to 0.5 mg/kg/inf cocaine availability for three consecutive sessions.

Drugs

Cocaine HCl was obtained from the National Institute of Drug Abuse, and was dissolved in 0.05% saline. D-84, a 3-hydroxy substituted piperadine analogue of GBR-12935, was synthesized according to methods described previously (Ghorai et al 2003). D-84 was solubilized in 20% β-cyclodextrin in sterile water. (Cavitron 82003, Cargill Food and Pharma specialists, Cedar Rapids, IA). During discrimination tests, D-84 was administered i.p 20 minutes before the start of the test session. This pre-treatment time was chosen because previous locomotor studies indicated that peak effects on locomotor activity occurred at 20 min post-injection. Cocaine was administered 10 minutes before the start of discrimination studies base on previous cocaine discrimination studies conducted in this laboratory. All drugs were administered in a volume of 1.0 ml/kg when administered i.p.

Data Analysis

For drug discrimination studies, the percentage of cocaine-lever responding (% CLR) was calculated for each subject by dividing the number of lever presses emitted upon the cocaine lever by the total number of presses emitted upon both levers and multiplying this quotient by 100. Individual values of % CLR were then averaged (±SEM). Complete generalization to the cocaine discriminative stimulus was inferred when % CLR was > 80%. Mean response rates for each test condition were calculated by dividing the total number of lever presses emitted upon both levers by the session duration (900 s) for each subject, and then these rates were averaged (±SEM). If a rat failed to make at least ten lever presses during a test session, its data were excluded from calculations of % CLR but were included for mean response rate determinations. This exclusion was made to prevent near-zero rates of responding from disproportionately influencing percent cocaine lever responding. ED50 values and their confidence intervals (CI) were calculated for % CLR and for reducing response rates using nonlinear regression analysis.

For self-administration substitution studies, data from the last day of substitution at each dose were used in the analyses. One-way repeated measures ANOVA, followed by Dunnett's post hoc tests, were used to compare self-infusions of drug to self-infusions of saline. For self-administration pretreatment studies, one-way repeated measures ANOVA were conducted at each dose of cocaine self-administered on cocaine intake (mg/kg/2-h session). Post-hoc analyses were conducted comparing cocaine intake during pretreatments with D-84 to vehicle pretreatment within each dose of cocaine self-administered using Dunnett's post tests. ED50 values and their confidence intervals (CI) for reducing cocaine intake were calculated using a variable slope, nonlinear regression analysis. In order to permit

calculations of ED50 values, a "dose" of 0.96 mg/kg (an order of magnitude lower than the lowest dose of D-84 tested) was assigned for the vehicle dose. ED50 values were assumed "incalculable" if their CIs ranged greater than two orders of magnitude. Statistical significance was assumed in all analyses if $p<0.05$. Statistical analyses and nonlinear regressions were performed using GraphPad Prism version 5.0 for Mac OSX, (GraphPad Software, San Diego, California USA).

Results

Effects of D-84 in Cocaine Discriminating Rats

Cocaine completely generalized to the 10 mg/kg cocaine training dose at doses of 10 and 30 mg/kg with an ED50 (CI) value for producing cocaine-lever responding of 2.85 mg/kg (2.1-3.5 mg/kg). When saline was tested, near-zero levels of cocaine-lever responding occurred (Fig. 2, Upper panel). At the highest dose of cocaine tested (30 mg/kg), rates of responding were decreased by ~50% relative to saline control levels (Fig. 1, lower panel). As D-84 dose increased, levels of %CLR increased until a maximum of 76% CLR occurred at a dose of 30.4 mg/kg, a dose which markedly reduced response rates, and complete generalization to the 10 mg/kg cocaine training dose never occurred (Fig.2 upper panel). Despite D-84's incomplete generalization to the cocaine stimulus, an ED50 (±CI) was calculable (8.2 (3.4-19.8) mg/kg).

Cocaine dose-dependently reduced response rates with an ED50 (CI) value of 13.83 (4.8-39.3) mg/kg. D-84 also dose-dependently reduced response rates with an ED50 (CI) value of 33.9 mg/kg (15-72) mg/kg (Fig.2 lower panel).

Self-administration of Cocaine and D-84 and the Effects of D-84 Pre-treatment on Cocaine Self-Administration in Rats

Fig. 3 (upper panel) shows the mean numbers of infusions obtained when cocaine and D-84 were available for self-administration. The relationship between self-administered cocaine infusions and dose was characterized by an inverted U-shaped curve with peak numbers of infusions occurring at the intermediate cocaine dose of 0.1 mg/kg/infusion, which was significantly greater than saline control numbers ($p<0.05$). D-84's self-administration was also characterized by a U-shaped curve relating infusions to dose with peak numbers of infusions occurring at 0.3 mg/kg/infusion, and which were significantly greater than vehicle control numbers ($p<0.05$). Fig. 3 (lower panel) shows mean drug intake (mg/kg/2-session) of self-administered cocaine and D-84. Mean drug intake for both cocaine and D-84 increased as a function of dose. Peak levels of D-84 (21.6±4.61) intake were nonsignificantly lower than peak levels of cocaine (24.3±3.34) intake.

Effects of D-84 Pre-treatment on Cocaine Intake

Fig. 4 shows the mean cocaine intake (mg/kg/2-h session) obtained following D-84 pretreatment as a function of self-administered cocaine dose. Compared to vehicle pretreatment, the highest dose of D-84, 30.4 mg/kg, significantly reduced cocaine intake at cocaine self-administered doses of 0.5 ($p=0.0167$) and 1.0 ($p=0.0254$) mg/kg/infusion. Generally, pre-treatment with low to intermediate doses of D-84 non-significantly decreased intake of low dose cocaine self-administration.

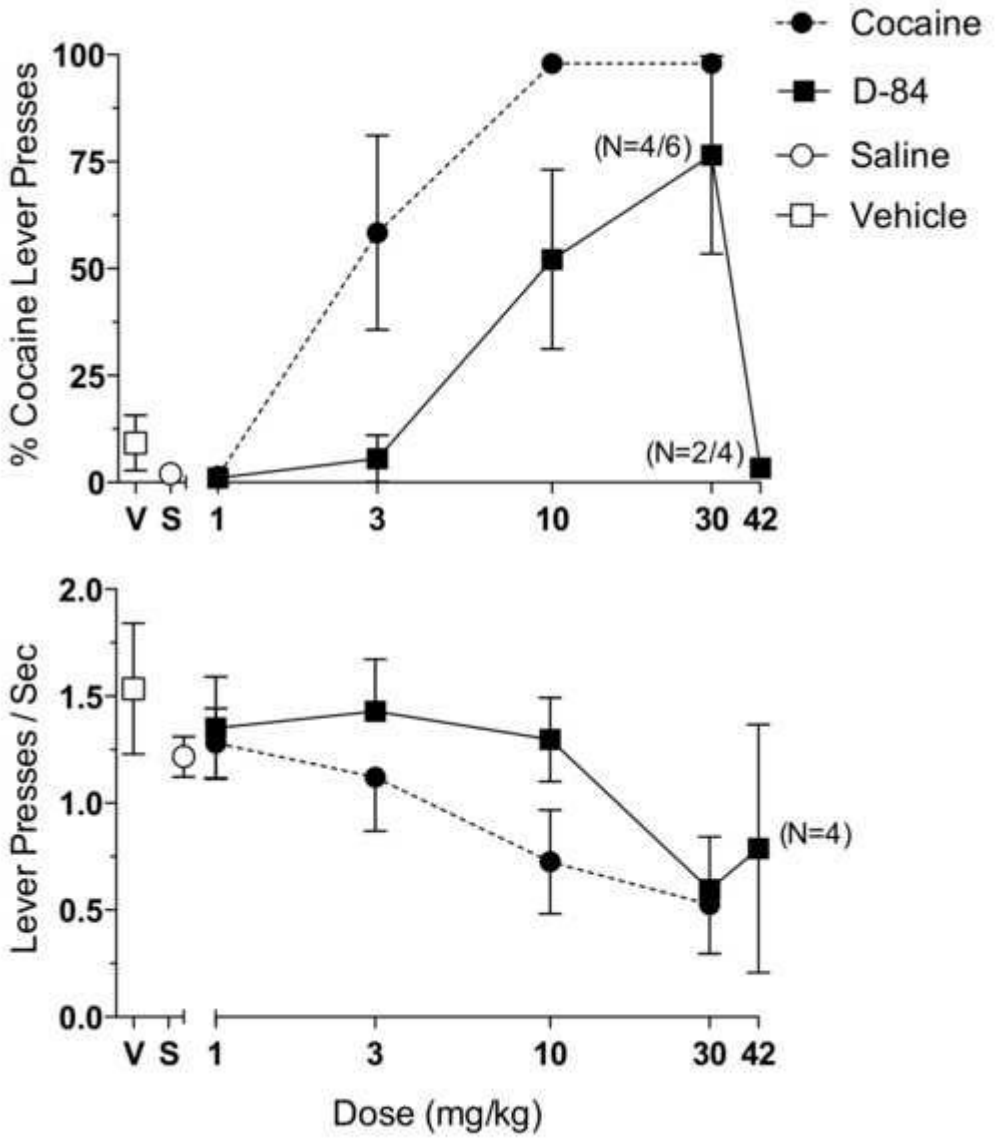


Fig. 2. Upper panel: Effects of cocaine and D-84 dose (mg/kg) on the percentage of cocaine lever responses in rats trained to discriminate 10 mg/kg cocaine. "V"=20% w/v cyclodextrin vehicle; "S"=saline; filled circles=cocaine; filled squares=D-84. Each symbol represents a mean of six rats, except N=4 and 2 at 30 and 42 mg/kg, respectively, because some rats failed to meet minimum response rate criteria (see text) and their data were not included for %CLR expressions but were included for expressions of response rate. Bars represent ±SEM. When ratios of N are provided at a dose the denominator indicates total rats tested at that dose, and the numerator indicates the number meeting response rate criteria. If a rat failed to meet response rate criteria at a lower dose, it was not tested at higher doses (e.g., only four rats were tested at 42 mg/kg because two had not met response rate criteria at 30 mg/kg).
Bottom panel: Mean numbers of total lever presses per sec. Each symbol represents a mean of six rats except N=4 at 42 mg/kg because two rats failed to meet response criteria at the preceding, lower dose (i.e., at 30 mg/kg) and were not tested at higher doses (see text).

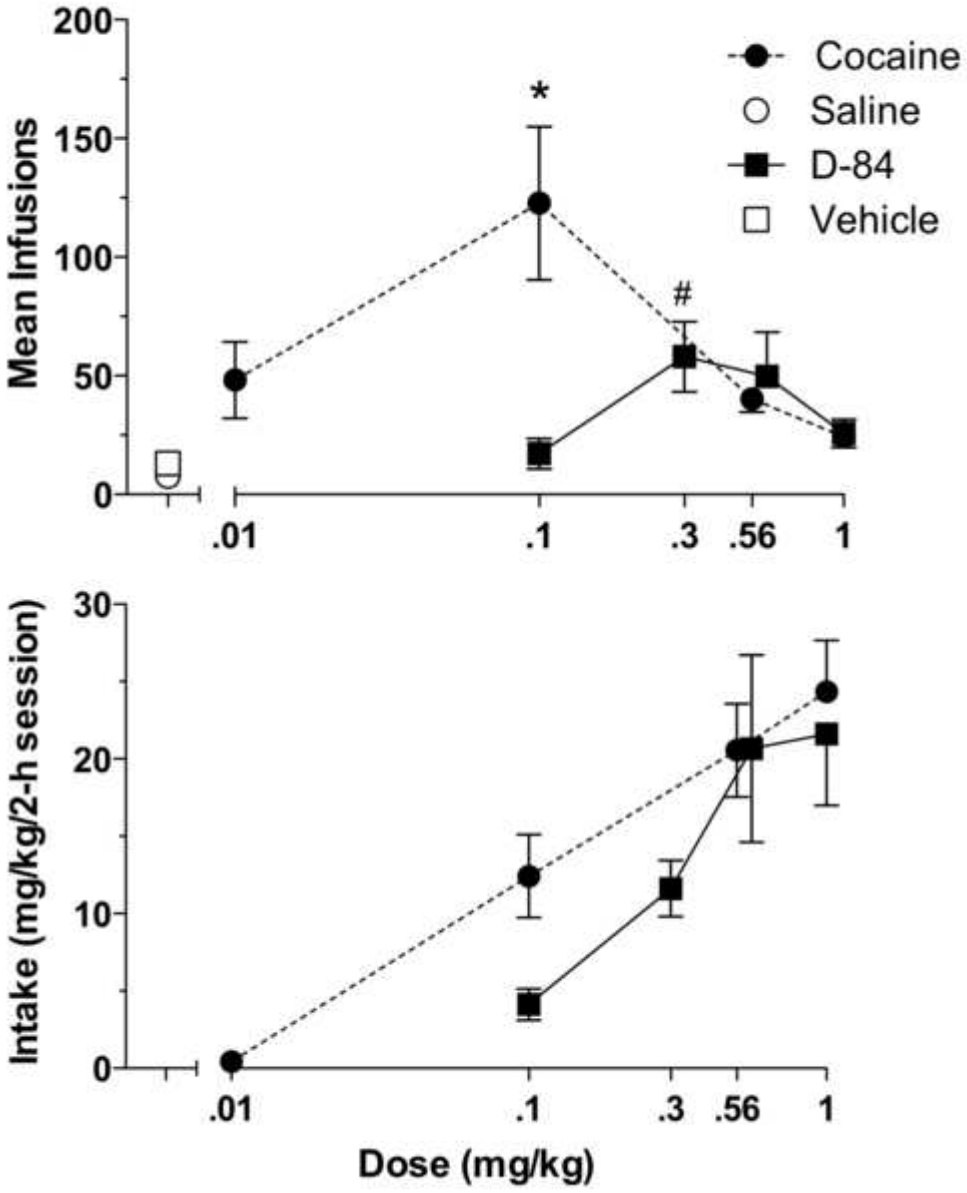


Fig. 3. Upper panel: Mean infusions of saline (unfilled circles) and D-84s vehicle (unfilled squares), and of cocaine (filled circles) and D-84 (filled squares) obtained as a function of dose. Brackets through data points indicate ±SEM. N=5 at all conditions. * = $p < 0.05$ as compared to saline; # = $p < 0.05$ as compared to vehicle.
Bottom panel: Mean drug intake (mg/kg/2-h session) as a function of dose. Other details as described for the upper panel.

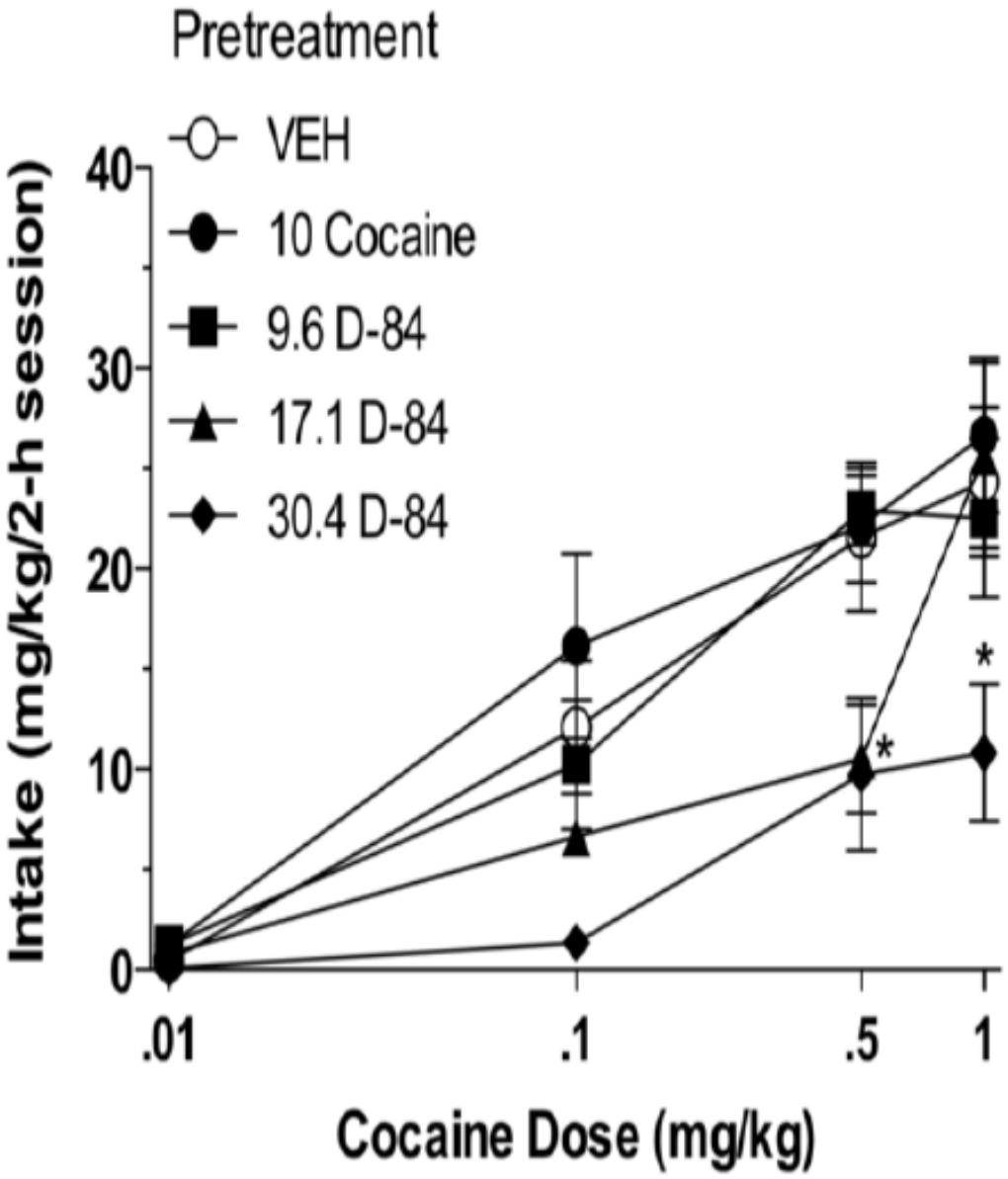


Fig. 4. Cocaine intake (mg/kg/2-h session) obtained following the various pretreatments as a function of self-administered cocaine dose. * = $p < 0.05$ as compared to vehicle pre-treatment. Bars through data points ±S.E.M.

Discussion

The objective of the current study was to characterize the behavioural effects of the selective DAT inhibitor, D-84, a 3-hydroxy-piperazine derivative of GBR-12935 (Ghorai, et al., 2003), in order to further determine its potential as a replacement therapy for cocaine dependence. The results showed that D-84 partially generalized to the cocaine discriminative stimulus, attenuated cocaine self-administration and was self-administered significantly higher than vehicle control levels.

Previous research has shown that binding potencies of DAT inhibitors correlate well with their reinforcing effects in self-administration (Ritz et al., 1987), and as a result, research into replacement therapies for cocaine dependence has focused on DAT inhibitors (F. I. Carroll et al., 2006; J.L Katz, Kopajtic, Agoston, & Newman, 2003). The fast onset and short duration of action of psychostimulant drugs likely contribute to their abuse liability, and it is thought that a replacement therapy with a slower onset and longer duration of action may compete with and effectively reduce craving for cocaine, but have a lower abuse potential than cocaine. This theory is based on the successful treatment of treating opiate dependence with the replacement therapy, methadone (Uchtenhagen, 2003). There have been several studies evaluating the effectiveness of DAT inhibitors as replacement therapies for cocaine-abusing subjects, but so far none have proven unequivocally effective or have had unacceptable toxicity. GBR-12909 has a high affinity for the DAT and results in non-human primates showed that it attenuates cocaine self-administration (Stafford, Rice, Lewis, & Glowa, 2000), however clinical trials were halted during phase I due to cardiac toxicity (Herman BH et al 2005). It is possible that a more selective DAT inhibitor with less NET activity may provide the right balance of cocaine-like effects but with reduced toxicity and therefore provide a usable replacement therapy for cocaine dependence.

To that end, D-84 was synthesized and subsequently identified as one of the most selective DAT inhibitors here-to-fore disclosed (Ghorai et al., 2003). In the current study, D-84 occasioned incomplete generalization (76%) in cocaine discriminating rats. This is consistent with previously published data showing that D-84 produces incomplete generalization (67%CLR) to cocaine in mice (Ghorai et al.,2003). Because in vitro binding efficacy at the DAT generally correlates well with in vivo efficacy for inhibiting DA uptake, and corresponding cocaine-like effects, it is surprising that D-84, being a potent and efficacious DAT inhibitor, did not occasion complete generalization to cocaine's discriminative stimulus. There are, however, other DAT compounds that fall into this category of being selective for DAT over serotonin or norepinephrine, without producing full generalization. Benztropine is a well known cocaine analogue that binds to the DAT with similar potency to cocaine but does not produce full generalization in cocaine discrimination studies (Newman, et al., 1994). It is thought that the decrease in cocaine-like behavioural effects of benztropine analogues is likely due to the antimuscarinic effects inherent in these compounds (Ranaldi & Woolverton, 2002). A broad spectrum binding evaluation of D-84 (NIDA Broad Spectrum Analysis 32154) indicated that it has low muscarinic activity (M1 Ki= 512 nM, M2 Ki= 816 nM) that unlikely limited its generalization to cocaine. D-84 also has activity at D4 receptors (Ki 28.9 nM), but it is difficult to determine how much of an influence this may have on D-84's behavioural effects.

A potential cocaine replacement therapy likely needs to share some of the subjective effects with cocaine, and D-84 does occasion partial cocaine-lever responding (maximum of 76% CLR). D-84's partial generalization to cocaine's discriminative stimulus could translate into a reduction of craving for cocaine if used as a medication. D-84 was self-administered, but was less potent than cocaine. Its self-administration suggests it likely would promote patient compliance. Finally, D-84's ability to reduce self-administration of higher doses of

cocaine suggests that it may substitute for cocaine when given as a pretreatment. This latter suggestion, however, needs qualification somewhat by the observation that D-84 also reduced food maintained response rates at high doses during discrimination tests, suggesting that some of the non-specific effects of D-84 could contribute to the attenuation of cocaine self-administration.

D-84 is less potent but longer acting than cocaine for stimulating locomotor activity in mice (Ghorai et al., 2003). D-84 is both less potent and efficacious than cocaine in producing cocaine's discriminative stimulus effects in mice (Ghorai et al., 2003) and in rats (present study). D-84 was also less potent than cocaine as a reinforcer in the present study. Considering D-84s general lower potency than cocaine, and observing that D-84 was self-administered at a lower peak infusion level than cocaine, and at a somewhat lower peak intake level as well, it seems likely that peak cocaine-like effects were never obtained with D-84 during the self-administration tests. Those observations raise the possibility that D-84 has less reinforcing efficacy, and consequent lower abuse potential, than cocaine. Additional studies, however, more directly addressing relative reinforcing efficacy between D-84 with cocaine and other known drugs of abuse (e.g., progressive ratio or choice studies) are needed to more definitively predict D-84s likely relative abuse potential.

Conclusions

The results of the current study show that D-84 is less potent and efficacious than cocaine in occasioning cocaine-like subjective effects, maintains self-administration at lower infusion levels, and can reduce cocaine self-administration when given as a pretreatment. These results show that D-84 possesses some favourable characteristics likely desirable for a replacement therapy in treating cocaine dependency, and encourages further study for its utility as a pharmacotherapeutic.

The goal of this section of the dissertation was to evaluate the behavioural characteristics of D-84, a novel selective DAT inhibitor, as a potential drug developmental candidate. Cocaine's abuse related effects are attributable to its inhibitory actions at the DAT (Ritz et al., 1987), and a potential treatment for cocaine dependence could be a DAT inhibitor with tolerable toxicity. The current results with D-84 are, so far, consistent with that desirable profile. However, although the discrimination, self-administration, and pre-treatment effects of D84 seemed favourable, they could be improved upon. Recent evidence has suggested that the serotonergic system plays a modulatory role in cocaine's behavioural effects (Xi, Gardner, 2008). The second part of this dissertation study focused on this serotonin component in an attempt to determine whether altering the level of SERT inhibition modulates the cocaine-like behaviour of high affinity DAT inhibitors. Introducing some SERT inhibition, to DAT inhibition, could lead to an improved profile as a potential cocaine replacement therapy, relative to a highly selective DAT inhibitor such as D-84.

INVESTIGATING THE INFLUENCE OF SERT INHIBITION ON THE COCAINE-LIKE BEHAVIOURAL EFFECTS OF DAT INHIBITORS

Serotonin –Dopamine Interactions

Selectivity at the DAT is predominantly responsible for cocaine's strong reinforcing effects (Ritz, et al., 1987), suggesting that a highly selective DAT inhibitor may have its own strong reinforcing effects and likely comparable abuse potential. This can be problematic in terms of developing compounds to act as potential replacement therapies. While it is advantageous for treatment compounds to share some behavioural effects with cocaine in order to treat and insure compliance by the patient, a potential treatment needs also to have less inherent abuse potential than cocaine itself.

Role of Serotonin in Modulating the Reinforcing Effects of Cocaine

Although there is sufficient evidence to support the hypothesis that cocaine produces its reinforcing effects by stimulating the mesolimbic dopamine pathway, it has other biochemical effects as well. Cocaine also binds to and inhibits uptake at the serotonin (SERT) and norepinephrine (NE) transporters (NET) (Koe, 1976; Reith, Sershen, Allen, & Lajtha, 1983). It has generally been determined that binding to NET does not play a major role in the reinforcing effects of psychomotor stimulants (Wee et al., 2006; Woolverton, 1987; Yokel & Wise, 1975) and might even have a role in producing the aversive effects of cocaine (Jones, Hall, Uhl, & Riley, 2010).

The 5-HT system has become implicated in the development of novel pharmacotherapies for drug abuse and addiction (Xi & Gardner, 2008). Focus has been

applied to the potential importance of 5-HT in modulating the reinforcing effects of cocaine. Serotonin (5-HT) neurons originate in the raphe nuclei in the midbrain and project to numerous other brain regions (Hoyer, Hannon, & Martin, 2002), including the mesolimbic DA pathway, where activation of 5-HT receptors within this reward pathway has shown to have a modulating effect on the DA system (Bubar & Cunningham, 2006). It has been determined that, of the 14 subtypes of 5-HT receptors, the 5-HT_{2A} and 5-HT_{2C} receptor families play major roles in modulating DA output (Higgins & Fletcher, 2003). It has been shown that 5-HT_2c receptors inhibit mesolimbic DA output, whilst 5-HT_{2A} receptor activation potentiates it suggesting that a net effect of activation or inhibition could occur depending on which receptor system has the predominant influence at the moment. Given the current literature, it seems that the serotonergic system in the brain plays an important role in modulating the effects of dopamine in the mesolimbic reward system. As further evidence of the serotonin system's role, it has also been shown that an increase in extra cellular 5-HT, after administration of the selective serotonin reuptake inhibitor (SSRI), fluoxetine, has an inhibitory effect on cocaine self-administration (M. E. Carroll, Lac, Asencio, & Kragh, 1990a; Howell & Byrd, 1995; Peltier & Schenk, 1993; Porrino et al., 1989). In addition, it has been shown that systemic administration of fluoxetine and 8-OH-DPAT, a 5-$HT1_A$ agonist, reduces low dose cocaine self-administration (M. E. Carroll, et al., 1990a; Peltier & Schenk, 1993). Loh and Roberts (1990) trained rats to self-administer cocaine on a progressive ratio (PR) schedule. Rats were then given intracerebral injections of the selective 5-HT neurotoxin, 5-7-dihydroxytryptamine. In comparison to sham-treated control animals, the break point for cocaine self-administration in 5-HT depleted animals was increased suggesting that decreased levels of brain 5-HT increased motivation for cocaine self-administration, possibly by altering the incentive value of cocaine (Loh & Roberts, 1990). A further study in squirrel monkeys pretreated with either a 5-HT uptake inhibitor or a

direct 5-HT agonist attenuated cocaine self- administration (Czoty, Ginsburg, & Howell, 2002).

Studies involving SSRI's and direct 5-HT receptor agonists and antagonists have provided further evidence to document that 5-HT has a dampening effect on cocaine's reinforcing effects and that serotonergic compounds themselves have very few cocaine-like behaviours. Drug discrimination studies with SSRI's have shown that they do not generalize to the cocaine discriminative stimulus and are not self-administered, suggesting that these compounds are not cocaine-like and likely have low abuse liability (Callahan & Cunningham, 1997; Cunningham & Callahan, 1991; Simon & Appel, 1997).

Taken together, these studies point to an important role of 5-HT in modulating the reinforcing effects of cocaine. Given the lack of reinforcing properties of 5-HT agonists, combined with the ability of SSRI's to reduce cocaine's positive reinforcing effects, it seems possible that the serotonergic system could be used to modulate the positive reinforcing effects of cocaine-like compounds in an effort to reduce their abuse potential.

Overall, these research reports indicate that increasing the level of 5-HT uptake inhibition, resulting in increases of 5-HT at its receptors, tends to decrease the reinforcing strength of cocaine. This, therefore, introduces the possibility that a DAT inhibitor possessing an optimum level of SERT inhibition could inherently have a decreased reinforcing efficacy while still retaining some cocaine-like activity to palliate the cocaine addict.

An added advantage to having increased serotonergic activity is the effect of serotonin on depression. A major side effect of cocaine withdrawal, and hence one reason for the high rate of relapse, is depression. If a DAT inhibitor possessed a sufficient level of SERT inhibition, it may not only reduce the abuse potential of the drug, but also, given the use of

SSRI's in the treatment of depression (Hamon, 1995), may help treat the depression seen upon cessation of cocaine use .

The results reported with D-84 in Chapter Two showed promise of its potential as a replacement therapy for cocaine. It has a long duration of activity, partially generalizes to cocaine and is self-administered but maintains rates of responding at levels lower than cocaine. All of these factors promote its potential as a candidate for drug development. Having said this, other evidence is available suggesting that other compounds could also be potential cocaine-dependence treatments. Taking into account all of the evidence currently available concerning the influence of serotonin on dopamine transmission, and consequentially on cocaine's reinforcing effects, it seems plausible that another possible replacement therapy for cocaine dependence, or an enhanced one, could be a DAT inhibitor that concurrently possessed an optimum level of SERT inhibition. The DAT component would allow a compound to retain some cocaine-like behavior, whilst the SERT influence would dampen the acute reinforcing effects of the compound and at the same time potentially help in treating depression produced upon withdrawal from cocaine use. Having said this, there are still many unanswered questions. Not all DAT inhibitors are considered equal, as discussed earlier, and cocaine is considered to be a triple amine uptake inhibitor that has high affinity for the SERT as well as the DAT. Obviously, given the positive reinforcing effects of cocaine, this level of SERT inhibition is clearly not having an inhibitory effect. This may imply that the level of SERT inhibition, and not the mere presence of SERT inhibition, may be key in producing a compound that retains some cocaine-like behavior but with enough SERT inhibition to attenuate abuse liability.

The aim of the following study was to examine the effects of selective DAT inhibitors that vary in their level of SERT inhibition, in order to characterize how a change in SERT inhibition influences the cocaine-like behavioural effects of high affinity DAT inhibitors.

Hypothesis

The central hypothesis of this study is that increasing the level of SERT inhibition will decrease the cocaine-like effects of DAT inhibitors. The goal of this project will be completed by fulfilling the following specific aims:

1: Identify individual DAT inhibitors with levels of SERT inhibition that can be identified as "low" (Ki >1500nM), "medium" (Ki between 70nM - 1500nM) and "high" (Ki <70nM) SERT inhibitors, and evaluate their effects in locomotor activity to determine how the level of serotonergic activity affects their locomotor activity.

2: Evaluate the effects of the selected drugs in cocaine discrimination procedures to determine how the level of serotonergic activity modulates their discriminative stimulus (i.e., subjective) and response rate effects.

3: Evaluate the effects of selected DAT inhibitors in self-administration procedures to identify how serotonergic levels affect self-administration of the test compounds.

Experimental Approach

The immediate objective of the following studies is to evaluate the role of SERT uptake inhibition in modulating the cocaine like-behavioural effects of DAT inhibitors. In particular, the goal is to determine if the level of SERT inhibition is regularly related to the magnitude of cocaine-like activity.

Three compounds with a similar high DAT uptake potency and efficacy were identified but which varied in their level of SERT inhibition. Drugs were categorized according to their level of SERT uptake inhibition. Compounds with SERT Ki values of $\leq$ 90nM were deemed "high" SERT activity drugs; SERT Ki values between 70-1500 nM were deemed "medium" SERT activity drugs; and those with Ki values $\geq$ 1500 nM were deemed "low" SERT activity drugs. These cut-off values were selected based upon general referrents to drugs in the relevant scientific literature, but more importantly following consultations with dissertation committee member, Maarten Reith, Ph.D.

Selection of Test Compounds

Table 1 identifies the compounds tested. The compounds were selected to be as similar as possible in their DAT inhibition but to vary widely in their level of SERT inhibition, with the objective of inferring that any differences in behavioural effects would be due to the influence of SERT inhibition. The compounds were also selected upon having low and similar levels of NET inhibition to minimize influence by noradrenergic activity.

D-84

D-84 is an optically active 3-hydroxy substituted piperidine derivative of GBR-12935 (Ghorai et al., 2003), and is, to date, one of the most selective DAT inhibitors currently available (NIDA 31254 Broad spectrum analysis). It has very low affinity for SERT (Ki = 1274 nM), and as such fills the role as a "high DAT, low SERT" compound. Previous research has shown it to have a similar onset of action in locomotor activity studies but a longer duration of activity when compared to cocaine (Ghorai, et al., 2003). This same study also showed incomplete generalisation to cocaine in discrimination tests in mice. Since it has

been determined that D-84 is a highly selective DAT inhibitor with extremely low SERT activity, D-84 was chosen as the "low" SERT compound. D-84 was behaviourally characterised as reported in chapter two, and the results provided information regarding its potential as a replacement therapy. It was selected as the "high DAT, low SERT" compound to help aid the objectives of the second section of this dissertation. The data for D-84 presented in this chapter are data that were collected and reported previously (Ghorai, et al., 2003) and is being presented here for comparison purposes.

GBR-12909

GBR-12909 is a high affinity DAT inhibitor that possesses affinity for the SERT (Ki DAT= 10.6nM, SERT= 73nM) and, by the rationale described previously, is considered to be a "medium" SERT inhibitor. Studies have shown that GBR-12909 increases extracellular DA in rats (Rothman et al., 1989) and pre-treatment with GBR-12909 reduces cocaine induced increases in extracellular DA (Rothman, et al., 1991). It has also been shown to generalize to cocaine in discrimination studies (Holtzman, 2001), to be self-administered in rats and non-human primates, and shows similar break points to cocaine in progressive ratio studies (Roberts, 1993). It is also the only drug of the current studies to have been assessed clinically for its potential as a cocaine dependence treatment (Vocci & Elkashef, 2005). Observing its level of SERT inhibition, it was selected as the candidate as a "high DAT, medium SERT" compound for the purposes of these experiments.

RTI-55

(–)-2β-Carbomethoxy-3β-(4-iodophenyl)tropane (RTI-55, β-CIT) is a potent phenyltropane DAT inhibitor (Boja et al., 1991) that also has potent uptake inhibition activity at the SERT (Ki DAT= 1.6nM , SERT= 3.8nM) (Boja, Mitchell, et al., 1992). RTI-55 is self-administered by rhesus monkeys and fully substitutes for cocaine in food reinforced discrimination studies (Weed, Mackevicius, Kebabian, & Woolverton, 1995). Other studies have shown RTI-55 to have cocaine-like discriminative stimulus effects in rats (Boja, Cline, et al., 1992; Cline, Terry, Carroll, Kuhar, & Katz, 1992). Since it is established that RTI-55 is a potent DAT inhibitor which also possesses potent inhibition activity at SERT, it was selected as the "high DAT, high SERT" compound for the purposes of this study. The uptake inhibition data for cocaine and the selected test compounds are presented in Table 1.

	DAT (nM)	SERT (nM)	NET (nM)	Category
COCAINE	270	180	221	MEDIUM SERT
D-84	4.05	1274		LOW SERT
GBR-12909	4.3	73	426	MEDIUM SERT
RTI-55	1.6	3.8	32	HIGH SERT

Table 1: Uptake Inhibition Activity (Ki (nM)) for Selected DAT Inhibitors. Cocaine and GBR-12909 (Matecka et al., 1996, Kuhar et al.,1999 Boos et al.,2006), RTI-55 (Boja et al., 1992), D-84 (NIDA Broad Spectrum Analysis)

Effects of DAT/SERT Inhibition on Locomotor Activity

Activation of postsynaptic dopamine receptors in the nucleus accumbens elevates locomotor activity in rodents (Pijnenburg & van Rossum, 1973; Sharp, Zetterstrom, Ljungberg, & Ungerstedt, 1987). Consequently, since psychomotor stimulants activate mesolimbic dopamine receptors, it is this action that is thought associated with the psychomotor stimulant related dose-dependent increases in locomotor activity in rodents (Wise & Bozarth, 1987).

It was therefore determined that, since the DAT is a major site of action for cocaine, it may play a necessary role in cocaine-induced locomotor activity (Uhl, Hall, & Sora, 2002). Studies have shown that acute administration of low to moderate doses of cocaine stimulates locomotor activity whilst higher doses are shown to decrease distance traveled due to increases in stereotyped behavior (Nielsen & Scheel-Kruger, 1988; Post, Weiss, Pert, & Uhde, 1987; Tyler & Tessel, 1979). DAT knockout (KO) mice have been shown to have higher than normal locomotor activity levels when compared to wild type mice, presumably due to the higher basal levels of DA. This same study showed that acute administration of cocaine increased locomotor activity in wild type mice but not in the DAT KO mice (Giros, Jaber, Jones, Wightman, & Caron, 1996; Sora et al., 1998), possibly because their baselines levels of DA were already higher than wild type mice making an increase after cocaine administration difficult to detect. These and other studies provide evidence to show that DAT inhibition is responsible for cocaine induced locomotor stimulation suggesting that all of the selected DAT inhibitors may stimulate locomotor activity. Other studies have shown that stimulation of the serotonergic system does not significantly affect locomotor activity (Fletcher, Sinyard, & Higgins, 2006; Fletcher, Sinyard, Salsali, & Baker, 2004), which may mean that increasing SERT inhibition will not have a pronounced effects in this assay, and

that the locomotor effects ultimately would be attributable to DAT activity. It should be noted that selection of the test compounds was made with their NET affinity in mind. NE produces its behavioural effects through activation of adrenergic receptors which are responsible, amongst other behaviours, for mediating contraction of vascular smooth muscle (Bylund et al., 1994). It is this action, along with the production of an enhanced QT interval that has lead to some NE-related cardiotoxicity, which has prevented compounds with pronounced NE activity from being developed into approved medications (Herman BH et al., 2005, Vocci FJ et al., 2005). However, developments over recent years have allowed for the production of compounds with subtype-selective effects, which may potentially help avoid cardio-related effects and have prompted NET-driven medication developments for stimulant abuse (Kampman et al., 2006; Sofuoglu, Brown, Babb, Pentel, & Hatsukami, 2000).

Locomotor activity determines whether a compound is generally behaviorally-active, but can also determine other characteristics of test compounds. Since the main aim of this experiment was to determine how altering the level of SERT effects the cocaine–like locomotor activity effects of the selected DAT inhibitors, locomotor activity results can provide valuable information as to how these DAT inhibitors differ from cocaine and indeed, each other. As mentioned previously, in terms of drug development, an effective therapy for cocaine dependence would need share some behavioural effects with cocaine, but show potential for having a lower abuse liability. Cocaine has a fast onset and short duration of activity thought to maximize its abuse potential, and it likley would be beneficial for treatment compounds to have a slower onset and longer duration of activity in an attempt to reduce their abuse liability. Research reports discussed earlier indicated that the serotonin system can modulate dopaminergic neurotransmission, therefore potentially alterring locomotor activity. Locomotor activity studies will determine the onset and duration of

activity of compounds, as well as peak total distance travelled, all of which can be compared to determine how changing the level of SERT inhibition alters these variables.

Hypothesis

With the understanding of DA's involvement in locomotion, it therefore seems reasonable to predict that since direct acting DA agonists stimulate locomotor activity, compounds that indirectly activate the DA receptors should also stimulate locomotion. Since it has been shown that cocaine, by inhibiting the DAT, leads to an increase in extracellular DA, and this in turn leads to stimulant effects on locomotion, it seems reasonable to assume that other indirectly acting DA agonists, like other DAT inhibitors, will also have a stimulant effect on locomotion. Based on binding and affinity data for the chosen drugs, the rank order of DAT inhibition is RTI-55> D-84> GBR-12909> Cocaine. We would predict that RTI-55 will have the greatest stimulant effect on locomotor activity since it has the highest DAT inhibition activity, however, it may also be possible that, since SERT inhibition has been shown to have an inhibitory effect on the stimulant-like effects of cocaine, the locomotor activity effects of these selected DAT inhibitors may vary based on their level of SERT inhibition. It also remains to be seen whether the level of DAT uptake inhibition has a greater effect on peak levels of locomotor, duration of action, or both and also whether the ratio of DAT/SERT inhibition influences locomotor activity.

Materials and Methods

Subjects

Adult, male Swiss-Webster mice weighing 25-35g were used in the locomotor activity studies. Mice were allowed to acclimate to the vivarium environment one week prior to the start of testing and were housed five per cage, with continuous access to food (Harlan Teklad,

Madison, Wisconsin) and water. All mice were drug naïve at the start of the study and did not have prior experience with the test chambers. The mice were housed in an AAALAC-accredited animal facility with a controlled temperature on a 12h light-dark cycle and all testing occurred during the light component.

Drugs

All drugs were injected i.p in a volume equivalent to 10ml/kg. The vehicle for cocaine and RTI-55 was sterile saline and the vehicle for GBR-12909 was 40% cyclodextrin in sterile water (Sigma Aldrich inc). Drugs (doses) tested were cocaine (1,10 and 30 mg/kg), D-84 (3.0, 10, 30, 56 mg/kg), RTI-55 (0.1, 1.0 and 10 mg/kg) and GBR-12909 (1, 30 and 56 mg/kg).

Apparatus and procedure

Locomotor activity tests were conducted in eight, commercially obtained, automated activity monitoring devices, enclosed in sound and light attenuating chambers (AccuScan Instruments, Columbus, OH). The interior of each device is divided into two separate 20 x 20 x 30 cm arena's permitting the independent and simultaneous measurement of two mice. Sixteen photobeam sensors are spaced 2.5 cm apart along the walls of the chamber and are used to detect movement.

Mice were injected and immediately placed into the chambers. A separate vehicle control group was tested during tests with each compound. Tests were 4 h in duration, and total distance traveled (cm) during each 10 min of the 240 min test session was recorded as the dependent measure.

Data Analysis

Data was analyzed using a two-way repeated measures ANOVA (Prism 5, GraphPad software, San Diego, CA). Post tests were performed using the Bonferroni test comparing dose to vehicle at each of the 24, 10-min time points. Differences are inferred when $P < 0.05$.

Results

Cocaine Increases Total Distance Travelled in a Dose Dependent Manner

Figure 5 shows the time course effects of cocaine over the 4 hour duration of the experiment. The figure shows the first 60 minutes on the left hand portion of the x axis whilst the remaining 3 hours are summed on the right portion of the x axis. Doses of 10 and 30 mg/kg cocaine produced peak effects of total distance travelled within the first 10 minutes. This elevation in locomotor activity returns to near vehicle control levels by the 60 minute time point. When the data was summed across the remaining three hours, cocaine did not produce a significant increase in total distance travelled ($P>0.05$) (Ghorai et al., 2003).

D-84 Increases Total Distance Travelled in a Dose Dependent Manner

Figure 6 shows the time course effects of D-84 over the 4 hour duration of the experiment. Thirty and 56 mg/kg D-84 stimulated locomotor activity with onset of effects occurring within the first 10 minutes post injection. Activity levels following injection of 30 mg/kg remained elevated throughout this period; when the data was summed over the remaining 3 hours activity levels were significantly above vehicle throughout. Peak effects occurred within the last 3 hours, with maximum total distance travelled reaching ~ 3500 cm. The highest dose of D-84, 56 mg/kg, initially produced increases in locomotor activity but following 20 minutes post injection, activity levels decreased to vehicle control levels. When

the 56 mg/kg data was summed over the remaining 3 hours the activity returned to levels that were elevated significantly above vehicle control levels. Therefore high dose D-84 significantly increased total distance travelled when compared to vehicle control levels ($P < 0.05$) and had a longer duration of action when compared to cocaine doses that significantly increased activity.

GBR-12909 Increases Total Distance Travelled in a Dose Dependent Manner

Figure 7 shows the effects of doses of GBR -12909 on total distance travelled over the 4 hour time course. 30 and 56 mg/kg GBR-12909 stimulated locomotor activity with an onset of activity at 10 mins and peak effects 20 minutes post injection. After 56 mg/kg GBR-12909 levels remained significantly ($p<0.05$) elevated compared to vehicle control until approximately 2 hours into the time course, after which activity levels returned to near vehicle control levels. 1 mg/kg GBR-12909 did not elevate total distance travelled above vehicle control levels at any point throughout the 4 hour time course. GBR-12909 produced a maximum level of total distance travelled of approximately 1600 cm which is approximately 50% less than the maximum level produced by cocaine and D-84.

RTI-55 Increases Total Distance Travelled in a Dose Dependent Manner

Figure 8 shows the effects of doses of RTI-55 on total distance travelled over the 4 hour time course. RTI-55, 0.1 mg/kg, did not affect total distance travelled compared to vehicle at any time point. RTI-55, 1 mg/kg, produced a peak effect (1400 cm total distance travelled) at 60 minutes and remained elevated, significantly above vehicle ($p<0.05$) control for the remainder of the 4 hour time course. 10 mg/kg RTI-55 produced a peak effect (~1500 cm total distance travelled) at 20 minutes, significantly above vehicle control levels ($p<0.05$),

but activity returned to near vehicle levels within 30 minutes post injection and remained comparable to vehicle for the remainder of the 4 hour time course.

Summary of Results

The aim of the current study was to determine the locomotor activating effects of the selected test compounds. All test compounds increased locomotor activity statistically above vehicle control levels ($p<0.05$) but there were differences in the peak level and duration of action of these increases. All the compounds were behaviourally active. Cocaine has a fast onset (10 minutes) and short duration of activity (<60 minutes). Cocaine produced a maximum level of total distance travelled of approximately 3000 cm. D-84 also has a fast onset of activity, similiar to cocaine, but has a much longer duration of activity and produced the highest levels of cumulative total distance travelled when compared to the other compounds tested (~3500 cm). GBR-12909 had a similar onset of activity compared to cocaine but showed approximately 50% lower peak levels of locomotor activity compared to cocaine and D-84 ($p<0.05$ when compared to cocaine and D-84). RTI-55 was the most potent compound, stimulating locomotor activity at lower doses than the other compounds. RTI-55 also showed the slowest onset of activity and also had the lowest peak activity levels ($p< 0.05$ when compared to cocaine and D-84). All of the test compounds had duration of activities longer than cocaine (D-84>RTI-55>GBR-12909>cocaine).

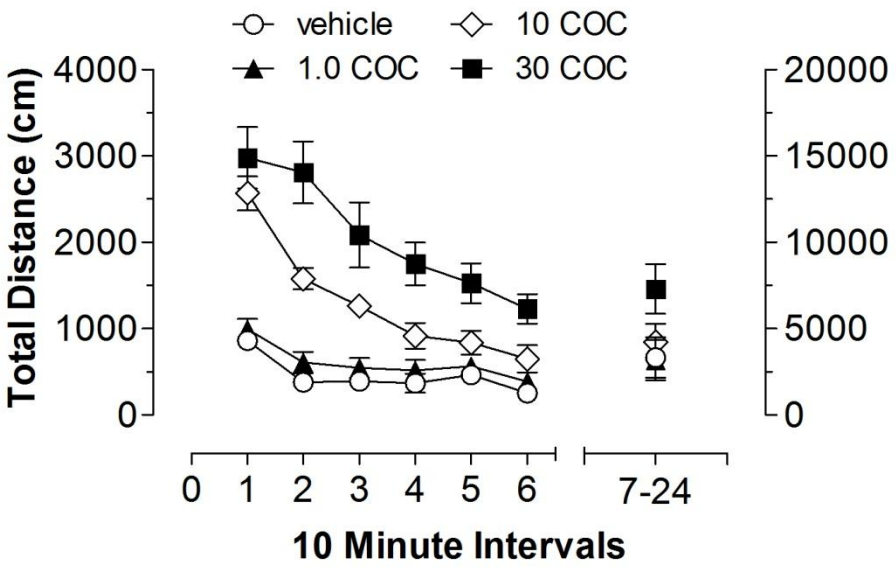


Figure 5: Effects of Cocaine on Locomotor Activity in Mice: The results show the effects of cocaine on total distance traveled over a 4 hour time course. Doses of 10 and 30 mg/kg cocaine produced peak total distance traveled at 10 minutes post injection. Levels remained elevated, above vehicle control levels, for the first hour of the 4 hour time course. By the 4 hour end point, all levels were returned to vehicle control levels (Ghorai et al., 2003).

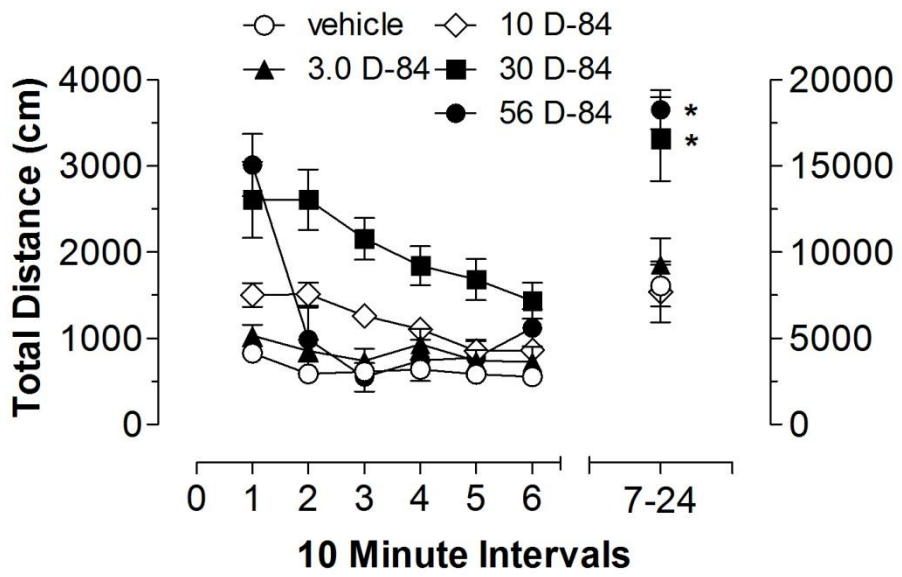


Figure 6: Effects of D-84 on Locomotor Activity in Mice: The graph shows the effects of D-84 on total distance traveled over a 4 hour time course. Peak effects were seen at ten minutes post injection, but by one hour doses of 3 and 10 mg/kg did not differ significantly from vehicle control levels. Doses of 30 and 56 mg/kg D-84 remained elevated above vehicle control levels (*p< 0.05) throughout the 4 hour time course. (Ghorai et al., 2003)

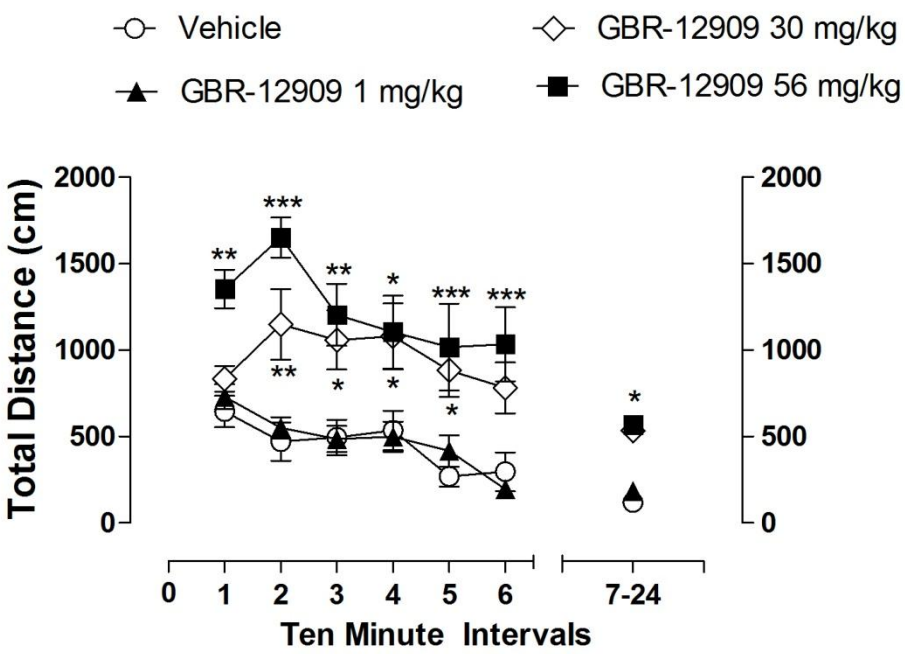


Figure 7: Effects of GBR-12909 on Locomotor Activity in Mice: The results show the effects of cocaine on total distance traveled over a 4 hour time course. Peak effects were seen at 10 (56 mg/kg) and 20 (30 mg/kg) minutes post injection and effects remained significantly elevated above vehicle controls levels until the 60 min time point (30 mg/kg). The highest dose of 56 mg/kg remained significantly elevated throughout the duration of the 4 hour time course. 1 mg/kg GBR-12909 did not significantly increase total distance traveled at any time point. (*=$p<0.05$, **=$p<0.01$ and ***=$p<0.001$ when compared to vehicle control using a repeated measures ANOVA followed by Dunnets post hoc tests).

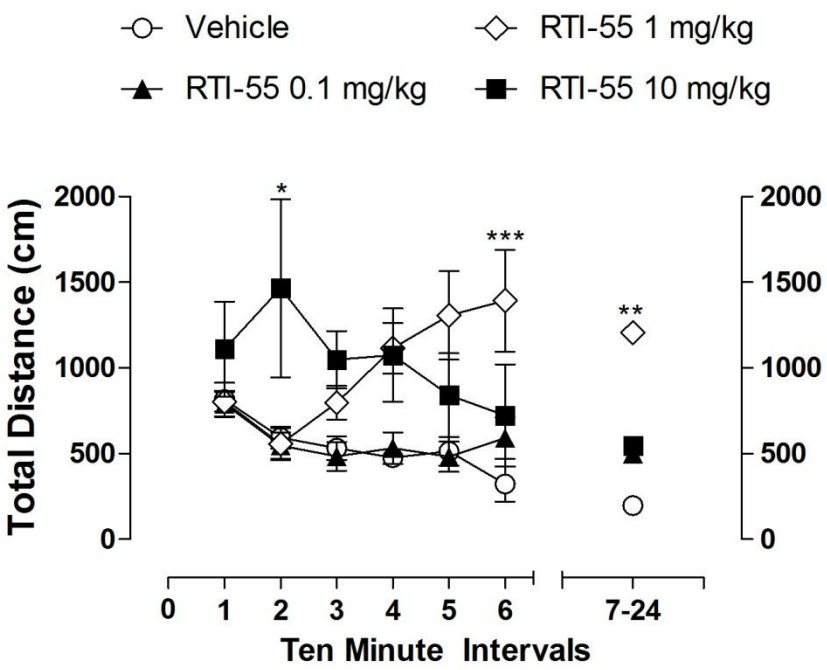


Figure 8: Effects of RTI-55 on Locomotor Activity in Mice: The results show the effects of RTI-55 on total distance traveled over a 4 hour time course. Peak effects occurred at 20 mins (10 mg/kg) and 60 mins (1 mg/kg) post injection. 1 mg/kg produced distance traveled significantly above vehicle control throughout the 4 hour time course. RTI-55, 10 mg/kg, returned to near vehicle levels by the 60 minute time point. 0.1 mg/kg did not significantly increase distance travelled above vehicle control at any time point.

Discussion

The overall aim of this experiment was to determine how changing the level of SERT inhibition influences the locomotor activity effects of the selected DAT compounds. Cocaine is considered to be a triple amine uptake inhibitor and is not selective for the DAT over the other monoamine transporters (Koe, 1976; Reith, et al., 1983). Cocaine stimulated locomotor activity, increasing total distance travelled in a dose dependent manner (Ghorai et al., 2003). It has a fast onset (10 minutes) and a relatively short duration of activity (~60 mins), both of which are thought to contribute to its high abuse liability (Volkow et al., 2000). D-84 is one of the most selective DAT inhibitors currently available (NIDA Broad Spectrum Analysis) and was shown to have a fast onset but a very long duration of activity when compared to cocaine. It produced dose dependent increases in total distance travelled that were statistically greater than vehicle control but these increases were non-significantly higher than the increases seen with cocaine. The hypothesis stated that the presence of SERT would decrease cocaine-like behavior. Results with D-84, which possesses very little SERT inhibition, may support this hypothesis since D-84, while non significant, exhibited a trend towards a higher stimulation than cocaine and had a much longer duration of action. If the hypothesis was correct, then the lack of SERT inhibition present in D-84 may be responsible for this trend towards greater motor stimulating effects of D-84 when compared to cocaine.

GBR-12909 has a high affinity for the DA transporter but also has intermediate potency at blocking the 5-HT transporter (Andersen, 1987). GBR-12909 had a similar onset of activity to cocaine but produced lower peak activity effects and had a longer duration of activity when compared to cocaine. This may support the hypothesis that increased SERT produces an inhibitory influence on DA neurons that leads to decreased firing and to a decrease in extracellular DA (Bubar & Cunningham, 2006; Higgins & Fletcher, 2003). A

decrease in extracellular DA may be responsible for the lower peak activity levels seen with GBR-12909. These results may further support the hypothesis that SERT inhibition may be playing an inhibitory role resulting in a decrease in the cocaine-like stimulant effects of the DAT inhibitor.

RTI-55 has the highest level of DAT inhibition but it also has the highest level of SERT inhibition compared to the other tested compounds (Boja, Mitchell, et al., 1992). It showed the lowest peak activity and the slowest onset of activity. RTI-55 produces peak effects approximately 50% lower than cocaine's peak effects. A high SERT inhibition will cause a greater increase in extracellular 5-HT, which could lead to inhibitory effects on dopamine neurotransmission (Czoty, et al., 2002). RTI-55 also showed an extremely long duration of activity (greater than 4 hours) compared to cocaine. The duration of activity, however, could be related to its dissociation from the DAT itself. Cocaine has a fast onset and offset of activity which is attributable to its fast association and disassociation from the DAT (Desai, Kopajtic, French, Newman, & Katz, 2005), and it is this action that is thought to be responsible for producing its robust behavioural effects. RTI-55 is a higher affinity DAT compound which may disassociate at a slower rate than cocaine, therefore producing its longer duration of action. The same could be said of all the DAT compounds tested since they, similarly, all have a longer duration of action than cocaine. Because it is thought that a slower onset of action of psychomotor stimulants may contribute to a lower abuse potential (Gorelick, 1998), the slower onset of RTI-55, combined with its longer duration of action, and lower peak locomotor activating effects, could be advantageous in terms of considering it a potential cocaine replacement therapy.

The serotonergic system consists of large family of 5-HT receptor subtypes that can modulate DA neurotransmission and, in particular, it has been shown that the 5-HT_{2A} and 5-

HT_{2C} receptor families play a key role in modulating the *in vivo* activity of psychomotor stimulants such as cocaine (Bubar & Cunningham, 2006). The serotonergic system is ideally placed in the CNS to be able to provide a modulatory influence over the DA system (Halliday & Tork, 1989) and it has been shown that stimulation of 5-HT receptors can change the firing rate of some DA neurons. Recently, in vitro studies have shown that activation of 5-HT_2 receptors can tonically inhibit firing of DA neurons (De Deurwaerdere, Navailles, Berg, Clarke, & Spampinato, 2004). These and other studies provide evidence to show that SERT inhibition can influence cocaine-like behavioural effects. The current results showed a correlation between increasing SERT inhibition and decreased peak locomotor activating effects. There was no such correlation between increased SERT inhibition and onset and duration of activity suggesting that these attributes may be unrelated to the presence of inhibitory SERT effects.

Conclusions

The aim of this experiment was to determine how increasing the level of SERT inhibition could alter cocaine-like locomotor effects. Increasing the level of SERT inhibition coincided with a decrease in peak locomotor activity levels. Given that all test compounds had very similar uptake inhibition affinities at the DAT but differed widely in their level of SERT inhibition, it can be speculated that this decrease in peak locomotor activating effect is directly related to modulation by the serotonergic system. Having said that, these differences in SERT inhibition may not be the only factor contributing to these behavioural activities. As mentioned earlier, DAT occupancy and binding kinetics could be playing a role, and although the compounds have similar, low affinities at NET compared to DAT and SERT, this potential influence on locomotor activity must be taken into account as well.

As previously mentioned, the goal of these experiments was to determine how increasing SERT affects the cocaine-like effects of high affinity DAT inhibitors. Locomotor stimulating effects are one effect of psychomotor stimulants, including cocaine (Tyler & Tessel, 1979). Cocaine and other psychomotor stimulants also produce discriminative stimulus effects (Callahan, De La Garza, & Cunningham, 1997), and drug discrimination is another paradigm for determining how increasing SERT may alter cocaine-like behavior. Therefore, the next study employed cocaine discrimination procedures to determine if increasing SERT inhibition affected the way the test compounds generalized to cocaine.

Effects of DAT/SERT Inhibitors in Cocaine Discriminating Rats

Psychomotor Stimulants and Drug Discrimination

Drug discrimination is a procedure that was developed as a means of assessing receptor mediated effects and potency of psychomotor drugs (Overton, 1977). It also was used to make inferences regarding the subjective effects of drugs and their abuse liability (Rosecrans, 1989; Stolerman, Mariathasan, & Garcha, 1991).

Two discrimination procedures that have been most widely used are the T-maze task and lever selection in a two lever operant choice procedure (Balster, 1991b). Using these procedures, the drug acts as an interoceptive stimulus that acquires control over a subject's responding. Animals are trained to respond appropriately according to the type of stimulus, drug or vehicle, presented to them prior to the start of the session. For example, Overton et al., (1961) trained animals to move to the correct side of an elevated T-maze in order to received food reinforcement contingent upon whether they were pretreated with a training drug or its vehicle. Animals can also be trained to lever press under intermittent schedules of reinforcement reinforced with food presentation in operant chambers (Appel et al., 1991; Balster, 1991a; Callahan, Bryan, & Cunningham, 1995). In this particular paradigm, the operant chamber has two levers, one designated as the drug lever and the other designated as the vehicle lever. During any given experimental session pressing only one lever is reinforced. The animal receives a drug or vehicle injection prior to the start of the session, and throughout the session only pressing one of the two levers will be reinforced. Pressing the vehicle lever, after a pre-session vehicle injection, indicates that the animal perceives the

vehicle effect as different from the drug effect. The percentage of drug lever responding (%DLR) indicates the degree of similarity between test drugs and the training stimulus. The response rate variable (RR) measures non-specific behavioural effects of the test compound.

Once animals are reliably trained to select injection-appropriate levers the animals can be tested using generalization tests. The animal is administered vehicle or any other dose of training drug or any dose of another drug. If the animal selects the drug lever, it is inferred that a stimulus generalization occurred between the effects of the test drug with those of the training drug. If the test drug evokes primarily vehicle-lever selection, then it is inferred that the test drug did not produce a stimulus that is qualitatively similar to the training drug (it did not produce stimulus generalization). Tests conducted with doses of the same training drug but at lower doses show generalization to occur in an orderly, dose-dependent fashion. Generalisation studies are therefore an effective method of determining how "training drug-like" test compounds are.

Cocaine's Discriminative Stimulus

Cocaine's discriminative stimulus effects have been studied for years (Jarbe, 1978) and the involvement of the dopaminergic system in cocaine's discriminative stimulus effects has been recognized for some time (Callahan, Appel, & Cunningham, 1991; Johanson & Fischman, 1989; Witkin, Nichols, Terry, & Katz, 1991) . Studies with agonists and antagonists have shown the involvement of the dopamine receptors D_1 and D_2 in producing cocaine's discriminative stimulus effects (Callahan, De la Garza, & Cunningham, 1994; Filip & Przegalinski, 1997; Witkin, et al., 1991), and it has been shown that lesioning the dopamine system with 6-hydroxy-dopamine decreasing extracellular DA attenuates cocaine's discriminative stimulus effects (Dworkin & Smith, 1988). Substitution tests with dopamine reuptake inhibitors such as bupropion and mazindol have shown complete substitution for

cocaine's discriminative stimulus (L. E. Baker, Riddle, Saunders, & Appel, 1993; Broadbent, Michael, Riddle, & Apple, 1991; Witkin, et al., 1991). Further studies have shown that agonists for D_1, D_2 and D_3 receptors all produce partial to full generalization to cocaine in discrimination studies (Acri et al., 1995; Callahan & Cunningham, 1993; Kantak, Edwards, & Spealman, 1995; Terry, Witkin, & Katz, 1994). It has also been shown that D_1 and D_2 receptor antagonists attenuate the discriminative stimulus effects of cocaine (L. E. Baker, et al., 1993; Callahan & Cunningham, 1993; Callahan, et al., 1994). These, and other studies, provide evidence for the role of DA in producing cocaine's discriminative stimulus cue.

Serotonergic Modulation of Cocaine's Discriminative Stimulus Effects

There is evidence indicating that SERT inhibition may play a minor role in cocaine discrimination. It is known that the raphe nuclei of the midbrain possess the 5-HT neurons that project to the forebrain including the DA mesolimbic system (Vertes, 1991). It has already been discussed that cocaine's behavioural effects are chiefly mediated through the DA mesolimbic pathways, but it should also be noted that the VTA and NA, within this system, receive input from 5-HT neurons suggesting a possible interaction between the serotonergic and dopaminergic systems (Gervais & Rouillard, 2000).

Studies have shown that selective reuptake inhibitors for 5-HT and NE do not generalize to the cocaine discriminative stimulus cue (L. E. Baker, et al., 1993; Callahan & Cunningham, 1995; Cunningham & Callahan, 1991; Spealman, 1995) but may play a modulatory role. Although fluoxetine, a SERT inhibitor, fails to generalize to cocaine, it enhances sub-threshold doses of cocaine, causing a leftward shift in the cocaine dose-response curve (Callahan & Cunningham, 1997) . This study also showed that agonists at 5-HT_{1A} receptors fail to substitute for the cocaine stimulus suggesting that this subtype of 5-HT receptor may not play a role in the discriminative stimulus effects of cocaine.

Further studies have shown that manipulation of the 5-HT_2 receptor family can modulate cocaine discrimination. Serotonin 5-HT_2 agonists do not themselves mimic the cocaine cue (Callahan & Cunningham, 1995; Peltier & Schenk, 1993). However, when the 5-$HT_{2C/1B}$ receptor antagonist, m-chlorophenylpiperazine (MCPP), is administered in combination with cocaine, the discriminative stimulus effects of cocaine are attenuated and a rightward shift in the cocaine dose response curve is produced (Callahan & Cunningham, 1995). MCPP produces its effects primarily through the 5-HT_{2C} receptor as opposed to the 5-HT_{1B} receptor (Conn & Sanders-Bush, 1987; Prisco, Pagannone, & Esposito, 1994), implicating a role for the 5-HT_{2C} receptor in the modulation of cocaine's discriminative stimulus effects. There is further evidence to suggest a role for the 5-HT_{2C} receptor system in modulating cocaine-like discrimination effects. Filip and colleagues showed that local infusion of a 5-HT_{2C} agonist enhances the discriminative stimulus effects of low dose cocaine whilst infusion of a 5-HT_{2C} antagonist dose dependently attenuates cocaine-like discriminative stimulus effects (Filip, Bubar, & Cunningham, 2006). These, and other studies provide evidence to suggest that the serotonergic system plays a modulatory role in cocaine's discriminative stimulus effects and shows the possibility of manipulating this systeme for advantages in developing compounds for treating cocaine dependence.

Several DAT inhibitors, while retaining some cocaine-like effects, do not possess effects predictive of a high abuse potential as seen with cocaine (Ferragud, et al., 2009; J. L. Katz et al., 2001; J.L Katz, et al., 2003). The reasons for this remain unresolved but one possible explanation is the binding activity of DAT compounds at sites other than the DAT. Considering serotonin may have an inhibitory influence over DA neurotransmission (Callahan & Cunningham, 1995), it may be plausible that one possible reason for the lack of robust reinforcing effects seen with certain DAT compounds could be due to an inhibitory

SERT component. It remains to be seen if there is a specific or optimum level of SERT inhibition required to reduce cocaine-like discriminative stimulus effects of DAT compounds and the goal of this experiment was to determine how increasing the level of SERT inhibition alters generalization of the test drugs to cocaines discriminative stimulus.

Hypothesis

DA plays a major role in the discriminative stimulus effects of cocaine (Johanson & Fischman, 1989) but it has also been shown that some DAT inhibitors incompletely generalize to cocaine in discrimination studies (J.L Katz, et al., 2003). With these observations in mind, it would be expected that the DAT test compounds may, at least partially, generalize to cocaine in discrimination studies. However, since it is also known that serotonin can produce attenuating effects on cocaine discrimination (Callahan & Cunningham, 1997), it is possible that increasing the level of SERT inhibition will decrease the cocaine-like discriminative stimulus effects produced by the test compounds.

Experimental Approach

In order to determine how altering the level of SERT inhibition affects generalization to cocaine, test dose response curves of cocaine, D-84, GBR-12909 and RTI-55 in rats trained to discriminate 10 mg/kg cocaine from saline in a two lever, food reinforced operant task were obtained. ED50's for cocaine lever responding and for reducing response rates were calculated along with percent cocaine lever selection (%CLR), which was used to determine generalization to cocaine's discriminative stimulus. Complete generalization was inferred when %CLR > 80%.

A change in species were used between locomotor activity and drug discrimination studies. Mice were used in locomotor studies because the lab had already an established

database of compounds, pharmacologically similar and dissimilar to the selected test compounds, to which the current data could contribute or be compared to. Rats were used in the discrimination studies to facilitate interpretation of data between discrimination and self-administration studies, and to help determine doses to be tested in self-administration experiments.

Materials and Methods

Subjects

Adult, male, experimentally-naive Long-Evans hooded rats (Harlan Sprague-Dawley, Indianapolis, IN) were used. Subjects were individually housed in an AAALAC accredited animal facility and given *ad libitum* food and water. Free feeding weights were obtained, and food (Harlan Teklad, Madison, Wisconsin) was subsequently restricted and rats were then maintained at 85% of their free feeding weights. Post session feeding was adjusted to maintain these weights throughout the study.

Studies were approved by the Institutional Animal Care and Use Committee of the Virginia Commonwealth University and conformed with NIH Guidelines for Care and Use of Laboratory Animals.

Drug Discrimination Apparatus and Procedure

Rat discrimination studies were conducted in two-lever operant conditioning chambers (Med-Associates Inc., St. Albans, VT) equipped with a house light and food dispenser that delivered 45 mg food pellets (Research Diets, Noyes Precision Pellets, New Brunswick, NJ). Scheduling of pellet deliveries and collection of data were accomplished by

a microcomputer and associated interface (Med-Associates Inc., St. Albans, VT, MED-PC® IV).

Drug discrimination training occurred during daily (M-F) 15-min experimental sessions. The rats were initially trained to press one of two levers under a fixed-ratio 1 (FR 1) schedule of reinforcement in which each lever press resulted in a pellet delivery. The response requirement was gradually increased to FR 10. During the next few sessions the rats were reinforced only for pressing the alternate lever until they pressed reliably under FR 10 scheduling conditions, after which drug discrimination training commenced. Rats were injected with 10 mg/kg cocaine or saline vehicle i.p., 10 min prior to the start of the session. For each rat, one lever was designated correct following drug administration and the other as correct following saline administration. The lever upon which the rats initially acquired the lever press response was designated as the saline-appropriate lever. All responses on the inappropriate lever were recorded, but had no programmed consequences. The lever on which the rats were initially trained and on which they acquired the lever-press response was designated as the vehicle-appropriate lever. Alternation of cocaine and saline injections proceeded according to a two-monthly cycle (Month #1: CSSCS, SCCSC, SCSCS, CSCSC; Month #2: SCCSS, CSCSC, CSSCC, SCSCS; in which C=cocaine S=saline). Lever pressing produced pellet delivery only on the injection-appropriate lever for that day. Incorrect presses reset the response requirement on the correct lever.

Substitution tests began once a rat met the following criteria: 1) the first completed fixed-ratio (FFR) occurred on the lever designated correct on at least eight of ten consecutive sessions; and 2) at least 80% of the total responses were emitted on the correct lever during those eight sessions. After these initial training criteria were met, testing could occur twice a week, on Tuesdays and Fridays, provided that the rats completed the FFR on the correct lever

during the most recent training drug and saline sessions; otherwise, a training day was administered. Test sessions were identical to training sessions except completion of the FR10 contingencies on either lever resulted in pellet delivery. Dose-response curves were collected first with cocaine (1-30 mg/kg) before substitution tests with D-84 (1-42 mg/kg), GBR-12909 (1-30 mg/kg) and RTI-55 (1-30 mg/kg) were conducted.

Drugs

All drugs were injected i.p in a volume equivalent to 1 ml/kg. The vehicle for cocaine and RTI-55 was sterile saline, and the vehicle for GBR-12909 was 40% cyclodextrin (Sigma Aldrich, St Louis, MO).

Data Analysis

The percentage of cocaine-lever responding (% CLR) was calculated for each subject by dividing the number of lever presses emitted upon the cocaine lever by the total number of presses emitted upon both levers and multiplying this quotient by 100. Individual values of % CLR were then averaged ($\pm$ SEM). Complete generalization to the cocaine discriminative stimulus was inferred when % CLR was $\geq$ 80%. Mean response rates for each test condition were calculated by dividing the total number of lever presses emitted upon both levers by the session duration (900 s) for each subject, and then these rates were averaged ($\pm$SEM). If a rat failed to make at least ten lever presses during a test session, its data were excluded from calculations of % CLR but were included for mean response rate determinations. This exclusion was made to prevent near-zero rates of responding from disproportionately influencing percent cocaine lever responding. ED_{50} values and their confidence intervals (CI) were calculated for % CLR and for reducing response rates using nonlinear regression analysis (Prism 5, GraphPad software, San Diego, CA).

Results

Cocaine Fully Generalizes to itself in a Dose Dependent Manner

Cocaine fully generalized to the 10 mg/kg cocaine training dose at doses of 10 and 30 mg/kg with an ED50 (CI) value for producing cocaine-lever responding (CLR) of 2.85 mg/kg (2.1-3.5 mg/kg). When saline was tested, near-zero levels of CLR occurred. The lowest dose of cocaine tested, 1.0 mg/kg, occasioned low levels (< 20%) of % CLR (Figure 1, Upper panel). At the highest dose of cocaine tested (30 mg/kg), rates of responding were decreased by ~50% relative to saline control levels (Figure 1, lower panel). Cocaine dose-dependently reduced response rates with an ED50 (CI) value of 13.83 (4.8-39.3) mg/kg. These results were presented graphically in Chapter 2.

D-84 Produces Incomplete Generalization to Cocaine

As D-84 dose increased, levels of %CLR increased D-84 (ED50 (CI) = 8.2 (3.4-19.8) mg/kg), but only occasioned a maximum of 76% CLR at a dose of 30.4 mg/kg, and never produced complete generalization to the 10 mg/kg cocaine training dose (Fig.1 upper panel). D-84 also dose-dependently reduced response rates with an ED50 (CI) value of 33.9 mg/kg (15-72) mg/kg (Fig.1 lower panel). These results were presented graphically in Chapter Two.

GBR-12909 Produces Complete Generalization to Cocaine

GBR-12909 completely generalized to the cocaine training dose of 10 mg/kg. GBR-12909 (30 mg/kg) produced 98% generalization to cocaine with an ED50 value (CI) for producing cocaine lever responding of 9.02 mg/kg (4.1-19.8 mg/kg). When vehicle was tested, near zero levels of % CLR occurred (Fig 9. Upper Panel). The highest dose of GBR-

12909 tested (30 mg/kg) caused non-significant reductions in response rates (Fig 9. Lower Panel)

RTI-55 Produces Complete Generalization to Cocaine

RTI-55 fully generalized to the training dose of cocaine. RTI-55 (10 mg/kg) produced 82.6% generalization to cocaine with an ED50 value for producing % CLR of approximately 1.3 mg/kg. When vehicle was tested, near zero levels of CLR occurred (Fig 10. Upper Panel). RTI-55 dose dependently reduced response rates with an ED50 (CI) value of 5.97 mg/kg (3.26-10.92 mg/kg) (Fig.10 Lower Panel). RTI-55 resulted in a left-ward shift in the dose response curve when compared to cocaine.

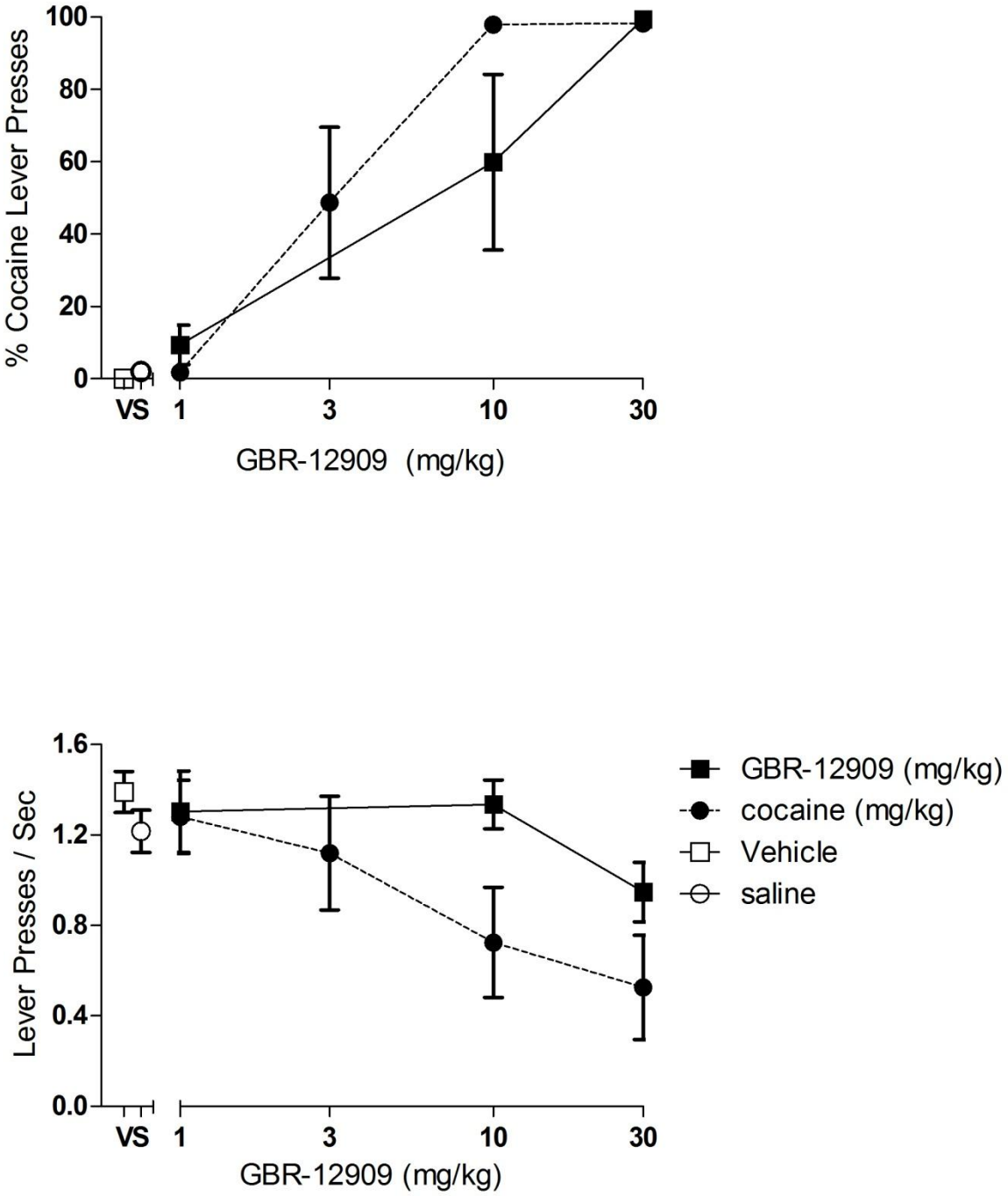
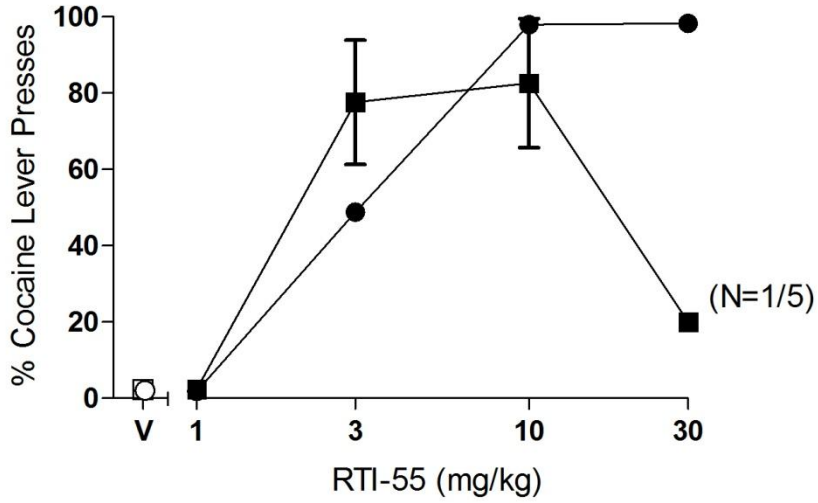


Fig 9. Upper panel: Effects of cocaine and GBR-12909 dose (mg/kg) on the percentage of cocaine lever responses in rats trained to discriminate 10 mg/kg cocaine. "V"= 40% cyclodextrin vehicle; "S" = saline; filled circles= cocaine; filled squares = GBR-12909. Each symbol represents a mean of six rats. Bars represent ±SEM. **Bottom panel:** Mean numbers of total lever presses per sec.



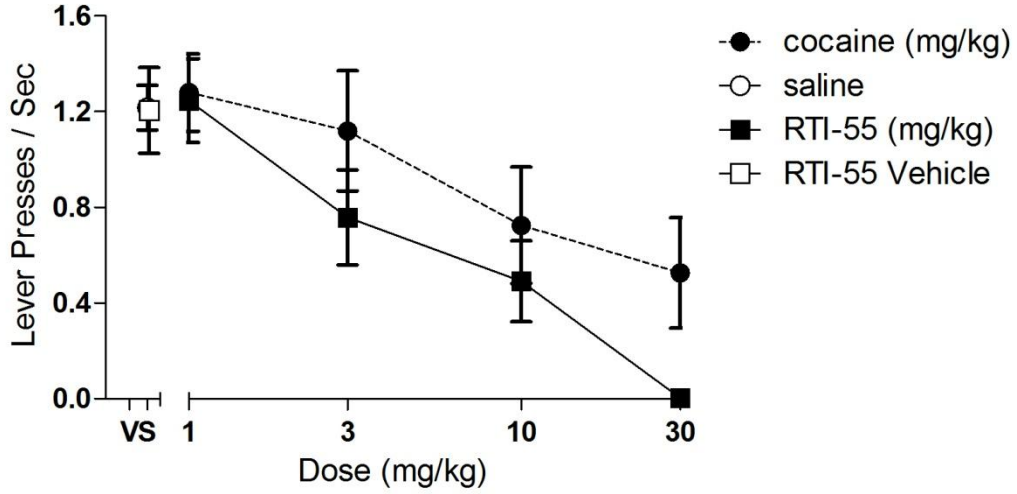


Fig 9. Upper panel: Effects of cocaine and RTI-55 dose (mg/kg) on the percentage of cocaine lever responses in rats trained to discriminate 10 mg/kg cocaine. "V"= sterile saline vehicle; "S" = saline; filled circles= cocaine; filled squares = RTI-55. Each symbol represents a mean of six rats except at the 30 mg/kg dose of RTI-55 where % CLR represents 1 of 4 rats tested (see data analysis text for explanation). Bars represent ±SEM. **Bottom panel:** Mean numbers of total lever presses per sec.

Discussion

Cocaine was shown to completely generalize to itself in these discrimination studies, with an ED_{50} value of 2.85 mg/kg. Reductions in response rates were seen with the highest dose tested. D-84 failed to show complete generalization in these studies. D-84 produced a maximum of only 76% CLR, which is consistent with previously published results showing incomplete generalization (67%) to cocaine in mouse discrimination studies (Ghorai, et al., 2003).

It has been suggested that SERT inhibition may attenuate cocaine-like behavioural effects of compounds (Callahan & Cunningham, 1997), but it is known that D-84 has little SERT inhibition activity (NIDA Broad Spectrum Analysis). Given that D-84 has little SERT inhibitory effects, it is likely that its lack of complete cocaine-like discriminative stimulus effects has little to do with a SERT modulatory component.

There are a number of DAT inhibitors available that have a high selectivity for the DAT, generalize to cocaine in discrimination studies, and yet likely possess a much lower abuse potential than cocaine itself (Bergman, Kamien, & Spealman, 1990; Ferragud, et al., 2009; J. L. Katz, et al., 1997). Psychomotor stimulants, like cocaine, are known to have a fast onset of activity which is thought to contribute to their abuse liability (Balster & Schuster, 1973; Kimmel, et al., 2007). There has also been a significant correlation between the subjective high reported following cocaine administration and high DAT occupancy levels (Volkow et al., 1997). D-84 may produce a lower DAT occupancy level, when compared to cocaine, which could be contributing to its lack of full generalization to cocaine in these discrimination studies. Further studies, possibly with PET neuro-imaging to determine DAT occupancy after D-84 administration, are needed to further investigate the lack of full generalization seen with of D-84.

GBR-12909 is considered, for the purposes of this study, to be an intermediate SERT uptake inhibitor. It produced full generalization to cocaine (98%) with an ED_{50} (CI) for occasioning cocaine-lever responding of 9 mg/kg (4.1-19.8 mg/kg). Response rates were not significantly reduced at any dose tested. These data confirm previously published studies that show GBR-12909 generalises to cocaine in discrimination studies (Cunningham & Callahan, 1991; Holtzman, 2001; Spealman, 1993). It may be possible to conclude that since both of these compounds have similar, intermediate SERT inhibition, that it is not surprising to see that both compounds produced similar generalization levels, although, based on ED_{50} values for occasioning cocaine lever responding, cocaine is more potent than GBR-12909.

RTI-55 is a high affinity DAT inhibitor which also possess high affinity for the SERT (Boja et al., 1990). RTI-55 produced full generalization to cocaine (83%) with an ED_{50} for occasioning cocaine-lever responding of 1.3 mg/kg. RTI-55, however, also produced robust decreases in rates of responding that may also be attributable to its serotonergic activity.

When comparing maximum % CLR, a trend towards decreasing cocaine-like effects as the level of SERT inhibition was increased occurred. When increasing the SERT inhibitory component, a drop in % CLR from 100% with cocaine (SERT Ki 304nM) to 98% with GBR-12909 (SERT Ki 4.3 nM) to 82% with RTI-55 (SERT Ki 3.8nM) occurred. Since the test compounds are similar in their level of DAT inhibition but differ in their level of SERT inhibition, it may be speculated that this downward trend is attributable to the increased serotonergic presence. This could possibly be examined by using 5-HT receptor antagonists to block serotonergic effects to determine how much generalization each DAT compound produces in the absence of the SERT inhibitory component. However, it also needs to be noted that the results produced with D-84 complicate this interpretation. D-84 is a novel, selective DAT inhibitor with very little SERT inhibitory presence. If the presence of SERT

inhibition was the only possible determinant for producing a decrease in cocaine generalization with compounds with high DAT inhibition, then it would be expected that D-84 would have occassioned complete generalization but didn't. Since the differences in generalization are not large, it is not possible to determine, from these data, what level of SERT, if any, is required to produce a complete attenuation of cocaine-like effects.

Conclusions

The goal of this experiment was to determine how an increase in SERT inhibition would affect the cocaine-like discriminative stimulus effects of the selected compounds. Although the magnitude of thsis effect wasn't as robust as expected, a general trend in decreased cocaine-like discriminative stimulus effects as the level of SERT inhibition was increased did occur. Since GBR-12909 and RTI-55 had similar activity at DAT, but differed in their level of SERT inhibition, one possibility is that this decrease in cocaine-like effects is due to the increased presence of serotonin producing an inhibitory effect on the DA system. The results with D-84, however, indicate that multiple mechanisms may operate to put limits on the maximum level of % CLR obtained.

Rats and non-human species can be trained to reliably self-administer cocaine and other psychomotor stimulants and the final set of experiments focuses on self-administration in rodents. The overall goal in these subsequent studies was to determine how increasing the level of SERT inhibition alters levels of self-administration of the selected compounds.

The Influence of SERT Inhibition on the Self-administration of High Affinity DAT Inhibitors

It was first reported in the 1960's that rats and non-human primates surgically implanted with catheters would self-administer a variety of drugs, including stimulants, opioids and alcohol (Deneau, Yanagita, & Seevers, 1969; Thompson & Schuster, 1964; Weeks, 1962). Animals reliably self-administer drugs that are commonly abused by humans (Johanson & Balster, 1978) and drug self-administration procedures in rodents and non-human primates have proven to be productive procedures for investigating the abuse liability of drugs (Balster, 1991b; Schuster & Johanson, 1974; Thompson & Unna, 1977).

During drug self-administration preparations, animals are implanted with indwelling venous catheters through which infusions of drugs can be delivered. Delivery of drug is dependent on the number and pattern of responses which, in part, is determined by the schedule of reinforcement and the availability of drug.

It has been demonstrated that cocaine is reliably self-administrated in rodents and non-human primates and that this positive reinforcing effect is, principally, due to its binding activity at the DAT, resulting in an increase in extracellular DA (Kuhar, Ritz, & Sharkey, 1988; Ritz, et al., 1987; Woolverton & Johnson, 1992). The role of DA in the self-administration of cocaine is further established by studies using DA receptor ligands. It has been shown that high efficacy selective D_1 agonists readily substitute for cocaine in rats and rhesus monkeys trained to self-administer cocaine (Self & Stein, 1992; Weed & Woolverton, 1995) and, to further confirm a role of DA in the self-administration of cocaine, it has been shown that when D_1 and D_2 antagonists are given as a pretreatment to animals self-administering cocaine, a rightward shift in the cocaine dose-response curve results, possibly

implying a decrease in the reinforcing effects of cocaine, or at least its potency (Bergman, et al., 1990).

The self-administration of synthesized DAT inhibitors has also been investigated (Howell, et al., 2007; Howell, et al., 2000; Tella, et al., 1997). For instance, RTI-336, a tropane analogue, is self-administered by rhesus monkeys at rates lower than responding maintained by cocaine (Howell, et al., 2007). These doses of RTI-336 that reliably maintained responding, also dose-dependently reduced cocaine self-administration when given as a pretreatment. GBR-12909, another DAT inhibitor, is reliably self-administered (Roberts, 1993; Tella, 1995) and has been shown to reduce cocaine self-administration in rhesus monkeys (Woolverton, Hecht, Agoston, Katz, & Newman, 2001). Benztropine and its analogues are potent DAT inhibitors, and they too are self-administered, although at lower levels than cocaine (Hiranita, et al., 2009; Woolverton, et al., 2001).

The above studies suggest a role for the DA system in the reinforcing effects of cocaine. However, there is evidence to suggest that the serotonergic system may play a modulatory role in the self-administration of cocaine as well (Walsh & Cunningham, 1997), possibly by having an inhibitory effect on DA neurotransmission (Alex & Pehek, 2007). Studies in non-human primates have demonstrated that SERT inhibitors can attenuate the reinforcing effects of cocaine. The SSRI's fluoxetine, alaproclate and clomipramine are not self-administered, and when fluoxetine is given as a pre-treatment it dose-dependently decreases cocaine self-administration in squirrel monkeys (Howell & Byrd, 1995). Fluoxetine also decreases self-administration of cocaine in rats (M. E. Carroll, et al., 1990a), and acute administration of fluoxetine has been shown to reduce the break point for cocaine in progressive ratio studies (Richardson & Roberts, 1991). As further support of the role of the serotonin system in modulating cocaine-like effects, acute administration of 8-OH DPAT, a

5-HT_{1A} receptor agonist, reduces responding for cocaine in a similar manner to fluoxetine possibly implying that the reduction in responding seen with fluoxetine may be due to to activity at the 5-HT_{1A} receptor (Peltier & Schenk, 1993). Other studies have looked at the effects of reducing brain 5-HT levels on cocaine self-administration. Administration of a 5-HT neurotoxin (5,7-dihydroxytryptamine), which depletes levels of 5-HT in the brain, increases the break point for cocaine self-administration when compared to vehicle control animals (Loh & Roberts, 1990). Pre-treatment with l-tryptophan, which increases brain 5HT levels, decreases the mean number of infusions obtained of low dose cocaine in rats (M. E. Carroll, Lac, Asencio, & Kragh, 1990b).

These studies provide evidence suggesting that the serotonergic system plays a role in modulating the behavioural effects of cocaine. It may be possible that a DAT inhibitor with an appropriate level of SERT inhibition may be self-administered, but at lower levels than cocaine. The aim of this experiment was to determine if increasing the level of SERT inhibition alters self-administration of high affinity DAT inhibitors and if so, if there is an optimum level of SERT inhibition that will attenuate cocaine-like self-administration behaviour.

Hypothesis

Previous results from studies of this dissertation have shown that the selected test compounds are behaviourally active and they all, at least partially, generalize to cocaine in discrimination studies. Based on these data and reports that have indicated that DAT inhibitors can, potentially, be self-administered, it is expected that cocaine and the test compounds will be self-administered at some level. The literature discussed previously also reported data indicating that SSRI's are not reliably self-administered. It is possible that as

the level of SERT inhibition is increased (from D-84>cocaine>GBR-12909>RTI-55) there will be a decrease in self-administration.

Methods and Materials

Subjects

Adult, male, experimentally-naive Long-Evans hooded rats (Harlan Sprague-Dawley, Indianapolis, IN) were used. Subjects were individually housed in an American Association of Animal Laboratory Care-accredited facility and given *ad libitum* food and water. Free feeding weights were obtained, and food (Harlan Teklad, Madison, Wisconsin) was subsequently restricted to 15 g a day until rats achieved a target weight of approximately 320g. The rats were maintained at this target weight throughout the study by adjustments in post-session feedings.

Self-administration Apparatus and Procedure

Self-administration tests were conducted in operant conditioning chambers housed inside individual, isolated and ventilated boxes (Med-Associates Inc., St. Albans, VT). The front wall of each chamber was equipped with two retractable levers with a white stimulus light above each lever. A 5-w house light and Sonalert® tone generator were located on the rear wall of the chamber.

Infusion Assembly System

Catheters were constructed from polyurethane tubing (Access Technologies, Skokie, IL; 0.044" O.D. X 0.025" WE.D.). The proximal 3.2 cm of the catheter was tapered by stretching following immersion in hot sesame oil. The catheters were prepared with a

retaining cuff approximately 3 cm from the proximal end of the catheter. A second larger retaining cuff was positioned approximately 3.4 cm from the proximal end of the catheter. Mid-scapula cannula/connectors (Plastics One, Roanoke, VA). The cannula/connectors consisted of a threaded plastic post through which passed an "L" shaped section of 22 gauge stainless steel needle tubing. The lower surface of the plastic post was affixed to a 2 cm diameter disc of Dacron mesh. During experimental sessions the exposed threaded portion of the infusion cannula was connected to an infusion tether consisting of a 35 cm length of 0.40 mm polypropylene tubing encased within a 30 cm stainless steel spring to prevent damage. The upper portion of the 0.40 polypropylene tubing was connected to a fluid swivel (Lomir Biomedical, Inc, Quebec, Canada) that was, in turn, attached via 0.40 polypropylene tubing to the infusion syringe.

Surgical Procedure

Following acclimation to the laboratory environment, indwelling venous catheters were implanted into the right external jugular vein. Surgical anesthesia was induced with a combination of 50 mg/kg ketamine and 8.7 mg/kg xylazine. Rats were additionally administered 8 mg/kg oral Baytril for three days post-surgery. The ventral neck area and back of the rat were shaved and wiped with betadine and isopropyl alcohol. The rat was placed ventral side down on the surgical table and a 3 cm incision was made 1 cm lateral from mid-scapula. A second 0.5 cm incision was then made mid-scapula. The rat was then placed dorsal side down on the operating table and a 2.5 cm incision was made longitudinally through the skin above the jugular area. The underlying fascia was bluntly dissected and the right external jugular vein isolated and ligated. A small cut was made into the vein using an iris scissors and the catheter was introduced into the vein and inserted up to the level of the larger retaining cuff. The vein encircling the catheter between the two cuffs was then tied

with silk suture. A second suture was then used to anchor the catheter to surrounding fascia. The distal end of the catheter was passed subcutaneously and attached to the cannula/connector that was then inserted subcutaneously through the larger incision. The upper post portion of the connector/cannula exited through the smaller mid-scapula incision. All incisions were then sprayed with a gentamicin/betamethasone valerate topical antibiotic and the incisions were closed with Michel wound clips.

Rats were allowed to recover from surgery for at least 5 days before self-administration training began. Periodically throughout training, methohexital (1.5 mg/kg) or ketamine (5 mg/kg) was infused through the catheters to determine patency as inferred when immediate anesthesia was induced. Between sessions the catheters were flushed and filled with 0.1 ml of a 25% glycerol/75% sterile saline locking solution containing: 250 units/ml heparin, 250 mg/ml ticarcillin, and 9 mg/ml clavulanic acid. If during the experiment a catheter was determined to be in-patent, the left external jugular was then catheterized and the rat was returned to testing.

Substitution of Test Compounds

Cocaine self-administration training sessions were 2 h in duration and were conducted 5 days a week (M-F), unless testing had begun, and then sessions were run continuously until testing was completed. Initially, each response (fixed ratio 1, FR1) on the right-side lever resulted in delivery of a 0.5-mg/kg cocaine infusion (0.18 ml/6 sec). For the duration of the infusion, the tone sounded and the stimulus lights above both levers flashed at 3 Hz. Active (right-side) lever presses during the infusions as well as all inactive (left-side) lever presses were recorded but were without scheduled consequences. When neither increasing nor decreasing trends in infusion numbers for three consecutive sessions had occurred, and the rats had obtained at least 15 infusions during each session for at least 12 sessions, cue

changes (stimulus light flashes above levers and Sonalert activations) during infusions were discontinued. Discontinuation of cue changes was initiated to permit rapid extinction of lever pressing during substitutions with doses of test drugs. After three sessions of 0.5mg/kg/infusion cocaine self-administration without cues during which neither increasing nor decreasing trends in infusion numbers occurred, saline was substituted as the available infusate until the number of infusions obtained during a session was less than 50% the mean of the most recent three cocaine sessions. Rats were then returned to 0.5 mg/kg/cocaine availability without cues until trends in infusion numbers did not occur across three consecutive sessions. Vehicle, and increasing doses of GBR-12909 (0.1, 0.3, 1.0 and 1.3 mg/kg/infusion) were then substituted for three consecutive sessions each. Following tests with GBR-12909, the rats were returned to 0.5 mg/kg/inf cocaine availability for three consecutive sessions.

In a second group of rats, the same procedure was repeated but RTI-55 doses (0.001, 0.01, 0.03 and 0.1 mg/kg/infusion) were substituted for cocaine.

Data Analysis

For self-administration substitution studies, data from the last day of substitution at each dose were used in the analyses. One-way repeated measures ANOVA, followed by Dunnett's post hoc tests, were used to compare self-infusions of drug from self-infusions of saline. A compound is considered to be self-administered when its self-administration infusions are statistically greater than vehicle control levels. Statistical significance was assumed in all analyses if $p<0.05$. Statistical analyses and nonlinear regressions were performed using GraphPad Prism version 5.0 for Mac OSX, GraphPad Software, San Diego, California USA.

Results

Self-administration of Cocaine and D-84

Fig. 2 (upper panel) shows the mean numbers of infusions obtained when cocaine and D-84 were available for self-administration. The relationship between self-administered cocaine infusions and dose was characterized by an inverted U-shaped curve with peak numbers of infusions occurring at the intermediate cocaine dose of 0.1 mg/kg/infusion, which was significantly greater than saline control numbers ($p<0.05$). D-84's self-administration was also characterized by a U-shaped curve relating infusions to dose with peak numbers of infusions occurring at 0.3 mg/kg/infusion, and which were significantly greater than vehicle control numbers ($p<0.05$). Fig. 2 (lower panel) shows mean drug intake (mg/kg/2-session) of self-administered cocaine and D-84. Mean drug intake for both cocaine and D-84 increased as a function of dose. Peak levels of D-84 (21.6±4.61) intake were non-significantly lower than peak levels of cocaine (24.3±3.34) intake.

Self-administration of GBR-12909

Fig.11 (upper panel) shows the mean numbers of infusions obtained when GBR-12909 was available for self-administration. The relationship between self-administered GBR-12909 infusions and dose was characterised by an inverted U-shaped curve with peak numbers of infusions occurring at an intermediate dose of 0.3 mg/kg/infusion, which was significantly greater than saline control numbers ($p< 0.05$). Fig.11 (lower panel) shows mean drug intake (mg/kg/2-hr session) of GBR-12909. Mean drug intake increased as a function of dose and peak levels of GBR-12909 intake (12.4 $\pm$ 2.5) were significantly lower than peak levels of cocaine (24.3±3.34) intake.($p<0.05$, unpaired t-test).

Self-Administration of RTI-55

Fig.12 (upper panel) shows the mean number of infusions obtained when RTI-55 was available for self-administration. The relationship between self-administered RTI-55 infusions and dose was characterised by an inverted U-shaped graph with peak numbers of infusions occurring at a dose of 0.01 mg/kg/infusion, which was significantly greater than saline control numbers ($P<0.05$). Fig.12 (lower panel) shows mean drug intake (mg/kg/2-hr session) of RTI-55. Mean drug intake increased as a function of dose. Peak levels of RTI-55 intake (2.14 ± 0.12) were significantly lower than peak levels of all other tested drugs ($p< 0.05$ as shown by repeated measures ANOVA).

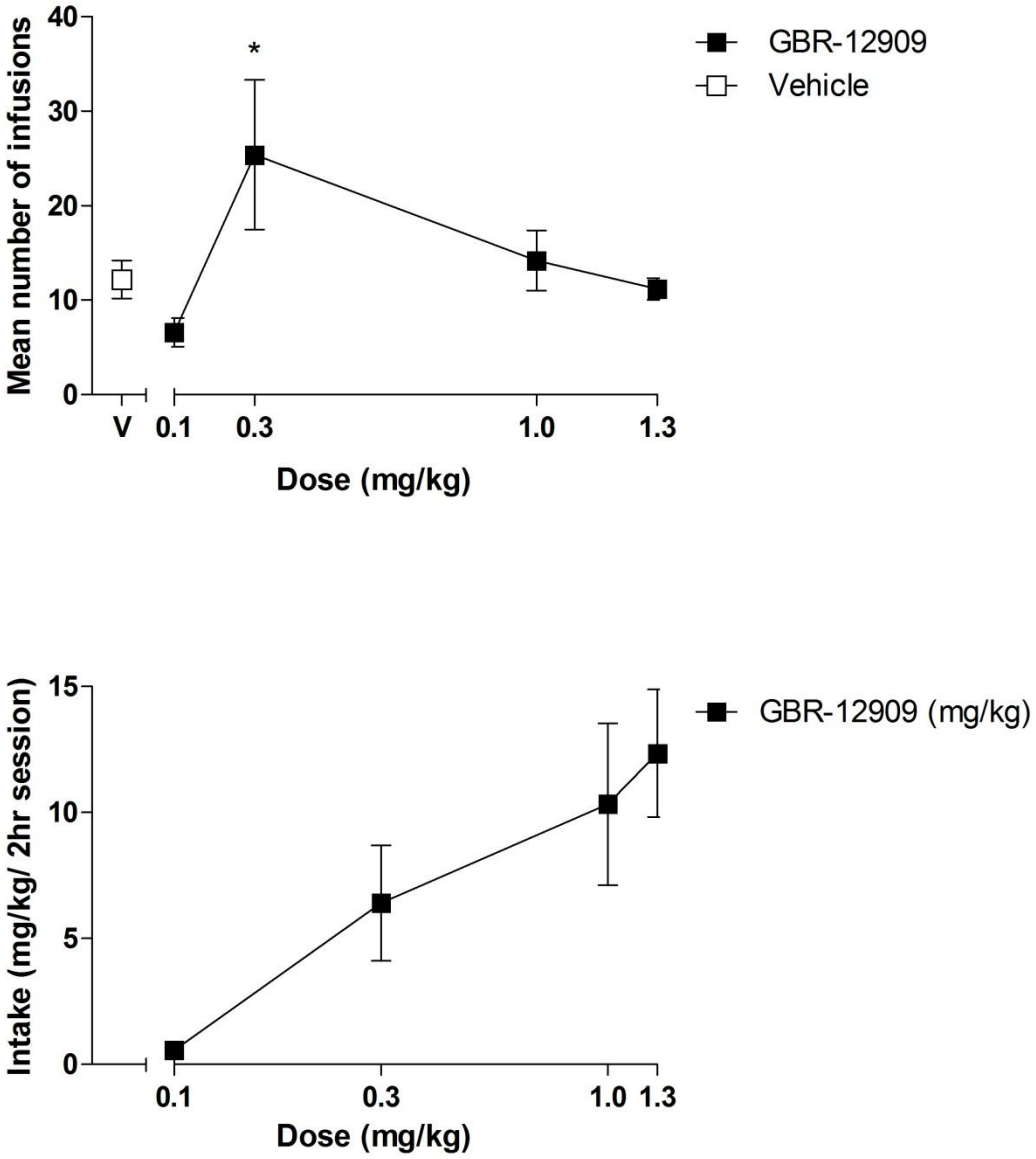


Fig. 11 Self-administration of GBR-12909: **Upper panel**: Mean infusions of GBR-12909 vehicle (unfilled squares), and GBR-12909 (filled circles) obtained as a function of dose. Brackets through data points indicate ±SEM. N=6 at all conditions. * = $p < 0.05$ as compared to vehicle. **Bottom panel:** Mean drug intake (mg/kg/2-h session) as a function of dose. Other details as described for the upper panel.

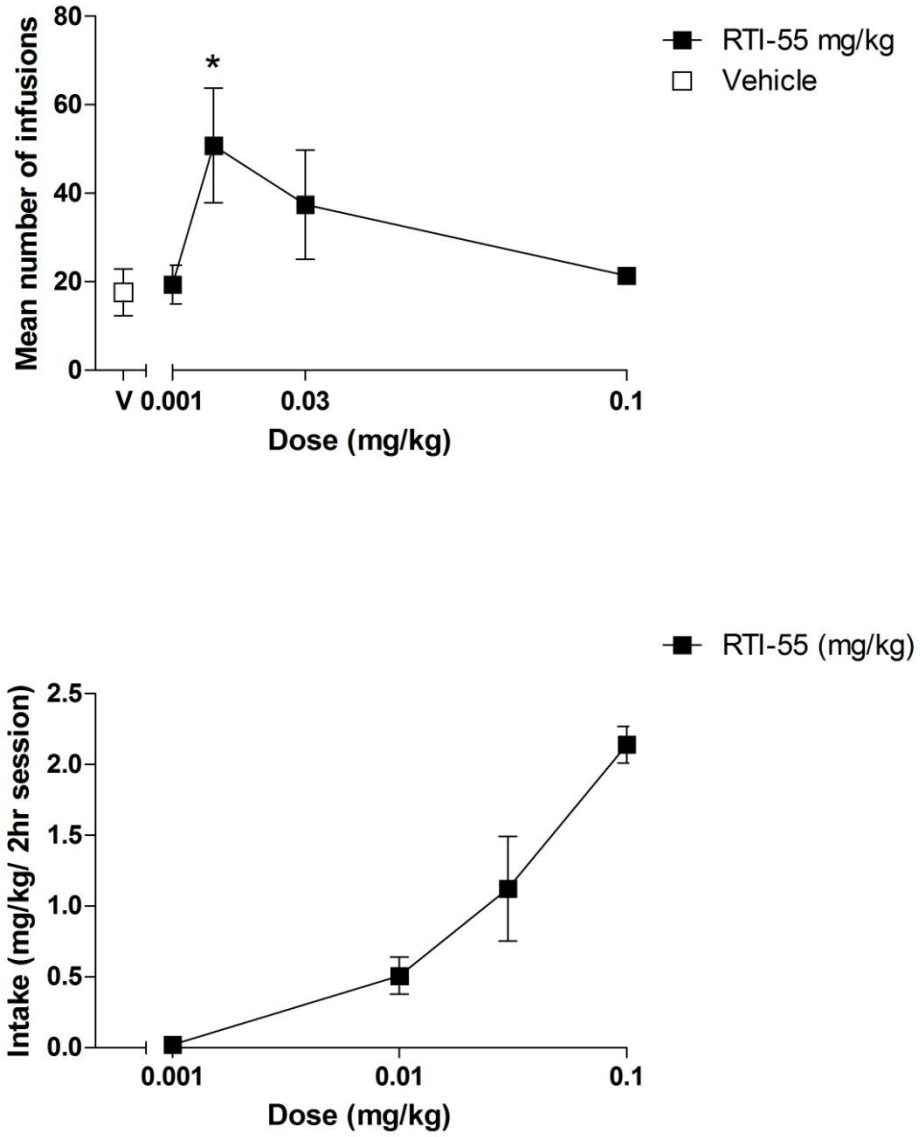


Fig. 12 Self-administration of RTI-55: **Upper panel**: Mean infusions of RTI-55 vehicle (unfilled squares), and RTI-55 (filled circles) obtained as a function of dose.
Brackets through data points indicate ±SEM. N=5 at all conditions. * = $p < 0.05$ as compared to vehicle. **Bottom panel:** Mean drug intake (mg/kg/2-h session) as a function of dose. Other details as described for the upper panel.

Discussion

The objective of this study was to determine if increasing the level of SERT inhibition altered the cocaine-like self-administration effects of the selected DAT inhibitors. This was achieved by the selection of three high affinity DAT inhibitors which varied, according to the rationale set out in the introduction, in their intrinsic potency of SERT inhibition. All three test compounds were reliably self-administered above vehicle control but maintained rates of responding at lower levels than when cocaine was available for self-administration. The three test compounds produced very similar mean infusions rates to each other. As the level of SERT inhibition increased in the order of D-84>GBR-12909>RTI-55, peak intake of drug decreased in the order of D-84>GBR-12909>RTI-55 and peak number of infusions decreased in the order of D-84>RTI-55>GBR-12909.

Cocaine is a non selective, triple amine uptake inhibitor and is robustly self-administered (Ritz, et al., 1987). D-84 is, to date, one of the most selective DAT inhibitors currently available (NIDA Broad Spectrum Analysis), and has negligible activity at the SERT. D-84 produced maximal infusion numbers lower than cocaine but similar to RTI-55 and GBR-12909. Given the observation that the presence of SERT inhibition can attenuate cocaine self-administration (Howell & Byrd, 1995), and that activity at DAT is likely responsible for cocaine's self-administration, the fact that D-84 is self-administered is not surprising and further suggests that DAT inhibition, by itself, is sufficient to maintain self-administration behavior. However, its incomplete generalization in cocaine discrimination studies suggests factors other than SERT inhibition may play a role in D-84's incomplete, cocaine-like profile. SERT inhibition has been shown to attenuate cocaine self-administration (M. E. Carroll, et al., 1990a) suggesting that the serotonergic system modulates cocaine self-administration behaviour. Cocaine possesses a higher uptake inhibition at the SERT than D-84 yet produces mean infusion numbers that exceed the mean infusion numbers produced

with D-84. If SERT inhibition was the lone determinant for limiting self-administration of high potency DAT inhibitor compounds, it may have been expected that D-84 would have produced higher infusion numbers than even cocaine and the other test compounds. While there are studies supporting the supposition that SERT inhibition attenuates cocaine self-administration, the current data suggest that SERT inhibition may not be the only factor. It has been reported that affinity at the DAT has a positive correlation with potency to maintain responding in self-administration experiments (Bergman, et al., 1989; Ritz, et al., 1987), but this does not explain the differences seen between cocaine and D-84, given that D-84 has a higher DAT uptake inhibition affinity than cocaine. Some DAT compounds, with a higher affinity for the DAT than cocaine, have a lower reinforcing efficacy and abuse potential when compared to cocaine (Ferragud, et al., 2009; Lile, et al., 2002; Lile et al., 2003; Woolverton, et al., 2001). It has been suggested that there are several binding sites on the DAT or multiple DAT conformations and that, potentially, different compounds bind to different sites/conformations. GBR-12909 and mazindol have been shown to bind to a site on the DAT that is different from the cocaine binding site suggesting that more than one binding site or conformation exists (Berger, Elsworth, Reith, Tanen, & Roth, 1990) and that these different binding domains/conformations may produce variations in cocaine-like effects of high affinity DAT inhibitors (J. L. Katz, Izenwasser, Kline, Allen, & Newman, 1999) (Chen & Reith, 2007; Chen, Zhen, & Reith, 2004). It may be possible that cocaine and the test drugs differ in their affinities for these different binding sites/conformations and that binding at the different sites/conformations may convey different levels of cocaine-like activity. Therefore, DAT uptake inhibition affinity alone may not be responsible for cocaine's abuse related effects, a consideration which may help explain the current data showing D-84 maintaining a lower mean rate of infusion when compared to cocaine, despite having a higher DAT uptake inhibition affinity.

GBR-12909 has intermediate inhibitory effects at the SERT and results from this study confirm previous findings that GBR-12909 is self-administered in pre-clinical animal models (Andersen, 1987; van der Zee, et al., 1980). RTI-55 is a high DAT, high SERT (Boja, Cline, et al., 1992) inhibitor and findings from the current study also confirm previous studies that it maintains rates of self-administration above vehicle control level in rhesus monkeys (Weed, et al., 1995) . Mean drug intake differed between the test compounds, descending in the following order D-84>GBR-12909>RTI-55. This trend follows the level of SERT inhibition in the test compounds with D-84 having the least and RTI-55 having the most SERT inhibition activity. This may suggest that an increase in SERT inhibition produces a decrease in drug intake. All three test compounds were self-administered above vehicle control levels, and definitive conclusions about their relative reinforcing efficacies cannot be determined from the current data. Studies more directly addressing relative reinforcing efficacy (e.g., progressive ratio or choice studies) are required before it is possible to make more definitive statements regarding the relative reinforcing efficacies of these compounds as a function of the level of SERT inhibition.

Based on the available literature, it is thought that the presence of serotonin, via uptake inhibition, can attenuate cocaine self-administration (Alex & Pehek, 2007; M. E. Carroll, et al., 1990a), and it is known that serotonergic agonists and SERT inhibitors are not self-administered themselves (Howell & Byrd, 1995). This suggests that compounds that promote an increase in serotonergic activity should have less cocaine-like effects and, presumably, as you increase this level of SERT inhibition from minimal levels in a selective DAT inhibitor like D-84, to high levels in a compound like RTI-55, there should be a descending level of cocaine-like effects. However, although the test compounds were self-administered less than cocaine, it seems that the large changes in SERT inhibition did not

produce comparable changes in cocaine-like behavioural effects between the drugs. This may suggest that an increase in SERT inhibition alone with these compounds was not sufficient to produce the predicted effects.

In the current studies RTI-55 maintained rates of responding at non-significantly higher levels than GBR-12909, despite having a higher uptake inhibition affinity at the SERT. This may imply that, whilst a level of SERT inhibition may be important for reducing cocaine-like behavioural effects (since they all were self-administered less than cocaine), it may not be the only factor involved. RTI-55 has a Ki value for inhibiting reuptake of DA of 1.6 nM compared to 10.6 nM for GBR-12909 and 4.05nM for D-84, and it has been established that there is a correlation between DAT affinity and self-administration (Kuhar, Ritz, & Boja, 1991; Ritz, et al., 1987). It is possible that these high DAT uptake inhibition affinities may be producing a higher level of DAT inhibition than can be attenuated by the level of available SERT inhibition which may explain the lack of significant differences in infusion numbers seen between the test compounds.

The level of DAT occupancy influences the reinforcing properties of compounds (Volkow, Wang, Fischman, et al., 1997). Human neuro-imaging studies have shown that there is a strong correlation between DAT occupancy and the subjective high reported following acute administration of cocaine (Volkow, Wang, Fischman, et al., 1997). Studies have shown that doses of cocaine that maintain peak response rates in self-administration result in DAT occupancy between 65% and 76% in rhesus monkeys (Wilcox et al., 2002). Further studies have shown that doses of GBR-12909 that decrease cocaine self-administration in rhesus monkeys result in DAT occupancy of approximately 50% (Lindsey et al., 2004). Since the test compounds all have high uptake inhibition affinity at the DAT, it may be possible that this high level of DAT inhibition could be producing a level of DAT occupancy that may be insurmountable by any level of SERT inhibition. Finally, the

pharmacokinetics of these compounds could be playing a role in their self-administration effects. It has been shown that a rapid onset is positively related to reinforcing and subjective effects of drugs (Balster & Schuster, 1973). It has also been shown that, when using FR schedules, as in this current study, drugs with a longer duration of activity tend to maintain lower rates of responding than drugs with shorter durations (Balster & Schuster, 1973; Winger, Stitzer, & Woods, 1975). As reported in chapter three, all of the current test compounds had durations of activity that were much longer than cocaine, and this pharmacokinetic effect may be playing a role in the differences in responding seen between cocaine and the test compounds.

The goal of this study was to determine if an increase in SERT inhibition decreased self-administration. Although the test compounds all maintained lower rates of responding than cocaine, they did not differ as greatly from each other as expected, despite the large differences in SERT inhibition. It may be possible that multiple ligand binding sites on the DAT, DAT receptor occupancy, or pharmacokinetics may, alone or in sum, be reducing the potential control that SERT inhibition may have on self-administration of these drugs, and therefore, while these data may help support previous findings that SERT inhibition does have an attenuating effect on cocaine-like self-administration behavior (M. E. Carroll, et al., 1990a, 1990b), the current results with D-84 underscore the complexity of the possible determinants which may exert such control.

SUMMARY OF RESULTS AND OVERALL DISCUSSION

This dissertation consisted of two components, the research for which were conducted sequentially, although the results were discussed concurrently (Table 2). The first component tested whether an extremely selective DAT inhibitor (D-84), being nearly devoid of serotonergic and noradrenergic activity, had a pharmacology which was suitable as a potential replacement therapy for cocaine dependency. The results demonstrated that D-84, possessed characteristics deemed favourable, although perhaps not ideal, as a cocaine replacement therapeutic. D-84 had a long duration of action in locomotor activity studies (>4 h), incompletely generalized to cocaine in discrimination studies (76%), attenuated cocaine self-administration and was self-administered above vehicle control levels. Incomplete generalization in the discrimination studies replicated earlier findings in the mouse (Ghorai, et al., 2003), strenthening its perception of partial generalization while extending it to another species. The second component of the dissertation examined whether manipulating the presence of serotonergic activity could be an additional approach for which to control cocaine-like effects of DAT inhibitors.

As described in Chapter Three, research has provided evidence suggesting that the serotonergic system can be manipulated for modulating the positive reinforcing effects of cocaine-like compounds (Callahan & Cunningham, 1997; Cunningham & Callahan, 1991; Simon & Appel, 1997). Attempting to understand if the proportional level of serotonergic activity can modulate cocaine-like behavioural effects of DAT inhibitors was the basis for the second part of this dissertation. The current results showed that D-84 (High DAT, low SERT), GBR-12909 (High DAT, medium SERT) and RTI-55 (High DAT, High SERT) compounds had longer durations of action than cocaine and showed that an increase in SERT

inhibition correlated with lower peak total distance travelled in locomotor activity studies. As SERT inhibition increased from D-84<cocaine<GBR-12909<RTI-55, peak locomotor activity decreased in reverse order from RTI-55>GBR-1209>cocaine>D-84, suggesting the possibility that an increase in SERT inhibition may be producing decreases in peak activating effects. D-84 and GBR-12909 had similar onsets and durations of action, despite differing greatly in their levels of SERT inhibition, and so it seems that other factors interacted with increasing SERT to control locomotor activity. RTI-55 possessed the highest level of SERT inhibition and showed the slowest onset of activity, starting at 20 min post-injection compared to 10 min for all other compounds. With the exception of D-84, all the test compounds completely generalized (>80%) to cocaine in discrimination studies and all the drugs were self-administered significantly above vehicle control levels, but maintained maximal rates of responding at levels lower than cocaine. In the absence of additional tests (e.g., progressive ratio or choice tests), however, it is not possible to state uncategorically the absolute reinforcing efficacy of each test drug in this study. As stated earlier, it has been hypothesized that potent affinity at the DAT is responsible for the reinforcing effects produced by cocaine (Ritz, et al., 1987), and observing that D-84, GBR-12909 and RTI-55 are self-administered, it is likely that their self-administration is due to their binding at the DAT. Additional studies are required in order to determine how increasing the level of SERT alters the reinforcing efficacy of the selected test compounds. This study provided data showing that the test compounds differed from cocaine in terms of their peak infusion numbers, although not significantly from each other given the analysis conducted, suggesting the need for additional studies to further isolate the exact influence of serotonin on these cocaine-like behaviours. It is possible that the selected compounds did not differ greatly enough in terms of their SERT inhibition affinities, and that more information could be gathered from repeating these studies with a more disparate group of test compounds.

Given the differences in SERT inhibition that the test compounds possess (see Tables 1 and 2), combined with the closeness of effects between the drugs in the discrimination and self-administration assays, it is not possible to deduce what SERT inhibition, by itself, is having on the recorded behaviours investigated in the current studies. DAT occupancy levels and binding kinetics should also be considered to potentially be playing an important role. It has been hypothesized that more than one binding site exists on the DAT available for cocaine and other ligands to bind, that different DAT conformations play a role in drug binding, or that additional relevant cocaine binding sites exist outside DAT (Berger, et al., 1990; Boja, Markham, Patel, Uhl, & Kuhar, 1992; Rothman et al., 1994). Differences in binding sites/conformations may potentially convey differences in behaviour which may help explain some differences in cocaine-like behaviour seen with the compounds. Izenwasser (1994) showed a positive correlation between DAT binding affinities and potencies for stimulating locomotor activity for cocaine and cocaine analogues. However, the same study failed to show a significant correlation between DAT binding potency and potency for inducing locomotor activity for DAT inhibitors from a different structural class from cocaine, suggesting that cocaine and other DAT inhibitors may produce locomotor activating effects through different binding mechanisms. Binding studies with the current test drugs in relation to locomotor activating effects could be helpful in determining how the compounds are influencing locomotor activity and could provide information as to how important the level of SERT inhibition is in comparison to binding profiles.

Since the results produced in the current discrimination and self-administration studies were not consistent with, at least in magnitude, with the experimental predictions, other potential explanations for the results should be examined. It was hypothesized that an increase in SERT inhibition would decrease peak locomotor activating effects, decrease generalization and decrease self-administration. The locomotor activity results generally

produced the expected findings, but results obtained with discrimination and self-administration were more equivocal. One other potential explanation for the results produced, independent of SERT inhibition, could be related to the level of DAT receptor occupancy produced by the test compounds or the level of increased extracellular DA produced on administration of the compounds. Studies using brain imaging techniques identified a positive relationship between an increase in neurotransmitter release in DA brain areas and the intensity of the subjective high associated with cocaine (Volkow et al., 1996; Volkow, Wang, & Fowler, 1997). Doses of cocaine that are commonly abused by human users have been shown to result in a DAT occupancy between 67% and 69% in baboons (Volkow, et al., 1996), and doses of cocaine that maintain peak rates of cocaine self-administration result in DAT occupancy of 65%-76% in rhesus monkeys (Wilcox, et al., 2002). Microdialysis studies have shown that drugs which fail to generalize to cocaine in discrimination studies do not significantly increase extra-cellular levels of DA in the nucleus accumbens (Desai, Paronis, Martin, Desai, & Bergman, 2010). This same study showed that acute administration of cocaine produces a rapid increase in DA levels whilst administration of GBR-12909 produces a slower, longer lasting peak. This confirms an earlier study that showed that GBR-12909 has a slower onset of action, possibly due to this slower increase in DA levels, which potentially could be a factor in the *apparent* low abuse liability possessed by GBR-12909 (Baumann et al., 2002). These factors need to be considered when discussing the present results since these factors could play a role that may supercede the importance of the level of SERT inhibition. Although there is evidence in the literature to suggest that increasing SERT inhibition can decrease cocaine-like effects, there are obviously other factors that need to be considered.

Cocaine produces its positive reinforcing effects through inhibitory action at the DAT (Ritz, et al., 1987; Spealman, et al., 1989), and as a consequence, much drug development has concentrated on using DAT inhibitors as potential replacement therapies (F. I. Carroll,

Howell, & Kuhar, 1999). One challenge for any replacement therapy for cocaine dependence is in developing effective compounds that have a safer abuse liability profile relative to cocaine. Cocaine has a fast onset of action which is considered to be a factor in its abuse liability (Balster & Schuster, 1973) and a short duration of action. It is thought that an effective treatment for cocaine dependence would benefit from possessing a slower onset and longer duration of action to minimize abuse while allowing for practical dosing schedules (F. I. Carroll, et al., 1999; Howell, et al., 2000). With this in mind, much focus of the search for a treatment for cocaine dependence has been on DAT inhibitors with a lower level of abuse liability than cocaine (F. I. Carroll, et al., 1999; Gorelick, 1998; Gorelick, et al., 2004). DAT inhibitors have been shown to generalize to cocaine in discrimination studies, albeit it at different degrees of substitution (Balster et al., 1991; J. L. Katz, et al., 2001; Newman, et al., 1994). Bupropion, for example, has high uptake inhibition at the DAT, and since this activity is known to be responsible for the high abuse liability of cocaine-like psychostimulants, it is not surprising that pre-clinical studies have shown bupropion to be self-administered in monkeys and rats at levels similar to cocaine (Bergman, et al., 1989; Tella, et al., 1997). These pre-clinical data, however, are not supported by the available epidemiological data that have shown bupropion to have been minimally abused during its many years as an approved anti-depressant and smoking cessation aid (Nieuwstraten et al., 2001, Wilkes, 2008). These, and other data, suggest that DAT inhibitors, whilst conveying cocaine-like effects in pre-clinical studies, may not necessarily always possess abuse liability. Results with D-84 provide data that show incomplete generalization to cocaine in discrimination tests, and raises the possibility that a high affinity and selective DAT inhibitor may possibly have a lower abuse liability than cocaine. Without explicitly design tests (e.g., progressive ratio or choice tests), however, it is not possible to make more solid predictions regarding the potential abuse liability of D-84.

Although a large body of the currently available data has focused on DAT inhibitors as potential replacement therapies for cocaine, there are alternative possibilities for drug developmental approaches. It has become evident that the serotonin system can modulate some of the behavioral effects mediated by the dopaminergic system (D. A. Baker, Tran-Nguyen, Fuchs, & Neisewander, 2001; Bubar & Cunningham, 2006; Burmeister, Lungren, & Neisewander, 2003; Callahan & Cunningham, 1995, 1997), and it was this knowledge that stimulated the research contained within the second component of this dissertation. The serotonergic system is ideally placed to modulate dopaminergic neurotransmission due to projections and innervations into the mesolimbic dopamine system, particularly the VTA and NA (Herve, Pickel et al. 1987; Phelix and Broderick 1995). It is possible that serotonin can act to decrease the firing rate of DA neurons causing a decrease in extracellular DA or by decreasing DA release from neurons (Di Mascio, Di Giovanni, Di Matteo, Prisco, & Esposito, 1998). The serotonergic system's ability to modulate cocaine-like behavioural effects may also be due to inhibiting the cocaine-induced increase in extracellular dopamine (Howell & Kimmel, 2008). It is possible that there is a optimum level of SERT inhibition that may limit the potential cocaine-like abuse liability effects of other DAT inhibitors and, a DAT inhibitor with an optimum level of SERT inhibition may be one way to produce a desired profile for a cocaine replacement therapy. This desired profile could be a compound with a high enough level of DAT inhibition to convey some cocaine-like behavioural effects, combined with a level of SERT inhibition that would constrain its abuse liability. Such a compound could potentially be an effective treatment for cocaine dependence but, to date, clinical evaluations have not identified a suitable candidate with this right balance of effects.

Evidence has suggested that there are multiple binding sites on or multiple conformations of the DAT (Berger, et al., 1990) and that cocaine binding may be better described by a two site binding model as opposed to a one site model (Madras, Fahey,

Bergman, Canfield, & Spealman, 1989). Low and high affinity binding sites have been identified and it has been determined that binding to the high affinity site is responsible for the abuse related effects seen with cocaine (J. L. Katz, et al., 1997), and in particular is thought to play a vital role in the discriminative stimulus effects of cocaine (Katz, Izenwasser et al., 2000). As mentioned earlier, certain DAT inhibitors bind to the DAT and inhibit DA uptake but do not possess effects predictive of a high abuse potential (J. L. Katz, et al., 2001; J.L Katz, et al., 2003). This could be explained by different compounds preferring different DAT conformations (Reith et al., 2001; Loland et al., 2008; Schmitt et al., 2008). With the dual binding sites observed for cocaine-like compounds (Madras, Fahey et al., 1989; Boja et al., 1992), it is possible that some DAT inhibitors bind with higher affinity to the low affinity site as opposed to the high affinity site resulting in low cocaine-like discriminative stimulus effects. This activity could help explain the effects seen with D-84 which is a highly selective DAT inhibitor yet fails to fully generalize to cocaine in discrimination studies. These low and high affinity binding sites on the DAT could be considered as an alternative explanation for the difference in effects produced with cocaine, RTI-55 and GBR-12909 when compared to D-84 and cocaine. It is possible that binding affinities of these compounds at the different binding sites on the DAT may play a predominant role in producing cocaine-like discriminative stimulus effects, thereby reducing the influence of SERT inhibition. If a reliable receptor binding protocol could be developed to quantifiably distinguish these different binding sites, studies comparing RTI-55, GBR-12909 and D-84 with cocaine could be carried out to compare individual *K*i values in such controlled binding experiments with ED_{50} values for occasioning cocaine generalization in an attempt to determine how the binding site may be influencing the discriminative stimulus effects of the test compounds. However, it should be noted that the pharmacological profile of the low-affinity binding site for cocaine-like compounds has not been clearly established, and that conditions reliably

producing a two-site binding phenomenon for such drugs has been elusive (Coffey & Reith, 1994; Reith & Coffey, 1994). Be that as it may, recent studies lending support for multiple sites on DAT for inhibitors and substrates have been inspired by the structural characterization of a bacterial homolog of the DAT, the leucine transporter (LeuT) (Yamashita et al., 2005). Shi et al. (2008) advanced evidence for two sites for the substrate leucine, one deep in the central cavity of the protein close the site for Na^+ (crucial for transport), and one more externally in the periplasmic vestibule. The distance between these sites, ~ 11-13 Å, fits the distance between the two heads of bivalent dopamine- and amphetamine-like ligands when bound and docked in human DAT (Schmitt, Mamidyala, Biswas, Dutta, & Reith, 2010). In this context, it is of interest that Nielsen et al. (2009) reported affinity gains for DAT, SERT, and NET of up to 45-fold for bivalent phenyl tropanes (of the same template as RTI-55) when tethered together with an ester linker of 10 atoms, again bridging binding sites separated by a distance of ~13 Å. The results point to a secondary binding site in DAT that has affinity for both DA-like substrates and phenyltropane-like inhibitors.

The second overall aim of this study was to determine if increasing SERT inhibition attenuated cocaine-like behavior in three different rodent assays. Whilst the results showed differences between cocaine and the test compounds in locomotor activity, in terms of peak effects and duration of action, differences in drug discrimination and self-administration were not as pronounced. In the discrimination studies, GBR-12909 (SERT Ki 73 nM) and RTI-55 (SERT Ki 3.8 nM) both completely generalized to cocaine despite their differences in SERT inhibition, and all three compounds were reliably self-administered above vehicle control levels. It may be harder than first thought to determine the exact influence SERT inhibition has over the cocaine-like behaviour produced by DAT inhibitors, at least when limited to the behavioural assays conducted in the current dissertation. Although the locomotor activity and

discrimination data showed a decrease in cocaine-like activity as SERT inhibition was increased, it is not possible to determine what level of SERT inhibition produces these attenuating effects. It is known that SSRI's are not self-administered (Gold & Balster, 1991) but can attenuate cocaine self-administration (M. E. Carroll, et al., 1990a) and can modulate cocaine's discriminative stimulus cue (Callahan & Cunningham, 1997; Cunningham & Callahan, 1991; Filip, et al., 2006) . It is also known that cocaine and other DAT inhibitors can stimulate locomotor activity (Cline et al., 1992), are self-administered and can generalize to cocaine in discrimination studies (Ritz, et al., 1987; Spealman, et al., 1989). This suggests that it may be possible to decrease the level of SERT inhibition from that equivalent in an SSRI to that associated with selective DAT inhibitor potentially resulting in a shift from non-cocaine like behavior to cocaine-like. Where that shift occurs has yet to be determined and the data from this study does not conclusively identify the level of SERT inhibition required in order to change a compound from cocaine-like to not cocaine-like. In fact, data with D-84 suggests multiple determinants are operative in controlling the level of cocaine-like behavioral expression in DAT inhibitors, and that SERT inhibition may only be one of them.

Determining the level of SERT inhibition needed to reduce cocaine-like effects may be complicated by the complexity of the serotonergic system itself. Data have shown that the 5-HT_2 system is important in modulating cocaine-like behavioural effects of DAT inhibitors (Filip, et al., 2006; Higgins & Fletcher, 2003), however, the 5-HT_{2A} and 5-HT_{2C} receptor systems seem to play opposing roles. Activation of the 5-HT_{2C} receptor system mediates an inhibitory effect on the dopaminergic systems (Di Matteo, De Blasi, Di Giulio, & Esposito, 2001), possibly through interaction with inhibitory GABA neurons (Eberle-Wang, Mikeladze, Uryu, & Chesselet, 1997), whilst activation of the 5-HT_{2A} system has been shown to enhance it (Alex & Pehek, 2007). An increase in extracellular 5-HT following SERT inhibition leads to activation of 5-$HT_{2A/C}$ receptors, and, given some of the opposing actions

just mentioned between 5-HT receptor subtypes, activation of these receptors may have opposing effects on dopaminergic neurotransmission, and hence, have opposing effects in modulating cocaine-like behaviour. Depending on the net activation of 5-HT_{2C} vs 5-HT_{2A}, an increase in serotonergic tone could either attenuate cocaine-like effects or enhance it, and it may be possible to elucidate the role of SERT inhibition in modulating cocaine-like effects if it was known which of the 5HT receptors are more influenced by this increase in extracellular 5-HT. These modulating effects of 5-$HT_{2A/2C}$ agonists have been previously reported with another CNS active compound, nicotine. Non-selective 5-$HT_{2A/2C}$ agonists have been shown in our laboratory to blunt the locomotor activating and discriminative stimulus effects of nicotine in rats (Batman, Munzar, & Beardsley, 2005) providing a further example by which SERT inhibition may attenuate dopaminergic-mediated activity.

The current study has reinforced reports indicating that DAT inhibitors stimulate locomotor activity, at least partially generalize to cocaine in discrimination studies, and can be self-administered. There are also reports indicating that SERT inhibitors can have an inhibitory influence over cocaine-like effects (Burmeister, et al., 2003; Callahan & Cunningham, 1995; M. E. Carroll, et al., 1990a). The results from the current studies are summarized in table two, and do not conclusively confirm that an increase in SERT inhibition is responsible for the decrease in cocaine-like effects, although the results do show a trend in that direction in the locomotor activity studies. All of the compounds were self-administered significantly above vehicle control levels but they differed in their maximum levels of drug intake. This effect may be related to the potency of the drugs in this assay. RTI-55 was the least potent of the drugs in stimulating locomotor activity but the most potent in self-administration (and discrimination) studies which may explain its very low drug intake levels in self-administration. However, this could also be related to its intrinsic reinforcing efficacy, but results from the current studies cannot categorically establish this without data

from additional studies. The results with RTI-55 could imply that SERT inhibition may be playing more of a role in modulating cocaine-like locomotor activity behaviour, as opposed to discrimination and self-administration behaviour, and may suggest that the effects of SERT inhibition may be dependent on the variable being measure.

It should be noted that not all selective DAT inhibitors are created equally. As mentioned previously, and as shown by discrimination results with D-84, not all selective DAT inhibitors produce complete cocaine-like behaviour. D-84 only partially generalized to cocaine in discrimination studies despite being a very selective and potent DAT inhibitor. Although it is possible that SERT is playing a role in attenuating cocaine-like effects of some DAT inhibitors, results obtained with D-84 indicate that SERT inhibition is not the only factor that can attenuate cocaine-like behaviour. SERT inhibition, pharmacokinetics, receptor occupancy and exact binding site may all be playing a role. All contributing factors may need to be considered in order to develop a successful replacement therapy in cocaine dependency.

As mentioned, this dissertation was divided into two sections with the first section concentrating on elaborating the pharmacology of a selective DAT inhibitor as part of a drug development effort. These data, while not conclusive, suggests that a selective DAT inhibitor such as D-84, may reduce cocaine self-administration and may possess a lower abuse liability than cocaine. Understanding how this compound produces its composite effects would help focus future drug developmental efforts.

The second component of this dissertation tested whether manipulating the level of SERT could be an effective approach for manipulating the expression of cocaine-like behavioral activity of DAT inhibitors. The current data (summarized in Table 2, following

this Discussion) shows that an increase in SERT inhibition correlates with a decrease in locomotor stimulating effects. These same data, with the exception of D-84, fail to show any significant effect of SERT inhibition on cocaine-like discrimination behaviour, and in fact, the discrimination results with D-84 complicates these interpretations. Finally, since all three test compounds were reliably self-administered above vehicle control levels, the data suggest that the differences in SERT inhibitory action in the currently tested compounds do not produce significant changes in self-administration.

Although the current data failed to determine if there is an optimum level of SERT inhibition required to attenuate the abuse related effects of cocaine, the hypothesis that SERT inhibition can, in fact, attenuate the reinforcing effects of cocaine, should still be kept in the forefront of research. Recent studies have suggested that introducing substitutions into the chemical structures of SSRI's such as fluoxetine, can produce a new generation of DAT inhibitors with a much lower apparent abuse potential than cocaine (Yoon, Cho, Yoon, Min, & Lee, 2009). This recent study strengthens the hypothesis that manipulating the serotonin system can reduce the cocaine-like positive reinforcing effects of DAT inhibitors, and this approach still may contribute to a replacement therapy for cocaine dependence.

Table Two: Overall Summary of Results

			Locomotor Activity		Drug Discrimination		Self-administration	
Compound	DAT (ki nM)	SERT (ki nM)	LMA peak distance travelled (cm)	LMA area under curve (cm_2)	DLR ED_{50} (mg/kg)	RR ED_{50} (mg/kg)	Dose at peak self-administered infusions ii (mg/kg/inf)	Maximum intake (mg/kg)
D-84	4.05	1274	3500	10312	8.2	33.9	0.3	21.6
Cocaine	478	304	3000	10276	2.85	13.83	0.1	24.3
GBR-12909	4.3	73	1600	6174	9.02	[i]	0.3	12.3
RTI-55	1.6	3.8	1500	5348	2.32	5.97	0.01	2.14

[1] GBR-12909 produced non-significant reductions in response rates at the dose range tested
[ii] Self-administration is defined as any dose where mean infusions> saline infusions

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VITA

Angela M Batman was born January 27th 1978 in Ipswich, England. Upon graduation from high school Angela attended the University of Sheffield where she obtained a Bachelor of Science degree in Pharmacology in May 2000. Angela came to Virginia Commonwealth University in August 2001 and enrolled in the Masters program in the Department of Pharmacology and Toxicology in the laboratory of Dr Patrick Beardsley. Angela's research work focused on the serotonergic modulation of nicotine's discriminative stimulus effects.

Upon completion of her Master's degree, Angela accepted a full time position in Dr Beardsley's lab where she used rat self-administration assays to screen compounds for their effectiveness in preventing cocaine reinstatement. In August 2006 Angela entered the Ph.D program in the Department of Pharmacology and Toxicology and her doctoral studies focused on researching replacement therapies for cocaine dependence. In addition to her graduate work, Angela continued to work full time dividing her energies between the cocaine reinstatement studies, metabolomic studies investigating the acute and chronic effects of methamphetamine and her doctoral work.

Manuscripts

Angela M Batman, M.S.; Aloke K Dutta, Ph.D.; Maarten E Reith, Ph.D.; Patrick M Beardsley, Ph.D. The Selective Dopamine Uptake Inhibitor, D-84, Substitutes for Cocaine in Self-Administration but is Self-Administered at Lower Levels, and only Partially Occasions it's Discriminative Stimulus. Eur J Pharmacology (2010) (Accepted pending revisions)

Kharkar, P. S., **Batman, A. M**, Zhen, J., Beardsley, P. M., Reith, M. E. and Dutta, A. K., Synthesis and biological characterization of (3R,4R)-4-(2-(benzhydryloxy)ethyl)-1-((R)-2-hydroxy-2-phenylethyl)-piperidin-3-ol and its stereoisomers for monoamine transporters. ChemMedChem (2009)

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[i] GBR-12909 produced non-significant reductions in response rates at the dose range tested
[ii] Self-administration is defined as any dose where mean infusions> saline infusions