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Determinants of Abuse-Related Effects of Monoamine Releasers in Rats

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Determinants of Abuse-Related Effects of Monoamine Releasers in Rats

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy at Virginia Commonwealth University

by

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List of Abbreviations

ADHD = attention deficit hyperactivity disorder
ANOVA = analysis of variance
DA = dopamine
DAT = dopamine transporter
FR = fixed ratio
FI = fixed interval
ICSS = intracranial self-stimulation
kg = kilogram
LC = locus ceruleus
MAR = monoamine releaser
MCR = maximum control rate
mg = milligram
ml = milliliter
NE = norepinephrine
NET = norepinephrine transporter
5HT = serotonin
IP = intraperitoneal
SC = subcutaneous
SEM = standard error of the mean
SERT = serotonin transporter
VTA = ventral tegmental area
List of Compounds

Chapters II and III:

- m-flouroamphetamine (PAL-353) - Releaser: NE ≥ DA >>> 5HT
- (+)-amphetamine (amphetamine) - Releaser: NE ≥ DA >>> 5HT
- (+)-phenmetrazine (phenmetrazine) - Releaser: NE ≥ DA >>> 5HT
- S-(+)-methamphetamine (methamphetamine) - Releaser: NE ≥ DA >>> 5HT
- m-methylamphetamine (PAL-314) - Releaser: NE ≥ DA >> 5HT
- p-methylamphetamine (PAL-313) - Releaser: NE ≥ DA = 5HT
- (+)-3,4-methylenedioxymethamphetamine ((+)MDMA) - Releaser: NE = DA ≤ 5HT
- Naphthylisopropylamine (PAL-287) - Releaser: NE = DA ≤ 5HT
- (±)-3,4-methylenedioxymethamphetamine (±MDMA) - Releaser: NE > DA = 5HT
- (-)-3,4-methylenedioxymethamphetamine ((-)MDMA) - Releaser: DA << NE ≤ 5HT
- (±)-fenfluramine (fenfluramine) - Releaser DA <<< NE < 5HT

Chapter IV:

- (αS)-6-Cl-5-Fl-α-methyl-1H-indole-1-ethanamine (Ro 60-0175) - 5HT$_{2C}$ agonist
- 6-Cl-2,3-dihydro-5-methyl-N-[6-[(2-methyl-3-pyridinyl)oxy]-3-pyridinyl]-1H-indole-1-carboxyamide (SB 242,084) - 5HT$_{2C}$ antagonist

Chapter V:

- (-)-Cocaine (cocaine) - Uptake inhibitor: NE ≥ DA ≥ 5HT
- 5-Cl-α-methyltryptamine (PAL-542) - Releaser: 5HT > DA >>> NE
- 5-Fl-α-ethyltraptamine (PAL-544) - Releaser: 5HT = DA > NE
- 6-α-methyltryptamine (PAL-571) - Releaser: 5HT ≥ NE > DA
- (−)-1-α-methyltryptamine (PAL-569) - Releaser: NE ≥ 5HT > DA
## Structures of Compounds by Chapter

### Chapter I and II Compounds:

- **PAL-353**
- **Amphetamine**
- **Phencyclidine**
- **Methamphetamine**
- **PAL-314**
- **PAL-313**
- **PAL-287**
- **MDMA**
- **Pfurtschennin**

### Chapter III Compounds:

- **Ro 60-0175**
- **SB 242,084**

### Chapter IV Compounds:

- **Cocaine**
- **PAL-544**
- **PAL-571**
- **PAL-542**
- **PAL-569**
Abstract

DETERMINANTS OF ABUSE-RELATED EFFECTS OF MONOAMINE RELEASERS IN RATS

By Clayton T. Bauer

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2013

Advisor: S. Stevens Negus, Ph.D.

Monoamine releasers constitute a class of compounds that promote release of dopamine, serotonin, and/or norepinephrine. These compounds have a range of different uses in the medical setting, including treatment of attention deficit hyperactivity disorder, narcolepsy, and obesity. A major limitation of many of these compounds (i.e. amphetamine, methamphetamine, phenmetrazine) is their propensity for abuse; however, not all monoamine releasers are abused (i.e. fenfluramine). The goal of this dissertation was to examine pharmacological determinants of abuse-related effects produced by monoamine releasers in two preclinical assays in rats: intracranial self-stimulation (ICSS) and drug discrimination. First, this work confirmed and expanded upon previous findings that selectivity for promoting release of dopamine versus serotonin is one determinant of abuse-related effects produced by monoamine releasers. This was accomplished by determining the behavioral effects of 11 different compounds that ranged in their selectivity for dopamine versus serotonin, and a correlation was found between the ability of a compound to facilitate ICSS and the selectivity of that compound for releasing dopamine versus serotonin. These data were then submitted to a rate-dependency analysis. Here, we found that all compounds produced rate-dependent effects, but that
the profile of these effects varied with a compound’s selectivity for dopamine versus serotonin. Next, the mechanism by which serotonin exerts its response rate-decreasing effects was investigated—specifically, the hypothesis that the 5HT$_{2C}$ receptor mediates serotonin’s abuse-limiting effects of monoamine releasers was tested. The data collected suggest that the 5HT$_{2C}$ receptor contributes to, but is not exclusively responsible for, the abuse-limiting effects produced by serotonin release. Finally, selectivity for norepinephrine versus dopamine was examined as a potential determinant of monoamine releaser abuse liability; results from these studies suggest that release of both dopamine and norepinephrine are required for expression of abuse-related effects in assays of ICSS and drug discrimination. These data provide a systematic examination of the determinants of the abuse-related effects produced by monoamine releasers and may contribute to development of medications with reduced abuse potentials.
Chapter I: Introduction

Background and Significance

Monoamine releasers constitute a class of compounds that promote the release of dopamine (DA), norepinephrine (NE) and serotonin (5HT) (Parada, 1988; Rothman, 2001). Amphetamine, the prototype drug in this class, has a long history of use as a medical treatment for indications including obesity, narcolepsy, and most recently attention deficit hyperactivity disorder (ADHD) (Setlik, 2009; Nishino, 2007; Bray, 1993; Rasmussen, 2008). The prevalence of amphetamine prescriptions in the U.S. nearly doubled between 2007 and 2011 as stimulant medications have been shown to be the most efficacious treatments for ADHD (Kutcher et al., 2004; Faraone and Glatt, 2010; Sembower et al., 2013); four and a half million prescriptions for extended release amphetamine were written in the second quarter of 2011 alone (Sembower et al., 2013).

In addition to amphetamine, several other monoamine releasers either are currently or have previously been clinically available by prescription, primarily for use as treatments for obesity (phenmetrazine, phendimetrazine, phentermine, and fenfluramine) and ADHD (methamphetamine) (Teter et al., 2006; Colman, 2005; Encinosa et al., 2005).

The most prominent issue limiting the clinical utility of these compounds is their high potential for abuse (Kollins, 2007; Jasinski and Kovacević-Ristanović, 2000). All of the compounds listed above are scheduled (distribution controlled by the Drug
Enforcement Agency), and non-medical use of prescription amphetamine is currently considered a major public health issue, especially among college students (US DOJ; Teter et al., 2006; Sembower et al., 2013). Additionally, many monoamine releasers also have long histories of abuse outside of the clinical setting (Kramer et al, 1967; Rasmussen, 2008; Teter et al., 2006). Abuse of amphetamines (amphetamine, methamphetamine) was described as early as the 1940’s and as of 2010, approximately 0.9% of the U.S. population 12 and older had used an illicit stimulant in the past month (Rasmussen, 2008; Anglin et al., 2000; SAMHSA, 2011). As efforts to curb amphetamine and methamphetamine use were increased by the Drug Enforcement Agency, a new series of “designer” drugs entered into the public arena; the most prevalent and well known of which is the monoamine releaser methylenedioxymethamphetamine (MDMA, “ecstasy”) (Chistophersen, 2000). Like, amphetamine and methamphetamine, MDMA has a high incidence of use in the US and around the world with more people initiating use of ecstasy than cocaine in 2011 (Chistophersen, 2000; SAMHSA, 2011); however, unlike amphetamine or methamphetamine, MDMA is traditionally thought to be non-addictive with most users foregoing frequent use of large quantities of MDMA (Jansen, 1999; Peroutka, 1990). Likewise, although more individuals used MDMA than methamphetamine in 2007, there were fewer emergency room visits related to MDMA than to methamphetamine around the same time (DAWN, 2007; SAMHSA, 2008). This suggests that intermittent MDMA use is less likely than intermittent methamphetamine use to engender patterns of drug use that require clinical intervention. Fenfluramine, although scheduled, appears to have
little to no abuse liability in humans (Chait et al., 1986; Griffith et al., 1975; Johanson and Uhlenhuth, 1982).

Of interest to the current dissertation is the apparent spectrum of abuse liability seen with the monoamine releasers: amphetamine > MDMA >> fenfluramine. This pattern of differential abuse has led to the evaluation of these compounds in pre-clinical assays to establish the determinants of monoamine releaser abuse liability. The primary finding to date is that co-release of DA/NE release is rewarding, but 5HT release is not. Clinically relevant, DA/NE-selective MARs (amphetamine, methamphetamine, and phenmetrazine) are readily self-administered in preclinical assays (Götestam and Andersson, 1975; McKenna and Ho, 1980). Conversely, a highly 5HT-selective releaser (fenfluramine) is not self-administered in preclinical studies (Wood and Tessel, 1974; Dahl and Götestam, 1989). Non-selective releasers of DA, NE, and 5HT, such as the racemate and isomers of 3,4-methylenedioxymeth-amphetamine (MDMA), display inconsistent reinforcing effects in pre-clinical drug self-administration assays relative to more DA-selective releasers (Beardsley et al., 1986; Fantegrossi et al., 2002; Wang and Woolverton, 2007; Schenk., 2009). Likewise, mixtures of a DA/NE-selective releaser (amphetamine) and a 5HT-selective releaser (fenfluramine) result in a decreased rate of self-administration in monkeys when compared to amphetamine alone (Wee and Woolverton, 2006). Finally, non-selective MARs produce weaker cocaine-like discriminative stimulus effects than DA-selective releasers (Wee et al., 2005; Rothman et al., 2005; Negus et al., 2007). These studies support the hypothesis that selectivity for DA/NE vs. 5HT is one determinant of abuse-related effects produced by MARs.
Another potential determinant for increased abuse potential of MARs is selectivity for DA versus NE. Although DA is well-established to be a key neurotransmitter in mediating abuse-related effects of monoamine releasers and other drugs (for review, Leshner and Koob, 1999), amphetamine and other abused monoamine releasers have slightly (2 to 3x) higher potency to release NE than DA (Rothman et al., 2001). Moreover, methamphetamine self-administration in rats was relatively resistant to pretreatment with DA-antagonists (Brennan et al., 2009), and ephedrine (a 19-fold NE-selective releaser) has been shown to maintain self-administration in monkeys (Anderson et al., 2001) and substitute for amphetamine (Young et al., 1998) and methamphetamine (Bondareva et al., 2002) in drug discrimination studies in rats. Perhaps the most compelling data on the importance of NE comes from human subjects where amphetamine-like discriminative stimuli produced by monoamine releasers correlate with potency to release NE, not DA (Rothman et al., 2001). This leads to the hypothesis that NE release is another determinant of the abuse-related effects produce by MARs; however, the role of DA vs. NE selectivity has been difficult to investigate further due to a lack of drugs that possess significant selectivity for DA or NE relative to the other catecholamine.

**Monoamine Releaser Pharmacology**

**Molecular Pharmacology**

The primary targets of monoamine releasers include the DA, 5HT, and NE transporters (DAT, SERT, and NET, respectively). These transporters are proteins made up of 12 transmembrane domains, and, under normal conditions, act as symporters transporting
one NA+, one Cl−, and either one 5HT or one NE molecule into the neuron (in the case of DA it is two NA+ and one Cl−) (Rudnick, 2006; Gu et al., 1996; Reith et al., 1997). It is these transporters that MARs enter to promote the release of DA, 5HT, or NE (Parada et al., 1988; Rothman et al., 2001; Fleckenstein et al., 2007). The precise mechanism by which this release takes place is still being debated in the literature with possible mechanisms including reversal of the monoamine transporters and increased synaptic release of the neurotransmitters (Fleckenstein et al., 2007; Sulzer, 2011; Daberkow et al., 2013).

**Figure I.1. Amphetamine promotes release of monoamine neurotransmitters from presynaptic terminals.**

As mentioned above, one of the variables that appears to determine abuse-related effects produced by MARs is the selectivity of a given compound for one or more of the monoamine transporters. *In vitro*, compounds like amphetamine are slightly more potent to release NE than DA, and more potent to release DA/NE than 5HT; compounds like MDMA are nearly equipotent at releasing DA, NE and 5HT; and fenfluramine is relatively impotent at releasing DA and NE, while potently releasing 5HT (Rothman et al., 2001; Rothman and Baumann, 2006). A summary of the compounds tested in this
dissertation and their relative potencies for releasing DA, NE and 5HT can be found on page vi, while specific potencies are provided in the respective chapters.

**Neurochemical Pharmacology**

In addition to *in vitro* measures of potency and selectivity, one can also look at *in vivo* measures of release, using microdialysis and fast scan cyclic voltammetry (Reith et al., 1997; Heien et al., 2004; Hernandez and Hobel, 1988). These assays also provide important information as to the effects of these drugs on neurochemistry. In particular, microdialysis has been used to evaluate the release of DA, 5HT, and to a lesser extent NE by many of the compounds listed in this dissertation (Rothman and Baumann, 2006; Baumann et al., 2000).

*In vivo*, “DA/NE-selective” compounds (based upon the aforementioned *in vitro* assays) such as PAL-353 and amphetamine have been shown to produce significant (>10 fold) increases in DA levels in the ventral striatum while producing little effect on 5HT levels (Rothman and Baumann, 2006; Baumann et al., 2000). One “non-selective” compound (PAL-287) appears to release DA and 5HT nearly equally in the pre-frontal cortex, while another non-selective compound (MDMA) appears to preferentially increase 5HT over DA in the anterior striatum (Rothman and Bauman, 2006; Koch and Galloway, 1997). The 5HT-selective compound fenfluramine produces >10 fold increases in baseline levels of 5HT with little to no effect on DA (Baumann et al, 2000). Although some compounds appear to be more or less selective *in vitro* than *in vivo* (ie. MDMA), there is generally a good correlation between a compound’s *in vitro* selectivity and the neurochemical changes produced *in vivo* (Baumann et al., 2011). While the
relationship between DA and NE selectivity has been less well studied, amphetamine has been shown to increase both DA and NE in the caudate and hippocampus, respectively (Kuczenski and Segal, 1997) with a greater percentage change seen with NE than DA. This is consistent with the in vitro selectivity data for amphetamine. Likewise, fenfluramine produces its greatest effect on 5HT, followed distantly by NE and DA - again, consistent with the in vitro selectivity data (Rothman et al., 2003). These data provide a solid basis upon which behavioral studies can be built. Correlations between behavior and selectivity for DA vs. 5HT and DA vs. NE will be two of the key issues addressed in this dissertation.

**Interactions of the monoaminergic systems**

The two dopaminergic pathways most closely associated with reward and motivation are the mesolimbic and mesocortical pathways; these pathways primarily originate in the ventral tegmental area (VTA), also known as A10, and project to the nucleus accumbens and pre-frontal cortex, respectively (Iversen et al., 2009). Of interest to the current dissertation are the connections and functional relationships of the noradrenergic and serotonergic systems to these dopaminergic reward pathways. Taken as a whole, the data presented below suggest that both NE and 5HT innervate and modulate the dopaminergic system.

**Serotonergic modulation of dopamine**

It is known that serotonergic neurons project to both the nucleus accumbens and ventral tegmental area from cell bodies in the raphe nuclei (Brown and Molliver, 2000; Van...
Bockstaele et al., 1994). There is also evidence that interruption of these serotonergic connections, through lesions of the dorsal raphe nucleus, leads to an increase in DA utilization in the nucleus accumbens (Hervé et al., 1979). Similarly, intraventricular injections of 5,7-dihydroxytryptamine (which result in broad lesions of the 5HT system) increase firing of dopaminergic neurons in the VTA by 37% (Guiard et al., 2008). These studies provide evidence that 5HT may exhibit tonic inhibition of DA under normal conditions, leading to the hypothesis that 5HT-selective releasers would further inhibit dopaminergic neurotransmission in these reward pathways.

**Figure I.2. Serotonergic modulation of the dopaminergic reward pathway.**

![Diagram of serotonergic modulation of the dopaminergic reward pathway.](image)

Adapted from Alex and Pehek, 2007
The receptors through which 5HT may be able to modulate abuse-related effects of DA have also been studied (for review, Alex and Pehek, 2007). One of these receptors, the 5HT$_{2C}$ receptor, appears to be especially relevant to 5HT’s ability to decrease dopaminergic activity. Anatomical studies have shown the presence of these Gq coupled receptors on GABAergic neurons in the substantial niagra/VTA (Eberle-Wang et al., 1997), and the 5HT$_{2C}$ receptor is also found in the nucleus accumbens and cingulate cortex (Eberle-Wang et al., 1997; Pompeiano et al., 1994). Neurochemical studies have shown that 5HT$_{2C}$ receptor activation can decrease basal levels of DA (Di Matteo et al., 2000), and behavioral studies with 5HT$_{2C}$ agonists have shown decreases in feeding (Somerville, et al., 2007; Grottick, et al., 2000), decreases in self-administration of the monoamine reuptake inhibitor cocaine (Manvich, et al. 2012a; Grottick, et al., 2000), and decreases in ICSS (expressed as increases in ICSS thresholds) (Hayes, et al., 2009). Conversely, antagonism (or inverse agonism) of the 5HT$_{2C}$ receptor has been shown to increase DA levels in the striatum (Alex et al., 2005), prevent 5HT agonist-induced decreases in striatal DA levels (Di Giovanni et al., 2000), and increase cocaine self-administration (Manvich, et al., 2012b). Taken together, these results support the hypothesis that 5HT, acting at 5HT$_{2C}$ receptors, can decrease dopaminergic activity and behaviors dependent on dopaminergic activity. However, the role of the 5HT$_{2C}$ receptor in mediating effects of monoamine releasers in particular is less clear.

**Noradrenergic modulation of dopamine**

There is also evidence of noradrenergic innervation of the dopaminergic system (Geisler and Zahm, 2005; Jones and Moore, 1977). Electrical stimulation of the locus coeruleus...
(LC) neurons increased levels of NE in the VTA and increased activity of DA neurons (Grenhoff et al., 1993). However, when exogenous NE was applied to the VTA, a decrease in firing rates of DA neurons was seen (Aghajanian and Bunney, 1977; White and Wang, 1984). Similar to the results of the latter study, lesions of the NE system by injection of 6-OHDA into the locus coeruleus increased firing of DA neurons in the VTA by 70% (Guiard et al., 2008). These data suggest that there may be both excitatory and inhibitory roles of NE on the activity of VTA dopaminergic neurons.

The receptors by which NE modulates DA at the level of the VTA are fairly well characterized. In particular, it appears that the \( \alpha \)-1 receptor is responsible for increases in DA neuron firing following NE administration while the \( \alpha \)-2 receptor mediates the inhibitory effects of NE (Grenhoff and Svensson, 1988; Grenhoff and Svensson 1989; Grenhoff and Svensson, 1993; Grenhoff et al., 1995). In addition to the \( \alpha \)-2 receptor, it appears that NE can act directly on D2 dopaminergic autoreceptors to produce inhibitory effects (Grenhoff et al., 1995; Lacey et al., 1987; Arencibia-Albite et al., 2007; Guiard et al., 2008). \( \beta \)-adrenoreceptors are not known to exist in the VTA (Grenhoff et al., 1995; Jones et al., 1990) and \( \beta \)-adrenergic compounds do not mediate the effects of NE in the VTA (Grenhoff et al., 1995).

**Research Strategy**

**Intracranial Self-Stimulation**

The primary assay used in this dissertation was intracranial self-stimulation (ICSS) in rats. ICSS is the general label for a family of operant conditioning procedures that use electrical brain stimulation as the reinforcing stimulus and was first described by Olds...
and Milner in 1954 (Olds and Milner, 1954; Stellar and Stellar, 1985; Shizgal and Murry, 1989; Lewis, 1993). This seminal experiment showed that rats would vigorously respond for electrical stimulation of certain brain areas. Over the past 40+ years, ICSS has offered an alternative approach to the more widely used assays of pre-clinical abuse liability assessment: self-administration (O’Connor et al., 2011; Ator and Griffiths, 2003) and place conditioning (Tzschentke, 2007) (Kornetsky and Esposito, 1979; Kornetsky et al., 1979; Vlachou and Markou, 2011; Wise, 1996). Relative to other commonly used reinforcing stimuli, such as food delivery or drug injections, electrical stimulation is distinguished by the speed of its delivery, the brevity of its effects, and the precise degree of control over its magnitude (Carlezon and Chartoff, 2007; Vlachou and Markou, 2011).

Although there are several different methods for performing ICSS (Stellar and Rice, 1989), the technique used in these studies was a frequency-rate procedure similar to that described by Carlezon and Chartoff (2007). Carlezon and Chartoff have extensively discussed the relative advantages provided by looking at ICSS frequency-rate curves in the study of reward. Advantages relative to other assays of drug reward (e.g. drug self-administration, place conditioning) include (a) the ability to examine test drug effects on different baseline rates of behavior, (b) the sensitivity of the assay to both abuse-related rewarding effects and abuse-limiting anhedonic effects, and (c) the ease of examining drug time course. ICSS does, however, suffer from a lack of face-validity as the reinforcer maintaining behavior is electrical stimulation, not the drug itself.

The advantage listed above that is of particular interest to our lab is the sensitivity of ICSS to both abuse-related (rate increasing) and abuse-limiting (rate decreasing) effects produced by a given test drug. This particular advantage is especially useful when
looking at the effects produced by a spectrum of releasers that include drugs which may be similar to amphetamine, which has been shown to produce predominately rate-increasing effects (Esposito et al, 1980; Kling-Petersen et al., 1994; Lin et al., 2000; Wise and Munn, 1995); MDMA, which has been shown to produce both increases in low rates of behavior and decreases in maximum rates (Lin et al., 1997); and/or fenfluramine which has only been shown to produce decreases in behavior (Olds and Yuwiler, 1992; Olds, 1995). The sensitivity of the frequency-rate procedure to the apparent abuse-limiting effects of 5HT will also permit further evaluation of the mechanisms by which these rate-decreasing effects are produced.

Finally, the other major advantage described by Carlezon and Chartoff is the range of baseline rates of behavior upon which drug effects can be evaluated. For example, stimulus parameters commonly used in ICSS studies employ stimulus delivery within milliseconds of a response, stimulus durations of 0.5 sec or less, and stimulus magnitudes that vary across frequency or amplitude increments as low as 0.05 log units. These characteristics have been exploited to develop ICSS procedures that can engender a wide range of baseline behavioral rates during relatively short (10 min) experimental sessions, thus making ICSS sensitive to rate-dependent effects produced by drugs. “Rate-dependency” in behavioral pharmacology describes a phenomenon wherein the effect of a drug on the rate of a behavior varies systematically as a function of the baseline, pre-drug rate of that behavior (Dews, 1958; Sanger and Blackman, 1976; Dews, 1981). This advantage of ICSS will be utilized later in this dissertation to explore the utility of rate-dependency analysis for the separation and stratification of the abuse-related behavior effects of drugs.
Drug Discrimination

A secondary behavioral procedure used in this dissertation was drug discrimination. Drug discrimination is an assay that attempts to relate the “subjective effects” (more precisely the interoceptive discriminative stimulus effects) of a novel drug to the subjective effects of a training drug (Schuster and Johanson, 1988; Dykstra et al., 1997; Oliveto et al., 1998). This procedure was described in its most popular two-key form by Kubena and Barry in 1969. Two-key drug discrimination is performed by conditioning an animal to respond on one key following saline administration or on a second key following a training drug. Responding on the appropriate key (saline key after saline administration, drug key after drug administration) results in the delivery of a food pellet. Following administration of a new compound, responding on the drug key indicates that the compound produces subjective effects similar to those produced by the training drug. This type of procedure is described in detail by Solinas et al. (2006).

The training drug used in this dissertation was cocaine. Cocaine, being a prominently abused psychomotor stimulant, has been shown to share discriminative stimulus effects with many other commonly abused psychomotor stimulants including amphetamine and methamphetamine (Callahan et al., 1991; La Garza, 1983; La Garza, 1985; Peltier et al., 1996). It is for this reason that cocaine discrimination has become an assay commonly used to determine whether a novel compound produces abuse-related discriminative stimulus effects similar to those of cocaine; additionally, subjective effects are thought to be one of the determinants of abuse, and compounds that share subjective effects may also share similar potentials for abuse (Spealman, 1992; also Ator and Griffiths, 2003). It is for this reason that cocaine was chosen as the test drug for these
studies – to probe the abuse-related subjective effects of established and novel monoamine releasers.

**Summary**

The background information provided here suggests that pharmacological selectivity to promote release of DA, NE, and/or 5HT is one determinant of the abuse-related effects produced by MARs. This dissertation will confirm and extend upon data supporting the hypothesis that selectivity for DA/NE versus 5HT is required for the expression of abuse-related effects produced by MARs in an assay of ICSS (Chapter II), and I will discuss these findings in terms of rate-dependence (Chapter III). Also examined will be studies attempting to elucidate the mechanism by which 5HT exerts its apparent abuse-limiting effects (Chapter IV). Finally, data will be presented addressing the hypothesis that selectivity for NE versus DA is required for the expression of abuse-related effects produced by MARs in assays of ICSS and cocaine discrimination (Chapter V). It is my hope that these data will help in the future development of safer medications for the treatment of ADHD, obesity, and/or narcolepsy.
**Chapter II: Use of Intracranial Self-Stimulation to Evaluate Abuse-Related and Abuse-Limiting Effects of Monoamine Releasers in Rats**

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**Introduction**

The purpose of these studies was to evaluate sensitivity of ICSS to differences in abuse-related effects produced by a series of 11 monoamine releasers that varied across a >8000-fold range in their pharmacological selectivity to promote release of DA/NE vs. 5HT. The work was designed to test two related hypotheses. First, we hypothesized that efficacy to facilitate ICSS would correlate with pharmacological selectivity to promote release of DA/NE vs. 5HT. This hypothesis was also examined by evaluating effects of drug mixtures that contained various proportions of a DA/NE-selective and a 5HT-selective releaser. Second, we hypothesized that efficacy to facilitate ICSS in rats would correlate with previously published efficacy to maintain drug self-administration in nonhuman primates responding under a progressive-ratio schedule. Confirmation of these hypotheses would clarify one of the pharmacological determinants (selectivity for DA/NE vs. 5HT) of the abuse-related effects produced by monoamine releasers and would further support the use of ICSS in abuse liability assessment.
Materials and Methods

Subjects

Thirty-eight adult male Sprague Dawley rats (Harlan, Frederick, MD, USA) were used. All rats had free access to water and were housed individually on a 12 hour light-dark cycle (lights on from 6 a.m. – 6 p.m.) in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. All rats also had free access to food and weighed between 314 and 375 grams at the time of surgery. Animal maintenance accorded with The National Institutes of Health guidelines on care and use of animal subjects in research (National Research Council, 2011). Experimental protocols were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee.

Assay of Intracranial Self-Stimulation

Surgery. Subjects were anesthetized with 2.5% isoflurane gas until unresponsive to toe-pincht. A stereotaxic device (Kopf, Tujunga, CA) was used to insert the cathode (0.25mm diameter) of a bipolar electrode into the left medial forebrain bundle at the level of the lateral hypothalamus (2.8mm posterior to Bregma, 1.7mm lateral to the midsaggital line, and 8.8 mm ventral to the skull). Three screws were placed in the skull, and the anode (0.125mm diameter, uninsulated) was wrapped around one of the screws to act as a ground. Dental acrylic was used to secure the electrode to the screws (and thus to the skull). Ketoprofen (5mg/kg) was used as a post-operative analgesic immediately and 24hrs after surgery. Animals were allowed to recover for at least 5 days before beginning ICSS training.
**Apparatus.** Operant chambers consisted of sound-attenuating boxes containing modular acrylic and metal test chambers (29.2x30.5x24.1 cm). Each chamber had a response lever (4.5 cm wide, 2.0 cm deep, 3.0 cm off the floor), three stimulus lights (red, yellow, and green) centered 7.6 cm above the response lever, a 2-W house light, and an ICSS stimulator (Med Associates, St. Albans, VT, USA). Bipolar cables routed through a swivel-commutator connected the stimulator to the electrode (Model SL2C, Plastics One, Roanoke, VA, USA). Med-PC IV computer software controlled all programming parameters and data collection (Med Associates, St. Albans, VT, USA).

**Training.** The behavioral procedure was similar to that described previously (Negus et al., 2010; Altarifi and Negus, 2011). In brief, the house light was illuminated during behavioral sessions, and lever press responding under a fixed-ratio 1 (FR 1) schedule produced delivery of a 0.5-s train of square-wave cathodal pulses (0.1ms/pulse). Stimulus lights over the lever were illuminated, and responding had no scheduled consequences, during delivery of the intracranial stimulus. During initial 60-min training sessions, stimulation intensity was set at 150µA, and stimulation frequency was set at 126 Hz. Stimulation intensity was then individually manipulated in each rat to identify an intensity that maintained a high rate of reinforcement (>30 stimulations/min). Once an appropriate intensity was identified, changes in frequency were introduced during sessions consisting of three consecutive 10-minute components, each of which contained 10 60-second trials. The stimulation frequency was 158 Hz for the first trial of each component, and frequency decreased in 0.05 log unit steps during the subsequent nine
trials to a final frequency of 56 Hz. Each trial began with a 10-s time out period, during which responding had no scheduled consequences, and 5 non-contingent stimulations at the designated frequency were delivered at 1-sec intervals during the last 5 sec of the time out. During the remaining 50 sec of each trial, responding produced both intracranial stimulation at the designated frequency and illumination of the lever lights under a fixed-ratio (FR) 1 schedule as described above. Training continued until frequency-rate curves were not statistically different over three days of training as indicated by lack of a significant effect of “day” in a two-way analysis of variance (ANOVA) with frequency and day as the two variables (see data analysis). All training was completed within 5 weeks of surgery.

Testing. For dose-effect studies, test sessions consisted of three consecutive “baseline” components followed first by a 20-min time out period and then by three consecutive “test” components. A single dose of a single drug was administered at the beginning of the time out, immediately after the baseline components and before the test components. All test drugs are listed in Table II.1. Also tested were mixtures of PAL-353 and fenfluramine in proportions of 1:1, 1:3 and 1:10 (PAL-353:fenfluramine). These proportions were based on the relative potencies of PAL-353 and fenfluramine to alter ICSS and were intended to permit assessment of PAL-353 in combination with relatively low, intermediate and high proportions of fenfluramine. Time course studies were also conducted with the highest dose of each individual compound (with the exception that the penultimate dose was tested with PAL-287 because the highest dose produced lethality in some rats). Time course test sessions consisted of three consecutive baseline components
followed immediately by drug injection and then by pairs of consecutive test components initiated 10, 30 and 100 min after drug injection. In some cases, additional pairs of test components were initiated 300 min or 24 hr after drug injection. Due to the different durations of action of PAL-353 and fenfluramine, time courses were not determined for the fixed-proportion mixtures. Test sessions were usually conducted on Tuesdays and Fridays, and three-component training sessions were conducted all other weekdays. The order of drug dose was varied across subjects using a Latin-Square design. Experiments with any one compound were completed before progressing to another experimental manipulation, and order of drug testing was irregular across rats. Tests with different compounds with a given rat were separated by at least one week, and during this inter-drug interval, a saline/vehicle test session was conducted to assure that injections and/or previous treatments did not alter ICSS measures.

Data Analysis. The primary dependent measure was the reinforcement rate in stimulations/trial. Raw reinforcement rates were normalized to the maximum control rate (MCR) for each subject on each day, where MCR was defined as the mean of the maximal rates observed during the second and third “baseline” components for that day. Therefore, %MCR was equal to [(response rate during a frequency trial) / (maximum control rate)] x 100. Data for each frequency were averaged across test components for each rat and then across rats to yield a “frequency-rate” curve for each experimental manipulation. Two-way ANOVA was used to compare frequency-rate curves, with ICSS frequency as one variable and dose or time as the second factor. A Holm-Sidak post-hoc
test followed all significant ANOVA’s, and p-values less than 0.05 were considered significant.

Two additional dependent measures were calculated to summarize data from frequency-rate curves. First, ICSS thresholds were calculated where possible using linear regression through data on the linear portion of the frequency-rate curve as described previously (Elmer et al., 2009; Pereira Do Carmo et al., 2009), with the exception that “threshold” was defined as the x-intercept rather than as frequency maintaining 50% of control rates, because the x-intercept is less vulnerable to perturbation by changes in maximal response rates (Miliaressis et al., 1986; Carlezon and Chartoff, 2007). For most ICSS curves, the linear portion of the curve was operationally defined as all data between 20% and 80% MCR, as well as the first point below 20% and the first point above 80% MCR. If peak asymptotic ICSS rates fell between 50-80% MCR, then the regression included only data up to the first point of the asymptote, defined operationally as the point at which further increases in stimulation frequency produced ≤10% increase in ICSS rate. To further protect against perturbations in threshold associated with global changes in response rates, regressions were not calculated and thresholds were not determined if ICSS rates failed to go below 20% MCR or above 50% MCR across the entire frequency range (e.g. see Carlezon and Chartoff, 2007). Thresholds were determined before and after drug administration on each day, and drug effects were expressed as % reduction in threshold relative to baseline thresholds for that day. As a second summary measure of ICSS, the total number of stimulations delivered across all frequencies within a component was summed. The mean number of total stimulations per component was determined before and after drug administration on each day, and
drug effects were expressed as the % baseline number of stimulations per component for that day. Because this second summary measure did not rely on fitting data to an equation, it did not require data selection or modification, and it could be applied to results from all treatment conditions.

Finally, correlations were evaluated between maximum facilitation of ICSS produced by each drug in this study and (a) in vitro selectivity to promote release of DA vs. 5HT (Rothman et al., 2001; Rothman et al., 2002; Rothman et al., 2005; Wee et al., 2005; Wang and Woolverton, 2007), and (b) maximal break point maintained under a progressive-ratio schedule of reinforcement in rhesus monkeys (Wee et al., 2005; Wang and Woolverton, 2007) (Table II.1). Maximal facilitation of ICSS was defined using each of the two summary measures as either (a) the maximal decrease in threshold, or (b) the maximal increase in total stimulations produced by any dose of each drug. Maximal drug effects on threshold and total stimulations were also correlated with each other. Data were analyzed by linear regression and a Pearson correlation test. A p-value <0.05 was determined to be significant for both the slope of the regression line and for the Pearson correlation test.

**Drugs**

(+) Amphetamine sulfate, S-(+)-methamphetamine HCl and (-)-cocaine HCl were provided by the National Institute on Drug Abuse Drug Supply Program (Bethesda, MD). (±)-Fenfluramine HCl was purchased from Sigma Chemical Co. (St. Louis, MO). All other compounds were synthesized as the fumarate salts by Dr. Bruce Blough (Research
Triangle Park, NC). All compounds were prepared in sterile saline and administered intraperitoneally (I.P.). Doses are expressed in terms of the salt forms above.

**Results**

Under baseline conditions, electrical brain stimulation maintained a frequency-dependent increase in ICSS rates (e.g. Figure II.1, open circles). The average±SEM MCR for these studies was 58.6 ± 1.34 stimulations per trial. In general, low rates of ICSS were maintained by low stimulation frequencies (1.75-1.90 log Hz). ICSS rates increased at intermediate stimulation frequencies (1.90-2.05 log Hz), and high, asymptotic ICSS rates were maintained by high stimulation frequencies (2.05-2.20 log Hz). The mean±SEM baseline threshold was 74.0 ± 0.8 Hz (1.87 log Hz). The mean±SEM number of total stimulations earned during baseline components was 276.09 ± 9.4 stimulations per component.

Figure II.1 (A-H) shows that the DA-selective monoamine releasers robustly facilitated ICSS across a broad range of doses. In this and all subsequent figures, drugs are ordered from top to bottom in order of decreasing selectivity for DA vs. 5HT. Left panels show drug effects on full frequency-rate curves, right panels show summary data expressed as drug effects on the percent baseline number of total stimulations per component. As shown in Table II.1, PAL-353, amphetamine, phenmetrazine, and methamphetamine each have ≥30 fold selectivity for promoting release of DA vs. 5HT, and all four of these drugs produced exclusively rate-increasing effects across a 10- to 30-fold range of doses. Amphetamine and methamphetamine were the highest potency compounds, (significant facilitation of ICSS at doses ≥ 0.032mg/kg), followed by PAL-
353 (≥0.32 mg/kg) and phenmetrazine (≥1.0 mg/kg). All drugs produced maximal increases in ICSS to at least 160% of the baseline number of reinforcers. Only phenmetrazine showed evidence of asymptotic stimulation across the dose range tested, with 10 mg/kg producing a lower mean increase in ICSS than the lower dose of 3.2 mg/kg. PAL-314 (Figure II.1 I,J), which has 6.5-fold selectivity to release DA vs. 5HT (Table II.1), produced only rate-increasing effects at lower doses (1.0 and 3.2 mg/kg); however, a higher dose of 10 mg/kg PAL-314 decreased higher rates of ICSS maintained by higher frequencies of brain stimulation (significant at 2.0-2.2 log Hz). Table II.2 shows that these drugs all decreased ICSS threshold values. However, thresholds could not be calculated at higher drug doses, because at these doses, ICSS rates exceeded 20% MCR at all brain stimulation frequencies.

Figure II.2 (A-H) shows the time courses of selected doses of each compound. PAL-353 (1.0 mg/kg), amphetamine (1.0 mg/kg), and phenmetrazine (3.2 mg/kg) produced peak facilitation at the earliest time point (10 min), and facilitation of ICSS was no longer apparent after 100 min (Fig. II.2 A-F). Methamphetamine (1.0 mg/kg) facilitated ICSS with a similarly rapid rate of onset but a longer duration of action, and methamphetamine effects were no longer apparent after 300 min (Fig. II.2 G,H). PAL-314 (10 mg/kg) produced only rate-decreasing effects after 10 min, but this transitioned to mixed rate-increasing and rate-decreasing effects after 30 min and exclusive rate-increasing effects after 100 min before effects fully dissipated by 300 min (Fig. II.2 I,J).

Figure II.3 (A-H) shows that releasers with lower selectivity to release DA vs. 5HT produced weaker facilitation of ICSS across a narrower range of doses than did more DA-selective releasers. Specifically, PAL-313, (+)MDMA, PAL-287 and
(−)MDMA produced maximal stimulation of ICSS to levels less than 130% of baseline, and none of these releasers produced exclusive facilitation of ICSS across more than a 3.2-fold range of doses before rate-decreasing effects emerged. The 5HT-selective releaser, fenfluramine, produced exclusively rate-decreasing effects at a dose of 3.2mg/kg (Fig. II.3 I,J). Table II.2 shows that all these drugs except fenfluramine decreased ICSS thresholds. Again, though, thresholds could not be calculated at high doses of some drugs either because ICSS rates exceeded 20% MCR at all frequencies ((+)MDMA) or because rates failed to achieve a minimum of 50% MCR at any frequency (PAL-287, fenfluramine).

Figure II.4 (A-J) shows the time courses of 3.2mg/kg of each compound except (−)MDMA (10mg/kg). For all drugs except fenfluramine, rate-decreasing effects were significant and tended to predominate at early time points, whereas rate-increasing effects were significant and tended to predominate at later time points. With the 5HT-selective releaser fenfluramine, the greatest decrease in responding was seen at 30min, and this effect was nearly gone by 24hrs (Fig. II.4 I,J). Fenfluramine did not facilitate ICSS at any dose or pretreatment time at any frequency of brain stimulation.

(±)MDMA (0.15-fold selective for DA vs. 5HT; Table II.1) was also tested and produced rate-increasing effects at 1.0mg/kg and both rate-increasing and rate-decreasing effects at 3.2mg/kg. A time course of 3.2mg/kg showed mixed rate-increasing and rate-decreasing effects at 10 and 30min, exclusively rate-increasing effects at 100min, and no effect at 300min (detailed data not shown, summary data included in Fig. II.6 and Table II.2).
Figure II.5 shows the effects of 1:1, 1:3 and 1:10 mixtures of the DA-selective releaser PAL-353 and the 5HT-selective releaser fenfluramine. The 1:1 mixture produced exclusive and dose-dependent rate-increasing effects over a 10-fold dose range and increased the % baseline reinforcers to a maximum of 151% (Fig. II.5 A,B). The 1:3 mixture produced only rate-increasing effects at the lower two doses and a maximum facilitation of ICSS to 150% baseline, but both rate-increasing and rate-decreasing effects were observed at the highest dose (Fig. II.5 C,D). The 1:10 mixture produced only rate-increasing effects at the lowest dose to a maximum of 120% baseline, both rate-increasing and rate-decreasing effects at the middle dose, and only rate-decreasing effects at the highest dose (Fig. II.5 E,F). Thus, increasing proportions of fenfluramine were associated with decreases in the maximum facilitation of ICSS and decreases in the range of doses across which facilitation was observed.

Figure II.6A shows the correlation between maximal facilitation of ICSS (expressed as maximal increase in total stimulations) and previously published data regarding in vitro selectivity to release DA vs. 5HT (Table II.1). The slope of the regression line was 29.6 ± 5.2 and was significantly different from zero (p=<0.0001). A Pearson correlation test showed a significant correlation (Pearson r=0.89, R²=0.78,p=0.0006). Fenfluramine was excluded from this figure because it did not facilitate ICSS at any dose or time and because precise selectivity could not be quantified due to low potency to release DA. Figure II.6B shows the correlation between maximal facilitation of ICSS and in vivo efficacy to main drug self-administration under a progressive-ratio procedure in rhesus monkeys (Table II.1). The slope of the regression line was 15.8 ± 3.0 and was significantly different from zero (p=<0.0001). A Pearson
correlation test also showed a significant correlation (Pearson r=0.80, $R^2=0.63$, $p=0.0320$). (-)MDMA and fenfluramine were excluded from the correlation because they did not facilitate ICSS in rats and/or did not reliably maintain self-administration in monkeys (self-administration by <50% of monkeys tested). PAL-287 and phenmetrazine were also excluded, because they have not been tested by Woolverton and colleagues under the progressive-ratio schedule of drug self-administration in rhesus monkeys.

Correlations were also determined using maximal reductions in ICSS thresholds from Table II.2. However, drug-induced changes in thresholds did not correlate significantly with either in vitro selectivity to release DA vs. 5HT ($p=0.32$) or with progressive-ratio break points in rhesus monkeys ($p=0.67$), and they also did not correlate with drug-induced changes in total stimulations ($p=0.98$).
**Summary**

This study determined effects of 11 monoamine releasers on ICSS in rats. There were two main findings. First, maximal degrees of ICSS facilitation (%Baseline Stimulations) correlated with *in vitro* selectivity to promote release of DA versus 5HT. Moreover, addition of a selective 5HT releaser (fenfluramine) to a selective DA releaser (PAL-353) produced a proportion-dependent attenuation of ICSS facilitation produced by the DA-selective releaser. These results suggest that serotonergic effects of monoamine releasers are sufficient to oppose and limit DA-mediated facilitation of ICSS. The second major finding of this study was that maximal degrees of ICSS facilitation in rats correlated with maximal break points maintained under a progressive-ratio schedule of reinforcement in rhesus monkeys. This latter correlation suggests that ICSS may be useful not only for qualitative identification of drugs with abuse potential, but also for quantitative stratification of relative abuse liability across different drugs. Taken together, these results support the use of ICSS in rats as an experimental tool to study the pharmacological determinants of abuse-related effects of monoamine releasers.
Table II.1: Previously published data on pharmacological selectivity and reinforcing efficacy of monoamine releasers; these data were correlated with ICSS data generated in the present study (Figure II.6). Each row shows (from left to right) drug name, in vitro EC$_{50}$ values (nM ± SD) to promote release of dopamine (DA) or 5HT (5HT), pharmacological selectivity expressed as the ratio of EC50 for 5HT release ÷ ICS50 for DA release, and maximum number of injections per session maintained in nonhuman primates responding under a progressive-ratio schedule of drug self-administration (Max inj/session in NHP SA).

<table>
<thead>
<tr>
<th>Drug</th>
<th>DA</th>
<th>5HT</th>
<th>Selectivity (5HT/DA)</th>
<th>Max. inj/session in NHP SA</th>
</tr>
</thead>
<tbody>
<tr>
<td>m-fluroamphetamine (PAL-353)</td>
<td>24.2 ± 1.1</td>
<td>1937 ± 202</td>
<td>80$^4$</td>
<td>11.9 ± 1.1$^4$</td>
</tr>
<tr>
<td>(+)-amphetamine (Amphetamine)</td>
<td>24.8 ± 3.5</td>
<td>1765 ± 94</td>
<td>71$^1$</td>
<td>13.8 ± 0.6$^3$</td>
</tr>
<tr>
<td>(+)-phenmetrazine (Phenmetrazine)</td>
<td>87.4 ± 7.8</td>
<td>3246 ± 263</td>
<td>37$^2$</td>
<td>Not Tested</td>
</tr>
<tr>
<td>S-(-)-methamphetamine (Methamphetamine)</td>
<td>24.5 ± 2.1</td>
<td>736 ± 45</td>
<td>30$^1$</td>
<td>13 ± 1$^5$</td>
</tr>
<tr>
<td>m-methylamphetamine (PAL-314)</td>
<td>33.3 ± 1.3</td>
<td>218 ± 22</td>
<td>6.5$^4$</td>
<td>11.6 ± 1.2$^3$</td>
</tr>
<tr>
<td>p-methylamphetamine (PAL-313)</td>
<td>44.1 ± 2.6</td>
<td>53.4 ± 4.1</td>
<td>1.2$^4$</td>
<td>8.9 ± 1.7$^3$</td>
</tr>
<tr>
<td>(+)-3,4-methylenedioxymethamphetamine ((+)MDMA)</td>
<td>142 ± 4.0</td>
<td>74 ± 3.0</td>
<td>0.52$^5$</td>
<td>10 ± 1$^5$</td>
</tr>
<tr>
<td>naphthylisopropylamine (PAL-287)</td>
<td>12.6 ± 0.4</td>
<td>3.4 ± 0.2</td>
<td>0.27$^7$</td>
<td>Not Tested</td>
</tr>
<tr>
<td>(±)-3,4-methylenedioxymethamphetamine ((±)MDMA)</td>
<td>376 ± 16</td>
<td>56.6 ± 2.1</td>
<td>0.15$^5$</td>
<td>9 ± 1$^5$</td>
</tr>
<tr>
<td>(-)-3,4-methylenedioxymethamphetamine ((-)MDMA)</td>
<td>3700 ± 100</td>
<td>340 ± 20</td>
<td>0.09$^5$</td>
<td>4.7 ± 0.8$^5$ *</td>
</tr>
<tr>
<td>(±)-fenfluramine (Fenfluramine)</td>
<td>&gt;10,000</td>
<td>79.3 ± 11.5</td>
<td>&lt;0.01$^4$</td>
<td>**</td>
</tr>
</tbody>
</table>

*Maintained responding in fewer than half of the monkeys tested.

**Does not maintain self-administration$^{16}$.

Citations: 1) Rothman et al., 2001; 2) Rothman et al., 2002; 3) Rothman et al., 2005; 4) Wee et al., 2005; 5) Wang et al., 2007.
Table II.2: Percent reduction in threshold produced by each dose of each drug relative to baseline. The last column shows the maximum decrease in threshold produced by each drug.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Veh</th>
<th>0.032</th>
<th>0.1</th>
<th>0.32</th>
<th>1.0</th>
<th>3.2</th>
<th>10.0</th>
<th>Δ_{max}</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAL-353</td>
<td>4.2</td>
<td>1.5</td>
<td>7.8</td>
<td>24.5</td>
<td>NC*</td>
<td>NC*</td>
<td>--</td>
<td>24.5</td>
</tr>
<tr>
<td>Amphetamine</td>
<td>4.3</td>
<td>8.3</td>
<td>7.4</td>
<td>32.1</td>
<td>NC*</td>
<td>--</td>
<td>--</td>
<td>32.1</td>
</tr>
<tr>
<td>Phenmetrazine</td>
<td>-3.4</td>
<td>--</td>
<td>--</td>
<td>-7.2</td>
<td>29.4</td>
<td>NC*</td>
<td>NC*</td>
<td>29.4</td>
</tr>
<tr>
<td>Methamphetamine</td>
<td>5.5</td>
<td>1.0</td>
<td>15.6</td>
<td>NC</td>
<td>NC</td>
<td>--</td>
<td>--</td>
<td>15.6</td>
</tr>
<tr>
<td>PAL-314</td>
<td>3.7</td>
<td>--</td>
<td>-0.1</td>
<td>11.2</td>
<td>20.6</td>
<td>NC</td>
<td>NC</td>
<td>20.6</td>
</tr>
<tr>
<td>(+)MDMA</td>
<td>-10.6</td>
<td>--</td>
<td>0.3</td>
<td>7.7</td>
<td>NC*</td>
<td>NC*</td>
<td>--</td>
<td>7.7</td>
</tr>
<tr>
<td>PAL-287</td>
<td>2.5</td>
<td>--</td>
<td>--</td>
<td>11.6</td>
<td>0.4</td>
<td>17.5</td>
<td>NC**</td>
<td>17.5</td>
</tr>
<tr>
<td>(±)MDMA</td>
<td>0.1</td>
<td>--</td>
<td>4.4</td>
<td>17.5</td>
<td>14.9</td>
<td>NC*</td>
<td>--</td>
<td>17.5</td>
</tr>
<tr>
<td>(-)MDMA</td>
<td>-0.1</td>
<td>--</td>
<td>--</td>
<td>12.2</td>
<td>16.6</td>
<td>28.3</td>
<td>27.4</td>
<td>28.3</td>
</tr>
<tr>
<td>Fenfluramine</td>
<td>2.9</td>
<td>--</td>
<td>--</td>
<td>-2.6</td>
<td>-13.7</td>
<td>NC**</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

NC = Not calculated
*No points fell below 20% of baseline.
**Maximum responding did not reach 50% of baseline.
Chapter II Figure Legends

Figure II.1. Effect of PAL-353, amphetamine, phenmetrazine, methamphetamine, and PAL-314 on ICSS. Left panels (A, C, E, G, I) show drug effects on full ICSS frequency-rate curves. Abscissae: Frequency of electrical brain stimulation in Log Hz. Ordinates: Percent maximum control reinforcement rate (%MCR). Drug name and doses are indicated in legends. Filled points represent frequencies at which reinforcement rates were statistically different from vehicle rates as determined by a two-way ANOVA followed by a Holm-Sidak post hoc test, p<0.05. Right panels (B, D, F, H, J) show summary ICSS data expressed as percent predrug baseline number of reinforcers delivered across all frequencies of brain stimulation. Abscissae: drug dose in mg/kg. Ordinates: Percent predrug baseline number of reinforcers. The drug and pretreatment time are shown for each panel. Upward/downward arrows indicate significant drug-induced increase/decrease in ICSS relative to vehicle for at least one brain stimulation frequency as determined by analysis of full frequency-rate curves. All data show mean ± SEM for 5-7 rats (except for 3.2mg/kg PAL-353, n=4). Statistical results for data in left panels are as follows: A. PAL-353 0.1-1.0 mg/kg (n=7): significant main effect of frequency [F(9,54)=88.6, p<0.001], dose [F(3,18)=38.8, p<0.001] and significant interaction [F(27,162)=7.9, p<0.001]. PAL-353 3.2 mg/kg (n=4): significant main effect of frequency [F(9,27)=17.1, p<0.001], dose [F(1,3)=12.0, p=0.04] and significant interaction [F(9,27)=18.8, p<0.001]. C. Amphetamine (n=6): significant main effect of frequency [F(9,45)=144.2, p<0.001], dose [F(4,20)=55.4, p<0.001] and significant interaction [F(36,180)=10.5, p<0.001]. E. Phenmetrazine (n=5): significant main effect of frequency [F(9,36)=22.8, p<0.001], dose [F(4,16)=4.9, p<0.009] and significant
Interaction \[F(36,144)=3.8, \ p<0.001\]. \textbf{G.} Effect of methamphetamine (n=5): significant main effect of frequency \[F(9,36)=56.2, \ p<0.001\], dose \[F(4,16)=40.4, \ p<0.001\] and significant interaction \[F(36,144)=6.6, \ p<0.001\]. \textbf{I.} PAL-314 (n=6): significant main effect of frequency \[F(9,45)=67.9, \ p<0.001\], dose \[F(4,20)=6.0, \ p=0.003\] and significant interaction \[F(36,180)=11.8, \ p<0.001\].

\textbf{Figure II.2.} Time courses of PAL-353, amphetamine, phenmetrazine, methamphetamine and PAL-314. Left panels (A, C, E, G, I) show drug effects on full ICSS frequency-rate curves. Right panels (B, D, F, H, J) show summary ICSS data expressed as percent predrug baseline number of reinforcers delivered across all frequencies. Other details as in Figure II.1. All data show mean ± SEM for 5-7 rats. Statistical results for data in left panels are as follows: A. PAL-353 (n=7): significant main effect of frequency \[F(9,54)=40.6, \ p<0.001\], time \[F(5,30)=29.5, \ p<0.001\], and significant interaction \[F(45,270)=3.3, \ p<0.001\]. C. Amphetamine (n=5): significant main effect of frequency \[F(9,36)=63.9, \ p<0.001\], time \[F(4,16)=48.2, \ p<0.001\], and significant interaction \[F(36,144)=3.5, \ p<0.001\]. E. Phenmetrazine (n=5): significant main effect of frequency \[F(9,36)=23.4, \ p<0.001\], time \[F(3,12)=18.6, \ p<0.001\], and significant interaction \[F(27,108)=4.5, \ p<0.001\]. G. Methamphetamine (n=5): significant main effect of frequency \[F(9,36)=36.4, \ p<0.001\], time \[F(4,16)=14.9, \ p<0.001\], and significant interaction \[F(36,144)=10.0, \ p<0.001\]. I. PAL-314 (n=5): significant main effect of frequency \[F(9,36)=47.8, \ p<0.001\], time \[F(4,16)=14.9, \ p<0.001\] and significant interaction \[F(36,144)=9.5, \ p<0.001\].
Figure II.3. Effect of PAL-313, (+)MDMA, PAL-287, (-)MDMA and fenfluramine on ICSS. Left panels (A, C, E, G, I) show drug effects on full ICSS frequency-rate curves. Right panels (B, D, F, H, J) show summary ICSS data expressed as percent predrug baseline number of reinforcers delivered across all frequencies. Other details as in Figure II.1. All data show mean ± SEM for 5-7 rats (except for 10.0mg/kg PAL-287, n=3). Statistical results for data in left panels are as follows: A. PAL-313 (n=5): significant main effect of frequency [F(9,36)=44.9, p<0.001] but not dose [F(3,12)=2.9, p=0.079]. There was a significant interaction [F(27,108)=3.8, p=<0.001]. C. (+)MDMA: significant main effect of frequency [F(9,36)=51.0, p<0.001], dose [F(4,16)=3.6, p=0.028] and significant interaction [F(36,144)=4.0, p<0.001]. E. PAL-287 0.32-3.2 mg/kg (n=5): significant main effect of frequency [F(9,36)=71.6, p<0.001], dose [F(3,12)=4.7, p<0.021] and significant interaction [F(27,108)=3.9, p=<0.001]. PAL-287 10.0 mg/kg (n=3): significant main effect of frequency [F(9,18)=27.0, p<0.001] but not dose [F(1,2)=3.1, p=0.222]. There was a significant interaction [F(9,18)=21.7, p=<0.001]. Additional rats were not tested at 10 mg/kg due to lethality. G. (-)MDMA: significant main effect of frequency [F(9,36)=72.4, p<0.001] but not dose [F(3,12)=3.4, p=0.052]. There was a significant interaction [F(27,108)=3.8, p<0.001]. I. Fenfluramine (n=7): significant main effect of frequency [F(9,54)=70.3, p<0.001], dose [F(3,18)=33.5, p<0.001] and significant interaction [F(27,162)=9.1, p=<0.001].
Figure II.3. Time courses of PAL-313, (+)MDMA, PAL-287, (-)MDMA and fenfluramine. Left panels (A, C, E, G) show time course of drug effects on full ICSS frequency-rate curves. Right panels (B, D, F, H) show summary ICSS data expressed as percent predrug baseline number of reinforcers delivered across all frequencies. Other details as in Figure II.1. All data show mean ± SEM for 5-7 rats. Statistical results for data in left panels are as follows: A. PAL-313 (n=6): significant main effect of frequency [F(9,45)=62.9, p<0.001], time [F(4,20)=3.6, p<0.001] and significant interaction [F(36,180)=8.0, p<0.001]. C. (+)MDMA (n=5): significant main effect of frequency [F(9,36)=36.6, p<0.001], time [F(4,16)=3.733, p=0.025], and significant interaction [F(36,144)=7.803, p<0.001]. E. PAL-287 (n=5): significant main effect of frequency [F(9,36)=85.0, p<0.001], time [F(5,20)=6.5, p<0.001] and significant interaction [F(45,180)=11.0, p<0.001]. G. (-)MDMA (n=6): significant main effect of frequency [F(9,45)=24.2, p<0.001], time [F(4,20)=5.5, p=0.004] and significant interaction [F(36,180)=3.6, p<0.001]. I. Fenfluramine (n=7): significant main effect of frequency [F(9,54)=41.1, p<0.001], time [F(5,30)=14.5, p<0.001] and significant interaction [F(45,270)=3.8, p<0.001].

Figure II.5. Effect of PAL-353/fenfluramine mixtures on ICSS. Left panels (A, C, E) show drug effects on full ICSS frequency-rate curves. Right panels (B, D, F) show summary ICSS data expressed as percent predrug baseline number of reinforcers delivered across all frequencies. Other details as in Figure II.1. All data show mean ± SEM for 5-6 rats. Statistical results for data in left panels are as follows: A. 1:1 PAL-
353/Fenfluramine (n=5): significant main effect of frequency [F(9,36)=66.5, p<0.001], dose [F(3,12)=43.2, p<0.001] and significant interaction [F(27,108)=10.4, p<0.001]. C. 1:3 PAL-353/Fenfluramine (n=6): significant main effect of frequency [F(9,45)=65.8, p<0.001], dose [F(3,15)=7.0, p<0.001] and significant interaction [F(27,135)=8.1, p<0.001]. E. 1:10 PAL-353/Fenfluramine (n=6): significant main effect of frequency [F(9,45)=54.2, p<0.001], dose [F(3,15)=21.6, p<0.001] and significant interaction [F(27,135)=10.3, p<0.001].

Figure II.6. Correlation of ICSS facilitation in rats with (A) in vitro selectivity to promote DA vs. 5HT release and (B) break points maintained under a progressive-ratio schedule of drug self-administration in rhesus monkeys. A. Abscissa: Log selectivity to release DA versus 5HT expressed as the log of selectivity values shown in Table II.1. Ordinate: Maximum facilitation of ICSS expressed as the maximum increase produced by any drug dose in percent pre-drug baseline number of reinforcers delivered across all brain stimulation frequencies (from figures 1 and 3, right panels). Fenfluramine was excluded from this figure because it did not facilitate ICSS at any dose or time and because precise selectivity could not be quantified due to low potency to release DA. B. Abscissa: Maximum break point maintained by any drug dose under a progressive-ratio schedule of drug self-administration in rhesus monkeys. Ordinate. Maximum facilitation of ICSS as in Panel A. (-)MDMA and fenfluramme were excluded from the correlation because they did not facilitate ICSS in rats and/or did not reliably maintain self-administration in monkeys (self-administration by <50% of monkeys tested). PAL-287
and phenmetrazine were also excluded, because they have not been tested by Woolverton and colleagues under the progressive-ratio schedule of drug self-administration in rhesus monkeys.
Figure II.1.
Figure II.2.
Figure II.3.

A. % MCR vs. Frequency (Log Hz)

B. PAL-313 Dose-Effect
20min Pretreatment

C. % MCR vs. Frequency (Log Hz)

D. 4MDMA Dose-Effect
20min Pretreatment

E. % MCR vs. Frequency (Log Hz)

F. PAL-297 Dose-Effect
20min Pretreatment

G. % MCR vs. Frequency (Log Hz)

H. 4MDMA Dose-Effect
20min Pretreatment

I. % MCR vs. Frequency (Log Hz)

J. Fenfluramine Dose-Effect
20min Pretreatment
Figure II.4.

A. MDMA Time Course 10mg/kg

B. PAL-313 Time Course 3.0mg/kg

C. MDMA Time Course 3.0mg/kg

D. PAL-287 Time Course 3.2mg/kg

E. (-)MDMA Time Course 10mg/kg

F. Fentramine Time Course 3.2mg/kg
Figure II.5.
Figure II.6.

A. Correlation between selectivity for dopamine release in vitro and ICSS in rats

B. Correlation between self-administration in monkeys and ICSS in rats
Chapter III: Rate-Dependent Effects of Monoamine Releasers on Intracranial Self-Stimulation in Rats

Accepted: Behavioural Pharmacology, April 2013

Introduction
The aim of the present report was to apply rate-dependency analysis to a previously published ICSS data set of 11 monoamine releasers (Chapter II). The drugs span a >8000-fold range of pharmacological selectivity for releasing DA/NE vs. 5HT, and includes relatively DA/NE-selective releasers (e.g. PAL-353 and amphetamine), the 5HT-selective releaser fenfluramine, and a series of releasers with graded selectivities between these extremes. Rate-dependency plots were generated from mean ICSS data for each dose of each drug as described previously with morphine (Altarifi and Negus, 2011), and these plots were then submitted to regression analysis to compare their -slopes and Y-intercepts. These parameters of the rate-dependency plots were correlated with dose within each drug and with pharmacological selectivity across drugs. Finally, the rate-dependent effects of PAL-353 and fenfluramine mixtures were also examined. The hypothesis was that these compounds would produce rate-dependent effects but that these rate-dependent effects would differ in their “parameters of rate-dependence.”
Materials and Methods

This study consisted of a reanalysis of data from Chapter II. See Chapter II for methodological details.

Data Analysis. The primary dependent measure was the reinforcement rate in stimulations/trial. Raw reinforcement rates were normalized to the maximum control rate (MCR) for each subject on each day, where MCR was defined as the mean of the maximal rates observed during the second and third “baseline” components for that day. Therefore, %MCR was equal to (response rate during a frequency trial) / (maximum control rate) x 100. Mean±SEM MCRs were 58.6±1.3 stimulations per trial. ICSS rates, expressed as %MCR, for each frequency were averaged across (a) the second and third baseline components and (b) across all three test components for each rat and then across rats to yield curves relating brain stimulation frequency to baseline and test ICSS rates for each experimental manipulation. These baseline and test data were then used to generate a rate-dependency plot where the x-axis was log baseline rate and the y-axis was log [(test rate) / (baseline rate) * 100]. Each rate-dependency plot consisted of 10 points for baseline and test rates maintained by each of the 10 different brain stimulation frequencies. Each plot was then submitted to linear regression analysis to determine two parameters: (1) the slope (expressed as -slope so that increasingly steep slopes were expressed as increasingly positive numbers), and (2) Y-intercept (expressed as the intercept at x=1, where the baseline rate equaled 10% MCR, and log baseline rate=1).

Although x=1 is a somewhat arbitrary choice of Y-intercept, it was chosen because of the
emphasis in the field on “threshold” measures of ICSS. The effects of a given dose of a
given drug were considered to be “rate-dependent” if the 95% confidence limits of the
slope did not include “0.” -Slope or Y-intercept parameters between doses of a given
drug or between drugs were considered to be significantly different if 95% confidence
limits did not overlap.

For each drug, the -slope and Y-intercept parameters were graphed as a function
of drug dose (log scale). In addition, rate-dependency across drugs was assessed by
correlating peak -slope (for any dose of each drug) or peak Y-intercept (for any dose of
each drug) with log in vitro selectivity to release of DA vs. 5HT. Data were analyzed by
linear regression and a Pearson correlation test. A p-value <0.05 was determined to be
significant for both the slope of the regression line and for the Pearson correlation test.
All regression analyses were conducted using Prism 5.0c for Mac OS X (GraphPad
Software, La Jolla, CA).

Results

Figure III.1A shows the effects of a 30-fold range of amphetamine doses on ICSS
frequency-rate curves (previously published - see Bauer et al. [2012] for time course,
statistics, and other details). Figure III.1B shows the same data graphed as rate-
dependency plots. Table III.1 shows that all 4 doses of amphetamine produced rate-
dependent effects (insofar as 95% confidence limits of the rate-dependency slopes did not
include “0”), but saline (vehicle) did not. Table III.1 also shows that amphetamine
produced dose-dependent increases in both -slope and Y-intercept parameters of rate-
dependency. The dose-dependency of amphetamine effects on parameters of the rate-dependency plots is also shown in Figure III.1C.

In Figure III.2, panels A-C show effects of representative high doses of the DA/NE-selective releaser PAL-353, the non-selective DA/NE/5HT releaser PAL-287, and the 5HT-selective releaser fenfluramine on ICSS frequency-rate curves (see Bauer et al. [2012] for other details). Figure III.2D shows rate-dependency plots for the effects of these three compounds, and linear regression analyses demonstrated that effects of PAL-353, PAL-287, and fenfluramine on ICSS were rate-dependent (see below and Table III.2). Figure III.3 shows the -slope and Y-intercept data produced by the rate-dependency plots as a function of dose for all doses of PAL-353, PAL-287, fenfluramine and 6 of the other releasers examined (amphetamine was previously shown in Fig. III.1; ±MDMA data (not shown) can be found in Table III.2).

To further examine the relationship between rate-dependency and pharmacological selectivity, the peak effects of each drug on -slope and Y-intercept parameters were correlated with in vitro measures of pharmacological selectivity. Table III.2 shows the peak effects of each drug on slope and Y-intercept parameters; note that, for some drugs, peak effects on these two parameters were produced by different doses. All drugs produced rate-dependent effects, but the profile of rate-dependency varied across drugs. Specifically, all drugs except fenfluramine produced negatively sloped rate-dependency plots, but the slopes and Y-intercepts varied across drugs. Fenfluramine produced a slope that was significantly different from zero (at a single intermediate dose [1.0 mg/kg] shown in Table III.2) thus fulfilling our definition of rate-dependency; however, the slope of this regression line was positive unlike the other drugs tested in this
procedure. Figure III.4 shows the correlation between these -slope and Y-intercept parameters and pharmacological selectivity (data for fenfluramine were excluded from the correlation because selectivity has not been precisely quantified due to very low potency to release DA (Rothman et al., 2001)). The correlation between selectivity and -slope was not statistically significant (Pearson r = 0.4164, R square = 0.1733, p-value = 0.2314), but the correlation between pharmacological selectivity and Y-intercept achieved significance (Pearson r = 0.7069, R square = 0.4997, p-value = 0.0223). The relationship between rate-dependency and pharmacological selectivity was also examined with mixtures of the DA-selective releaser PAL-353 and the 5HT-selective releaser fenfluramine. Figure III.5 panels A-C show effects of representative high doses of 1:1, 1:3 and 1:10 mixtures of PAL-353 and fenfluramine on ICSS frequency-rate curves (previously published - see Bauer et al. [2012] for other details). Figure III.5, panel D, shows the rate-dependency plots for these doses of these mixtures. Figure III.6 shows the relationship between mixture dose and the rate-dependency parameters –slope and Y-intercept for each mixture. Table III.3 shows the peak effects on slope and Y-intercept produced by any dose of each mixture. All three mixtures produced rate-dependent effects at some dose insofar as 95% confidence limits of the rate-dependency slopes did not include “0.” Peak slopes of the rate-dependency plots were not different across mixtures; however, the 1:1 mixture produces the greatest increase in Y-intercept values, and mixtures with higher fenfluramine proportions produced progressively smaller increases in Y-intercept.
Summary

This study applied rate-dependency analysis to behavioral effects produced by the prototypic monoamine releaser amphetamine and 10 other monoamine releasers that varied in their selectivity for releasing DA/NE vs. 5HT on ICSS in rats. Under saline control conditions, this "frequency-rate" ICSS procedure maintained a range of baseline rates that was qualitatively similar to the range of baseline rates engendered by FI schedules that have traditionally played a key role in previous research on rate-dependency of drug effects (Sanger and Blackman, 1976). Consequently, behavior produced under this ICSS procedure was readily amenable to rate-dependency analysis of drug effects. There were three main findings. First, all monoamine releasers produced effects that met the criterion for rate-dependence (i.e. slope of rate-dependency plot significantly different from “0” for at least one dose). Moreover, drug effects on the rate dependency parameters -slope and Y-intercept were generally dose-dependent with two exceptions – the 5HT-selective releaser fenfluramine and non-selective releaser PAL-287. Second, the Y-intercept parameter also correlated with in vitro pharmacological selectivity to release DA vs. 5HT, and Y-intercepts were also higher for PAL-353/fenfluramine mixtures that included higher vs. lower proportions of the DA-selective releaser PAL-353. Lastly, a correlation between peak Y-intercept and breakpoints under a progressive-ratio procedure in nonhuman primates was significant (p-value = 0.0314), suggesting that the Y-intercept parameter of rate-dependency in ICSS may have utility in abuse liability assessment. Taken together, these findings extend to ICSS the range of conditions under which amphetamine and related monoamine releasers produce rate-dependent effects and suggest applications for rate-dependency analysis including a novel
methods of looking at ICSS “thresholds” and the potential use of this measure to predict abuse liability.
Table III.1: Slope and Y-intercept values (95% confidence limits) for rate-dependency plots of amphetamine effects on ICSS shown in Figure III.1C. Note that slope values in Figure III.1C are graphed as “-slope” to yield increasingly positive numbers for increasingly steep slopes.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Slope</th>
<th>Y-intercept</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0.0182</td>
<td>1.936</td>
</tr>
<tr>
<td></td>
<td>(-0.2217 to 0.2581)</td>
<td>(1.748 to 2.124)</td>
</tr>
<tr>
<td>0.032 Amphetamine</td>
<td>-0.3357†</td>
<td>2.290*</td>
</tr>
<tr>
<td></td>
<td>(-0.5020 to -0.1695)</td>
<td>(2.170 to 2.411)</td>
</tr>
<tr>
<td>0.1 Amphetamine</td>
<td>-0.4112†</td>
<td>2.365</td>
</tr>
<tr>
<td></td>
<td>(-0.7089 to -0.1135)</td>
<td>(2.099 to 2.631)</td>
</tr>
<tr>
<td>0.32 Amphetamine</td>
<td>-0.7528†*</td>
<td>2.736*</td>
</tr>
<tr>
<td></td>
<td>(-1.013 to -0.4927)</td>
<td>(2.515 to 2.958)</td>
</tr>
<tr>
<td>1.0 Amphetamine</td>
<td>-0.9340†*</td>
<td>2.903*</td>
</tr>
<tr>
<td></td>
<td>(-0.9741 to -0.8939)</td>
<td>(2.869 to 2.936)</td>
</tr>
</tbody>
</table>

†Indicates significant rate-dependence as indicated by slope values that do not include “0” in the 95% confidence limits.

*Indicates significantly different from vehicle as indicated by non-overlapping confidence intervals.
Table III.2: Peak slope and Y-intercept parameters (95% confidence limits) from the rate-dependency plots of each drug. Note that peak values for the two rate-dependency parameters were sometimes obtained at different drug doses. Pharmacological selectivity of each drug to release DA vs. 5HT is also shown. These values were generated from in vitro synaptosome preparations taken from rat caudate nuclei (DA) or whole brain minus caudate and cerebellum (5HT) (references below).

<table>
<thead>
<tr>
<th>Drug</th>
<th>Selectivity</th>
<th>Dose</th>
<th>Slope</th>
<th>Y-intercept</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PAL-353</strong></td>
<td>80³</td>
<td>1.0</td>
<td>-0.6888</td>
<td>2.715</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(-0.8901 to -0.4874)</td>
<td>(2.587 to 2.844)</td>
</tr>
<tr>
<td><strong>Amphetamine</strong></td>
<td>71¹</td>
<td>1.0</td>
<td>-0.9340</td>
<td>2.903</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(-0.9741 to -0.8939)</td>
<td>(2.869 to 2.936)</td>
</tr>
<tr>
<td><strong>Phenmetrazine</strong></td>
<td>37²</td>
<td>3.2</td>
<td>--</td>
<td>2.723</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(2.628 to 2.817)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>-0.7550</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(-0.8566 to -0.6534)</td>
<td></td>
</tr>
<tr>
<td><strong>Methamphetamine</strong></td>
<td>30¹</td>
<td>1.0</td>
<td>-0.8838</td>
<td>2.849</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(-0.9326 to -0.8349)</td>
<td>(2.815 to 2.884)</td>
</tr>
<tr>
<td><strong>PAL-314</strong></td>
<td>6.5⁴</td>
<td>3.2</td>
<td>--</td>
<td>2.750</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(2.678 to 2.822)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>-0.8907</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(-0.9525 to -0.8289)</td>
<td></td>
</tr>
<tr>
<td><strong>PAL-313</strong></td>
<td>1.2⁴</td>
<td>1.0</td>
<td>--</td>
<td>2.377</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(2.288 to 2.466)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.2</td>
<td>-0.5480</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(-0.6450 to -0.4509)</td>
<td></td>
</tr>
<tr>
<td><strong>(+)MDMA</strong></td>
<td>0.52³</td>
<td>3.2</td>
<td>-0.7982</td>
<td>2.600</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(-0.8512 to -0.7452)</td>
<td>(2.563 to 2.637)</td>
</tr>
<tr>
<td><strong>PAL-287</strong></td>
<td>0.27³</td>
<td>0.32</td>
<td>--</td>
<td>2.263</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(2.085 to 2.440)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.2</td>
<td>-0.3850</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(-0.5315 to -0.2386)</td>
<td></td>
</tr>
<tr>
<td><strong>±MDMA</strong></td>
<td>0.15³</td>
<td>3.2</td>
<td>-9.092</td>
<td>2.739</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(-9.765 to -0.8419)</td>
<td>(2.686 to 2.793)</td>
</tr>
<tr>
<td><strong>(-)MDMA</strong></td>
<td>0.09³</td>
<td>10</td>
<td>-0.6109</td>
<td>2.379</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(-0.7893 to -0.4324)</td>
<td>(2.256 to 2.502)</td>
</tr>
<tr>
<td><strong>1.0 Fenfluramine</strong></td>
<td>&lt;0.01¹</td>
<td>1.0</td>
<td>0.3542</td>
<td>1.599*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.2628 to 0.4456)</td>
<td>(1.537 to 1.661)</td>
</tr>
</tbody>
</table>

*Fenfluramine produced a positive slope. Therefore, “peak” effect is lower than 2.0.

Table III.3: Peak slope and Y-intercept (and 95% confidence limits) for mixtures.

<table>
<thead>
<tr>
<th>Proportion PAL-353:Fenfluramine</th>
<th>Dose of PAL-353</th>
<th>Slope</th>
<th>Y-intercept</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1 Mixture</td>
<td>1.0</td>
<td>-0.9132 (-0.9303 to -0.8962)</td>
<td>2.862 (2.850 to 2.874)</td>
</tr>
<tr>
<td>1:3 Mixture</td>
<td>1.0</td>
<td>-0.9075 (-0.9944 to -0.8206)</td>
<td>2.692 (2.629 to 2.755)$^a$</td>
</tr>
<tr>
<td>1:10 Mixture</td>
<td>0.1</td>
<td>--</td>
<td>2.370 (2.268 to 2.471)$^a$</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>-0.8040 (-0.9283 to -0.6797)</td>
<td>--</td>
</tr>
</tbody>
</table>

$^a$Significantly different from 1:1 Mixture.
Chapter III Figure Legends

Figure III.1. Rate-dependent effects of amphetamine on ICSS. A. Previously published figure (Bauer, 2012) showing the effects of amphetamine on ICSS frequency-rate curves. Abscissa: Frequency of stimulation expressed at Log Hz. Ordinate: ICSS rate expressed as % Maximum Control Rate (%MCR). B. Transformation of data from Panel A into rate-dependency plots showing linear regression lines drawn through points generated by vehicle and 4 doses of amphetamine. Abscissa: Log (baseline rate). Ordinate: Log (% baseline rate). Line at Y=2.0 indicates no change from baseline rates, while line at X=1.0 indicates the position of the Y-intercept values used in Panel C. C. -slope and Y-intercept values generated by regression lines in Panel B are plotted against dose of amphetamine. Abscissa: Dose of amphetamine in mg/kg (log scale). Left ordinate: -Slope. Right ordinate: Y-intercept.

Figure III.2. Rate-dependent effects of selected doses of PAL-353, PAL-287, and fenfluramine on ICSS. A-C. Effects of 1.0 mg/kg PAL-353, 3.2 mg/kg PAL-287 and 3.2 mg/kg fenfluramine on ICSS frequency-rates curve compared to the pre-drug baselines. Abscissae: Brain stimulation frequency expressed as Log Hz. Ordinates: ICSS rate expressed as % Maximum Control Rate (%MCR). D. Transformation of data from Panels A-C into rate-dependency plots showing linear regression lines drawn through points generated by the three drugs. Abscissa: Log (baseline rate). Ordinate: Log (% baseline rate).
Figure III.3. Rate-dependency parameters as a function of dose for nine of the monoamine releasers tested. Abscissae: dose in mg/kg (log scale). Left ordinates: Slope. Right ordinates: Y-intercept. Amphetamine (shown previously in Fig. III.1) and ±MDMA are omitted.

Figure III.4. Rate-dependency parameters as a function of pharmacological selectivity to release dopamine vs. serotonin. Abscissae: Pharmacological selectivity expressed as log (in vitro potency to release 5HT ÷ in vitro potency to release DA) as shown in Table III.2. Ordinate (panel A): Peak –slope for any dose of each drug as shown in Table III.2. Ordinate (panel B): Peak Y-intercept for any dose of each drug as shown in Table III.2.

Figure III.5. Rate-dependent effects of selected doses of PAL-353/fenfluramine mixtures on ICSS. A-C. Effects of 1:1, 1:3, and 1:10 mixtures of PAL-353/fenfluramine on ICSS frequency-rate curves compared to the pre-drug baselines. Abscissae: Brain stimulation frequency in log Hz. Ordinate: ICSS rate expressed as % Maximum Control Rate (%MCR). D. Transformation of data from Panels A-C into rate-dependency plots showing linear regression lines drawn through points generated by the mixtures. Abscissa: Log (baseline rate). Ordinate: Log (% baseline rate).

Figure III.6. Rate-dependency parameters as a function of dose for 1:1, 1:3 and 1:10 mixtures of PAL-353 and fenfluramine. Abscissae: dose of PAL-353 in the mixture
Figure III.7. Correlation between breakpoint in monkey self-administration and Y-intercept data generated by rate-dependency analysis of ICSS data from rats.

Abscissa: Maximum break point maintained by any drug dose under a progressive-ratio schedule of drug self-administration in rhesus monkeys. Ordinate: Peak Y-intercept produced by linear regressions of data on a rate-dependency plot. *(-)MDMA and fenfluramine did not reliably maintain self-administration in all monkeys under this paradigm. Monkey self-administration data taken from Wee et al., 2005; Wang and Woolverton, 2007; unpublished observations (fenfluramine).
Figure III.1.
Figure III.2.
Figure III.3.
Figure III.4.
Figure III.5.
Figure III.6.
Figure III.7.

Pearson $r = 0.7984$

$p$-value = 0.0314
Chapter IV: Role of the 5HT$_{2C}$ Receptor in Abuse-Limiting Effects of Monoamine Releasers on Intracranial Self-Stimulation in Rats.

In preparation

Introduction

The purpose of the present study was to evaluate the role of 5HT$_{2C}$ receptors in mediating abuse-related and abuse-limiting effects produced by monoamine releasers in an ICSS procedure in rats. Specifically, rats were trained to lever press for electrical brain stimulation under a “frequency-rate” procedure, in which brain stimulation frequency was varied during each daily session to generate a wide range of baseline ICSS rates. We have reported previously that DA-selective releasers produce an abuse-related increase in low rates of responding maintained by low brain stimulation frequencies, whereas 5HT-selective releasers produce an abuse-limiting decrease in high rates of responding maintained by high brain stimulation frequencies, and non-selective DA/5HT releasers simultaneously produce both facilitation of low ICSS rates and depression of high ICSS rates (Chapter II). In this study, effects on ICSS produced by a DA-selective releaser (amphetamine), a 5HT-selective releaser (fenfluramine), and two non-selective DA/5HT releasers (PAL-287, (+)MDMA) were determined alone and after pretreatment with the 5HT$_{2C}$ antagonist SB 242,084. We hypothesized that the 5HT$_{2C}$ antagonist would block
abuse-limiting effects of non-selective and 5HT-selective releasers and increase expression of DA-mediated abuse-related effects of the non-selective releasers.

**Materials and Methods**

**Subjects**

Twenty-six adult male Sprague Dawley rats (Harlan, Frederick, MD, USA) were used. All rats had free access to water and were housed individually on a 12 hour light-dark cycle (lights on from 6 a.m. – 6 p.m.) in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. All rats also had free access to food and weighed between 298 and 350 grams at the time of surgery. Animal maintenance accorded with The National Institutes of Health guidelines on care and use of animal subjects in research (National Research Council, 2011). Experimental protocols were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee.

**Assay of Intracranial Self-Stimulation**

The Surgery, Apparatus, and Training details of this study are the same as those reported previously [Chapter II].

**Testing.** For dose-effect studies, test sessions consisted of three consecutive “baseline” components followed first by a 25-min time out period and then by three consecutive “test” components. A single dose of a single drug was administered 20 min before the test component. For experiments in which vehicle or SB 242,084 was used as a
pretreatment, the pretreatment injection was given 5 min prior to the injection of the test compound (i.e. immediately after the baseline components).

The study was conducted in three phases. In Phase 1, effects of the 5HT\textsubscript{2c} agonist Ro 60-0157 (0.32-3.2 mg/kg) and the 5HT\textsubscript{2c} antagonist SB 242,084 were determined alone and in combination. Specifically, Ro 60-0157 (0.32-3.2 mg/kg) was examined alone to evaluate the degree to which agonist activity at 5HT\textsubscript{2c} was sufficient to depress ICSS, and SB 242,084 (0.032-1.0 mg/kg) was examined as a pretreatment to 3.2 mg/kg Ro 60-0157 to evaluate the potency of SB 242,084 as a 5HT\textsubscript{2c} antagonist. In Phase 2, effects of a confirmed 5HT\textsubscript{2c} antagonist dose of SB 242,084 (1.0 mg/kg) were examined on ICSS depression produced by the 5HT-selective releaser fenfluramine (3.2 mg/kg) and on ICSS facilitation produced by the DA-selective releaser amphetamine (0.32 mg/kg). Effects of SB 242,084 on ICSS depression produced by the kappa opioid agonist U69,593 (0.56 mg/kg) were also examined as a negative control. Finally, in Phase 3, effects of 1.0 mg/kg SB 242,084 were examined as a pretreatment to the non-selective DA/5HT releasers PAL-287 (naphthylisopropylamine; 1.0-5.6 mg/kg) and (+)MDMA (methylenedioxymethamphetamine; 1.0 and 3.2 mg/kg). Generally, test drug doses were based on results from previous studies (Negus et al., 2010; Bauer et al., 2013). Each test drug was evaluated in a group of 5-9 rats. Some subjects were used to evaluate more than one test drug so long as baseline ICSS rates remained stable, and in these cases, all experiments with a given test drug ± SB 242,084 were completed before experiments with another test drug were initiated and all treatments were counterbalanced. The sizes of experimental groups are reported in the figure legends. Test sessions were usually
conducted on Tuesdays and Fridays, and three-component baseline training sessions were conducted on all other weekdays.

**Data Analysis.** The primary dependent measure was the reinforcement rate in stimulations/trial. Raw reinforcement rates were normalized to the maximum control rate (MCR) for each subject on each day, where MCR was defined as the mean of the maximal rates observed during the second and third “baseline” components for that day. Therefore, %MCR was equal to [(response rate during a frequency trial) / (maximum control rate)] x 100. Data for each frequency were averaged across test components for each rat and then across rats to yield a “frequency-rate” curve for each experimental manipulation. Two-way ANOVA was used to compare frequency-rate curves, with ICSS frequency as one variable and treatment as the second factor. A Holm-Sidak post-hoc test followed all significant ANOVA’s, and p-values less than 0.05 were considered significant. As a second summary measure of ICSS, the average number of stimulations per component delivered across all frequencies was determined before and after drug administration on each day, and drug effects were expressed as the % baseline number of stimulations per component for that day.

**Drugs**

(+)‐Amphetamine sulfate was provided by the National Institute on Drug Abuse Drug Supply Program (Bethesda, MD). (±)‐Fenfluramine HCl was purchased from Sigma Chemical Co. (St. Louis, MO). SB 242,084 and Ro 60-0175 were purchased from Tocris (Bristol, UK). Naphthylisopropylamine (PAL-287) and (+)‐
methylenedioxymethamphetamine ( (+)MDMA) were synthesized as the fumarate salts by Dr. Bruce Blough (Research Triangle Park, NC). Amphetamine, fenfluramine, PAL-287 and (+)MDMA isomers were prepared in sterile saline. SB 242,084 was prepared in 20% PEG in saline. Ro 60-0175 was prepared in 4% DMSO in saline. All compounds were administered intraperitoneally (I.P.). Doses are expressed in terms of the salt forms above.

Results

Under vehicle conditions, electrical brain stimulation maintained a frequency-dependent increase in ICSS rates (e.g. Figure IV.1, open circles). The average ± SEM MCR for these studies was 62.20 ± 1.89 stimulations per trial. In general, low rates of ICSS were maintained by low stimulation frequencies (1.75–1.90 log Hz). ICSS rates increased in frequency-dependent manner with high stimulation frequencies (2.05-2.20 log Hz) maintaining the highest rates. The mean ± SEM number of total stimulations earned during baseline components was 327.15 ± 16.82 stimulations per component.

Figure IV.1 shows that the 5HT2C agonist Ro 60-0175 produces a dose-dependent downward shift of the ICSS frequency-rate curve (1A) along with a corresponding decrease in the total stimulations earned relative to the pre-test baseline (1B). The rate-decreasing effects of 3.2mg/kg Ro 60-0175 were dose-dependently blocked by pretreatment with the 5HT2C antagonist SB 242,084, with doses of 0.32 and 1.0 mg/kg SB 242,084 producing a significant blockade of the rate-decreasing effects produced by
3.2 mg/kg Ro 60-0175 (1C, D). The highest dose of SB 242,084 tested (1 mg/kg) did not significantly alter the ICSS rate-frequency curve when administered alone.

Figure IV.2 shows that 1.0 mg/kg SB 242,084 also blocked rate-decreasing effects produced by the 5HT-selective releaser fenfluramine (2A), but not those of the the kappa agonist U69,593 (2B), and SB 242,084 also failed to alter ICSS facilitation produced by the DA-selective releaser amphetamine (2C). Panel D shows the corresponding effects of these compounds on the total number of reinforcers earned relative to baseline in the absence (unfilled bars) and presence (filled bars) of SB 242,084.

Figure IV.3 shows that the non-selective DA/5HT releaser PAL-287 produced facilitation at 3 frequencies (1.75, 1.85 and 1.90 Log Hz) after 1.0mg/kg administration (3A), and this facilitating effect of PAL-287 was significantly enhanced by pretreatment with 1.0 mg/kg SB 242,084 at one stimulation frequency (1.95 Log Hz). Higher doses of 3.2 and 5.6 mg/kg PAL-287 continued to produce significant facilitation of low ICSS rates maintained by lower brain stimulation frequencies while also recruiting dose-dependent and significant decreases in high ICSS rates maintained by high frequencies (3B, C). The rate-decreasing effects were attenuated by pretreatment with SB 242,084 at one stimulation frequency (2.2 Log Hz) for 3.2 mg/kg PAL-287. At the highest dose of PAL-287 (5.6 mg/kg), SB 242,084 pretreatment significantly attenuated the rate-decreasing effects and significantly enhanced the rate-increasing effects at all but the lowest frequency (4C).

Figure IV.4 shows the effects of SB 242,084 on effects produced by (+)MDMA, another non-selective DA/5HT releaser. When administered after vehicle pretreatment, (+)MDMA also produced facilitation of low ICSS rates maintained by low brain
stimulation frequencies while producing dose-dependent decreases in high ICSS rates maintained by high stimulation frequencies. Effects of the lowest dose of (+)MDMA (1.0 mg/kg; 4A) were not altered by pretreatment with SB 242,084. A dose of 1.8 mg/kg (+)MDMA (4B) produced greater facilitation at a single frequency (1.85 Log Hz) following SB 242,084 pretreatment without significant attenuation of the rate-decreasing effects. At the highest dose of (+)MDMA (3.2 mg/kg), SB 242,084 pretreatment significantly attenuated the rate-decreasing effects and significantly enhanced the rate-increasing effects at all frequencies (4C).
Summary

This study evaluated the effects of a 5HT$_{2C}$ antagonist on monoamine releasers that varied in their selectivity for releasing DA vs. 5HT in rats responding for ICSS. There were three main findings. First, a 5HT$_{2C}$ agonist (Ro 60-0175) was shown to be sufficient to produce rate-decreasing (“abuse-limiting”) effects in ICSS, and this effect was blocked by pretreatment with a 5HT$_{2C}$ antagonist (SB 242,084). Similar rate-decreasing effects were also produced by the 5HT-selective releaser fenfluramine, and a dose of SB 242,084 that was sufficient to antagonize the rate-decreasing effects of Ro 60-0175 was also effective at blocking the rate-decreasing effects of fenfluramine. These results suggest that activation of the 5HT$_{2C}$ receptor is necessary for the expression of abuse-limiting effects produced by 5HT release. The second major finding was that pretreatment with SB 242,084 was able to significantly attenuate the rate-decreasing effects and unmask significantly greater rate-increasing effects produced by the non-selective DA and 5HT releaser PAL-287. Although similar effects were seen with another non-selective DA and 5HT releaser (+)MDMA, SB 242,084 pretreatment was not able to attenuate the rate-decreasing effects to the same degree. These data suggest that 5HT$_{2C}$ receptors partially mediate the abuse-limiting effects produced by non-selective monoamine releasers, and blockade of this receptor may result in an enhancement of the abuse-related effects produced by these drugs.
Chapter IV Figure Legends

Figure IV.1. Effects of the 5HT2C agonist Ro 60-0175 and antagonist SB 242,084.

Left panels (A, C) show drug effects on full ICSS frequency-rate curves. Abscissae: Frequency of electrical brain stimulation in Log Hz. Ordinates: Percent maximum control reinforcement rate (%MCR). Drug name and doses are indicated in legends. Filled points represent frequencies at which ICSS rates after drug treatment were statistically different from vehicle rates as determined by a two-way ANOVA followed by a Holm-Sidak post hoc test, p<0.05. Right panels (B, D) show summary ICSS data expressed as percent predrug baseline number of reinforcers per component delivered across all frequencies of brain stimulation. Abscissae: drug dose in mg/kg. Ordinates: Percent predrug baseline number of reinforcers per component. Upward/downward arrows indicate significant drug-induced increases/decreases in ICSS relative to vehicle for at least one brain stimulation frequency as determined by analysis of full frequency-rate curves in panels A and C. All data show mean ± SEM for 5 rats unless otherwise noted; statistical analysis was run for only 4 animals in Panel A because the fifth animal lost its electrode before completion of the highest dose. Statistical results for data in left panels are as follows: Panel A. Ro 60-0175 (0.32-3.2mg/kg): significant main effect of frequency [F(9,27) = 32.36, p<0.0001], dose [F(3,9) = 12.88, p=0.0013] and significant interaction [F(27,81) = 4.665, p<0.0001]. Panel C. SB 242,084 (0.032-1.0mg/kg) ± Ro 60-0175 (3.2mg/kg): significant main effect of frequency [F(9,36) = 87.34, p<0.0001], dose [F(6,24) = 9.421, p<0.0001] and significant interaction [F(54,216) = 2.159, p<0.0001].
Figure IV.2. Effects of the 5HT2C antagonist SB 242,084 on effects produced by fenfluramine, U69,593, and amphetamine. Panels (A-C) show drug effects on full ICSS frequency-rate curves. Abscissae: Frequency of electrical brain stimulation in Log Hz. Ordinates: Percent maximum control reinforcement rate (%MCR). Filled points represent frequencies at which ICSS rates after SB 242,084+test drug were statistically different from rates after vehicle+test drug as determined by a two-way ANOVA (repeated measures) followed by a Holm-Sidak post hoc test, p<0.05. Gray lines represent vehicle-vehicle treatment conditions, and asterisks denote points where vehicle+test drug significantly differed from vehicle+vehicle. Panel D shows summary ICSS data expressed as percent predrug baseline number of reinforcers per component delivered across all frequencies of brain stimulation. Abscissa: drug dose in mg/kg. Ordinate: Percent predrug baseline number of reinforcers per component. Upward/downward arrows indicate significant drug-induced increases/decreases in ICSS relative to test drug alone for at least one brain stimulation frequency as determined by analysis of full frequency-rate curves. Statistical results for data in panels A-C are as follows: Panel A. Fenfluramine (n=6) (3.2mg/kg) ± SB 242,084 (1.0mg/kg): significant main effect of frequency \([F(9,45) = 101.1, p<0.0001]\), treatment \([F(2,10) = 6.073, p=0.0188]\) and interaction \([F(18, 90) = 1.845, p=0.0314]\). Panel B. U69,593 (n=5) (0.56mg/kg) ± SB 242,084 (1.0mg/kg): significant main effect of frequency \([F(9,36) = 49.49, p<0.0001]\) but not treatment \([F(2,8) = 4.292, p=0.0542]\). However, there was a significant interaction \([F(18,72) = 1.783, p=0.0445]\). Panel C. Amphetamine (n=5) (0.32mg/kg) ± SB 242,084 (1.0mg/kg): significant main effect of frequency \([F(9,36) = 49.49, p<0.0001]\) but not treatment \([F(2,8) = 4.292, p=0.0542]\). However, there was a significant interaction \([F(18,72) = 1.783, p=0.0445]\).
47.23, p<0.0001] and treatment [F(2,8) = 8.288, p=0.0112], and there was a significant interaction [F(18,72) = 2.957, p=0.0006].

**Figure IV.3. Effects of the 5HT2C antagonist SB 242,084 on effects produced by the non-selective DA/5HT releaser PAL-287.** Panels (A-C) show effects on full ICSS frequency-rate curves. Abscissae: Frequency of electrical brain stimulation in Log Hz. Ordinates: Percent maximum control reinforcement rate (%MCR). Filled points represent frequencies at which reinforcement rates were statistically different from PAL-287 alone rates as determined by a two-way ANOVA (repeated measures) followed by a Holm-Sidak post hoc test, p<0.05. Gray lines represent vehicle-vehicle treatment conditions, and asterisks denote points where effects of PAL-287 alone significantly differed from effects of vehicle. Panel D shows summary ICSS data expressed as percent predrug baseline number of reinforcers delivered across all frequencies of brain stimulation. Abscissa: PAL-287 dose in mg/kg. Ordinate: Percent predrug baseline number of reinforcers. Upward/downward arrows indicate significant drug-induced increases/decreases in ICSS relative to PAL-287 alone for at least one brain stimulation frequency as determined by analysis of full frequency-rate curves. All data show mean ± SEM for 9 rats. Statistical results for data in panels A-C are as follows: Panel A. PAL-287 (1.0mg/kg) ± SB 242,084 (1.0mg/kg): significant main effect of frequency [F(9,72) = 161.9, p<0.0001] and treatment [F(2,16) = 14.58, p=0.0002], and there was a significant interaction [F(18,144) = 2.952, p=0.0002]. Panel B. PAL-287 (3.2mg/kg) ± SB 242,084 (1.0mg/kg): significant main effect of frequency [F(9,72) = 88.09, p<0.0001] and treatment [F(2,16) = 4.395, p=0.0301], and there was a significant interaction [F(18,144)
significant main effect of frequency \[F(9,72) = 16.60, p<0.0001\] and treatment \[F(2,16) = 26.06, p=0.0006\], and there was a significant interaction \[F(18,144) = 1.268, p<0.0001\].

**Figure IV.4. Effects of the 5HT2C antagonist SB 242,084 on effects produced by the non-selective DA/5HT releaser (+)MDMA.** Panels (A-C) show drug effects on full ICSS frequency-rate curves. Abscissae: Frequency of electrical brain stimulation in Log Hz. Ordinates: Percent maximum control reinforcement rate (%MCR). Drug name and doses are indicated in legends. Filled points represent frequencies at which reinforcement rates were statistically different from (+)MDMA alone rates as determined by a two-way ANOVA (repeated measures) followed by a Holm-Sidak post hoc test, \(p<0.05\). Gray lines represent vehicle-vehicle treatment conditions, and asterisks denote points where (+)MDMA alone significantly differed from vehicle. Panel D shows summary ICSS data expressed as percent predrug baseline number of reinforcers per component delivered across all frequencies of brain stimulation. Abscissa: drug dose in mg/kg. Ordinate: Percent predrug baseline number of reinforcers per component. Upward/downward arrows indicate significant drug-induced increases/decreases in ICSS relative to drug alone for at least one brain stimulation frequency as determined by analysis of full frequency-rate curves. All data show mean ± SEM for 9 rats. Statistical results for data in panels A-C are as follows: Panel A. (+)MDMA (1.0mg/kg) ± SB 242,084 (1.0mg/kg): significant main effect of frequency \[F(9,72) = 100.9, p<0.0001\] and treatment \[F(2,16) = 12.42, p=0.0006\], and there was a significant interaction
[F(18,144) = 2.632, p<0.0001]. Panel B. (+)MDMA (1.8mg/kg) ± SB 242,084 (1.0mg/kg): significant main effect of frequency [F(9,72) = 60.77, p<0.0001] but not treatment [F(2,16) = 1.228, p=0.3191]. There was a significant interaction [F(18,144) = 7.077, p<0.0001]. Panel C. (+)MDMA (3.2mg/kg) ± SB 242,084 (1.0mg/kg): significant main effect of frequency [F(9,72) = 3.230, p<0.0001] but not treatment [F(2,16) = 3.154, p=0.0700]. There was a significant interaction [F(18,144) = 25.50, p=0<0.0001].
Figure IV.2

A. 

- Veh-Vehicle
- Veh-Fenfluramine
- SB-Fenfluramine

B. 

- Veh-Vehicle
- Veh-U69,563
- SB-U69

C. 

- Veh-Vehicle
- Veh-Amph
- SB-Amph

D. 

- Vehicle
- 1.0 SB

Frequency (Log Hz)

% MCR

% Baseline Reinforcers

3.2 Fen
3.2 Fen
0.56 U69
0.56 U69
0.52 Amph
0.52 Amph
Figure IV.3

A.

B.

C.

D.

- Veh-Vehicle
- Veh-1.0 PAL-287
- SB-1.0 PAL-287

- Veh-Vehicle
- Veh-3.2 PAL-287
- SB-3.2 PAL-287

- Veh-Vehicle
- Veh-5.6 PAL-287
- SB-5.6 PAL-287

- Vehicle
- 1.0 SB

% MCR

% Baseline Reinforcers

Frequency (Log Hz)

PAL-287 Dose (mg/kg)
Figure IV.4

A.  
- Veh-Veh
- Veh-1.0 +MDMA
- SB-1.0 +MDMA

B.  
- Veh-Veh
- Veh-1.8 +MDMA
- SB-1.8 +MDMA

C.  
- Veh-Veh
- Veh-3.2 +MDMA
- SB-3.2 +MDMA

D.  
- Vehicle
- 1.0 SB

% MCR vs. Frequency (Log Hz)

% Baseline Reinforcers Vehicle +MDMA Dose (mg/kg)
Chapter V: Effects of Monoamine Releasers with Varying Selectivity for Releasing Dopamine versus Norepinephrine in Assays of Cocaine Discrimination and Intracranial Self-stimulation in Rats.

In preparation

Introduction

The current study used assays of cocaine discrimination and ICSS in rats to evaluate a series of monoamine releasers that varied >200-fold in their in vitro selectivity for releasing DA vs. NE while having similar potencies to release 5HT (Table V.1). There were two hypotheses. First, we predicted that cocaine-like discriminative stimulus effects would vary systematically as a function of DA vs. NE selectivity, with peak substitution produced by compounds that, like amphetamine, display similar potencies to release DA vs. NE. Second, we hypothesized that compounds producing cocaine-like discriminative stimulus effects would also facilitate ICSS, whereas those not able to produce cocaine-like discriminative stimuli would not. Confirmation of these hypotheses could contribute new insight into pharmacological determinants of abuse-related effects produced by monoamine releasers.
Materials and Methods

Subjects

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, or Harlan, Frederick, MD) were used for all studies. Animals in the discrimination assay were fed after behavior sessions and were maintained between 300-370g. Other animals had ad libitum access to standard rodent laboratory chow. All animals had continuous access to water and were housed individually on a 12 hour light-dark cycle, and studies were conducted during the light cycle. All experimental protocols accorded with the guidelines of the National Research Council (National Research Council, 2011) and were approved by the Institutional Animal Care and Use Committee.

In Vitro Assay of Monoamine Release (performed by Baumann and colleagues)

Selectivities of PAL-542, PAL-544, PAL-571, and PAL-569 to evoke release via the rat monoamine transporters (rSERT, rNET, and rDAT) were examined in rat brain synaptosomes essentially as previously described (Cozzi et al., 2012). Rats were euthanized with CO2, decapitated, and the brains were rapidly removed and dissected on ice. Rat whole brain minus cerebellum (for rSERT and rNET release assays) or rat striatum (for rDAT release assays) was homogenized in ice-cold 10% sucrose containing 1 μM reserpine. For rSERT release assays, 100 nM nomifensine and GBR12935 were added to the sucrose solution to block uptake of [3H]5-HT into NE and DA terminals. For the rNET release assays, 100 nM GBR12935 and citalopram were added to block [3H]MPP+ uptake into DA and 5-HT terminals. For the rDAT release assays, 100 nM desipramine and citalopram were added to block [3H]MPP+ uptake into NE and 5-HT
terminals. Tissues were homogenized with a Potter-Elvehjem homogenizer (12 strokes), then the homogenates were subjected to centrifugation at 1000×g for 10 min at 4 °C. The pellet was discarded and the supernatant containing the synaptosomes was retained on ice until use. Synaptosomes were preloaded with 5 nM [3H]5-HT to test for release via rSERT or with 5 nM [3H]MPP+ to test for release via rNET and rDAT. To load, synaptosomes were incubated in a polypropylene beaker with stirring at 25 °C for 60 min with 5 nM [3H]5-HT or [3H]MPP+ in Krebs-phosphate buffer containing (mM): NaCl (154.4), KCl (2.9), CaCl2 (1.1), MgCl2 (0.83), d-glucose (5), sodium ascorbate (5.7), pargyline (0.05), reserpine (0.001), pH=7.4. The appropriate combination of transporter blockers (citalopram, desipramine, nomifensine, or GBR12935; 100 nM) was present depending on the transporter under study. After incubation to steady state, 850 μL of preloaded synaptosomes were added to 12×75 mm polystyrene test tubes or 96-well polypropylene plates that contained 150 μL test drug in uptake buffer plus 1 mg/mL bovine serum albumin. Incubations were maintained at 25 °C. After 5 min (rSERT assays) or 30 min (rNET and rDAT assays), the release reaction was terminated by dilution with 4 mL wash buffer (10 mM Tris–HCl containing 0.9% NaCl at 25 °C, pH=7.4) followed by rapid vacuum filtration through glass fiber filters (Whatman GF/B) using a cell harvester (Brandel, Gaithersberg, MD, USA). Filters were rinsed twice with 4 mL wash buffer, dried briefly under vacuum, and then transferred to 24-well plates. Scintillation cocktail (0.6 mL) was added to each well, and after an overnight incubation, the retained tritium was assessed at 40% efficiency by liquid scintillation counting. EC50 values for transporter assays were determined using the nonlinear least-squares curve fitting program MLAB-PC (Civilized Software, Bethesda, MD, USA). Selectivities were
calculated as ratios of EC50 values to promote release via transporters for DA vs. NE 
(EC$_{50}$ DA/EC$_{50}$ NE) and for DA vs. 5HT (EC$_{50}$ DA/EC$_{50}$ 5HT).

**Cocaine Discrimination**

**Apparatus.** Studies were conducted in 10 rats using procedures similar to those described 
previously (Lamas et al., 1998; Caine et al., 2000). Operant chambers consisted of 
sound-attenuating boxes containing modular acrylic and metal test chambers (Med 
Associates, St. Albans, VT). Each chamber had two response levers (4.5 cm wide, 2.0 
cm deep, 3.0 cm off the floor), a panel of three stimulus lights (red, yellow, and green) 
centered 7.6 cm above each response lever, and a pellet dispenser located between the 
two levers (Med Associates, St. Albans, VT, USA). Med-PC IV computer software 
controlled all programming parameters and data collection (Med Associates, St. Albans, 
VT, USA).

**Training Procedure.** To facilitate the acquisition of food-maintained operant responding, 
an overnight behavioral session was conducted in which 5 mg food pellets were available 
under a fixed-time 4 min; fixed-ratio (FR) 1 conjoint schedule of reinforcement. Under 
this conjoint schedule, food pellets were delivered under a 4 min fixed-time schedule. In 
addition, and independently of the fixed-time schedule, rats could earn additional pellets 
by responding on the lever under an FR 1 schedule of reinforcement. After the overnight 
behavioral session, rats were trained to discriminate 5.6 mg/kg cocaine intraperitoneal 
(IP) from saline in a two-lever, food-reinforced drug discrimination procedure. 
Discrimination training was conducted 5 days/week during daily sessions consisting of a
10-min response period, during which stimulus lights were illuminated over both levers, and rats could earn up to 25 food pellets by responding under a FR10 schedule of food presentation. If all 25 pellets were delivered before 10 min elapsed, then stimulus lights were extinguished and responding had no further scheduled consequences. Saline or 5.6 mg/kg cocaine was administered IP 10 min prior to the start of the operant session. Saline or cocaine was administered in a double alternating pattern across days. Following administration of saline, only responding on the saline-appropriate lever produced food, whereas following administration of 5.6 mg/kg cocaine, only responding on the cocaine-associate lever produced food. Saline and cocaine-associated levers were counterbalanced between animals such that the right lever was associated with saline for half the rats and with cocaine for the other rats. Responses on the incorrect lever reset the response requirement on the correct lever. The criteria for accurate discrimination were 1) ≥75% injection-appropriate responding for the first ratio requirement, 2) ≥90% injection-appropriate responding overall, and 3) criteria one and two were met for at least 5 of 6 consecutive days of training.

Testing Procedure. Test sessions were identical to training sessions except that completion of the response requirement on either lever produced food, and drugs were administered as described below. Test sessions were typically conducted on Tuesdays and Fridays and only conducted if criteria 1 and 2 above for accurate discrimination had been met during the preceding training session. The pretreatment time for d-amphetamine and cocaine dose-effect determinations was 20min. The pretreatment time for all other dose-effect determinations was 10min. With two exceptions (amphetamine,
fenfluramine), the drug dose tested in time course studies was the lowest dose to produce
full substitution (>85% cocaine-appropriate responding) or to eliminate responding in one
or more animals during dose-effect studies. The amphetamine (1.0mg/kg) and
fenfluramine (3.2mg/kg) time courses doses were chosen to allow comparison to
previously published effects on ICSS (Chapter II). For time course studies, different
pretreatment times (10-300 min) were tested in different test sessions. Dose-effect studies
were conducted before time course studies, and both dose and pretreatment time were
tested in ascending order. In addition, all tests with a given compound in a given rat
before initiation of testing with another compound.

Data Analysis. The primary dependent measures for each test session were percent
cocaine-appropriate responding ([number of responses on cocaine-associated lever ÷ total
responses] * 100) and rate of responding (total responses/total time stimulus lights
illuminated) for the entire test session. Drugs that produced ≥85% cocaine-appropriate
responding were considered to produce full substitution, whereas drugs that produced
≤15% cocaine-appropriate responding were considered to produce no substitution; 16-
84% cocaine-appropriate responding was considered partial substitution. Response rates
were reported as % Baseline Saline Rate ([rate for test session ÷ average rate from all
saline training days before all test sessions with that drug] * 100). Percent cocaine-
appropriate responding was calculated and included in analysis only if at least one ratio
requirement was completed.
**Intracranial Self-Stimulation**

The Surgery, Apparatus, Training, and Testing details of this study are the same as those reported previously [Chapter II]. The Data Analysis section is the same as reported in Chapter IV.

**Drugs**

Structures of monoamine releasers can be found in on page vii. (+)Amphetamine hemisulfate and (-)cocaine HCl were provided by the National Institute on Drug Abuse Drug Supply Program (Bethesda, MD). Racemic fenfluramine HCl was purchased from Sigma Chemical Co. (St. Louis, MO). All other compounds were synthesized by Dr. Bruce Blough (Research Triangle Park, NC) as their fumarate salts. All compounds were prepared in sterile saline and administered intraperitoneally (IP).

**Results**

Table V.1 shows *in vitro* potency to promote DA, 5HT and NE release for three reference compounds (amphetamine, PAL-287 and fenfluramine) and four novel compounds (PAL-542, PAL-544, PAL-571 and PAL-569). Data for reference compounds were published previously (Rothman et al., 2001; Rothman et al., 2005). The novel compounds all displayed similar selectivities to promote DA vs. 5HT release, characterized by modestly higher potencies (approximately 2-6 fold) for the 5HT transporter. However, these compounds displayed more than a 200-fold range of selectivities to promote DA vs. NE release. The most DA-selective compound was PAL-
542 (64-fold higher potency to release DA vs. NE), and the least DA-selective compound was PAL-569 (13-fold lower potency for release via the DA vs. NE transporter).

For the assay of cocaine discrimination, Table V.2 shows performance on training days preceding test days with each drug. Figure V.1 shows dose-effect curves for each monoamine releaser to produce cocaine-appropriate responding, and time course data for selected compounds are also shown. Table V.3 shows the number of animals that earned at least one reinforcer for each test with each drug and that thereby contributed to data in Figure V.1. Amphetamine produced dose-dependent substitution for cocaine (Fig. V.1A), and time course studies indicated that 1.0 mg/kg amphetamine produced full substitution from 10-30 min but not after 100 min (data not shown). Conversely, fenfluramine produced less than 21% cocaine-appropriate responding at all doses (Fig. V.1A) and time points (data not shown). PAL-287 doses up to 3.2 mg/kg produced only partial substitution for cocaine in dose-effect studies using a 10 min pretreatment time, and a higher dose of 5.6 mg/kg eliminated responding in all subjects (Fig. V.1A, Table V.3). However, in time course studies, 5.6 mg/kg PAL-287 produced partial substitution that peaked after 100 min at 75% cocaine-appropriate responding (Fig. V.1C). All compounds that did not fully substitute were tested up to doses that decreased rates (Supplemental Figure V.1A).

Of the four novel compounds tested, the relatively non-selective compound PAL-571 (2-fold selective for NE vs. DA) produced partial substitution in a dose- (Fig. V.2B) and time-dependent manner (Fig. V.2D). In contrast, the most DA- vs. NE-selective releaser (64-fold selective; PAL-542), the 4-fold DA vs. NE-selective releaser PAL-544, and the most NE vs. DA-selective releaser (13-fold selective; PAL-569) failed to
substitute for cocaine at any dose (Fig. V.2B) or time (data not shown). All compounds were tested up to doses that decreased response rates (Supplemental Figure V.1B).

Table V.4 shows the proportion of rats in which each drug produced full substitution (>85% cocaine-appropriate responding) at any dose or time. Amphetamine substituted in all rats tested, PAL-287 and PAL-571 substituted in 3/5 and 2/5 rats tested, respectively, and none of the other compounds substituted for cocaine in any rat at any dose or pretreatment time.

Effects of amphetamine, PAL-287 and fenfluramine on ICSS were reported previously (Bauer et al., 2013), and briefly, amphetamine dose-dependently facilitated ICSS, fenfluramine depressed ICSS, and PAL-287 facilitated low ICSS rates while depressing high ICSS rates. Only the novel releasers were examined here. The average MCR (± SEM) under baseline conditions was 63.00 ± 4.92, and the average total reinforcers earned across trials (± SEM) was 281.50 ± 26.01. Figure V.2 shows the dose-effect function (A) and time course (C) of PAL-571 on ICSS frequency-rate curves, and panels B and D show summary data for the dose-effect and time course studies, respectively. PAL-571 failed to facilitate ICSS at any frequency during the dose-effect determination using a 20min pretreatment time, although doses of 1.0 and 3.2 mg/kg significantly depressed ICSS at 2.0-2.05 log Hz and 2.0-2.2 log Hz, respectively. During the time course test, 3.2 mg/kg PAL-571 only decreased ICSS from 10-100 min, but these rate-decreasing effects were no longer apparent after 300 min, and at this time, PAL-571 significantly facilitated ICSS at one frequency (1.95 log Hz) after 300 min. The other releasers produced exclusively rate-decreasing effects during both dose-effect tests (Figure V.3) and time course tests (data not shown).
Figure V.4 shows uses data from Table V.1 to plot DA/NE and DA/5HT selectivity for releasers examined in this study. Black-filled symbols indicate compounds that produced both 1) full substitution for cocaine in all animals in drug discrimination and 2) facilitated ICSS without significant depression of maximal rates. Grey-filled symbols indicate compounds that produced 1) full substitution in at least one animal and 2) facilitated ICSS at some dose or time with depression of maximal rates also seen. Unfilled symbols indicate compounds that failed to substitute for the discriminative stimulus effects of cocaine at any dose or time and also failed to significantly facilitate ICSS at any dose or time.
Summary

This study investigated the role of DA/NE selectivity as a determinant of abuse-related effects of monoamine releasers in rats. There were three main findings. First, a series of four compounds was identified (PAL-542, PAL-544, PAL-571 and PAL-569) that displayed similar selectivities to promote substrate release via DA and 5HT transporters but a >200-fold range of selectivities to promote release via DA and NE transporters. This set of compounds provided an opportunity to examine the effect of DA/NE selectivity, although the relatively high potency of all compounds to also release 5HT served as a complicating factor. Second, within this range of compounds, evidence for abuse-related cocaine-like discriminative stimulus effects and ICSS facilitation was obtained only for PAL-571, which has similar potencies to promote release via DA and NE transporters. Compounds with greater DA selectivity (PAL-542, PAL-544) or greater NE selectivity (PAL-569) were ineffective to produce either cocaine-like discriminative stimulus effects or ICSS facilitation. Lastly, effects of PAL-571 were qualitatively similar to effects of PAL-287, another compound with similar DA/NE and DA/5HT selectivities, and less than effects of amphetamine, which has similar DA/NE selectivity but much greater DA/5HT selectivity. Taken together, these studies suggest that both DA and NE are necessary for the expression of abuse-related effects by monoamine releasers in these assays.
**Table V.1**: Potency (nM ± SEM) and selectivity of compounds to promote substrate release through DA, 5HT and NE transporters in an *in vitro* synaptosome preparation.

Selectivity numbers ≤1 indicate higher potency to promote DA release DA transporters than 5HT or NE release. Data for amphetamine, PAL-287 and fenfluramine were published previously.

<table>
<thead>
<tr>
<th>Drug</th>
<th>EC$_{50}$ DA</th>
<th>EC$_{50}$ 5HT</th>
<th>EC$_{50}$ NE</th>
<th>Selectivity DA/5HT</th>
<th>Selectivity DA/NE</th>
</tr>
</thead>
<tbody>
<tr>
<td>(+)amphetamine$^1$ (Amphetamine)</td>
<td>24.8 ± 3.5</td>
<td>1765 ± 94</td>
<td>7.07 ± 0.95</td>
<td>0.014</td>
<td>3.508</td>
</tr>
<tr>
<td>naphthylisopropylamine$^2$ (PAL-287)</td>
<td>12.6 ± 0.4</td>
<td>3.4 ± 0.2</td>
<td>11.1 ± 0.9</td>
<td>3.706</td>
<td>1.135</td>
</tr>
<tr>
<td>(±)-fenfluramine$^1$ (Fenfluramine)</td>
<td>&gt;10,000</td>
<td>79.3 ± 11.5</td>
<td>739 ± 57</td>
<td>&gt;126.1$^*$</td>
<td>&gt;13.53$^*$</td>
</tr>
<tr>
<td>1-(5-Cl-1H-indol-3-yl) propan-2-amine (PAL-542)</td>
<td>54 ± 2</td>
<td>16 ± 2</td>
<td>3434 ± 516</td>
<td>3.375</td>
<td>0.016</td>
</tr>
<tr>
<td>1-(5-flouro-1H-indol-3-yl) propan-2-amine (PAL-544)</td>
<td>32 ± 1</td>
<td>19 ± 1</td>
<td>126 ± 18</td>
<td>1.684</td>
<td>0.254</td>
</tr>
<tr>
<td>1-(1H-indol-5-yl) propan-2-amine (PAL-571)</td>
<td>173 ± 10</td>
<td>28 ± 3</td>
<td>79 ± 19</td>
<td>6.179</td>
<td>2.190</td>
</tr>
<tr>
<td>(R)-1-(1H-indol-1-yl) propan-2-amine (PAL-569)</td>
<td>1062 ± 54</td>
<td>177 ± 20</td>
<td>81 ± 9</td>
<td>6.000</td>
<td>13.11</td>
</tr>
</tbody>
</table>

Citations: 1) Rothman et al., 2001 SYNAPSE; 2) Rothman et al., 2005 JPET. Selectivity for all other compounds provided by Rothman and colleagues. *Fenfluramine selectivity is approximate using 10,000nM as the potency for releasing DA.
Table V.2: Average (± SEM) % Cocaine-appropriate responding for saline and cocaine training days immediately preceding test days for each treatment group, as well as raw saline rates for the training days over which the dose-effect studies were conducted.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>% Cocaine-appropriate responding on cocaine days preceding test days</th>
<th>% Cocaine-appropriate responding on saline days preceding test days</th>
<th>Raw saline responses /sec</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cocaine</td>
<td>98.37 ± 0.54</td>
<td>4.69 ± 1.44</td>
<td>0.60 ± 0.10</td>
</tr>
<tr>
<td>Amphetamine</td>
<td>97.30 ± 0.86</td>
<td>1.50 ± 0.96</td>
<td>0.77 ± 0.17</td>
</tr>
<tr>
<td>PAL-287</td>
<td>98.96 ± 0.79</td>
<td>0.92 ± 0.87</td>
<td>1.17 ± 0.24</td>
</tr>
<tr>
<td>Fenfluramine</td>
<td>99.50 ± 0.43</td>
<td>0.67 ± 0.49</td>
<td>1.13 ± 0.17</td>
</tr>
<tr>
<td>PAL-542</td>
<td>98.03 ± 0.92</td>
<td>1.10 ± 0.60</td>
<td>0.97 ± 0.14</td>
</tr>
<tr>
<td>PAL-544</td>
<td>98.04 ± 1.32</td>
<td>1.57 ± 1.06</td>
<td>0.93 ± 0.15</td>
</tr>
<tr>
<td>PAL-571</td>
<td>99.73 ± 0.18</td>
<td>0.83 ±0.45</td>
<td>0.91 ± 0.18</td>
</tr>
<tr>
<td>PAL-569</td>
<td>99.70 ± 0.20</td>
<td>1.17 ± 0.25</td>
<td>1.24 ± 0.12</td>
</tr>
</tbody>
</table>
Table V.3: Number of animals earning at least one reinforcer (numerator) in each group of animals (N=denominator) for each dose (mg/kg) and pretreatment time. The allocation of animals in the numerator comprise the data points in Figure V.2. Time courses were run using the highest dose tested.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (mg/kg)</th>
<th>Pretreatment Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1</td>
<td>0.32</td>
</tr>
<tr>
<td>Amphetamine</td>
<td>5/5</td>
<td>5/5</td>
</tr>
<tr>
<td>PAL-287</td>
<td>--</td>
<td>5/5</td>
</tr>
<tr>
<td>Fenfluramine</td>
<td>--</td>
<td>6/6</td>
</tr>
<tr>
<td>PAL-542</td>
<td>5/5</td>
<td>5/5</td>
</tr>
<tr>
<td>PAL-544</td>
<td>--</td>
<td>5/5</td>
</tr>
<tr>
<td>PAL-571</td>
<td>--</td>
<td>6/6</td>
</tr>
<tr>
<td>PAL-569</td>
<td>5/5</td>
<td>5/5</td>
</tr>
</tbody>
</table>
Table V.4. Mean (range) of cocaine-appropriate responding and percentage of control response rate at the dose and time producing either full substitution in the greatest number of animals or highest average substitution (for compounds which failed to fully substitute in any animal).

<table>
<thead>
<tr>
<th>Drug</th>
<th>No. Substituting</th>
<th>Dose (Time)</th>
<th>% Cocaine Responding</th>
<th>% Control Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphetamine</td>
<td>5/5</td>
<td>1.0 mg/kg (20min)</td>
<td>98.4 (97-100)</td>
<td>77.8 (18.5-237.7)</td>
</tr>
<tr>
<td>PAL-287</td>
<td>3/5</td>
<td>5.6 mg/kg (100min)</td>
<td>68.8 (14-100)</td>
<td>60.9 (15.9-40.1)</td>
</tr>
<tr>
<td>Fenfluramine</td>
<td>0/6</td>
<td>3.2 mg/kg (100min)</td>
<td>10.9 (0.6-32.9)</td>
<td>20.6 (20.6)</td>
</tr>
<tr>
<td>PAL-542</td>
<td>0/5</td>
<td>3.2 mg/kg (100min)</td>
<td>11.9 (0-27)</td>
<td>20.6 (5.5-40.6)</td>
</tr>
<tr>
<td>PAL-544</td>
<td>0/5</td>
<td>1.0 mg/kg (10min)</td>
<td>2.6 (0.8-2.7)</td>
<td>87.1 (34.0-142.0)</td>
</tr>
<tr>
<td>PAL-571</td>
<td>2/5</td>
<td>3.2 mg/kg (10min)</td>
<td>39.2 (0-100)</td>
<td>13.6 (0-20.6)</td>
</tr>
<tr>
<td>PAL-569</td>
<td>0/5</td>
<td>3.2 mg/kg (30min)</td>
<td>40.3 (0-70.4)</td>
<td>35.3 (0.9-91.5)</td>
</tr>
</tbody>
</table>
**Chapter V Figure Legends**

**Figure V.1: Effects of compounds in drug discrimination.** Percent cocaine-appropriate responding for all compounds tested in rats trained to discriminate 5.6 mg/kg cocaine from saline (N=5 or 7 per group; Table V.2 for details). Panels A and B: Abscissa represents drug dose (mg/kg), and ordinate represents average % cocaine-appropriate responding (± SEM). Panels C and D: Abscissa represents time after administration (min) and ordinate represents average % cocaine appropriate responding (± SEM).

**Figure V.2: Effect of PAL-571 on responding in ICSS.** Left panels (A, C) show drug effects on full ICSS frequency-rate curves. Abscissae: Frequency of electrical brain stimulation in Log Hz. Ordinates: Percent maximum control reinforcement rate (%MCR). Drug name and doses (A) or time points (B) are indicated in legends. Filled points represent frequencies at which reinforcement rates were statistically different from vehicle rates as determined by a two-way ANOVA followed by a Holm-Sidak post hoc test, p<0.05. Right panels (B, D) show summary ICSS data expressed as percent pre-drug baseline number of reinforcers delivered across all frequencies of brain stimulation. Abscissae: drug dose in mg/kg (B) or time in min (D). Ordinates: Percent pre-drug baseline number of reinforcers. The drug and pretreatment time are shown for each panel. Upward/downward arrows indicate significant drug-induced increase/decrease in ICSS relative to vehicle (dose-effect) or baseline (time course) for at least one brain stimulation frequency as determined by analysis of full frequency-rate curves. PAL-571 DEC: Significant main effect of frequency \[F(9,36) = 133.8, p<0.0001\], dose \[F(3,12) = \]
35.28, \( p<0.0001 \), and there was a significant interaction [\( F(27,108) = 8.940, p<0.0001 \)].

PAL-571 time course: Significant main effect of frequency [\( F(9,36) = 78.95, p<0.0001 \)],
time [\( F(4,16) = 8.503, p=0.0007 \)], and there was a significant interaction [\( F(36,144) = 4.542, p<0.0001 \)]. N=5 for all points.

**Figure V.3: Summary of drug effects on ICSS for PAL-542, PAL-544, and PAL-569.** Panels show summary ICSS data expressed as percent pre-drug baseline number of reinforcers delivered across all frequencies of brain stimulation. Abscissae: drug dose in mg/kg (B). Ordinates: Percent pre-drug baseline number of reinforcers. Downward arrows indicate significant drug-induced increase/decrease in ICSS relative to vehicle (dose-effect) for at least one brain stimulation frequency as determined by analysis of full frequency-rate curves (not shown) using a two-way ANOVA followed by a Holm-Sidak post hoc test, \( p<0.05 \). PAL-542: Significant main effect of frequency [\( F(9,36) = 91.13, p<0.0001 \)], dose [\( F(3,12) = 16.49, p=0.001 \)], and significant interaction [\( F(27,108) = 2.777, p=0.0001 \)]. PAL544: Significant main effect of frequency [\( F(9,36) = 94.84, p<0.0001 \)], dose [\( F(3,12) = 196.0, p<0.0001 \)], and significant interaction [\( F(27,108) = 27.72, p<0.0001 \)]. PAL-569: Significant main effect of frequency [\( F(9,36) = 103.2, p<0.0001 \)] but not dose [\( F(3,12)=2.997, p=0.0729 \)]. There was a significant interaction [\( F(27,108) = 1.753, p=0.0230 \)]. N=5 for all points.

**Figure V.4. In vitro selectivity to release dopamine, serotonin, and norepinephrine.** Abscissa: selectivity for DA vs. 5HT \( (IC_{50}DA/IC_{50}5HT) \). Ordinate: selectivity for DA vs. 5HT \( (IC_{50}DA/IC_{50}5HT) \). Black symbols denote drugs that 1) fully substituted for cocaine
in all animals tested at some dose or time point and 2) facilitated ICSS without depressing maximum rates. Grey symbols denote drugs that 1) fully substituted for cocaine in at least one, but not all animals at some dose or time and 2) facilitated ICSS at some dose or time but with significant depression of maximum rates. Unfilled symbols represent drugs that did not 1) substitute for cocaine in any animals at any dose or time nor 2) produced facilitation at any dose or time in ICSS.

**Supplemental Figure V.1.** Percent saline response rates for all compounds tested in rats trained to discriminate 5.6 mg/kg cocaine from saline (N=5 or 7 per group; Table V.2 for details). Response rates during training days conducted over the course of a given drug’s test days were used as the control rates of responding. Test day data are expressed as a percentage of the average response rates of those saline days. Panels A and B: Abscissa represents drug dose (mg/kg), and ordinate represents average rate as a percentage of the saline training rate (± SEM).
Figure V.1.
Figure V.2.

A. 

% MCR

0 25 50 75 100 125

Frequency (Log Hz)

- Vehicle
- 0.32 PAL-571
- 1.0 PAL-571
- 3.2 PAL-571

B. 

% Baseline Reinforcers

0 50 100 150 200

PAL-571 Dose (mg/kg)

Veh 0.32 1.0 3.2

PAL-571 Dose-Effect 20min Pre-treatment

C. 

% MCR

0 25 50 75 100 125

Frequency (Log Hz)

- Baseline
- 10min
- 30min
- 100min
- 300min

D. 

% Baseline Reinforcers

0 50 100 150 200

Time after administration

10min 30min 100min 300min

PAL-571 Time Course 3.2mg/kg
Figure V.3.
Figure V.4.
Supplemental V.1.

A.

B.

[Graphs showing the rate of saline training compared to drug dose (mg/kg) for different drugs: Amphetamine, PAL-287, Fentanyl, PAL-542, PAL-544, PAL-571, PAL-569.]
Chapter VI: Discussion

Introduction

Monoamine releasers constitute a class of compounds with a wide range of clinical utility, but their use is limited by their potential for abuse. The data presented in this dissertation have provided evidence that selectivity for 5HT over DA may limit abuse and that this abuse-limiting effect of 5HT is at least partially mediated through the 5HT$_{2C}$ receptor. The role of selectivity for DA vs. NE was also evaluated, and the data collected suggest that co-release of both DA and NE is necessary for the expression of abuse-related effects. Taken as a whole, the data presented in this dissertation have both confirmed previous findings and provided new insights into the determinants of abuse liability of monoamine releasers, which may inform efforts to develop novel medications with reduced potential for abuse.

Chapters II and III

Role of selectivity for DA vs. 5HT in ICSS

Results of the studies in Chapters II and III confirm and extend previous studies that have examined effects of monoamine releasers on intracranial self-stimulation in rats across a heterogeneous range of conditions. In agreement with the present findings, both
amphetamine (Esposito et al., 1980; Kling-Petersen et al., 1994; Lin et al., 2000; Wise and Munn, 1995) and methamphetamine (Elder et al., 1965) have been shown to facilitate ICSS. For example, amphetamine (0.5-2.0 mg/kg, IP) decreased the threshold of medial forebrain bundle stimulation required to maintain ICSS in a frequency-rate procedure in rats similar to the procedure used here, although this earlier study did not fully characterize amphetamine potency or time course (Wise and Munn, 1995). Also in agreement with the present studies, fenfluramine has been shown previously to depress ICSS (Olds and Yuwiler, 1992; Olds, 1995). In these earlier studies with fenfluramine, stable baseline rates of ICSS were maintained by a single magnitude of brain stimulation (i.e. a single frequency and intensity of stimulation), and 20 mg/kg (IP) fenfluramine decreased ICSS dramatically for at least 13 hours; however, other doses of fenfluramine were not studied, and fenfluramine effects on low rates of ICSS maintained by low stimulation magnitudes were not assessed to evaluate the potential for fenfluramine to facilitate ICSS. Finally, (±)MDMA (0.5-4 mg/kg, IP) was previously studied under an intensity-rate ICSS procedure (i.e. with manipulation of brain stimulation intensity rather than frequency), and similar to the present studies, (±)MDMA produced a mixture of decreases in peak response rates maintained by high stimulation amplitudes (rate-decreasing effects) and increases in low rates of responding maintained by low stimulation amplitudes (rate-increasing effects) (Lin et al., 1997).

The present studies extend on these earlier findings in three ways. First, these studies used a single procedure to evaluate effects produced by multiple doses of 11 monoamine releasers that varied along a continuum of pharmacological selectivity to promote release of DA and 5HT. This permitted direct comparison of dose-effect curves.
for a range of compounds under a standard set of experimental conditions. For example, both amphetamine and (+)MDMA produced abuse-related facilitation of ICSS, but amphetamine produced facilitation across a broader range of doses and to a higher maximal degree than (+)MDMA. This in turn permitted correlation of ICSS data to *in vitro* biochemical data, and there was a significant correlation between maximal facilitation of ICSS and biochemical selectivity to release DA vs. 5HT. Second, these studies compared effects of manipulating DA/5HT selectivity of individual compounds with effects of manipulating proportion of DA- or 5HT-selective releasers in fixed-proportion mixtures of compounds. Results with mixtures supported results with individual compounds in suggesting that promotion of 5HT release is sufficient to oppose and limit dopaminergically mediated ICSS facilitation.

Finally, these studies evaluated time course of all compounds. Most drugs displayed comparable onsets of action (within 10 min) and durations of action (100-300 min), although consistent with earlier ICSS studies (Olds and Yuwiler, 1992; Olds, 1995), fenfluramine had a slightly slower onset and longer duration of action than the other releasers. Time course studies also permitted comparison of the duration of rate-increasing and rate-decreasing effects produced by weakly selective compounds (e.g. PAL-314, PAL-313, PAL-287 and the MDMA enantiomers). For all these compounds, rate-decreasing effects required higher doses and had shorter durations than rate-increasing effects. As a result, the highest levels of ICSS facilitation with these compounds were observed early after administration of low doses but later after administration of higher doses. The limited ability of these compounds to facilitate ICSS soon after their administration may be related to their weaker reinforcing efficacy in drug
self-administration studies (see below), because early-onset effects of drugs or other consequent stimuli play a stronger role than delayed effects as determinants of reinforcement (Lattal, 2010; Woolverton et al., 2012).

Correlation of monoamine releaser effects in assays of ICSS with drug self-administration in non-human primates and abuse in humans

One rationale for selecting the test compounds (varying in selectivity for DA vs. 5HT) used in these studies was that most had been tested previously in rhesus monkeys responding under a progressive-ratio schedule of drug self-administration (Wee et al., 2005; Wang and Woolverton, 2007). Drug self-administration by rhesus monkeys is a strong predictor of abuse liability in humans, and it has historically played an important role in preclinical abuse liability assessment for regulatory purposes (Balster and Bigelow, 2003). Moreover, progressive-ratio schedules of reinforcement may be especially useful in stratifying the relative reinforcing efficacy of different drugs (Richardson and Roberts, 1996; Stafford et al., 1998; Stoops, 2008), and the monoamine releasers studied here maintained a wide range of different breakpoints in rhesus monkeys responding under a progressive-ratio schedule (Wee et al., 2005; Wang and Woolverton, 2007). Consequently, these compounds afforded a unique opportunity to compare drug effects on ICSS in rats with effects of the same drugs in a well-established nonhuman primate assay of preclinical abuse liability assessment. The correlation of results across assays was significant, suggesting that ICSS in rats may function as a good predictor of reinforcing effects of monoamine releasers in monkeys, and by extension, of abuse liability in humans. Previous studies have provided qualitative evidence to
suggest that ICSS procedures are predictive of abuse liability (Vlachou and Markou, 2011; Wise, 1996). This study extends on this earlier work by demonstrating a quantitative relationship between abuse-related effects in ICSS and drug self-administration procedures. Data for four other drugs could not be included in the correlation because either (a) they did not reliably maintain self-administration under the progressive-ratio procedure so that a breakpoint could not be determined ((-)-MDMA, fenfluramine), or (b) they have not been tested in monkeys with the progressive-ratio procedure (phenmetrazine, PAL-287). However, phenmetrazine produced relatively high maximal facilitation of ICSS and also maintained self-administration in nonhuman primates under fixed-ratio schedules of reinforcement (Griffiths et al., 1976; Corwin et al., 1987), whereas PAL-287 produced weaker maximal facilitation of ICSS and did not reliably maintain self-administration in rhesus monkeys under a fixed-ratio schedule of reinforcement (Rothman et al., 2005). Thus, results with these compounds also support the general correspondence of results from rat ICSS and monkey self-administration procedures.

Although monkey self-administration is a strong predictor of human abuse and allows for a quantitative correlation with ICSS, we can also attempt a direct comparison of abuse-related effects in ICSS with abuse in humans. Although not quantifiable, there is a qualitative correlation between the abuse liability of these compounds in humans and the effects seen in ICSS. As described in Chapter I, there appears to be a spectrum of abuse with monoamine releasers where amphetamine/methamphetamine > MDMA >> fenfluramine. A similar pattern was seen in ICSS where amphetamine and methamphetamine produced pronounced facilitation over a broad range of doses while
MDMA produced modest facilitation over a narrow range of doses and fenfluramine produced no facilitation at any dose. These data also support the general correspondence of results from rat ICSS as a predictor of abuse liability in humans. Briefly, it is worth noting that the clinical situation is not as clear as may be suggested here. Recall from Chapter I that although MDMA has not typically been described as “addictive,” the prevalence of MDMA initiation was greater than that of cocaine in 2011 indicating things other than the direct reinforcing effects of the drug (as measured by self-administration) or reward (thought to be measured by ICSS) are likely involved in human consumption.

**Rate-dependence in ICSS**

Baseline rates of behavior have been shown to be one potential determinant of drug effects on schedule-controlled behavior. Two monoamine releasers that have been studied extensively to this end are amphetamine and methamphetamine. For example, one seminal study showed that methamphetamine increased low baseline rates of responding at relatively low doses, while decreasing higher response rates at higher doses, in pigeons responding under various schedules of reinforcement (Dews, 1958). Similarly, another study found that efficacy of amphetamine to increase response rates was inversely proportional to the baseline rate of responding in pigeons responding under various schedules with FI components (Barrett, 1974). These types of findings led to the hypothesis that effects produced by amphetamine and analogues of amphetamine (such as methamphetamine) may be largely governed by the frequency of that behavior’s occurrence under baseline conditions – low rates would be more susceptible to drug-induced increases in behavior, and high rates would be more susceptible to drug-induced
decreases in behavior. This hypothesis has been supported by research done in rats responding for food under multiple schedules of reinforcement, where amphetamine (3.0mg/kg) increased the rate of responding under a differential-reinforcement-of-low-rates (DRL) schedule (which generated low baseline rates of responding) while a comparable dose (3.2mg/kg) produced a decrease in response rates maintained under an FR30 schedules of reinforcement (which generated higher baseline rates of responding) (Sidman, 1956; Owen, 1960). Similarly, one can evaluate responding under FI schedules to distinguish periods of the interval when responding is low, intermediate, and high as the interval progresses. In studies with rats, pigeons and monkeys, amphetamine and/or methamphetamine tended to increase low rates of responding early in the fixed interval but decrease high rates of responding late in the fixed interval (Harris et al., 1978; Smith, 1964; Kelleher and Morse, 1968). It is also worth noting that, in these studies, drugs were generally more potent to increase low rates of responding than to decrease high rates.

The current studies using an ICSS procedure in rats confirm and extend these earlier findings in three ways. First, amphetamine and methamphetamine predominately increased low ICSS rates maintained by low brain-stimulation frequencies while having little to no effect on high ICSS rates maintained by high brain-stimulation frequencies. Both drugs produced effects on ICSS that met the criterion for rate-dependence (i.e. slope of the linear regression on the rate-dependency plot significantly differed from “0”).

Second, amphetamine and methamphetamine effects on rate-dependency parameters were dose-dependent, with increases in dose producing increases in both -slope and Y-intercept values. Amphetamine has previously been shown to produce dose-dependent
changes in rate-dependency parameters under other conditions (Harris et al., 1978). Lastly, the current studies expanded assessment of rate-dependency from the prototype amphetamines to nine other monoamine releasers that varied across a >8000-fold range in their pharmacological selectivity to release DA/NE vs. 5HT, and to mixtures of DA-selective and a 5HT-selective releaser. At least one dose of each monoamine releaser and mixture produced rate-dependent effects, and this permitted correlation between rate-dependency parameters (-slope, Y-intercept) and pharmacological selectivity. Peak-slope values did not correlate with either pharmacological selectivity to release DA vs. 5HT or with proportion of the DA- vs. 5HT-selective releasers. By contrast, peak Y-intercept values (a measure of maximum reinforcement rate at 10% baseline responding) did correlate significantly with pharmacological selectivity to release DA and were significantly higher for PAL-353/fenfluramine mixtures that contained higher vs. lower proportions of PAL-353. These findings suggest that rate-dependent effects on ICSS may be shared across a wide range of monoamine releasers, and the vertical position of the rate-dependence plot (as defined by Y-intercept) may be an attribute of rate-dependence that could be used to distinguish drugs.

**Implications of rate dependence for mechanisms of drugs effects on ICSS**

In assays of ICSS, responding is maintained by delivery of electrical brain stimulation, and effects of monoamine releasers and other drugs on ICSS are often interpreted as reflective of drug-induced changes in sensitivity to the reinforcing stimulus (Esposito et al., 1978; Wise 1980). From this perspective, drug effects on ICSS are considered to be dependent primarily on the magnitude of the reinforcing stimulus and on drug-induced
changes in detection of that reinforcing stimulus. However, the observation of rate-
dependent drug effects in the present studies suggests that monoamine releaser effects on 
ICSS may be dependent instead on baseline rates of responding, and at least to some 
degree, independent of reinforcer magnitude. This conclusion resonates with earlier 
studies finding, for example, that amphetamine produced rate-dependent effects on rates 
of fixed-interval responding maintained by either food presentation or by stimulus-shock 
avoidance (e.g. Kelleher and Morse, 1968), suggesting the potential for independence of 
drug effects from reinforcer type. Moreover, other direct and indirect DA agonists can 
increase low rates of responding maintained in drug self-administration assays either by 
low cocaine doses or by vehicle, suggesting the potential for independence of drug effects 
from reinforcer magnitude (Barret et al., 2004; Panlilio et al., 1998; Caine et al., 1999). 
Lastly, recent studies with ICSS have also provided evidence to suggest that stimulant 
effects reflect processes other than enhanced sensitivity to the electrical stimulus (e.g. 
decreased sensitivity to the “cost” of responding [Hernandez, 2010]). Results of the 
present studies suggest that rate-dependency may provide one source of reinforcer-
independent factors that influence drug effects on ICSS.

Rate-dependency in ICSS as an index of abuse-related effects. Both clinical studies 
(Brauer et al., 1996) and preclinical drug self-administration studies (Wee et al., 2005; 
Wang and Woolverton, 2007) suggest that abuse-related effects of monoamine releasers 
vary as a function of pharmacological selectivity to release DA vs. 5HT. In Chapter III, 
the peak Y-intercept parameter of rate-dependency plots in ICSS also correlated with 
pharmacological selectivity to release DA vs. 5HT. Taken together, these findings
suggest that peak Y-intercept of rate-dependent drug effects on ICSS in rats may serve as a predictor of other preclinical and clinical measures of abuse liability. To assess that possibility, Figure III.7 shows the correlation between peak Y-intercept for seven drugs tested in this study and peak breakpoint maintained by the same seven drugs in rhesus monkeys responding under a progressive-ratio schedule (Wee et al., 2005; Wang and Woolverton, 2007). These data produced a statistically significant correlation (Pearson r = 0.7984, R squared = 0.6374, p-value = 0.0314). As mentioned in the total stimulations correlation above, four compounds were not included in this correlation. Two were not tested in the progressive-ratio procedure (phenmetrazine, PAL-287) but qualitatively fit the correlation (phenmetrazine is abused and has a relatively high Y-intercept; PAL-287 is not and has a relatively low Y-intercept), and two did not maintain progressive-ratio responding ((-)-MDMA, fenfluramine). Thus, the significant correlation presented in Fig. III.7, together with the qualitative examples provided above, suggests that peak Y-intercept values from rate-dependency plots may serve as a dependent measure in ICSS that is predictive of reinforcing efficacy in assays of drug self-administration. This possibility is further supported by recent studies with the mu opioid receptor agonist morphine (Altarifi and Negus, 2011). In this study, the same Y-intercept metric of rate dependency also increased as a function of a variable (chronic opioid treatment) that is known to enhance other measures of mu agonist abuse liability (e.g. break points in progressive-ratio drug self-administration procedures in nonhuman primates). Additional work would be needed to assess the predictive validity of Y-intercept in ICSS results for breakpoint in drug self-administration results across other classes of drugs or other conditions.
Comments on Data Analysis

Three approaches were used in these studies to generate summary measures of drug effects on ICSS for correlation with other neurochemical and behavioral data. One approach used regression analysis to determine drug-induced changes in threshold frequencies required to maintain ICSS. The second approach evaluated drug-induced changes in the total number of stimulations delivered across all frequencies, and this latter measure integrated both drug-induced increases in low rates of ICSS maintained by low stimulation frequencies and drug-induced decreases in high rates of ICSS maintained by high stimulation frequencies. The third approach utilized linear regression of the points on a rate-dependency plot to generate a “parameter of rate-dependence” (Y-intercept). All three approaches agreed in showing that all drugs except fenfluramine facilitated ICSS. However, only the total-stimulations and Y-intercept correlations yielded a measure of ICSS efficacy that correlated with in vitro selectivity to release DA vs. 5HT and with in vivo efficacy to maintain self-administration in rhesus monkeys. By contrast, the threshold measure did not correlate with in vitro pharmacological selectivity or in vivo reinforcing efficacy in monkeys. This dissociation likely reflects constraints on the range of conditions across which threshold analysis can be applied. For example, in the present study, thresholds could often not be calculated after administration of high drug doses because ICSS rates were greater or less than criterion rates across the entire frequency range. Such drug-induced “vertical” shifts in ICSS frequency-rate curves are often interpreted as evidence of motor effects that alter response capability, as opposed to lateral shifts often interpreted as hedonic effects that selectively alter reward-related effects produced by stimulating brain reward substrates (Miliaressis et al., 1986; Carlezon
The constraints imposed by threshold analysis have provided useful boundaries that protect against confounding motor effects in research using ICSS to examine hedonic effects of experimental manipulations on brain reward substrates. However, the present results suggest that these constraints may pose an obstacle to quantitative prediction of rewarding effects and abuse liability, perhaps because expression of rewarding effects and abuse liability involves an integration of both hedonic and motor effects. The significant correlations of the total-stimulation and Y-intercept measures of efficacy to facilitate ICSS with both in vitro pharmacological selectivity and in vivo efficacy in non-human primate self-administration assays suggests that these metrics would be most useful in predicting expression and pharmacological determinants of the abuse liability of existing and novel monoamine releasers. The utility of these measures for predicting reinforcing effects and abuse liability of drugs from other pharmacological classes remains to be determined.

Chapter IV
Role of the 5HT$_{2C}$ receptor in the rate-decreasing effects of monoamine releasers

Results of the studies performed in Chapter IV confirm and extend previous studies that have examined the effects of the 5HT$_{2C}$ receptor on intracranial self-stimulation in rats. In agreement with the past findings, activation of the 5HT$_{2C}$ receptor was sufficient to decrease responding for ICSS, and this effect could be antagonized with a 5HT$_{2C}$ antagonist (Katsidoni et al., 2011). Several other findings were also in agreement with previously published studies: the DA-selective releaser amphetamine facilitated responding in ICSS (Chapter II; Wise and Munn, 1995), the 5HT-selective releaser
fenfluramine decreased responding for ICSS (Chapter II; Olds and Yuwiler, 1992; Olds, 1995), and the kappa opioid agonist U69,593 decreased responding for ICSS (Negus et al., 2010). These previous studies were extended by examining the role of the 5HT$_{2C}$ receptor in the effects produced by these compounds. 5HT$_{2C}$ antagonism blocked the rate-decreasing effects produced by fenfluramine without altering the rate-decreasing (abuse-limiting) effects of U69,593 or the rate-increasing effects of amphetamine. These results demonstrate the selectivity of the antagonist to block rate-decreasing effects mediated by 5HT release without altering either the rate decreasing-effects mediated by activation of the kappa opioid receptor or the rate-increasing effects mediated by DA release.

Previous ICSS studies using the non-selective releasers PAL-287 and/or MDMA, performed by our laboratory and others (Chapter II; Lin et al., 1997), have shown these compounds to produce both rate-increasing (abuse-related) effects and rate-decreasing (abuse-limiting) effects. Although no other ICSS studies have been done with PAL-287, the current study showed that, similar to fenfluramine, 5HT$_{2C}$ antagonism was sufficient to block most of the rate-decreasing effects produced by PAL-287. Unlike fenfluramine, this antagonism also produced a greater increase in facilitation of low frequencies. Thus the data collected with SB 242,084 as a pretreatment to fenfluramine and PAL-287 would support the hypothesis that the 5HT$_{2C}$ receptor plays a major role in the abuse-limiting effects produced by 5HT release. In keeping with this hypothesis, one previous study examined the effect of MDMA in the presence and absence of a non-selective 5HT antagonist (methysergide) and showed that the antagonist blocked the rate-decreasing effects produced by MDMA without changing the threshold levels of responding (Lin et
al., 1997). This finding implicated 5HT receptors in mediating the rate-decreasing effects of MDMA in ICSS, but did not identify the specific 5HT receptor subtype involved. The present study showed that pretreatment with a selective 5HT$_{2C}$ antagonist attenuated but did not fully block (+)MDMA’s rate-decreasing effects. Similar to results gathered with PAL-287, greater facilitation was seen in the presence of SB 242,084 pretreatment. The partial blockade of the rate-decreasing (and unmasking of greater rate-increasing) effects achieved with SB 242,084 supports the role of the 5HT$_{2C}$ receptor in mediating abuse-limiting effects, but, taken together with the full blockade produced in the methysergide study, indicates that other serotonergic receptors also contribute to the rate-decreasing effects produced by (+)MDMA. It is interesting that antagonism of the 5HT$_{2C}$ receptor appeared sufficient to almost entirely block the rate-decreasing effects produced by PAL-287 but not (+)MDMA despite these compounds sharing very similar selectivities to release DA vs. 5HT (Rothman et al., 2005; Wang et al., 2007). This difference may be partly explained by the fact that, besides releasing DA and 5HT, PAL-287 also acts as a potent, low efficacy agonist (Emax = 20%) at the 5HT$_{2C}$ receptor (Rothman et al., 2007), whereas (+)MDMA appears to have low affinity for this receptor (Nash et al., 1994).

**Implications for the 5HT$_{2C}$ receptor in abuse liability**

Our lab has previously shown that % baseline reinforcers in ICSS correlated with progressive ratio breakpoint in nonhuman primates (Chapter II). The present results would predict that pretreatment with a 5HT$_{2C}$ antagonist would increase the breakpoints maintained by PAL-287, and to a lesser degree (+)MDMA, while having no effect on amphetamine. In particular, high doses of PAL-287 (Figure IV.3, Panel D), which would
not normally have been associated with abuse-related effects (white bars), may now have an increased potential for abuse in the presence of a 5HT\textsubscript{2C} antagonist (black bars). Similarly, moderate doses of (+)MDMA (Figure IV.4, Panel C) produced greater ICSS facilitation (1.0mg/kg) or fewer rate-decreasing effects (1.8 and 3.2mg/kg) in the presence of SB 242,084. These data support the hypothesis that the serotonergic component of non-selective monoamine releasers act to produce abuse-limiting effects, particularly via the 5HT\textsubscript{2C} receptor.

Two unique clinical findings also suggest a correlation between 5HT\textsubscript{2} receptors and abuse of MDMA. First, the 5HT\textsubscript{2} family of receptors has been purported to be associated with acute adverse effects produced by MDMA administration (Liechti et al., 2000). In this study, healthy volunteers were given MDMA with or without ketanserin (a non-selective 5HT\textsubscript{2} receptor antagonist) pretreatment. Individuals receiving the antagonist pretreatment reported fewer acute adverse effects of MDMA such as “inactivation” or “dazed state”. Second, a study of MDMA use among adolescents in the United States reported that young women were more likely to use MDMA than young men (Wu et al., 2010). Interestingly, ovarian hormones decrease 5HT\textsubscript{2C} mRNA expression in non-human primates (Gundlah et al., 1999). Taken together, one might hypothesize that women may be more likely to use MDMA because they have fewer 5HT\textsubscript{2C} receptors and thus experience fewer acute adverse effects. However, one study looking at the subjective effects of MDMA showed that women actually experience more acute adverse effects of MDMA relative to men (Liechti et al., 2001). Therefore, the relationship between the 5HT\textsubscript{2C} receptor, sex, and abuse of MDMA is not clear-cut. In summary, although there is some evidence clinically and pre-clinically that the 5HT\textsubscript{2C}
receptor partially mediates the abuse-limiting effects of produced by 5HT release, much work is left to be done.

Chapter V

Role of selectivity for DA vs. NE in drug discrimination and ICSS

Results of the present studies confirm and extend previous studies looking at the cocaine-like discriminative stimulus effects of monoamine releasers in rats. First, amphetamine dose- and time-dependently substituted for cocaine as previously described in numerous studies in both rats (McKenna and Ho, 1980; Colpaert et al., 1978; D’Mello and Stolerman, 1977) and monkeys (La Garza and Johnson, 1983; Negus et al., 1998). Likewise, amphetamine and cocaine share similar subjective effects in people (Oliveto et al., 1998; Fischman et al. 1976). Also in agreement with previous studies in both rats and monkeys, fenfluramine failed to fully substitute for cocaine (Negus et al., 2007; Wood and Emmett-Oglesby, 1988; McKenna and Ho, 1980). PAL-287 has previously been shown to substitute fully for cocaine in 4/5 monkeys (Negus et al., 2007) but did not substitute for amphetamine in rats (Glennon et al., 1984); in the current study, PAL-287 fully substituted for cocaine in 3/5 rats.

Results from the current study extend previous studies on the cocaine-like discriminative stimulus proprieties of monoamine releasers by looking at 4 novel compounds (PAL-542, PAL-544, PAL-571 and PAL-569) ranging >200 fold in their in vitro selectivity for releasing DA vs. NE while having similar potencies to release 5HT (Table V.1). Although these studies were likely limited by the high potency of these compounds to release 5HT (see discussion below), these data suggest that similar
potencies to release DA and NE (with a slight preference for NE) is required for
expression of cocaine-like discriminative stimulus effects by monoamine releasers in rats.
In particular, the ~60 fold DA-selective compound PAL-542 and 4-fold DA-selective
PAL-544 did not substitute for cocaine at any dose or time. Similar effects were obtained
with the 13-fold NE-selective compound PAL-569. However, PAL-287 (non-selective)
and PAL-571 (2-fold NE-selective) did substitute in a subset of animals (3/5 and 2/5,
respectively) at 100 min and 10 min, respectively.

Amphetamine, PAL-287 and fenfluramine had been previously studied by this lab
and others (Chapter II) and as such, were not re-tested in ICSS. However, dose-effect
and time course studies were done for all four novel monoamine releasers (PAL-542,
PAL-544, PAL-571 and PAL-569) in ICSS. PAL-542, PAL-544, and PAL-569 all
resembled the effects of fenfluramine in ICSS with no facilitation at any dose or time.
Only PAL-571 produced any facilitation, and the facilitation seen was modest (a single
significant frequency at the latest time point [300min]). Thus the results seen in ICSS
with amphetamine, PAL-287, and fenfluramine in addition to the 4 novel compounds
correspond closely with the results gathered in drug discrimination. Specifically, robust
abuse-related effects were seen with amphetamine (full substitution for cocaine and
facilitation of ICSS across a wide range of doses); modest abuse-related effects were seen
with PAL-287 and PAL-571 (partial substitution for cocaine and mixed
depression/facilitation of ICSS); and no abuse-related effects were seen with
fenfluramine, PAL-542, PAL-544, and PAL-569 (no substitution for cocaine or
facilitation of ICSS).

**Limitations presented by high potency to release 5HT and implications for abuse**
The data presented in the Chapter II suggest increased selectivity for 5HT vs. DA may limit the expression of abuse-related effects. In particular, Chapter II showed that the levels of ICSS facilitation achieved with DA-selective drugs were greater than those achieved with non-selective or 5HT-selective compounds. Similarly, results from drug discrimination described in Chapter V suggested that non-selective compounds (PAL-287) or 5HT selective compounds (fenfluramine) do not substitute fully for cocaine, whereas compounds that selectively release DA relative to 5HT (amphetamine) do substitute fully. Unfortunately, compounds with low potency to release 5HT and variable potencies to release DA vs. NE do not exist, and all four of the novel monoamine releasers tested in Chapter V have relatively high potencies to release 5HT. As such, it is possible that the expression of abuse-related effects (substitution for cocaine or facilitation of ICSS) associated with these compounds’ ability to release DA or NE may have been partially (PAL-571) or fully (PAL-542, PAL-544, PAL-569) masked by the abuse-limiting (rate-decreasing) effects produced by high levels of 5HT release. Likely due to the high potency of the novel drugs to release 5HT, the compounds evaluated in this chapter would not be expected to have high abuse liabilities. None produced very significant facilitation of ICSS, and none produced full substitution for cocaine in all animals.

**Sufficiency and necessity of DA and NE in drug discrimination and ICSS**

Data supporting a role for DA in abuse-related effects of monoamine releasers are strong. For example, DA-selective uptake inhibitors like GBR12909 or RTI-113 substitute for amphetamines (Desai et al., 2010) and facilitate ICSS (Rosenberg et al., 2013).
Similarly, DA agonists (both D1 [A77636] and D2 [quinpirole]) have been shown to produce abuse-related effects in ICSS (facilitation) and drug discrimination (substitution for cocaine) (Carr and Kim, 2001; Howell et al., 2000; Callahan et al., 1991; Witkin et al., 1991; Terry et al., 1994). Both discriminative stimulus and ICSS facilitating effects of amphetamines can be blocked by DA antagonists like pimozide (Ho and Huang, 1975; Gallistel and Karras, 1984).

Data supporting a role for NE in abuse-related effects of monoamine releasers are weaker. For example, the NE-selective uptake inhibitor nisoxetine substituted for amphetamine but not cocaine in monkeys (Kamien and Woolverton, 1989; Kleven et al., 1990), and ephedrine (19-fold NE-selective releaser) has been shown to substitute for amphetamine in rats (Young et al., 1998) and partially substitute for cocaine in monkeys (Gold and Balster, 1996). Neither NE-selective uptake inhibitors (nisoxetine and nortryptaline) nor a NE agonist (clonidine) facilitated ICSS in rats (Rosenberg et al., 2013; Gallistel and Freyd, 1987). In the only study to examine effects of a NE antagonist on abuse-related effects of a monoamine releaser, the α1 antagonist prazocin failed to block discriminative stimulus effects of methamphetamine (Munzar and Goldberg, 1999).

These previous studies support, to some degree, the results of the current experiments showing an important role of both DA and NE in the abuse-related effects of monoaminergic drugs. However, the studies mentioned above showing significant substitution for cocaine and amphetamine by ephedrine (19-fold NE-selective) would suggest that PAL-569 (13-fold NE-selective) should have at least partially substituted for cocaine in rats – although, as discussed, the high potency of PAL-569 to release 5HT is a potentially significant confound. Another potential confound is the evidence in the
literature for ephedrine to release significant amounts of DA in addition to NE, indicating
that its in vitro selectivity may not match its in vivo release (Wellman, et al., 1998;
Rothman et al., 2001).

**Future Directions**

Although the studies described in this dissertation could lead to a plethora of future
directions, in keeping with my MD/PhD training, I would like to focus on potential future
studies that could be performed if I carried the results from this dissertation into the
clinic. In particular, I have a serious interest in pursuing the role of the 5HT_{2C} receptor in
the abuse potential of MDMA. As described throughout this dissertation, there is some
evidence that the 5HT_{2C} receptor may mediate abuse-limiting effects of non-selective
releasers. Therefore, if the 5HT_{2C} receptor is involved in limiting the abuse-potential of
MDMA, would individuals with less functional polymorphisms of the 5HT_{2C} receptor
gene also be more likely to abuse MDMA? Two different polymorphisms of the 5HT_{2C}
receptor gene have been described as having opposite effects on weight maintenance.
First, Westberg et al. (2002) showed that a coding region polymorphism resulting in a
cysteine to serine substitution resulted in teenage girls being overly susceptible to weight
loss relative to their peers; while another lab showed a polymorphism in the promoter
region (cysteine to threonine substitution) that was associated with obesity in women
(Pooley et al., 2004). Would functionally opposite receptor polymorphisms such as
these result in individuals who were either hyper- or hypo-sensitive to the abuse-limiting
effects of MDMA that may be mediated through the 5HT_{2C} receptor? Reciprocally, do
those individuals who take larger doses of MDMA more frequently have less functional or fewer 5HT₂C receptors?

In a similar vein, lorcaserin (a 5HT₂C agonist) has recently been approved by the FDA as a prescription anorectic (Reuters.com, 2012). This compound has been shown to be efficacious in helping patients lose weight in a multicenter clinical trial (Smith et al., 2010). If 5HT₂C agonists decrease DA-release, would a 5HT₂C receptor agonist such as lorcaserin act at 5HT₂C receptors to produce abuse-limiting effects that might oppose DA-mediated, abuse-related effects of monoamine uptake inhibitors (e.g. cocaine) or releasers (e.g. amphetamine)? Would it increase the adverse effects often associated with MDMA? Such studies are of critical importance if lorcaserin achieves widespread use in weight management clinics. These are just two examples of psychopharmacology experiments that could easily be done in the clinic and which would nicely correlate with the preclinical work done in Chapter IV of this dissertation.
Appendix J: From raw data to frequency-rate curve for 0.32 mg/kg amphetamine

Baseline Session

Test Session

1. Discard first component

2. Calculate MCR = (60 + 58)/2 = 59

3. Average each

MCR normalize to frequency and components and three last session

4. Average each

10.00
8.17
6.43
5.00
3.67
2.69
2.00
1.25
1.00
0.60
0.50
0.33
0.25
0.17
0.10
Appendix 2: Comparison of raw vs. normalized rates of reinforcement.
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