2015

Structural Determinants of Abuse-Related Neurochemical and Behavioral Effects of Para-Substituted Methcathinone Analogs in Rats

Julie S. Bonano
Virginia Commonwealth University

Follow this and additional works at: https://scholarscompass.vcu.edu/etd
Part of the Pharmacology Commons

© The Author

Downloaded from
https://scholarscompass.vcu.edu/etd/3911

This Dissertation is brought to you for free and open access by the Graduate School at VCU Scholars Compass. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of VCU Scholars Compass. For more information, please contact libcompass@vcu.edu.
Structural Determinants of Abuse-Related Neurochemical and Behavioral Effects of Para-Substituted Methcathinone Analogs in Rats

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

by

Julie S. Bonano
Bachelor of Science, University of North Carolina at Chapel Hill, 2010

Advisor: S. Stevens Negus, Ph.D.
Professor of Pharmacology and Toxicology
Virginia Commonwealth University

Virginia Commonwealth University
Richmond, Virginia
March, 2015
Acknowledgments

The work contained within this dissertation would not have been possible without the endless support of multiple individuals. First and foremost, I’d like to thank my amazing husband, John Bonano, for being my rock and for serving as a constant reminder that I can accomplish my dreams. To my loving parents, Jon and Debbie Suyama, thank you for supporting me in all my endeavors and for making me the woman I am today. To my “seeblings,” Crystal LeBlanc, Ryan and Jonathan Suyama, thanks for believing in me through it all. I would also like to extend my most sincere gratitude to my advisor Steve Negus, my co-advisor Lou De Felice, and the MD/PhD program director Gordon Archer for their advice, guidance and financial and academic support. Likewise, Matt Banks was instrumental in my mastery of microdialysis and in the publication of my manuscripts. Thanks, also, to my wonderful committee members, Richard Glennon, Keith Shelton, Sammanda Ramamoorthy, and Dace Svikis, for sharing their unique research perspectives and contributing to the development and completion of the work described in this dissertation. Finally, I would like to extend a special thanks to my friends and coworkers for their support in and out of the lab, particularly Kelen Freitas, Mike Leitl, Tyler Steele, Krasi Cameron, and Ernesto Solis. I am eternally grateful for the quality of individuals I have in my life. I love and thank you all.
Table of Contents

List of Abbreviations ............................................................................................................... iv
List of Compounds .................................................................................................................. v
Structures of Compounds ......................................................................................................... vi
Abstract ................................................................................................................................... vii
Chapter I: Introduction ............................................................................................................. 1
Chapter II: Abuse-Related and Abuse-Limiting Effects of Methcathinone and the Synthetic “Bath Salts” Cathinones Analogs Methyleneoxyxpyrovalerone (MDPV), Methylone and Mephedrone on Intracranial Self-Stimulation in Rats .............................................................................................................. 41
Chapter III: Quantitative Structure-Activity Relationship (QSAR) Analysis of the Pharmacology of Para-Substituted Methcathinone Analogs ................................................................................. 56
Chapter IV: Abuse-Related Neurochemical Effects of Para-Substituted Methcathinone Analogs in Rats: Microdialysis studies of Nucleus Accumbens Dopamine and Serotonin ........................................................................................................... 78
Chapter V: Discussion ............................................................................................................... 96
Chapter VI: References ............................................................................................................ 123
List of Abbreviations

ANOVA = analysis of variance
DA = dopamine
DAT = dopamine transporter
FR = fixed ratio
ICSS = intracranial self-stimulation
IP = intraperitoneal
MAR = monoamine releaser
MCR = maximum control rate
MFB = medial forebrain bundle
mg/kg = milligrams per kilogram
NAc = Nucleus accumbens
NE = norepinephrine
NET = norepinephrine transporter
QSAR = quantitative structure-activity relationship
SEM = standard error of the mean
SERT = serotonin transporter
VTA = ventral tegmental area
5-HT = serotonin
List of Compounds

Chapter II:
Methcathinone (MCAT)
Methylenedioxyphenylethylamine (MDPV)
Methylone (methylenedioxyamphetamine; MDMC)
Mephedrone (4-CH₃ MCAT)

Chapter III:
Methcathinone (MCAT)
Flephedrone (4-F MCAT)
Mephedrone (4-CH₃ MCAT)
Methedrone (4-OCH₃ MCAT)
4-Chloromethcathinone (4-Cl MCAT)
4-Bromomethcathinone (4-Br MCAT)
4-Fluoromethcathinone (4-F MCAT; 4-Flmap)

Chapter IV:
Amphetamine (AMPH)
Fenfluramine (FEN)
Methcathinone (MCAT)
Flephedrone (4-F MCAT)
Mephedrone (4-CH₃ MCAT)
Methedrone (4-OCH₃ MCAT)
4-Chloromethcathinone (4-Cl MCAT)
4-Bromomethcathinone (4-Br MCAT)
Structures of Compounds

Chapter II:

Methcathinone  
MDPV  
Methylone  
Mephedrone

Chapters III and IV:

MCAT, $R = H$
4-F MCAT (Flephedrone), $R = F$
4-CH$_3$ MCAT (Mephedrone), $R = CH_3$
4-OCH$_3$ (Methedrone), $R = OCH_3$
4-Cl MCAT, $R = Cl$
4-Br MCAT, $R = Br$
4-CF$_3$ MCAT, $R = CF_3$

Chapter IV:

Amphetamine  
Fenfluramine
Abstract

STRUCTURAL DETERMINANTS OF ABUSE-RELATED NEUROCHEMICAL AND BEHAVIORAL EFFECTS OF PARA-SUBSTITUTED METHCATHINONE ANALOGS IN RATS

By Julie S. Bonano

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

Virginia Commonwealth University, 2015

Advisor: S. Stevens Negus, Ph.D.

Methcathinone (MCAT) is the β-ketone analog of methamphetamine, and like its amphetamine analog, MCAT functions as a monoamine releaser that selectively promotes the release of dopamine (DA) and norepinephrine (NE) over serotonin (5-HT). MCAT produces amphetamine-like psychostimulant effects and is classified as a Schedule I drug of abuse by the United States Drug Enforcement Administration (DEA). Recently, synthetic MCAT analogs have emerged as designer drugs of abuse in Europe and the United States and have been marketed under deceptively benign names like “bath salts” in an attempt to evade legal restriction. These dangerous, recently emergent and novel drugs of abuse display varying selectivity to promote release of DA/NE vs. 5-HT, and selectivity for DA neurotransmission is believed to correlate with abuse liability. The goal of this dissertation was to conduct preclinical research to examine structural determinants of abuse-related behavioral and neurochemical effects produced by a series of synthetic MCAT analogs. Specifically, this project focused on one feature of the methcathinone scaffold: the para substituent of the benzene ring. A series of six novel MCAT analogs will be examined
to evaluate how physicochemical parameters (steric, $E_s$; electronic, $\sigma_p$; lipophilic, $\pi_p$) of the \textit{para} substituent influence \textit{in vitro} monoamine transporter selectivity as well as \textit{in vivo} neurochemical and behavioral effects. Results from this body of work implicate steric factors as being particularly important in determining a compound’s abuse-related neurochemical and behavioral effects. Thus, these data not only offer an improved understanding of the mechanism of abuse-related drug effects produced by synthetic MCAT analogs, but also help in the generation of homology models of the human DA and 5-HT transporters (DAT and SERT, respectively).
I. Synthetic Cathinone Abuse: a Clinical Problem

Khat (*Catha edulis*) is a perennial crop indigenous to the Eastern Horn of Africa and the Arabian Peninsula where it has been used for millennia for its central stimulant effects (Alles et al., 1961). The khat plant grows as a bush or tree and contains leaves that are chewed or brewed into a tea. The social context surrounding khat consumption closely resembles that of coffee consumption in Western society. In fact, khat’s cultivation and use in Ethiopian and southwestern Arabian regions predates the use of coffee, with its first historical reference dating back to the early 14th century (Cerulli, 1936; Ukers, 1955). The khat plant itself was first classified in 1762 by botanist Forskål who noted its cultivation and reported its use within Yemen as a plant of “medicinal virtues” (Alles et al., 1961). For centuries, attempts were made at elucidating the chemical structure of the active compounds in khat. In 1887, Flückiger and Gerock found that caffeine was not present in the plant and could not be responsible for the plant’s pharmacological stimulant effects. They did, however, discover trace amounts of a basic material that they called “katine” (Fluckiger and Gerock, 1887).

It is now known that cathinone is the primary psychoactive component of the khat plant. Although cathinone was not isolated from khat until 1975 (U.N. Document, 1975), its N-methyl derivative methcathinone (MCAT) was synthesized decades before in 1928 as a precursor to ephedrine (Hyde et al., 1928). Structurally, cathinone and MCAT are the β-ketone analogs of amphetamine and methamphetamine (see Figure I.1), and they function similarly as monoamine
 releasers that selectivity promote release of dopamine (DA) and norepinephrine (NE) over serotonin (5-HT) (Wagner et al., 1982; Nielsen and Schechter, 1985; Kalix and Glennon, 1986; Cozzi et al., 1999, 2013). Both cathinone and MCAT produce amphetamine-like stimulant effects (Glennon et al., 1987; Dal Cason et al., 1997) and are classified as Schedule I drugs of abuse by the Drug Enforcement Administration (DEA). Federal scheduling of cathinone and MCAT in the early 1990s reduced their use and availability, but it also stimulated a wave of synthesis of synthetic cathinone derivatives that emerged as designer drugs in the U.K. in 2009 and the U.S. in 2010. These novel synthetic cathinones were marketed as legal alternatives to cocaine, MDMA and the amphetamines, given deceptive names like “bath salts,” and labeled “not for human consumption” to evade legal restriction (Spiller et al., 2011).

Methylenedioxyxypyrovalerone (MDPV), mephedrone and methylone are three cathinone derivatives that were amongst the most common constituents of the first wave of “bath salts.” Bath salts are generally sold as a white or brown powder on the internet or in drug paraphernalia shops and produce euphoric effects, as well as increased sociability and sex drive (NIDA, 2014). In addition to these euphoric effects, bath salts can produce dangerous side effects, including paranoia, agitation, and hallucinatory delirium (Hohmann et al., 2014). Although synthesis of synthetic cathinones has been described for decades (mephedrone in 1929, Saem de Burnaga Sanchez, 1929; MDPV in 1967, Boehringer and Sohn, 1967; methylone [MDMC] in 1996, Jacob and Shulgin, 1996; Dal Cason et al., 1997), their presence was largely ignored until their marketing and abuse as legal alternatives to MDMA were first reported on Internet drug websites in 2003 (Morris, 2010). Following an increase in
their prevalence over subsequent years, as well as an increase in associated negative health consequences (i.e. emergency department visits, calls to poison control centers) (Spiller et al., 2011), MDPV, mephedrone and methylone were emergency scheduled by the DEA in October 2011. After preclinical testing substantiated claims of abuse potential, these three bath salts constituents were moved to permanent Schedule I classification (DEA, 2011). While Schedule I status of these bath salts compounds has reduced access to and consumption of early synthetic cathinones (Stogner and Miller, 2013), it has also triggered the emergence of a second wave of further derivatized synthetic cathinones (Thornton et al., 2012; Marinetti and Antonides, 2013).

Methedrone and flephedrone are two additional synthetic cathinones that emerged in the second wave of bath salts. These derivatives have already been identified by US Drug Courts, in toxicology screens, and in the international drug market (Prosser and Nelson, 2012; Leffler et al., 2014), and both were added to the growing list of Schedule I drugs of abuse in April 2014. Studies on the effects of various synthetic MCAT derivatives on motor behavior and a functional observation battery in mice showed that methedrone and flephedrone share important pharmacological properties with other Schedule I synthetic cathinones (i.e. mephedrone, MDPV) (Marusich et al., 2012), supporting their recent classification as Schedule I drugs. The increasing prevalence of \textit{para}-substituted MCAT analogs in the drug market and in toxicology screens provides significant rationale for investigating neurochemical and behavioral effects of other \textit{para}-substituted derivatives that may be the next drugs of abuse to emerge, such as 4-
chloromethcathinone (4-Cl MCAT), 4-bromomethcathinone (4-Br MCAT), and 4-trifluoromethylmethcathinone (4-TFMMC, 4-CF3 MCAT).

**Figure I.1**

**Structural Comparisons between Amphetamines and Cathinones**

![Structural Comparisons between Amphetamines and Cathinones](image)

*Structural comparison of amphetamine and cathinone analogs. Cathinones are β-keto amphetamines, differing from their amphetamine analogs by the presence of a carbonyl group at the beta position relative to nitrogen. Amphetamines and cathinones exist as one of two enantiomers, designated (S)-(+) and (R)-(−). Enantiomers are a class of stereoisomers in which each drug isomer is a non-superimposable mirror image of the other, differing only its ability to rotate plane-polarized light. Racemic mixtures, containing equal parts of each enantiomer, are investigated in this dissertation.*
Synthetic cathinones are most commonly ingested orally ("bombing") or by insufflation in doses of ~100-200mg and are consumed in binge-like patterns with an average of six doses across a nine-hour period (German et al., 2014). Because of their stimulant and hallucinogenic properties, synthetic cathinones are often used as alternatives to MDMA, cocaine and amphetamine. As with many drugs of abuse, subjective reports of synthetic cathinone intoxication are highly variable. One account of mephedrone intoxication from the Erowid Experience Vaults describes the high as a positive experience: “The euphoria was absolutely crazy. I looked in the mirror, my eyes were saucers. I felt giddy from how amazing I felt... The stuff is dangerous in how much I can fiend it, though.” Another account of mephedrone intoxication, however, describes more disconcerting drug effects: “What I experienced was rather disappointing in so much that it wasn’t anything like MDMA, though my pupils were gigantic, my heart was racing, my teeth were grinding, I was freezing one moment then sweating the next. I couldn’t focus my eyes on anything... and as the night went on, my eyes would dart around from one hallucination to the next until I realized those things/shadows weren't really there.”

Moreover, various case reports of bath salts intoxication have demonstrated health hazards associated with use and abuse of synthetic cathinones. A recent case series involving five patients who presented to the emergency department (ED) after ingesting bath salts describes the constellation of symptoms as similar to other sympathomimetic drugs with “delirium, hallucinogenic-delusional symptoms, extreme agitation, combativeness, and rhabdomyolysis” (Imam et al., 2013). All five patients were male, and their ages ranged from 28-42 years old. Four of the five
patients had past medical histories of psychiatric disease (e.g. bipolar, depression, anxiety, ADHD), and all presented with elevated heart rate and respiratory rate. Interestingly, despite all five patients admitting to bath salts ingestion, three patients had negative urine drug screens, one tested positive for cannabinoids, and one tested positive for cocaine. These negative drug screens for bath salts constituents suggest that hospitals either lack the analytical methods required to detect synthetic cathinones and their metabolites in urine, or patients have taken drugs that they believed to be bath salts when, in fact, they did not. In either scenario, the discrepancy contributes to skewed epidemiology and, more than likely, an overall underestimation of bath salts use.

II. Anatomy and Function of Monoamine Systems

Monoamines, receptor location and binding, and neuronal projections

Synthetic cathinones produce their behavioral effects primarily by acting on neural circuits in the brain that use the monoamine neurotransmitters dopamine (DA), serotonin (5-HT) and norepinephrine (NE). Studies of the organization of monoaminergic neuronal systems and their projections throughout the brain date back to the early 1960s when the two primary catecholamines DA and NE were first identified (Carlsson et al., 1962). Shortly thereafter, the first detailed report on the distribution of DA-, NE- and 5-HT-containing neurons in the rat brain was published (Dahlstroem and Fuxe, 1964), in which twelve groups of catecholamine cells (labeled A1-A12) were identified. Then, with the advent of immunohistochemistry for the catecholamine-synthesizing enzymes tyrosine hydroxylase (TH), dopa-
decarboxylase, and dopamine-β-hydroxylase in the 1970s, more detailed mapping of the catecholaminergic systems (particularly dopaminergic projections) became possible. While the presence of TH at detectable levels remains one of the most sensitive and consistent markers for labeling DA neurons, it is neither selective nor completely reliable; TH is also present in noradrenergic neurons, and TH expression in DA neurons can vary over time and in response to functional demand (Björklund and Dunnett, 2007).

DA, synthesized from the non-essential amino acid tyrosine, is the predominant catecholamine neurotransmitter in mammals, and its immediate precursor is L-DOPA. After synthesis, DA is sequestered into synaptic vesicles where it is stored until an action potential is fired within the dopaminergic neuron, which causes vesicular docking and release into the synaptic cleft. Once DA enters the synapse, it functions as a neurotransmitter by binding to and activating DA receptors. DA receptors are seven transmembrane G-protein coupled receptors (GPCRs), and at least five DA receptor subtypes (D₁-D₅) exist: two D₁-like receptor subtypes (D₁ and D₅), which are Gₛ-coupled and stimulate adenylyl cyclase, and three D₂-like subtypes (D₂, D₃, D₄), which are Gₛ-coupled and inhibit adenylyl cyclase (Missale et al., 1998). DA receptors are widely expressed throughout the CNS, and they are involved in the control of locomotion, cognition, emotion and endocrine regulation. The D₁ receptor is the most widespread and most highly expressed of the DA receptors, and D₁ mRNA is most abundant in caudate, nucleus accumbens, and olfactory tubercle (Dearry et al., 1990). The D₅ receptor is less prevalent in the rat brain compared to D₁, and D₅ mRNA predominates in cerebral cortex, lateral
thalamus and striatum (Choi et al., 1995). Furthermore, while the D₂ receptor is expressed in all major brain regions receiving dopaminergic projections (e.g. striatum and nucleus accumbens), D₃ receptors have a more specific distribution pattern within limbic areas (Bouthenet et al., 1991). Lastly, D₄ receptors are most highly expressed in frontal cortex, olfactory bulb, hypothalamus and thalamus (O’Malley et al., 1992). DA receptors are also found in the peripheral nervous system (PNS), but are largely localized to the kidney, vasculature and pituitary where they function primarily to modulate cardiovascular and renal function and tone.

The primary sources of dopaminergic neurons are the substantia nigra (SN; A9) and ventral tegmental area (VTA; A10). As depicted below in Figure I.2, DA neurons originating in the SN project primarily to the caudate-putamen along the nigrostriatal pathway, while those originating in the VTA project mainly to the nucleus accumbens and prefrontal cortex along mesolimbic and mesocortical pathways, respectively (Oades and Halliday, 1987). Although the designation of these three historical DA pathways is convenient, it is likely an oversimplification, as cells of origin in the SN-VTA complex are intermixed such that SN contains neurons that also innervate cortical and limbic regions, and VTA contains DA neurons projecting to ventral striatum and parts of the caudate-putamen (Björklund and Dunnett, 2007).
Figure 1.2

Distribution of dopaminergic projections in the rat brain. Dopaminergic neuronal cell bodies in the SN project predominantly to the caudate-putamen and comprise the nigrostriatal pathway, while DA neurons in the VTA project primarily to the nucleus accumbens and neocortex along the mesolimbic and mesocortical pathways, respectively. Image from homepage.psy.utexas.edu/homepage/class/Psy332/Salinas/Neurotransmitters/Slide08.GIF.

Norepinephrine (NE) is the other primary catecholamine neurotransmitter in mammalian brains and, like DA, is synthesized from tyrosine prior to packaging into synaptic vesicles. DA is the direct precursor to NE and is converted to NE by the enzyme dopamine-β-hydroxylase. NE functions by binding to and activating adrenergic receptors, another class of GPCRs that serve as targets not only for NE but also for epinephrine. The two main types of adrenergic receptors, α and β, are further divided into subtypes: α₁ (Gq-coupled), α₂ (G1-coupled), and β₁-β₃ (allGs-coupled).
coupled; β2 also couples to Gi) (Insel, 1996). Adrenergic α1 receptors are located on vascular smooth muscle, gastrointestinal (GI) and urinary sphincters, and arrector pili muscles where they play a critical role in the ‘fight or flight’ sympathetic response, while α2 receptors are located on secretory terminals of noradrenergic neurons where they function as a negative feedback mechanism. The β1 adrenergic receptor is predominantly expressed in cardiac tissue where activation by NE increases heart rate and cardiac muscle contractility. β2 receptors are found in smooth and striated muscle where they contribute to sympathetic responsiveness, and the β3 receptor is located mainly in adipose tissue where it plays a key role in lipolysis and thermogenesis. Synthesis of NE occurs primarily in neuronal cell bodies within the locus ceruleus (LC), and noradrenergic neurons project from the LC to the lateral tegmentum, hippocampus, amygdala, entorhinal cortices, thalamus and neocortex (Von Bohlen und Halbach and Dermietzel, 2006). These noradrenergic projections are shown in Figure I.3.
Distribution of noradrenergic projections in the rat brain. The LC contains ~1,500 NE cells that project to the ipsilateral forebrain, and axonal branching permits a single neuron to have nerve terminals in various brain regions. The only major region that is not innervated by neurons from the LC is the region containing the basal ganglia.


Unlike DA and NE, which are catecholamines derived from tyrosine, serotonin (5-HT) is a monoamine neurotransmitter that belongs to the tryptamine class and is biochemically derived from the essential amino acid tryptophan. 5-HT and its receptors are primarily localized to the GI tract, blood platelets and, notably, throughout the CNS (Baumgarten and Grozdanovic, 1997). 5-HT receptors are a group of receptors that are activated by their endogenous ligand 5-HT to influence a variety of biological processes, including anxiety, cognition, learning, memory, mood and sleep, amongst others. Seven 5-HT receptor classes are known to exist (5-HT1-7), and all are GPCRs with the exception of 5-HT3, which is a ligand-gated ion channel.
These seven receptor classes include a total of 14 5-HT receptor subtypes: 5-HT₁A, 1B, 1D, 1E and 1F, 5-HT₂A, 2B and 2C, 5-HT₃, 5-HT₄, 5-HT₅A and 5B, 5-HT₆ and 5-HT₇ (Hoyer et al., 2002). 5-HT receptor subtypes that are most commonly implicated in addiction include 5-HT₁A, 5-HT₁B, 5-HT₂A, 5-HT₂C and 5-HT₃. 5-HT₁A receptors are diffusely distributed throughout the CNS and, in the raphe nuclei, they function as autoreceptors to inhibit serotonergic neuronal firing. 5-HT₁B receptors are also expressed in the CNS, but they are concentrated in the basal ganglia, striatum and frontal cortex (Hoyer et al., 2002). 5-HT₂A receptors are widely distributed in peripheral and central tissues; within the CNS, 5-HT₂A receptors are primarily located in cortex, claustrum and basal ganglia. 5-HT₂C receptor distribution, on the other hand, is confined to the CNS and choroid plexus. Lastly, 5-HT₃ receptors are present in the CA1 pyramidal cell layer of the hippocampus, the dorsal motor nucleus of the solitary tract, and the area postrema (Laporte et al., 1992). The raphe nuclei serve as the primary source of 5-HT, and serotonergic neurons project from the raphe to the caudate-putamen, globus pallidus, amygdala, limbic forebrain and neocortex (Steinbusch, 1981). Specifically, nine groups of serotonergic cell bodies have been identified and designated B1-B9 (Dahlstroem and Fuxe, 1964), and serotonergic projections from these nuclei are depicted below in Figure 1.4.
Although monoamine systems are relatively distinct and differentiable, both functionally and anatomically, there is also a great degree of interconnectivity between them (see Figure I.5). Various studies over the last few decades have demonstrated the existence of important functional interactions between DA, NE and 5-HT systems. For example, serotonergic systems have been shown to negatively regulate NE and DA systems through 5-HT$_{2A}$ and 5-HT$_{2C}$ receptor-mediated mechanisms, respectively (Szabo and Blier, 2002; De Deurwaerdère et al., 2004). Conversely, noradrenergic systems have been shown to exert positive and negative influences on 5-HT systems through $\alpha_1$- and $\alpha_2$-adrenergic receptors, respectively (Baraban and Aghajanian, 1980).
Interacting systems: functional connectivity of monoaminergic neurons. Direct and indirect connections between DA, NE and 5-HT neurons are mediated by various receptor types, and monoamine systems can interact in at least two ways. First, neurons containing one neurotransmitter type can be directly modulated by inputs from neurons containing a different neurotransmitter. For example, DA neurons may express 5-HT receptors that mediate serotonergic modulation of DA release. Second, neurons containing different monoamines can provide convergent input on downstream neurons. For example, a target neuron may express both DA and 5-HT receptors that mediate convergent and interacting input from DA and 5-HT neurons. Image from Monoamine Neurocircuitry Review by Hamon and Blier, 2013.
Monoamine Transporters

Plasma membrane and vesicular monoamine transporters (see Figure I.7) play a key role in regulating the effects of DA, NE and 5-HT. Plasmalemmal monoamine transporters belong to the soluble carrier 6 (SLC6) family and use the electrochemical gradients of sodium (Na\(^+\)) and chloride (Cl\(^-\)) as the driving force to mediate the rapid uptake of substrate (i.e. monoamine neurotransmitter) from the extracellular space following synaptic release. A simplified view of the ionic stoichiometry of plasmalemmal monoamine transporters is that DA and NE transporters (DAT and NET, respectively) translocate one neurotransmitter with 2 Na\(^+\) and 1 Cl\(^-\); while the 5-HT transporter (SERT) transports 5-HT with 1 Na\(^+\) and 1 Cl\(^-\) (Benarroch, 2013). This fixed stoichiometry is based largely upon Michaelis-Menten kinetics in which neurotransmitter uptake is plotted as a function of neurotransmitter concentration; however, these M-M curves are generated under conditions of constant ionic (Na\(^+\) and Cl\(^-\)) concentrations, and experiments are performed without controlling for voltage. Consequently, while this notion of fixed stoichiometry at plasmalemmal transporters is easy to understand and generally accepted within the scientific community, it is likely a vast oversimplification of transporter function. Alternative views suggest that changes in membrane potential (i.e. voltage) and/or transporter substrate concentrations contribute to variable “channel modes” of monoamine transport that produce coincident changes in ionic stoichiometry (Pramod et al., 2013). An “alternating access” model of monoamine transport shown in Figure I.6 illustrates the dynamic nature of plasmalemmal monoamine transporters, which supports the concept of variable stoichiometry.
Figure 1.6

Diagram of alternating access and channel modes of monoamine transport. Substrate and ions have access to the binding site of outward facing transporters (i). Once inside the transporter, the outer gate closes and generates an occluded state (ii), which ultimately transitions to an inward facing state (iii) that releases substrate and ions into the cytosol. The transporter may also undergo a transition to a channel state in which outward and inward facing gates are open simultaneously, allowing for a less restricted, variable stoichiometry flow of substrate and ions. Figure from Pramod et al., 2013.
DAT, NET and SERT are expressed in the plasma membrane of dendrites and axons of their respective monoaminergic neurons, and these transporters serve as the major mechanism for terminating monoaminergic signaling. Vesicular monoamine transporters (VMATs), on the other hand, are members of the SLC18 family and utilize the vesicular pH gradient as the driving force for sequestration of cytoplasmic DA, NE and 5-HT into the synaptic vesicle. While plasmalemmal transporters function to clear monoamines from the synapse and recycle them to maintain vesicular stores, VMATs function to sequester monoamines within synaptic vesicles at presynaptic axon terminals and thus play a major role in regulating synaptic homeostasis (Benarroch, 2013).
Plasma membrane and vesicular monoamine transporters belong to the soluble carrier (SLC) superfamily and play a major role in regulating effects of DA, NE and 5-HT. Plasmalemmal transporters are expressed in the dendrites and axons and mediate reuptake of synaptically released neurotransmitter from the extracellular space. VMATs are expressed within monoaminergic axons and mediate sequestration of cytoplasmic neurotransmitter into synaptic vesicles. Image from Benarroch, 2013.

III. Pharmacology of Monoamine Releasers and Uptake Inhibitors

Monoamine releasers, like the amphetamines, are drugs that function as substrates at monoamine transporters to promote release of DA, NE and/or 5-HT in an action potential-independent manner (Rothman et al., 2001). Monoamine uptake
inhbitors like cocaine, on the other hand, function as transporter blockers to impede removal of synaptic DA, NE and/or 5-HT after their release in response to an action potential; thus, uptake inhibitors are action potential-dependent under normal physiological conditions (O’Brien, 2006). Although monoamine releasers and uptake inhibitors act through different mechanisms, they yield the same end result of elevating brain monoamine levels. Various monoamine releasers and uptake inhibitors (e.g. d-amphetamine, phentermine, methylphenidate, fluoxetine) are available for clinical use in the treatment of disorders like ADHD, obesity, anxiety and depression, but accessibility to many of these drugs is tightly regulated due to their potential for abuse.

At the level of the synapse, monoamine releasers and uptake inhibitors exert opposite effects at plasmalemmal monoamine transporters, yet produce the same outcome of increasing extracellular monoamine levels. Monoamine uptake inhibitors, like cocaine and MDPV, prevent transporter-mediated reuptake of synaptic monoamines into presynaptic terminals by acting as competitive blockers that bind membrane transporters and inhibit transport of endogenous neurotransmitters (Izenwasser, 2004; Baumann et al., 2013). The mechanism of action of monoamine releasers, like amphetamine and methcathinone, is more complicated and controversial. First and less debated is the notion that monoamine releasers are actively transported into the presynaptic neuron by acting as substrates at plasmalemmal transporters (Connor and Kuczynski, 1986). Upon gaining access to the cytoplasm of the presynaptic neuron, the actions of monoamine releasers are more contentious. Perhaps the most commonly held belief
is that monoamine releasers function within the presynaptic neuron to disrupt packaging of newly synthesized and/or recycled neurotransmitters from the cytosol into vesicles by VMATs. This leads to a depletion of DA vesicular stores and an increase in cytosolic monoamine levels that promotes reverse transport through plasma membrane monoamine transporters back into the synapse, independent of vesicular fusion and exocytotic efflux (Sulzer et al., 1995; Kahlig et al., 2005; Lüscher, 2012; see Figure I.8). A more recent, alternative hypothesis suggests that transport of monoamine releasers (i.e. amphetamine) as substrates through membrane transporters can actually produce local depolarization of the presynaptic neuron, causing vesicular docking, fusion, and neurotransmitter release in a similar fashion to “normal,” exocytotic monoamine release generated by action potentials (Ramsson et al., 2011a, 2011b; Avelar et al., 2013). Additional studies have supported and extended upon this alternative hypothesis by showing that amphetamine not only enhances evoked DA responses to current pulse trains, but also increases the amplitude, duration and frequency of spontaneous DA transients (i.e. naturally occurring, non-electrically evoked, phasic increases in extracellular DA) (Daberkow et al., 2013).
Cocaine, a classic monoamine uptake inhibitor, blocks plasmalemmal transporter-mediated reuptake of monoamine neurotransmitters from the synapse into the presynaptic neuron, causing an increase in extracellular levels of neurotransmitter. Amphetamine, a monoamine releaser, acts as a plasmalemmal transporter substrate such that the drug molecule itself passes through the transporter, thereby gaining access to the presynaptic neuron and exerting effects that induce release of neurotransmitter into the synapse. Image from Basic & Clinical Pharmacology, 12th Edition, McGraw Hill.

A major distinguishing feature between any given monoamine releaser or uptake inhibitor is its differential selectivity for DAT, NET and SERT. For releasers, potency at DAT and NET are generally similar; however, potency to promote release via DAT/NET can vary widely from potency to act at SERT. The selectivity of
monoamine releasers and uptake inhibitors can be measured *in vitro* using synaptosomes collected from neural tissue, such as that from rat brain. In a rat brain synaptosome preparation, tissue is homogenized to break apart constituent neurons and then centrifuged to isolate plasmalemmal spheres (i.e. synaptosomes) that are separated from their neuronal cell bodies but that still contain plasmalemmal proteins, including monoamine transporters. Releasers and uptake inhibitors can then be evaluated for their potencies and efficacies to either (a) promote release of radiolabeled monoamine tracers that have been preloaded into the synaptosomes, or (b) block uptake of radiolabeled monoamines applied to the external solution bathing the synaptosomes. Results from *in vitro* synaptosome studies illustrate that DAT/NET vs. SERT selectivity can vary drastically for monoamine releasers (see Table I.1). For uptake inhibitors, there are compounds that function as selective inhibitors for each plasmalemmal transporter, as well as clinically relevant non-selective inhibitors (see Table I.2).

**Table I.1**

<table>
<thead>
<tr>
<th>Monoamine Releaser</th>
<th>DAT EC₅₀ (nM)</th>
<th>NET EC₅₀ (nM)</th>
<th>SERT EC₅₀ (nM)</th>
<th>DAT-vs.-SERT Selectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphetamine</td>
<td>24.8±3.5</td>
<td>7.2±0.4</td>
<td>1765±94</td>
<td>71</td>
</tr>
<tr>
<td>MDMA</td>
<td>376±16</td>
<td>77.4±3.4</td>
<td>56.6±2.1</td>
<td>0.15</td>
</tr>
<tr>
<td>Fenfluramine</td>
<td>&gt;10,000</td>
<td>739±57</td>
<td>79.3±11.5</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

*EC₅₀* values to release DA, NE and 5-HT, and DAT-vs.-SERT selectivities, for representative monoamine releasers (Rothman et al., 2001).
### Table I.2

<table>
<thead>
<tr>
<th>Monoamine Uptake Inhibitor</th>
<th>DAT IC$_{50}$ (nM)</th>
<th>NET IC$_{50}$ (nM)</th>
<th>SERT IC$_{50}$ (nM)</th>
<th>DAT-vs.-SERT Selectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>RTI-113 $^a$</td>
<td>3.0</td>
<td>31</td>
<td>229</td>
<td>76</td>
</tr>
<tr>
<td>Nisoxetine* $^b$</td>
<td>505±50</td>
<td>0.460±0.20</td>
<td>158±29</td>
<td>0.31</td>
</tr>
<tr>
<td>Citalopram $^c$</td>
<td>20,485±923</td>
<td>4,332±295</td>
<td>2.40±0.09</td>
<td>0.0001</td>
</tr>
<tr>
<td>Cocaine $^d$</td>
<td>478±25</td>
<td>779±30</td>
<td>304±10</td>
<td>0.64</td>
</tr>
</tbody>
</table>

IC$_{50}$ values to inhibit uptake of DA, NE and 5-HT, and DAT-vs.-SERT selectivities for representative monoamine uptake inhibitors.

*IC$_{50}$ values have not been reported for the selective NE reuptake inhibitor nisoxetine. Instead, affinities ($K_i$, nM±SEM) at monoamine transporters are included.

$a$ Kuhar et al., 1999, $^b$ Kula et al., 1999, $^c$ Rothman et al., 2001, $^d$ Matecka et al., 1996

In order to characterize neurochemical effects produced by drugs of abuse, including monoamine releasers like amphetamine and uptake inhibitors like cocaine, investigators have focused on measuring the impact of various drugs on brain monoamine levels in the nucleus accumbens (NAc), the major terminal of the mesolimbic dopaminergic system, using in vivo assays like microdialysis. Intracerebral microdialysis is an experimental technique that, in combination with high performance liquid chromatography and electrochemical detection (HPLC-ECD), can be used to recover and measure endogenous extracellular monoamines in discrete brain regions (Zetterström et al., 1983). Early studies of the neurochemical effects of various drugs of abuse on extracellular levels of DA in the striatum and NAc showed that virtually all drugs abused by humans (e.g. opiates, ethanol,
nicotine, amphetamine and cocaine) increased DA levels in both regions, but particularly in the accumbens (Di Chiara and Imperato, 1988). Subsequent studies confirmed that various classes of abused drugs, particularly monoamine releasers and uptake inhibitors, elevate accumbal DA levels (Carboni et al., 1989) and extended on those findings by demonstrating that alterations in other accumbal monoamine levels (i.e. especially 5-HT) also influence a drug’s abuse potential. In fact, studies have suggested that drug-induced increases in accumbal 5-HT levels are not associated with abuse and may actually inhibit DA neurotransmission (Baumann et al., 2011, 2012).

With regards to neurochemical effects of monoamine releasers specifically, amphetamine has been repeatedly shown to produce selective increases in NAc DA levels without significantly increasing accumbal 5-HT (Ichikawa et al., 1998; Kankaanpää et al., 1998). Fenfluramine, on the other hand, selectively increases accumbal 5-HT without impacting DA levels (Benloucif and Galloway, 1991; Shoaib et al., 1997). Mixed-action monoamine releasers like MDMA (Gough et al., 1991) and methylone (Baumann et al., 2012) produce increases in both DA and 5-HT levels in nucleus accumbens. Figure 1.9 illustrates neurochemical effects of amphetamine, MDMA and fenfluramine on extracellular NAc DA and 5-HT levels, as determined by microdialysis in preliminary experiments that I conducted (Bonano et al., in submission).
Figure 1.9

**Effects of amphetamine (AMPH), methylenedioxymethamphetamine (MDMA), and fenfluramine (FEN) on DA and 5-HT levels in the NAc of awake rats expressed as a percentage of baseline neurotransmitter levels.** Left panels indicate temporal changes in % baseline DA, while right panels indicate changes in % baseline 5-HT. Arrow indicates time of drug administration. Filled symbols indicate statistical significance (P<0.05) compared to vehicle conditions within a given time point. Figure from Bonano et al., in submission.
Substantial evidence exists to suggest that elevations in synaptic 5-HT levels attenuate the stimulant properties of DA-selective monoamine releasers and uptake inhibitors. For one, elevations in synaptic 5-HT have been shown to exert an inhibitory influence on mesolimbic DA neurons (Rothman and Baumann, 2006). In particular, as depicted and described previously in Figure 1.5, 5-HT can inhibit DA neurons directly or by producing convergent effects that inhibit DA effects on downstream, postsynaptic neurons. For example, a study investigating the effects of a null mutation that eliminates expression of 5-HT\textsubscript{2C} receptors found that mutant mice had increased activity of substantia nigra pars compacta (SNc) dopaminergic neurons, elevated baseline DA levels in dorsal striatum (DSt), and enhanced sensitivity to behavioral effects of d-amphetamine (a DA-selective monoamine releaser) and GBR 12909 (a DA-selective uptake inhibitor) (Abdallah et al., 2009). Another group investigated 5-HT\textsubscript{1B} receptor knockout mice to assess the function of 5-HT\textsubscript{1B} receptors in modulating synaptic transmission of the dorsal raphe, ventral midbrain and nucleus accumbens, and they concluded that 5-HT\textsubscript{1B} receptors function as auto- and heteroreceptors to exert presynaptic inhibition of monoamine transmitter release in the CNS (Morikawa et al., 2000). Furthermore, supplementing dietary tryptophan (5-HT precursor) in rats has been shown to attenuate amphetamine self-administration (Smith et al., 1986). Studies have also reported that increasing brain 5-HT activity can attenuate the reinforcing effects of cocaine (Czoty et al., 2002). Additionally, cocaine analogs with higher SERT potency support lower rates of self-administration than cocaine analogs with lower SERT potency.
(Roberts et al., 1999). Together, these findings suggest that serotonergic systems exert an inhibitory effect on dopaminergic systems.

An implication of these findings is that abuse liability of monoamine releasers might be determined in part by their relative selectivity to promote release of DA/NE vs. 5-HT, and published data from our lab support a relationship between pharmacological selectivity for DA/NE vs. 5-HT release and behavioral efficacy to produce abuse-related facilitation of intracranial self-stimulation (ICSS) (Bauer et al., 2013b; Bonano et al., in press). ICSS is an experimental tool that has been used to study abuse liability of stimulants, among other classes of drugs (Kornetsky and Esposito, 1979; Wise, 1996; Bonano et al., 2014b; Negus and Miller, 2014). The idea that specialized neural circuits, or “reward pathways,” exist largely originated based on early observations of ICSS; that is, rats would work vigorously to earn electrical brain stimulation delivered to some brain targets (Olds and Milner, 1954). Olds began using the phrase “reward neuron” in the late 1950s to discuss such neural circuits. Now, the mesolimbic DA pathway (VTA to NAc) is widely regarded as the central “reward pathway,” implicating midbrain DA neurons as major mediators of the rewarding effects of drugs of abuse (Wise and Bozarth, 1987; Koob, 1992; Ikemoto and Bonci, 2014). In ICSS, subjects are trained to lever press for pulses of brain stimulation delivered via microelectrodes implanted in reward-related brain regions like the medial forebrain bundle (see Figure I.10), and different frequencies or intensities of brain stimulation maintain different rates of lever-press responding. Many drugs of abuse increase (“facilitate”) low rates of ICSS maintained by low frequencies or intensities of brain stimulation; thus, ICSS
facilitation is often interpreted as an abuse-related drug effect. ICSS shows substantial congruence with other preclinical measures of abuse liability, such as drug self-administration and conditioned place preference (CPP) (Negus and Miller, 2014; Vlachou and Markou, 2011).

**Figure I.10**

The medial forebrain bundle (MFB), a fiber tract that passes through the lateral hypothalamus (LH), is a common target for electrical brain stimulation in ICSS studies.

*A) Sagittal and B) coronal sections of the rat brain indicate MFB location in orange.*

*Electrical stimulation of MFB in ICSS is believed to activate excitatory glutamatergic inputs from LH to DA neuron cell bodies in the ventral tegmental area (VTA), as shown*
in panel C. In VTA, glutamate binds ionotropic glutamate receptors and increases the firing rate of DA neurons whose cell bodies reside there. Images adapted from ICSS Review by Negus and Miller, 2014.

An additional benefit of ICSS is its ability to discriminate both abuse-related and abuse-limiting drug effects in a single procedure. Monoamine releasers and reuptake inhibitors can simultaneously produce DA-mediated, abuse-related effects (facilitation of low ICSS rates) and 5-HT-mediated, abuse-limiting effects (depression of high rates of ICSS). For example, DA-selective releasers like amphetamine produce exclusive facilitation of ICSS across a broad range of doses (Bauer et al., 2013b). Conversely, the 5-HT-selective releaser fenfluramine produces exclusive depression of ICSS (Olds and Yuwiler, 1992; Bauer et al., 2013b). Mixed-action DA/5-HT releasers like MDMA produce simultaneous facilitation of low ICSS rates and depression of high ICSS rates (Bauer et al., 2013b). These results, depicted in Figure I.11, support the assertion that ICSS is capable of discerning abuse-related and abuse-limiting drug effects, and that a drug’s selectivity to promote DA vs. 5-HT release correlates with its profile of abuse-related facilitation and/or abuse-limiting depression of ICSS. Furthermore, these findings lend additional, behavioral support to the assertion that serotonergic systems are capable of exerting an inhibitory effect on dopaminergic systems.
Effects of representative doses of amphetamine (1.0 mg/kg), MDMA (3.2 mg/kg), and fenfluramine (3.2 mg/kg) on ICSS behavior. Amphetamine, a DA-selective releaser, produces exclusive facilitation of ICSS, whereas fenfluramine, a 5-HT-selective releaser, produces exclusive depression of ICSS. MDMA, a nonselective releaser, produces a mixed behavioral profile of rate-increasing and rate-decreasing effects on ICSS. Filled points represent frequencies at which reinforcement rates were statistically different from vehicle rates as determined by two-way ANOVA followed by Holm-Sidak post hoc test, P<0.05. Figure adapted from Bauer et al., 2013b.

IV. Pharmacology of Synthetic Cathinones

In the late 1980s, cathinone and MCAT were shown to induce release of radioactivity from [3H]DA-prelabeled tissue from rat caudate in a manner similar to that observed with amphetamine and methamphetamine (Glennon et al., 1987). Later studies found that MCAT is potent both as a monoamine releaser and as an uptake inhibitor, and that its selectivity favors DA and NE vs. 5-HT (Cozzi et al., 1999, 2013). Despite its potent effects as an uptake inhibitor, MCAT’s relatively higher potency as a monoamine transporter substrate suggests that it produces
neurochemical and behavioral effects primarily as a result of its capacity to induce monoamine release. Three early synthetic cathinone bath salts that were amongst the first to be investigated preclinically were mephedrone, methylone and MDPV. One study showed that mephedrone and methylone both function as substrates for monoamine transporters (i.e. monoamine releasers), with a release profile comparable to MDMA (Baumann et al., 2012). Subsequently, using frog oocytes that had been transfected with cRNA to express hDAT, mephedrone was shown to produce inward currents that resembled depolarization produced by monoamine releasers (i.e. transporter substrates) like amphetamine, whereas MDPV produced outward currents that resembled cocaine-induced hyperpolarization characteristic of monoamine uptake inhibitors (i.e. transporter blockers) (Cameron et al., 2013a, 2013b). Studies by Simmler et al. (2013) demonstrated that mephedrone was nearly equipotent as a releaser and inhibitor of DA and 5-HT, but was much more potent as an inhibitor of NET. Conversely, MDPV was a potent inhibitor of DAT and NET with weak inhibition at SERT, and MDPV had no detectable function as a releaser at any monoamine transporter (Simmler et al., 2013), consistent with the “uptake inhibitor-like” electrophysiological signature demonstrated by Cameron et al. (2013a).

In terms of behavior, mephedrone, methylone and MDPV have been shown to induce abuse-related, amphetamine-like stimulant effects including locomotor activation, facilitation of ICSS, maintenance of self-administration, and substitution for abused drugs in assays of drug discrimination (summary provided in Table I.3). First, mephedrone, methylone and MDPV have repeatedly been reported to induce
hyperlocomotion in rodents (Baumann et al., 2012; López-Arnau et al., 2012; Marusich et al., 2012; Fantegrossi et al., 2013). In a locomotor study in rats, which compared various synthetic cathinones (mephedrone, methylone, methedrone, MDPV, 3-fluoromethylmethcathinone, 4-fluoromethylmethcathinone), MDPV was the most potent and mephedrone was the least potent at inducing locomotion (Marusich et al., 2012). Despite their potency differences, MDPV and mephedrone displayed similar efficacies to increase locomotion (measured by total beam breaks in open field activity chambers), whereas methedrone produced weak effects on locomotor stimulation (Marusich et al., 2012). Another locomotor study performed in mice examined MDPV, mephedrone, methylone, 4-fluoromethylmethcathinone (flephedrone), butylone and naphylone and found that MDPV and mephedrone were nearly equipotent at producing hyperlocomotion, but MDPV produced much longer lasting effects (Gatch et al., 2013).

In ICSS studies published during the early stages of this dissertation project, synthetic cathinones were reported to increase response rates and reduce thresholds for electrical brain stimulation. MDPV (0.1-2.0 mg/kg, i.p.) produced ICSS facilitation and reduced current-intensity threshold in a discrete trial procedure in male Sprague-Dawley rats (Watterson et al., 2012b), consistent with abuse potential. Mephedrone (1-10 mg/kg, i.p.) produced mixed effects on frequency-rate ICSS curves in male C57BL/6J mice, with a facilitation of low ICSS rates maintained by low brain-stimulation frequencies, but also a reduction in maximal rates (Robinson et al., 2012). This overall profile of effects is similar to results shown in Figure I.11 for MDMA. Methylone (0.1-10mg/kg, i.p.) showed a
dose-dependent trend to decrease ICSS current-intensity thresholds, but these reductions were not statistically significant (Watterson et al., 2012a).

Synthetic cathinones have also been reported to produce abuse-related behavioral effects in assays of drug self-administration. MCAT maintained self-administration in baboons (Kaminski and Griffiths, 1994), and MDPV showed potent stimulant effects and was self-administered in rats (Watterson et al., 2012b; Aarde et al., 2013b). Likewise, mephedrone (Hadlock et al., 2011; Aarde et al., 2013a) and methylone served as reinforcers and produced dose-dependent acquisition of self-administration in rats (Watterson et al., 2012a).

Fewer synthetic cathinones have been investigated in drug discrimination; however, results are largely consistent in showing that several synthetic cathinones substitute for other psychostimulant drugs of abuse, primarily amphetamine. Cathinone and MCAT substituted for (+)-amphetamine in rats (Kalix and Glennon, 1986; Glennon et al., 1987). Interestingly, methylone substituted for (+)-amphetamine in rats, but 3,4-methylenedioxycathinone (the N-desmethyl analog of methylone) did not (Dal Cason et al., 1997). Furthermore, in rats trained to discriminate mephedrone, MDMA fully substituted while methamphetamine and cocaine produced only partial substitution (Varner et al., 2013). Results from behavioral studies of various synthetic cathinones’ effects published before or during my dissertation studies are summarized in Table I.3.
<table>
<thead>
<tr>
<th>Drug</th>
<th>Pharmacology</th>
<th>Dose/Route</th>
<th>Sex/ Strain/ Species</th>
<th>Behavioral Effects</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDPV</td>
<td>Monoamine uptake inhibitor, DAT&gt;&gt;SERT</td>
<td>1-30 mg/kg, i.p.</td>
<td>Male ICR mice</td>
<td>▲ locomotion</td>
<td>Marusich et al. 2012</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.3-30 mg/kg, inj?</td>
<td>Male Swiss-Webster mice</td>
<td>▲ locomotion, ED$_{50}$= 1.26mg/kg</td>
<td>Gatch et al. 2013</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1-2mg/kg, i.p. 0.05-0.2 mg/kg/inf.</td>
<td>Male Sprague-Dawley rats</td>
<td>▲ ICSS, Self-administered</td>
<td>Watterson et al. 2012b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.05mg/kg/inf., i.v.</td>
<td>Male Wistar rats</td>
<td>Self-administered, avg: 0.9 mg/kg/h</td>
<td>Aarde et al. 2013b</td>
</tr>
<tr>
<td>Methylenone (MDMC)</td>
<td>Monoamine releaser, DAT=SERT</td>
<td>3-56 mg/kg i.p.</td>
<td>Male ICR mice</td>
<td>▲ locomotion</td>
<td>Marusich et al. 2012</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1-30 mg/kg</td>
<td>Male Swiss-Webster mice</td>
<td>▲ locomotion, ED$_{50}$=1.48mg/kg</td>
<td>Gatch et al. 2013</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1-10 mg/kg, i.p. 0.05-0.5 mg/kg/inf., i.v.</td>
<td>Male Sprague-Dawley rats</td>
<td>▲ ICSS, Self-administered</td>
<td>Watterson et al. 2012a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1-3 mg/kg, i.p.</td>
<td>Male Sprague-Dawley rats</td>
<td>Substituted for 1.0mg/kg (+)-Amph discrimin-stimulus, ED$_{50}$= 2.36 mg/kg</td>
<td>Dal Cason et al. 1997</td>
</tr>
<tr>
<td>Mephedrone</td>
<td>Monoamine releaser, DAT=SERT</td>
<td>3-56 mg/kg i.p.</td>
<td>Male ICR mice</td>
<td>▲ locomotion</td>
<td>Marusich et al. 2012</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.3-30 mg/kg</td>
<td>Male Swiss-Webster mice</td>
<td>▲ locomotion, ED$_{50}$=1.38mg/kg</td>
<td>Gatch et al. 2013</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1-10 mg/kg i.p.</td>
<td>Male C57BL/6J mice</td>
<td>▲↓ ICSS</td>
<td>Robinson et al. 2012</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.24mg per 10uL infusion, i.v.</td>
<td>Male Sprague-Dawley rats</td>
<td>Self-administered, ▲ drug intake 1.77 (d1) to 6.78mg (d8)</td>
<td>Hadlock et al. 2011</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0mg/kg/ infusion, i.v.</td>
<td>Male Sprague-Dawley and Wistar rats</td>
<td>Self-administered, 95% (S-D) and 98% (Wistar) reward-lever discrimination</td>
<td>Aarde et al. 2013a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.2 mg/kg, i.p.</td>
<td>Male Long-Evans rats</td>
<td>3.2mg/kg MDMA fully substituted for Meph discriminative stimulus</td>
<td>Varner et al. 2013</td>
</tr>
<tr>
<td>Flephedrone</td>
<td>Monoamine releaser, DAT&gt;SERT</td>
<td>10-56 mg/kg, i.p.</td>
<td>Male ICR mice</td>
<td>▲ locomotion</td>
<td>Marusich et al. 2012</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.3-30 mg/kg</td>
<td>Male Swiss-Webster mice</td>
<td>▲ locomotion, ED$_{50}$=2.04mg/kg</td>
<td>Gatch et al. 2013</td>
</tr>
</tbody>
</table>
In conclusion, existing data suggest that emerging synthetic cathinones function as monoamine releasers and/or uptake inhibitors similarly to established drugs of abuse, like amphetamine and cocaine. At the time when my dissertation work started, relatively little was known about the pharmacology of these emerging drugs of abuse, or about the determinants of their abuse-related effects. In the subsequent section, an overview of my dissertation studies is provided, which outlines the manner in which a series of para-substituted MCAT analogs were systematically investigated not only to determine in vitro and in vivo effects of these compounds on neurochemistry and behavior, but also to better understand the structural determinants and molecular mechanisms underlying these abuse-related effects.

V. Quantitative Structure-Activity Relationship (QSAR) Analysis

Quantitative structure-activity relationship (QSAR) analysis served as an organizing principle for the analysis of data from my project. QSAR studies are used to correlate physicochemical parameters of drug molecules with functional outcomes of drug administration, both in vitro and in vivo (Glennon and Young, 2011). For the studies included in this dissertation, the para substituent on the benzene ring of the MCAT scaffold, R, was manipulated to include substituents that varied systematically along three physicochemical dimensions [steric (E_s), electronic (σ_p), and lipophilic (π_p)] (Figure I.12). Taft’s steric parameter E_s was one of the first steric parameters employed in QSAR analysis and was derived by measuring rates of hydrolysis of various esters (Taft 1952, 1953). Hydrolysis rates
are dependent upon the contribution of both steric strain and steric hindrance; thus, this integration of steric parameters into a single metric ($E_s$) provides a simple and useful tool for conducting preliminary QSAR analyses. Large $E_s$ values indicate low functional steric bulk of the substituent, and increasingly negative $E_s$ values indicate progressively greater magnitudes of steric bulk. The lipophilic value $\pi_p$ provides a measure of the degree to which the addition of a given substituent alters partitioning of the compound between aqueous and organic solvents. Thus, $\pi_p$ signifies the relative rate at which a compound penetrates lipid membranes compared to the unsubstituted parent compound (Cammarata and Rogers, 1971). Finally, the electronic value $\sigma_p$ provides a measure of the change in free-energy that accompanies formation of complexes between ligands (e.g. drugs) and their target proteins (e.g. monoamine transporters) (Yoshida et al., 2012). Substituents with positive $\sigma_p$ values are electron-withdrawing, whereas substituents with negative $\sigma_p$ values are electron-donating, and the absolute value of $\sigma_p$ signifies the degree to which the substituent produces an electronic effect.
Chemical structure of the MCAT scaffold and physicochemical parameters of the para substituent, R. Values for physicochemical parameters (Taft’s steric parameter, $E_S$; electron-withdrawing capacity, $\sigma_p$; lipophilicity, $\pi_p$) were taken from Wolff (1980).

### VI. Overview of Dissertation Studies

The overall goal of this dissertation was to investigate expression and pharmacological determinants of abuse-related effects produced by emerging synthetic cathinone drugs of abuse. This was done by examining quantitative structure-activity relationships (QSARs) for MCAT and six para-substituted MCAT analogs on various experimental endpoints, including: (1) *in vitro* potency to function as substrates for DAT and SERT in rat brain synaptosomes, (2) *in vivo* modulation of ICSS in male Sprague Dawley rats, (3) *in vivo* potency to release DA and 5-HT in the nucleus accumbens in male Sprague-Dawley rats, and (4) *in vitro* potency to produce DAT- and SERT-mediated currents in a frog-oocyte expression
system. QSAR studies are used to correlate physicochemical parameters of drug molecules with functional outcomes of drug administration, both in vitro and in vivo (Glennon and Young, 2011). Since each MCAT analog was designed to contain a different para substituent with unique steric (Eₚ), electronic (σₚ), and lipophilic (πₚ) features, differences in these physicochemical parameters could be used to determine correlations between the structure of each compound and the neurochemical and behavioral effects it produces.

Chapter II of this dissertation describes results from my first publication (Bonano et al., 2014b). Because of the recent and dramatic increase in abuse of synthetic cathinones in the U.S., we conducted preclinical behavioral studies to investigate the expression of abuse-related effects produced by MCAT and by the early “bath salts” constituents that were emergency scheduled by the DEA in 2011. Specifically, this study examined behavioral effects of MCAT, MDPV, methylone and mephedrone in rats using ICSS.

One key finding from this early study on ICSS modulation by synthetic cathinones was that MCAT and mephedrone produced strikingly different behavioral effects despite having similar molecular structures that differed only in the identity of the para substituent (-H for MCAT; -CH₃ for mephedrone). Previous studies from our group suggested that differential effects of MCAT and mephedrone might be related to their differential effects at DA vs. 5-HT transporters (Bauer et al., 2013b). Accordingly, the remainder of my dissertation focused on use of QSAR analysis to investigate the role of the para substituent of the MCAT scaffold as a
determinant of effects produced by MCAT analogs on behavior and on neurochemical and electrophysiological measures of DAT and SERT function.

Chapter III describes results from a subsequent publication on the abuse-related in vitro neurochemical and in vivo behavioral effects of para-substituted MCAT analogs in rats (Bonano et al., 2014a). This study examined QSARs for MCAT and six para-substituted MCAT analogs on (a) in vitro potency to promote monoamine release via DAT and SERT, and (b) in vivo modulation of ICSS. Correlations were evaluated for neurochemical and behavioral effects relative to each other and to steric (Eₜ), electronic (σ_p) and lipophilic (π_p) parameters of the para substituents.

Chapter IV describes the in vivo neurochemical effects of these same para-substituted MCAT analogs on extracellular accumbal DA and 5-HT levels using microdialysis in rats. As a follow-up to the studies described in Chapter III, which identified steric volume as a critical determinant of in vitro DAT-vs.-SERT selectivity, correlations were evaluated between steric volume of the para substituents of tested MCAT analogs and the efficacy of those analogs to increase DA vs. 5-HT levels in the nucleus accumbens (NAc) of awake rats.

The major conclusion of my work is that steric features of the para substituent on the MCAT scaffold are key determinants of the abuse-related stimulant effects of MCAT analogs, including the bath salts constituent mephedrone. This dissertation not only extends the body of literature on designer drugs of abuse by investigating abuse-related behavioral effects of prominent bath salts constituents (Chapter II), but it also provides evidence to support physicochemical
determinants of both in vitro and in vivo neurochemical and behavioral effects of a novel series of systematically derivatized MCAT analogs (Chapters III & IV). Overall, the data contained within the body of this dissertation will provide an improved understanding of the mechanism(s) of synthetic MCAT analogs’ abuse-related effects, which might guide both regulatory control of emerging designer drugs and development of novel strategies for treating bath salts intoxication to improve clinical outcomes associated with bath salts use and abuse. Furthermore, these data will aid in the analysis of docking interactions between MCAT analogs and their molecular targets (DAT and SERT), as well as in the generation of hypothetical structural models of DAT and SERT.
Chapter II: Abuse-Related and Abuse-Limiting Effects of Methcathinone and the Synthetic “Bath Salts” Cathinones Analogs Methyleneoxypyrovalerone (MDPV), Methylone and Mephedrone on Intracranial Self-Stimulation in Rats


Introduction

The goal of the present study was to compare the potency and time course of ICSS effects produced by methcathinone (MCAT) and the three recently scheduled "bath salts" cathinone analogs: MDPV, methylone and mephedrone. Recent studies have reported facilitation of ICSS by MDPV in rats (Watterson et al. 2012) and mephedrone in mice (Robinson et al. 2012); however, effects of MCAT and methylone on ICSS have not yet been described, nor have ICSS effects of these structurally, pharmacologically and epidemiologically-related drugs been directly compared. Based on the in vitro selectivity of these compounds to promote release or block reuptake of DA vs. 5-HT, we predicted that MCAT and MDPV would display the greatest efficacy to produce abuse-related facilitation of ICSS, whereas methylone and mephedrone would produce mixed effects that would include both DA-mediated facilitation of low ICSS rates and 5-HT-mediated depression of higher ICSS rates.
Materials and Methods

Subjects

Eighteen adult male Sprague-Dawley rats (Harlan, Frederick, MD, USA) weighing 314-387 g at the time of surgery were individually housed and maintained on a 12 h light/dark cycle with lights on from 6:00 a.m. to 6:00 p.m. Rats had free access to food and water except during testing. Animal maintenance and research were in compliance with the National Institutes of Health guidelines for the care and use of animal subjects in research (National Academy of Sciences, 2011) and adhered to guidelines of the Committee for Research (National Research Council, 2003). All animal use protocols were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee.

Intracranial self-stimulation (ICSS) Procedure

Surgery. Rats were anesthetized with isoflurane (2.5-3% in oxygen; Webster Veterinary, Phoenix, AZ, USA) until unresponsive to toe-pincher prior to implantation of stainless steel electrodes (Plastics One, Roanoke, VA, USA). The cathode of each bipolar electrode was 0.25 mm in diameter and covered with polyamide insulation except at the flattened tip, whereas the anode was 0.125 mm in diameter and uninsulated. The cathode was stereotaxically implanted into the left medial forebrain bundle (MFB) at the level of the lateral hypothalamus (2.8 mm posterior to bregma, 1.7 mm lateral to midsagittal suture, 8.8 mm ventral to skull). Three screws were placed in the skull, and the anode was wrapped around one screw to serve as the ground. The skull screws and electrode were secured to the skull with dental acrylic. Ketoprofen (5 mg/kg) was used for post-operative analgesia
immediately and 24 h after surgery. Animals were allowed to recover for at least 7 days prior to commencing ICSS training.

**Apparatus.** Experiments were conducted in sound-attenuating boxes that contained modular acrylic and metal test chambers (29.2 x 30.5 x 24.1 cm) equipped with a response lever (4.5 cm wide, 2.0 cm deep, 3 cm off the floor), three stimulation lights (red, yellow, and green, positioned 7.6 cm directly above the response lever), a 2 W house light and an ICSS stimulator (Med Associates, St. Albans, VT, USA). Electrodes were connected to the stimulator via a swivel commutator (Model SL2C, Plastics One, Roanoke, VA, USA). The stimulator was controlled by Med-PC IV computer software that also controlled programming parameters and data collection (Med Associates).

**Training.** Following initial shaping of lever press responding, rats were trained under a fixed-ratio 1 (FR 1) schedule of brain stimulation using a behavioral procedure identical to that previously described (Bauer et al. 2013). During behavioral sessions, each lever press resulted in the delivery of a 0.5 s train of square wave cathodal pulses (0.1 ms pulse duration) and illumination of the stimulus lights over the lever. Stimulation intensity and frequency were set at 150 µA and 126 Hz, respectively, during initial 60 min training sessions. Stimulation intensity was then individually adjusted for each rat to the lowest value that sustained ICSS rates > 30 stimulations/min. This intensity (130-240 µA across rats) was then held constant for the remainder of the study, and frequency manipulations were introduced. Sessions involving frequency manipulations consisted of three sequential 10 min components. During each component, a descending series of 10
frequencies (158 to 56 Hz in 0.05 log increments) was presented, with each
frequency available for a 1 min trial. Each frequency trial consisted of a 10 s time-
out, during which five non-contingent “priming” stimulations were delivered at the
frequency of stimulation that would be available during that trial, followed by a 50 s
“response” period, during which responding produced electrical stimulation under a
FR 1 schedule as described above. Training continued until rats reliably responded
for only the first three to six frequency trials of each component over a period of at
least three consecutive training days.

**Testing.** Studies with the racemates of MCAT (0.1-1.0 mg/kg), MDPV (0.1-3.2
mg/kg), methylone (0.32-10 mg/kg) and mephedrone (1.0-10 mg/kg) were
conducted in two phases. In the first phase, a 1.0 to 1.5 log unit range of doses was
tested for each drug with the goal of testing a dose range from an ineffective dose to
a high dose that maximally facilitated ICSS. Dose ranges were based on extant
literature (Aarde et al. 2013;Baumann et al. 2012a; Cozzi et al. 2013; Hadlock et al.
2011; Shortall et al. 2012; Watterson et al. 2012) and our own empirical results. For
these dose-effect studies, test sessions consisted of three sequential “baseline”
components followed by a 30 min time-out period and then by three sequential
“test” components. A single dose of test drug was administered intraperitoneally
(i.p.) at the beginning of the time-out period. In the second phase, a time course was
determined for effects produced by the highest dose of each compound. Time course
test sessions consisted of three consecutive baseline components followed by
immediate drug injection, and then by pairs of consecutive test components
beginning after 10, 30, 100 and 300 min. In the case of MDPV, which had a longer
duration of action, an additional pair of test components was initiated 24 h after drug injection. Test sessions were completed on Tuesdays and Fridays, and three-component training sessions were conducted on all other weekdays. The order of testing with vehicle and drug doses was varied across subjects using a Latin-square design. MCAT, MDPV and mephedrone were tested in separate groups of six rats each, and methylone was tested in five rats from the group that initially received methcathinone (the sixth rat lost its headcap and could no longer be tested). Methylone testing began one week after completion of methcathinone testing to minimize potential for carryover effects, and a vehicle test session was conducted in this interval to confirm stable responding.

**Data Analysis.** The primary dependent variable was reinforcement rate in stimulations per minute during each frequency trial. To normalize these data, raw reinforcement rates from each trial in each rat were converted to percent maximum control rate (%MCR), with MCR defined as the mean of the maximal rates observed during the second and third baseline components of any given session in any given rat. Thus, %MCR values for each trial were calculated as (reinforcement rate during a frequency trial) / (MCR) x 100. For each test session, data from the second and third baseline components were averaged to yield a baseline frequency-rate curve, and data from test components were averaged to generate test frequency-rate curves. Baseline and test curves were then averaged across rats to yield mean baseline and test curves for each manipulation. For statistical analyses, results were compared by repeated measures two-way ANOVA with ICSS frequency as one factor.
and either dose or time as the second factor. A significant ANOVA was followed by the Holm-Sidak *post hoc* test and the criterion for significance was set at *P*<0.05.

Two other analytic strategies were also used to provide summary measures for stratification of drug efficacies to facilitate ICSS (Altarifi and Negus 2011; Bauer et al. 2013; Bauer et al. in press). The first approach calculated the total number of stimulations delivered per component across all 10 frequency-trials. Test data were normalized to individual baseline data using the equation:

\[
\text{\% baseline total stimulations per component} = \left( \frac{\text{mean total stimulations per test component}}{\text{mean total stimulations per baseline component}} \right) \times 100.
\]

Data were then averaged across rats in each experimental condition. The second approach employed rate-dependency analysis to provide a measure of the degree of facilitation of low ICSS rates maintained by low brain stimulation frequencies. Specifically, baseline and test frequency-rate curves were used to generate rate-dependency plots where the x-axis was log baseline rate and the y-axis was log \([(test \ rate/baseline \ rate) \times 100]\). Each rate-dependency plot consisted of 10 points for baseline and test rates maintained by each brain stimulation frequency. These plots were then subjected to linear regression analysis to determine two parameters: (1) the slope (expressed as a-slope such that increasingly steep slopes were 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). Summary measures across conditions were considered to be significantly different if 95% confidence limits did not overlap between drugs. Note that ICSS thresholds were not used to compare drug effects for reasons discussed previously (Bauer et al. 2013).
Drugs

(±)-Methcathinone HCl and (±)-3,4-methylenedioxyamphetamine HCl (methylone) were prepared as previously reported (Glennon et al. 1987; Dal Cason et al. 1997). (±)-3,4-Methylenedioxyamphetamine HCl (MDPV) and (±)-4-methylmethcathinone HCl (mephedrone) were available from a recent investigation (Cameron et al. 2013a) and were prepared as previously described (Köppe et al. 1969; de Durnaga and Sanchez 1929, respectively). Compounds were prepared in sterile saline and delivered i.p.

Results

Electrical brain stimulation maintained a frequency-dependent increase in ICSS rates under baseline conditions (e.g. “vehicle” data in Figure II.1). Across the 18 rats used in these studies, the average ± SEM baseline MCR was 64.4 ± 1.98 stimulations per trial, and the mean ± SEM number of total baseline stimulations was 330 ± 20.7 stimulations per component. Figure II.1 shows dose-effect data for MCAT (0.1-1.0 mg/kg), MDPV (0.32-3.2 mg/kg), methylone (1.0-10 mg/kg) and mephedrone (1.0-10 mg/kg). Two-way ANOVA indicated significant main effects of frequency and dose and significant frequency x dose interactions for all drugs, and interaction effects are reported below for each drug. MCAT [F(27,135)= 6.43, P<0.0001] and MDPV [F(27,135)= 5.11, P<0.0001] produced dose-dependent facilitation of low ICSS rates maintained by low brain stimulation frequencies with no evidence at these doses and pretreatment times of depression of high ICSS rates maintained by high brain stimulation frequencies. Methylone [F(36,144)= 7.94,
P<0.0001] also produced a dose-dependent facilitation of low ICSS rates maintained by low stimulation frequencies; however, the highest dose of 10 mg/kg methylone also significantly decreased high ICSS rates. Lastly, mephedrone [F(27,135)= 13.9, P<0.0001] produced weak facilitation of low ICSS rates while dose-dependently depressing high ICSS rates maintained by high brain stimulation frequencies. MCAT was the most potent compound to alter ICSS (significant effects at doses ≥0.1 mg/kg), followed by MDPV (≥0.32 mg/kg), and methylone and mephedrone(≥1.0 mg/kg). Lower doses of MDPV (0.1 mg/kg) and methylone (0.32 mg/kg) were also tested but had no effect (data not shown).

Figure II.2 shows the time course of effects produced by the highest dose of each compound. Two-way ANOVA indicated significant main effects of frequency and time and significant frequency x time interactions for all drugs, and interaction effects are reported below for each drug. MCAT (1.0 mg/kg; [F(36,180)= 5.23, P<0.001]) produced maximal facilitation of ICSS at the earliest time point (10 min), and significant ICSS facilitation was no longer apparent after 300 min. MDPV (3.2 mg/kg; [F(45,225)= 5.52, P<0.0001]) produced maximal facilitation of ICSS maintained by low stimulation frequencies after 10 min, but it also depressed ICSS at the two highest stimulation frequencies at this same early time point. At later times, MDPV produced only ICSS facilitation, and this facilitation was still significant after 300 min (1.85-1.95 log Hz) and 24 hr (1.85 log Hz; data not shown). Methylone (10 mg/kg; [F(36,144)= 8.93, P<0.0001]) yielded a mixed profile of effects with both rate-increasing and maximal rate-decreasing effects at 10 min. Rate-decreasing effects were no longer significant after 30 min, but significant rate-increasing effects
persisted at 30 and 100 min. Mephedrone (10 mg/kg; [F(36,180)= 6.26, P<0.0001]) produced only rate-decreasing effects in the time course study. ICSS depression peaked at 10 min and was no longer significant after 300 min.

Figure II.3 shows dose-dependence and time course of each compound expressed as total number of stimulations per component, a summary measure that integrates both rate-increasing and rate-decreasing drug effects on ICSS. All drugs produced dose-dependent changes in this metric (Figure II.3A), and Table II.1 compares peak effects of each drug. By this metric, the rank order of efficacy was MCAT ≥ MDPV ≥ methylone ≥ mephedrone. All drugs had a rapid onset, and effects at 10 min provided further evidence for efficacy differences between MCAT, MDPV and methylone. Durations of action were MDPV > methylone > MCAT = mephedrone.

Figure II.4 shows results of rate-dependency analysis to assess efficacy of ICSS facilitation. Figure II.4A shows rate-dependency plots for each dose of MCAT. Figure II.4B shows rate-dependency plots for doses of other drugs that produced peak Y-intercepts. Figure II.4C shows dose-dependent effects of each drug on Y-intercept, and Table II.1 compares peak increases in Y-intercept produced by each drug. The rank order of efficacy was MCAT ≥ MDPV ≥ methylone > mephedrone.

**Summary**

This study compared effects produced by MCAT and three recently scheduled bath salts cathinone analogs, MDPV, methylone and mephedrone, on ICSS in rats. There were three main findings. First, all four analogs facilitated ICSS at some
dose. To the extent that facilitation of ICSS is suggestive of a drug’s abuse potential, these findings are consistent with abuse liability for all four compounds. Second, the compounds differed in their relative efficacy to facilitate ICSS, with a rank order of MCAT ≥ MDPV ≥ methylone > mephedrone based on the maximal Y-intercept in rate-dependency analysis. Third, the compounds differed in their time courses. All compounds displayed a rapid onset of action, but the methylenedioxy-substituted compounds MDPV and methylone had longer durations of action.
Table II.1

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose</th>
<th>Maximum % Baseline Stimulations</th>
<th>Dose</th>
<th>Maximum Y-Intercept</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methcathinone</td>
<td>1.0</td>
<td>192 (147-237)</td>
<td>1.0</td>
<td>0.86 (0.80-0.92)</td>
</tr>
<tr>
<td>MDPV</td>
<td>3.2</td>
<td>150 (105-196)</td>
<td>3.2</td>
<td>0.84 (0.82-0.86)</td>
</tr>
<tr>
<td>Methylone</td>
<td>10</td>
<td>148 (117-180)</td>
<td>10</td>
<td>0.78 (0.72-0.84)</td>
</tr>
<tr>
<td>Mephedrone</td>
<td>3.2</td>
<td>102* (87-118)</td>
<td>10</td>
<td>0.36* (0.30-0.43)</td>
</tr>
</tbody>
</table>

Efficacy to facilitate ICSS as indicated by maximal drug effects on (a) % Baseline Number of Stimulations per Component, and (b) Y-intercept of rate-dependency plots.

The dose producing the maximum effect on each measure is also indicated. Doses are expressed in mg/kg, and data for % Baseline Stimulations and Y-Intercepts are given as mean (95% CL). Values were considered to be significantly different if 95% confidence limits did not overlap.

*Significantly different from methcathinone as indicated by non-overlapping 95% confidence limits.
Effects of (±)-methcathinone, (±)-MDPV, (±)-methylone and (±)-mephedrone on full ICSS frequency-rate curves. Abscissae: frequency of electrical brain stimulation in log Hz. Ordinates: percent maximum control reinforcement rate (%MCR). Drug doses are indicated in legends in units of mg/kg. Filled points represent frequencies at which reinforcement rates were statistically different from vehicle rates as determined by two-way ANOVA followed by Holm-Sidak post hoc test, P<0.05. All data show mean ± SEM for five rats (methylone) or six rats (all other drugs).
Figure II.2

Time courses of (±)-methcathinone, (±)-MDPV, (±)-methylone and (±)-mephedrone effects on full ICSS frequency-rate curves. Drug doses are expressed in mg/kg and are indicated in front of the drug name in the title of each panel. Filled points represent frequencies at which reinforcement rates were statistically different from vehicle rates as determined by two-way ANOVA followed by Holm-Sidak post hoc test, P<0.05. All data show mean ± SEM for five rats (methylone) or six rats (all other drugs).
Summary of effects of (±)-methcathinone, (±)-MDPV, (±)-methylenone and (±)-mephedrone on ICSS expressed as percent pre-drug baseline number of stimulations delivered across all frequencies of brain stimulation. Left panel (a) compares potencies and efficacies of drugs. Abscissa: drug dose in mg/kg. Ordinate: percent pre-drug baseline number of ICSS reinforcers. Right panel (b) compares time course profiles of drugs. Abscissa: pretreatment time in min. Ordinate: percent pre-drug baseline number of ICSS reinforcers.
Rate-dependency analysis of methcathinone and its derivatives effects on ICSS. (a, b) Abscissae: log baseline ICSS rate. Ordinates: log percent baseline ICSS rate. Horizontal line at Y=2.0 indicates no change from baseline rates. Vertical line at X=1.0 indicates the position of the Y-intercept values used in Panel c. (c) Abscissa: drug dose in mg/kg. Ordinate: Y-intercept from linear regression analysis of rate-dependency plots. All points show mean data for 5-6 rats.
Chapter III: Quantitative Structure-Activity Relationship (QSAR) Analysis of the Pharmacology of Para-Substituted Methcathinone Analogues


Introduction

The goal of this study was to use quantitative structure-activity relationship (QSAR) analysis (Glennon and Young, 2011) to evaluate molecular determinants of abuse-related neurochemical and behavioral effects of MCAT and six para-substituted MCAT analogs. Figure III.1 shows the chemical structure of the MCAT scaffold, which served as the base molecule for all analogs investigated in this study. The para substituent on the benzene ring of the MCAT scaffold, R, was manipulated to include substituents that varied systematically along three physicochemical dimensions [steric ($E_s$), electronic ($\sigma_p$), and lipophilic ($\pi_p$)], and Table III.1 shows quantitative measures for each substituent on each parameter. The effects of each drug were examined on two experimental endpoints: (1) in vitro potency to promote monoamine release through DA and 5-HT transporters (DAT and SERT, respectively) in rat-brain synaptosomes, and (2) in vivo modulation of ICSS in rats. These two endpoints were selected for study in part because ICSS in rats is useful for predicting abuse potential of drugs in humans (Negus and Miller, 2014), and previous data with a different set of monoamine releasers indicated a correlation
between *in vitro* DAT-vs.-SERT selectivity and *in vivo* efficacy to produce an abuse-related facilitation of ICSS in rats (Bauer et al., 2013b). QSAR analysis was accomplished by correlating measures for each physicochemical parameter with data from neurochemical and behavioral studies. We predicted that *in vitro* DAT-vs.-SERT selectivity would correlate with maximal ICSS facilitation, and that one of the physicochemical parameters would correlate with both DAT-vs.-SERT selectivity and maximal ICSS facilitation. Results support this hypothesis and suggest that “steric bulk” of the *para* substituent, as quantified by Taft’s steric parameter ($E_s$), is a key determinant of abuse-related neurochemical and behavioral effects of *para*-substituted MCAT analogs.

**Materials and Methods**

**Drugs**

This study examined effects of MCAT and six analogs with different substitutions at the *para*, or “4,” position on the phenyl ring. For MCAT, the *para* substituent is hydrogen, and for the purposes of this study, the MCAT analogs are designated using the nomenclature “4-R MCAT,” with “R” being the substitution for hydrogen at the *para* position. In some cases, these compounds also have other generic names or other chemical names based on the abbreviation “MAP” (for methylaminopropiophenone, the chemical name for MCAT), and all compounds were synthesized as their racemic HCl salts using previously published procedures. Specifically, the following compounds were studied, and alternative names and published syntheses are shown in parentheses: MCAT (Findlay et al., 1981), 4-F
MCAT (flephedrone; Archer, 2009), 4-OCH₃ (methedrone; Lespagnol and Hallot, 1954), 4-Cl MCAT (Trepanier and Sprançmanis, 1964), 4-Br MCAT (4-BMAP; Foley and Cozzi, 2003), 4-CH₃ MCAT (mephedrone; McDermott et al., 2011), and 4-CF₃ MCAT (4-TFMAP; Cozzi et al., 2013). All compounds were dissolved in sterile saline for i.p. injection.

**In Vitro Release Assays**

All *in vitro* synaptosome studies were conducted by our collaborators Michael Baumann and John Partilla of the NIDA Intramural Research Program in Baltimore, MD. However, I conducted the correlational analyses with data generated from these studies.

**Subjects.** Male Sprague-Dawley rats (Charles River, Wilmington, MA, USA) weighing 250-350 g were housed three per cage with free access to food and water and maintained on a 12 h light/dark cycle with lights on from 7:00 a.m. to 7:00 p.m. Animal facilities were accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, and procedures were carried out in accordance with the Institutional Animal Care and Use Committee and the National Institutes of Health guidelines on care and use of animal subjects in research (National Research Council, 2011).

**Procedure.** Rats were euthanized by CO₂ narcosis, and brains were processed to yield synaptosomes as previously described (Rothman et al., 2003). For release assays, 9 nM [³H]1-methyl-4-phenylpyridinium ([³H]MPP⁺) was used as the radiolabeled substrate for DAT, whereas 5 nM [³H]5-HT was used as a substrate for SERT. All buffers used in the release assay methods contained 1 µM reserpine to
block vesicular uptake of substrates. The selectivity of release assays was optimized for a single transporter by including unlabeled blockers to prevent the uptake of $[^3\text{H}]\text{MPP}^+$ or $[^3\text{H}]5$-HT by competing transporters. Synaptosomes were preloaded with radiolabeled substrate in Krebs-phosphate buffer for 1 h (steady state). Release assays were initiated by adding 850 µl of preloaded synaptosomes to 150 µl of test drug. Release was terminated by vacuum filtration, and retained radioactivity was quantified by liquid scintillation counting.

Previous studies have used these procedures to evaluate MCAT, 4-CH$_3$ MCAT, and 4-CF$_3$ MCAT. For this study, MCAT effects were redetermined, and effects of the additional analogs 4-F MCAT, 4-OCH$_3$ MCAT, 4-Cl MCAT and 4-Br MCAT were also determined. Correlational analysis described below used data from the present study and data for 4-CH$_3$ MCAT and 4-CF$_3$ MCAT from previous studies (Baumann et al., 2012; Cozzi et al., 2013).

**Data Analysis.** Statistical analyses were carried out using GraphPad Prism (v. 6.0; GraphPad Scientific, San Diego, CA, USA). EC$_{50}$ values for stimulation of release were calculated based on non-linear regression analysis. DAT-vs.-SERT selectivity was also calculated as SERT EC$_{50}$ ÷ DAT EC$_{50}$, such that larger ratios indicate higher DAT selectivity.

**Reagents.** $[^3\text{H}]5$-HT (specific activity = 30 Ci mmol-1) was purchased from Perkin Elmer (Shelton, CT, USA). $[^3\text{H}]\text{MPP}^+$ (specific activity = 85 Ci mmol-1) was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). All other chemicals and reagents were acquired from Sigma-Aldrich (St. Louis, MO, USA).
Intracranial Self-Stimulation (ICSS)

**Subjects.** Male Sprague-Dawley rats (Harlan, Frederick, MD, USA) weighing at least 300 g at the time of surgery were individually housed and maintained on a 12 h light/dark cycle. Methods for surgery, training, testing and data analysis were generally identical to those described in the ICSS Methods section of Chapter II. Any important differences in methods for this study are described below.

**Training.** Rats were trained and tested under a FR 1 schedule of brain stimulation using a behavioral procedure identical to that described in Chapter II. Stimulation intensity was individually adjusted for each rat to the lowest value that sustained a high rate of reinforcement (> 30 stimulations/min), and this intensity (100-290 µA across rats) was held constant for the remainder of the study.

**Testing.** The MCAT analogs tested in this study were 4-F MCAT, 4-OCH₃ MCAT, 4-Cl MCAT, 4-Br MCAT, and 4-CF₃ MCAT. Two additional compounds, MCAT and 4-CH₃ MCAT, were studied previously (Bonano et al., 2014b; see Chapter II), and data from those studies were included in correlational analyses described below. Each drug was studied in dose-effect and time-course procedures. In dose-effect studies, a 1.0 to 1.5 log unit range of doses was tested for each drug with the goal of testing a dose range from a low dose that produced little or no effect to a high dose that produced maximal facilitation of ICSS. In time-course studies, the highest dose of each compound was tested. The order of testing with vehicle and drug doses was varied across subjects using a Latin-square design, and experiments with any single compound were completed prior to beginning tests with another compound. Furthermore, tests with different drugs in a single rat were separated by at least 1
week, and a saline vehicle test session was conducted during this period to ensure that injections or drug exposures did not alter individual ICSS baselines.

**Data Analysis.** ICSS data were analyzed as described previously (Bauer et al., 2013b; Negus and Miller, 2014). The primary dependent variable in this ICSS procedure was the reinforcement rate in stimulations per minute during each frequency trial. To normalize data, raw reinforcement rates from each trial in each rat were converted to %MCR. Results from test sessions were compared by repeated measures two-way ANOVA, with ICSS frequency as one factor and dose or time as the second factor. A significant ANOVA was followed by Holm-Sidak *post hoc* test, *P*<0.05. As a summary measure of drug effects on ICSS, the total number of stimulations delivered per component across all 10 frequency-trials was also calculated for each dose and time point. Test data were normalized to individual baseline data and were then averaged across rats in each experimental condition.

**Correlational Analysis**

Correlations were evaluated between *in vitro* and *in vivo* drug effects using linear regression and a Pearson correlation test as described previously for a different set of monoamine releasers (Bauer et al., 2013b). Specifically, *in vitro* selectivity to promote DAT- vs. SERT-mediated release was compared to maximal *in vivo* facilitation of ICSS (defined as the maximum increase in % baseline total stimulations per component produced by any dose of a given drug). Correlational analysis was also used to assess quantitative structure-activity relationships (QSAR) between physicochemical features and the *in vitro* and *in vivo* effects of each drug (Glennon and Young, 2011). Specifically, steric (Eₛ), electronic (σₛ) and lipophilic
(πₚ) constants of \textit{para} substituents on the MCAT scaffold (Wolff, 1980) were compared to \textit{in vitro} potency for DAT- and SERT-mediated release, \textit{in vitro} DAT-vs.-SERT selectivity, and maximal \textit{in vivo} facilitation of ICSS. The measure used to represent steric bulk of \textit{para} substituents in this study was Taft’s steric parameter, \(E_s\), which was developed to reflect the steric influence of substituents on the rate of hydrolysis; thus, Taft’s steric constant represents a functional measure of steric bulk. Correlations and statistical analyses were carried out using Prism 6.0 (GraphPad Scientific, San Diego, CA, USA), and correlations were considered statistically significant if \(P<0.05\).

\textbf{Results}

\textit{In Vitro} Monoamine Release Mediated by DAT and SERT

All seven compounds considered in this study produced concentration-dependent increases in DAT-mediated \(^{3}\text{H}\)MPP⁺ release and SERT-mediated \(^{3}\text{H}\)5-HT release from rat brain synaptosomes. Data for 4-CH₃ MCAT and 4-CF₃ MCAT were published previously (Baumann et al., 2012; Cozzi et al., 2013), and Figure III.2 shows data for the other five compounds. Table III.1 shows EC₅₀ values for each compound to promote DAT- and SERT-mediated monoamine release. DAT-vs.-SERT selectivity is also reported for each compound. DAT-vs.-SERT selectivity varied across a more than 4000-fold range, with MCAT functioning as the most DAT-selective compound and 4-CF₃ MCAT functioning as the most SERT-selective compound.
**Intracranial Self-Stimulation**

Electrical brain stimulation maintained a frequency-dependent increase in ICSS rates under baseline conditions. Across the 33 rats used in these studies, the average ± SEM baseline MCR was 61 ± 2 stimulations per trial, and the mean ± SEM number of total baseline stimulations across all frequencies was 291 ± 14 stimulations per component.

All seven compounds considered in this study produced dose-dependent changes in ICSS. Data for MCAT and 4-CH₃ MCAT were reported previously (Bonano et al., 2014b) and were described above in Chapter II. Figure III.3 shows dose-effect data for the other five compounds. Two-way ANOVA indicated significant main effects of frequency and dose and significant frequency x dose interactions for all drugs, and only the interaction effects are reported below for each drug. 4-F MCAT [F(36,144)= 10.54, P<0.0002] produced dose-dependent facilitation of low ICSS rates maintained by low brain stimulation frequencies with no evidence at these doses and pretreatment times of depression of high ICSS rates maintained by high brain stimulation frequencies. 4-OCH₃ MCAT [F(36,180)= 5.55, P<0.0001] , 4-Cl MCAT [F(36,180)= 7.31, P<0.0001], and 4-Br MCAT [F(36,180)= 5.30, P<0.0001] also produced facilitation of low ICSS rates maintained by low stimulation frequencies; however, higher doses of these compounds also significantly decreased high ICSS rates. Lastly, 4-CF₃ MCAT [F(27,135)= 1.69, P=0.027] produced exclusive depression of high ICSS rates maintained by high brain stimulation frequencies.

Figure III.4 shows the time course of effects produced by the highest tested dose of each compound. Two-way ANOVA indicated significant main effects of
frequency and time and significant frequency x time interactions for all drugs, and only the interaction effects are reported below for each drug. 4-F MCAT (3.2 mg kg⁻¹; [F(36,144)= 5.28, P<0.0001]) produced facilitation of ICSS within 10 min after administration, and significant ICSS rate-increasing effects were no longer apparent by 300 min. 4-OCH₃ MCAT (32 mg kg⁻¹; [F(36,144)= 8.34, P<0.0001]) produced only depression of ICSS after 10 min, but both rate-increasing effects and rate-decreasing effects were apparent after 30 and 100 min. 4-Cl MCAT (10 mg kg⁻¹; [F(36,180)=7.10, P<0.0001]) and 4-Br MCAT (10 mg kg⁻¹; [F(45,225)=4.25, P<0.0001]) produced rate-decreasing effects that peaked after 10 min and were still significant after 300 min. Rate-increasing effects were apparent only 300 min after administration of this high dose of 4-Cl MCAT and at no time after 4-Br MCAT. 4-CF₃ MCAT (10 mg kg⁻¹; [F(36,180)=1.96, P=0.002]) produced exclusive depression of ICSS from 10 to 100 min, and these effects were no longer apparent after 300 min.

**Correlational Analysis**

Figure III.5 shows that *in vitro* selectivity for DAT- vs. SERT-mediated release correlated with *in vivo* efficacy to facilitate ICSS (Fig. III.5A). Figure III.5, together with Table III.2, also shows results of QSAR analysis. Significant correlations were found between steric bulk (Eₛ) and both *in vitro* DAT- vs. SERT selectivity (Fig. III.5B) and *in vivo* efficacy to facilitate ICSS (Fig. III.5C). For both of these QSAR correlations, the largest outlier was 4-OCH₃ MCAT, which had lower DAT- vs. SERT selectivity and produced weaker ICSS facilitation than would have been predicted based on the correlation with Eₛ. There was no significant correlation between steric bulk and *in vitro* potency at either DAT or SERT individually, and lipophilicity (ᵦₚ)
and electronic ($\sigma_p$) parameters did not correlate significantly with any in vitro or in vivo endpoint (Table III.2).

**Summary**

This study compared in vitro neurochemical and in vivo behavioral effects produced by MCAT and six para-substituted analogs. There were three main findings. First, all seven compounds functioned as substrates at both DAT and SERT, and DAT-vs.-SERT selectivity varied >4000-fold across compounds. Second, all drugs dose-dependently altered ICSS, and efficacy to produce an abuse-related facilitation of ICSS also varied across compounds. In agreement with a previous study using a different set of compounds (Bauer et al., 2013b), in vitro DAT-vs.-SERT selectivity correlated with in vivo efficacy to facilitate ICSS ($R=0.92$, $P=0.003$). Finally, QSAR analysis identified a significant correlation between the steric parameter ($E_S$) of the para substituents and both in vitro DAT-vs.-SERT-selectivity ($R=0.78$, $P=0.04$) and in vivo facilitation of ICSS ($R= 0.81$, $P=0.03$). These results suggest that steric bulk of the para substituent of the MCAT scaffold is a key determinant of both in vitro DAT-vs.-SERT selectivity and in vivo expression of abuse-related behavioral effects in an ICSS procedure.

Steric bulk can be further broken down into definitive parameters of volume, width, and length to more closely investigate how structural characteristics of the para substituent influence in vitro and in vivo activity of MCAT analogs. As a follow-up to the initial QSAR investigation described here in Chapter III, which identified $E_S$ as a critical determinant of DAT selectivity and abuse-related facilitation of ICSS,
collaborators conducted additional correlations between volume, maximum width, minimum width and length and the experimental endpoints described above (Sakloth et al., 2014). Results from these correlations concluded that steric volume correlates most strongly with *in vitro* and *in vivo* abuse-related effects, even better than $E_S$ (Figure III.6). This finding suggests that steric volume is a particularly important component of steric contributions to abuse-related drug effects produced by *para*-substituted MCAT analogs.
Table III.1

<table>
<thead>
<tr>
<th>Drug Name</th>
<th>Physicochemical Parameter a</th>
<th>In Vitro Release EC50 b</th>
<th>Maximal ICSS Facilitation d</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Eₚ</td>
<td>σₚ</td>
<td>πₚ</td>
</tr>
<tr>
<td>MCAT</td>
<td>1.24</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>4-F MCAT</td>
<td>0.78</td>
<td>0.06</td>
<td>0.14</td>
</tr>
<tr>
<td>4-OCH₃ MCAT</td>
<td>0.69</td>
<td>-0.27</td>
<td>-0.02</td>
</tr>
<tr>
<td>4-Cl MCAT</td>
<td>0.27</td>
<td>0.23</td>
<td>0.71</td>
</tr>
<tr>
<td>4-Br MCAT</td>
<td>0.08</td>
<td>0.23</td>
<td>0.86</td>
</tr>
<tr>
<td>4-CH₃ MCAT</td>
<td>0.00</td>
<td>-0.17</td>
<td>0.56</td>
</tr>
<tr>
<td>4-CF₃ MCAT</td>
<td>-1.16</td>
<td>0.54</td>
<td>0.88</td>
</tr>
</tbody>
</table>

Physicochemical parameters, in vitro release activities, and in vivo behavioral ICSS effects of MCAT and its para-substituted analogs.

a Physicochemical parameters of para substituent (functional steric bulk, Eₚ; electron-withdrawing capacity, σₚ; lipophilicity, πₚ) as reported in Wolff, 1980.

b EC50 values derived from concentration-effect curves in Figure III.2, or published previously for 4-CH₃ MCAT (Baumann et al., 2012), 4-CF₃ MCAT (Cozzi et al., 2013).

c DAT selectivity calculated as SERT EC50 ÷ DAT EC₅₀.

d Maximal ICSS facilitation expressed as maximum increase in % Baseline Stimulations per Component from Figure III.3, or published previously for MCAT and 4-CH₃ MCAT (Bonano et al., 2014b).
Table III.2

<table>
<thead>
<tr>
<th>Physicochemical Parameter a</th>
<th>log DAT EC₅₀</th>
<th>log SERT EC₅₀</th>
<th>log DAT Selectivity</th>
<th>Maximal ICSS Facilitation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eₛ</td>
<td>R = -0.71</td>
<td>R = 0.58</td>
<td>R = 0.78*</td>
<td>R = 0.81*</td>
</tr>
<tr>
<td></td>
<td>P = 0.07</td>
<td>P = 0.17</td>
<td>P = 0.04</td>
<td>P = 0.03</td>
</tr>
<tr>
<td>σₚ</td>
<td>R = 0.37</td>
<td>R = -0.10</td>
<td>R = -0.29</td>
<td>R = -0.24</td>
</tr>
<tr>
<td></td>
<td>P = 0.42</td>
<td>P = 0.84</td>
<td>P = 0.53</td>
<td>P = 0.61</td>
</tr>
<tr>
<td>πₚ</td>
<td>R = 0.26</td>
<td>R = -0.64</td>
<td>R = -0.52</td>
<td>R = -0.66</td>
</tr>
<tr>
<td></td>
<td>P = 0.57</td>
<td>P = 0.12</td>
<td>P = 0.23</td>
<td>P = 0.11</td>
</tr>
</tbody>
</table>

Results of correlational analysis between physicochemical parameters, in vitro potencies at DAT and SERT, and maximal ICSS facilitation

*Significant correlation

a Functional steric bulk, Eₛ; electron-withdrawing capacity, σₚ; lipophilicity, πₚ
Figure III.1

Chemical structure of MCAT scaffold. R-site of para substituent, which was systematically varied to generate MCAT analogs.
Figure III.2

Effects of test drugs on DAT- and SERT-mediated monoamine release in rat brain synaptosomes. Abscissae: log concentration of drug (molar). Ordinates: percent maximum release. All points show mean ± SD for N=3 separate experiments. EC$_{50}$ values shown in Table III.1 were derived from these concentration-effect curves. Data collected by M. Baumann and J. Partilla of the NIDA Intramural Research Program.
Figure III.3

Effects of test drugs on ICSS in rats. Left panels 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 doses are
indicated in boxed legends in units of mg/kg. Filled points represent frequencies at which reinforcement rates were statistically different from vehicle rates as determined by two-way ANOVA followed by Holm-Sidak post hoc test, P<0.05. Right panels show summary ICSS data for drug effects across all frequencies. Abscissae: drug dose in mg/kg. Ordinates: percent baseline number of stimulations per component delivered across all brain stimulation frequencies. 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. All data show mean ± SEM for five (4-F MCAT) or six rats (all other drugs). Maximal ICSS facilitation values shown in Table III.1 were taken from the right panels for each drug.
Figure III.4

Time courses of drug effects on ICSS in rats. The title of each panel shows the drug and test dose in units of mg/kg. Left panels show full ICSS frequency-rate curves. Time points are indicated in the boxed legend in units of minutes. Filled points represent
frequencies at which reinforcement rates were statistically different from baseline rates as determined by two-way ANOVA followed by Holm-Sidak post hoc test, P<0.05. Right panels show summary ICSS data for drug effects across all frequencies. Abscissae: time after treatment in min. All data show mean ± SEM for five rats (4- OCH₃ MCAT and 4-F MCAT) or six rats (all other drugs). Other details as in Figure III.3.
Correlational analysis across experimental endpoints and structural parameters from Table III.1. Panel A shows the correlation between in vitro selectivity for DAT- vs. SERT-mediated monoamine release (abscissa) and maximum in vivo ICSS facilitation (ordinate). Panel B shows the correlation between steric bulk (E_s, abscissa) and in vitro DAT selectivity (ordinate).
vitro selectivity for DAT- vs. SERT-mediated monoamine release (ordinate). Panel C shows the correlation between steric bulk ($E_s$, abscissa) and maximum in vivo ICSS facilitation (ordinate). All points show mean data for 5-6 rats.
Figure III.6

Relationship between the volume of the para substituent and: in vitro selectivity for DAT-vs.-SERT-mediated monoamine release (left panel), in vivo ICSS facilitation (right panel). Figure adapted from Sakloth et al., 2014.
Chapter IV: Abuse-Related Neurochemical Effects of Para-Substituted Methcathinone Analogs in Rats: Microdialysis Studies of Nucleus Accumbens

Dopamine and Serotonin

Bonano JS, Glennon RA, Lazenka MF, Negus SS, and Banks ML. In preparation for submission to *Neuropharmacology*.

**Introduction**

The goal of this study was to examine abuse-related neurochemical effects of MCAT and five *para*-substituted MCAT analogs on dopamine (DA) and serotonin (5-HT) levels in the nucleus accumbens (NAc) of live, wake rats. Abuse-related neurochemical drug effects were investigated using microdialysis in male Sprague-Dawley rats implanted with guide cannula targeting the NAc. Microdialysis is an experimental technique that can be used to recover and measure endogenous extracellular monoamines in discrete brain regions (Zetterström et al., 1983), and early studies on the neurochemical effects of various drugs of abuse on extracellular DA levels in the striatum and NAc showed that virtually all abused drugs (e.g., opiates, ethanol, nicotine, amphetamine, cocaine) increase DA levels in both regions, but particularly in the accumbens (Di Chiara and Imperato, 1988). Subsequent studies confirmed that various classes of abused drugs, especially monoamine releasers and uptake inhibitors, elevate accumbal DA levels (Carboni et al., 1989) and extended on those findings by demonstrating that alterations in other accumbal monoamine levels (i.e. 5-HT) also influence a drug's abuse potential. We
hypothesized that MCAT and each of its para-substituted analogs would increase extracellular levels of DA and/or 5-HT in the NAc of live, wake rats, and that these in vivo neurochemical measures of abuse potential (i.e. selectivity to induce release of DA vs. 5-HT in NAc) would correlate with: a) in vitro selectivity for monoamine release via DAT-vs.-SERT; b) in vivo facilitation of ICSS; and, based on the results of QSAR analysis described in Chapter III, c) steric volume of the para substituent on the MCAT scaffold.

**Materials & Methods**

**Subjects**

Adult male Sprague-Dawley rats (Harlan, Frederick, MD, USA) weighing at least 300 g at the time of surgery were individually housed and maintained on a 12 h light/dark cycle with lights on from 6:00 a.m. to 6:00 p.m. Rats had ad libitum access to food and water except during microdialysis experiments. Animal facilities were accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, and experimental procedures were approved by the Institutional Animal Care and Use Committee in accordance with guidelines (8th edition) for the care and use of animal subjects in research (National Research Council, 2011).

**Drugs**

This study determined neurochemical effects of MCAT and five para-substituted MCAT analogs. All MCAT analogs studied in Chapter III, with the exception of 4-CF₃ MCAT, were also examined in microdialysis studies. In addition,
prior to initiation of experiments with MCAT analogs, D-amphetamine hemisulfate (Sigma Aldrich, St. Louis, MO) and (±)-fenfluramine HCl (Sigma Aldrich) effects were determined as control compounds for DA-selective and 5-HT-selective monoamine release, respectively. All compounds were dissolved in sterile saline for intraperitoneal (i.p.) injection, and all doses are expressed as the salt forms listed above.

**Microdialysis**

**Surgery.** Rats (N=61) were anesthetized with 3.0% isoflurane in oxygen until unresponsive to toe-pinches and secured in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA, USA). Guide cannula (8mm long, 0.5mm outer diameter; CXG-8, Eicom, San Diego, CA, USA) were implanted bilaterally and terminated 1mm above the nucleus accumbens (NAc) (coordinates: 1.5mm anterior to bregma, 1.8mm lateral to midsagittal suture, 6.0mm ventral to dura). The guide cannulas were secured to the skull using screws (Plastics One, Inc., Roanoke, VA, USA) and orthodontic resin (Butler Schein, Dublin, OH, USA). A dummy cannula (CXD-8, Eicom) was inserted into each guide cannula to maintain cannula patency. Animals were allowed at least 7 recovery days prior to initiating microdialysis testing.

**Procedure.** On test days, rats were briefly anesthetized with 3.0% isoflurane in oxygen, one of the dummy cannulas was removed, and a microdialysis probe (10mm long, CX-I-8-2, Eicom) with a 2mm artificial cellulose “cuprophan” membrane (50kDa molecular weight cut-off) at its tip was inserted into a 8mm guide cannula such that it extended 2mm beyond the end of the guide cannula and into the NAc. The probe was connected to a two-channel liquid swivel (TCS2-23,
Eicom), and the rat was placed into an acrylic experimental cage (30 cm³). Microdialysis probes were perfused with artificial cerebrospinal fluid (aCSF) (147mM NaCl, 2.8mM KCl, 1.2mM CaCl₂, 1.2mM MgCl₂) at a rate of 1µL/min. Mobile phase consisted of 2% methanol (EMD, Gibbstown, NJ, USA), 100mM phosphate buffer (Sigma Chemicals, St. Louis, MO, USA), 500mg/L 1-decane sodium sulfonate (TCI America, Montgomeryville, PA, USA), and 50mg/L EDTA-2Na⁺ (Dojindo Laboratories, Kumamoto, Japan). Dialysate samples were collected into a 50µL injector loop at 10min intervals using an online auto-injector (EAS-20s, Eicom) and immediately analyzed for DA and 5-HT concentrations by high-pressure liquid chromatography (HPLC) coupled to electrochemical detection (HTEC-500, Eicom). DA and 5-HT were separated using a C18-reverse phase column (PP-ODS II, Eicom) and were detected using a graphite working electrode and an Ag vs. AgCl reference electrode with an applied potential of +450mV. Preliminary experiments conducted by probe immersion into a known standard concentration of DA indicated a lag time of ~20min for dialysate to traverse the tubing from the probe to the electrochemical detector at the 1µL/min flow rate. DA and 5-HT were identified according to characteristic retention times of the standard solution and concentrations were quantified by comparison with peak heights of the standard concentration curve (0.01-100pg per 10µL) generated prior to drug administration in each microdialysis experiment. The lower limit of neurotransmitter detection was 0.1pg. DA and 5-HT levels were determined to be stable after 6 consecutive baseline samples were obtained with <25% variability around the running mean of both neurotransmitters. Testing was conducted using drug doses based on previous
behavioral studies from our laboratory (Bauer et al., 2013b; Bonano et al., 2014a, 2014b). Specifically, amphetamine (0.1-1.0mg/kg), fenfluramine (1.0-3.2mg/kg), MCAT (0.32-3.2mg/kg), and 5 para-substituted MCAT analogs (4-F, 4-Cl, 4-CH₃, and 4-Br MCAT, 1-10mg/kg; 4-OCH₃ MCAT, 3.2-32mg/kg) were administered i.p., and dialysate samples were collected for 180min after drug administration. Rats were tested no more than four times (twice per cannula; at least two weeks between re-assessment of a given site). Specifically, rats were administered a drug dose and tested in microdialysis no more than once per week, and accessing a guide cannula site occurred every two weeks at most. Rats’ drug exposures varied, such that any given rat may have been tested with multiple doses of the same drug or with different drugs. However, data for any one dose of any one drug were always collected in different rats (N=5-6 for each dose of each drug). Furthermore, a single set of vehicle (saline, i.p.) tests were conducted, and these data were used for all drug and dose comparisons. At the completion of all experiments, rats were euthanized with CO₂, and brains were removed and stored in 10% formalin. Probe placement was verified by gross visual inspection of unstained brain sections. Only rats with correct probe placements were included in data analyses.

**Data Analysis.** The primary dependent variables were extracellular DA and 5-HT concentrations in each dialysate fraction expressed as a percent of the average of the 6 mean baseline concentrations before drug or vehicle administration for each experiment. These individual normalized DA and 5-HT concentrations were then averaged across rats to yield group mean results for graphical presentation. Results were analyzed by two-way repeated measures ANOVA, with drug dose and
time as the two main factors. A significant drug dose × time interaction was followed by Holm-Sidak post hoc test, and the criterion for significant was set at P<0.05.

In addition to the analysis above, peak increases in DA and 5-HT concentrations produced by each dose of each drug were used to generate dose-effect graphs (maximum % baseline neurotransmitter vs. drug dose). Linear regression analysis of the ascending limb of the dose-effect curve for a given drug was used to extrapolate the dose (mg/kg) of that drug required to elevate DA and 5-HT levels to 250% of baseline (DA ED\text{250} and 5-HT ED\text{250}, respectively). In vivo DA/5-HT selectivity for each drug was defined by the ratio DA ED\text{250} ÷ 5-HT ED\text{250}.

**Correlational Analysis**

Correlations were evaluated between in vivo neurochemical selectivity to increase accumbal DA vs. 5-HT and each of the following measures: 1) in vitro selectivity to promote DAT- vs. SERT-mediated release (Fig. IV.5A), 2) abuse-related facilitation of ICSS (Fig. IV.5B), and 3) steric volume (Fig. IV.5C). Correlations were determined using linear regression and a Pearson correlation test. Correlations and statistical analyses were carried out using Prism 6.0 for Mac (GraphPad Scientific, San Diego, CA, USA), and correlations were considered statistically significant if P<0.05.

**Results**

Across microdialysis experiments, baseline (mean ± S.E.M.) NAc extracellular DA and 5-HT levels were 0.83 ± 0.04pg and 0.85 ± 0.05pg per 9μL, respectively.
Figure IV.6 shows placements of microdialysis guide cannula for all rats included in data analyses.

**Effects of Amphetamine and Fenfluramine on NAc DA and 5-HT levels**

Both amphetamine and fenfluramine produced time-dependent increases in extracellular NAc DA and/or 5-HT levels, and these data are summarized in Figure IV.1. Two-way ANOVA indicated significant main effects of dose and time and a significant dose x time interaction for amphetamine to increase DA levels, whereas two-way ANOVA indicated a significant main effect of time, but not dose, and a significant dose x time interaction for fenfluramine to increase 5-HT levels. Only the interaction effects are reported below for each drug to selectively elevate its relevant neurotransmitter (i.e. DA for amphetamine, 5-HT for fenfluramine). Under vehicle conditions (i.p. saline), no significant changes in NAc DA or 5-HT levels were observed over the 180-min observation period. Amphetamine (0.1-1.0mg/kg), a DA-selective monoamine releaser with ~70-fold selectivity for releasing DA vs. 5-HT *in vitro* (Rothman et al., 2001), increased NAc DA concentrations to a maximum of 621% from baseline \[F(51,204)= 4.577, P<0.0001\], compared to a maximal 5-HT increase of 161% from baseline \[F(51,204)= 1.535, P=0.02\]. Conversely, fenfluramine (1.0-3.2mg/kg), a 5-HT-selective releaser with <0.01-fold selectivity for DA vs. 5-HT (Rothman et al., 2001), increased NAc 5-HT concentrations to maximum of 597% from baseline \[F(34,136)= 2.546, P<0.0001\], compared to a maximal DA increase of 152% from baseline [no significant interaction].
Effects of MCAT and its para-substituted analogs on NAc DA and 5-HT levels

MCAT and all five para-substituted MCAT analogs produced significant increases in NAc DA and/or 5-HT levels. Figure IV.2 illustrates the neurochemical effects of MCAT, 4-F MCAT (flephedrone) and 4-Cl MCAT on accumbal DA and 5-HT levels. Two-way ANOVA indicated significant main effects of dose and time and significant dose x time interactions for all drugs. Only the interaction statistics are reported below for each drug. MCAT (0.32-3.2mg/kg) produced selective dose- and time-dependent increases in extracellular DA concentrations to 518% of baseline levels [F(34,136)= 5.749, P<0.0001], compared to no statistically significant effects on 5-HT concentrations at any dose tested. 4-F MCAT (1.0-10mg/kg) produced dose- and time-dependent increases in DA levels [F(51,204)= 5.291, P<0.0001] to a maximum of 745% of baseline, and in 5-HT levels [F(51,204)= 11.11, P<0.0001] to a maximum of 1,567% of baseline. Similarly, 4-Cl MCAT (1.0-10mg/kg) produced dose- and time-dependent increases in DA [F(51,204)= 5.694, P<0.0001] to 1,261% of baseline, and in 5-HT [F(51,204)= 9.710, P<0.0001] to 1,834% of baseline concentrations. Interestingly, for these two halogenated MCAT analogs, increases in accumbal 5-HT levels exhibited a rapid onset and offset, with 5-HT levels peaking within 20min (controlling for ~20min lag time) and then precipitously declining. Increases in accumbal DA levels, on the other hand, exhibited a slower onset and sustained elevation, reaching peak levels towards the end of the 180-min experimental sessions.

Figure IV.3 shows the effects of 4-CH$_3$ MCAT (mephedrone), 4-Br MCAT, and 4-OCH$_3$ MCAT (methedrone) on extracellular NAc DA and 5-HT concentrations.
Two-way ANOVA indicated significant main effects of dose and time and a significant dose x time interaction for mephedrone at each neurotransmitter. Two-way ANOVA indicated a significant main effect of time and a significant dose x time interaction for 4-Br MCAT at both neurotransmitters, but only indicated a significant main effect of dose for 4-Br MCAT at DA (i.e. no main effect of dose for 4-Br MCAT at 5-HT). Lastly, two-way ANOVA indicated significant main effects of dose and time for 4-OCH₃ MCAT at each neurotransmitter, but a significant dose x time interaction only existed at 5-HT (i.e. no significant interaction for 4-OCH₃ MCAT at DA). Significant interaction effects are reported below for each drug at each monoamine when applicable. 4-CH₃ MCAT (1.0-10mg/kg) increased NAc concentrations of both DA [F(34,136)= 2.896, P<0.0001] and 5-HT [F(34,136)= 15.61, P<0.0001] to a maximum of 523% and 1061% of baseline, respectively. 4-Br MCAT (1.0-10mg/kg) increased extracellular concentrations of both DA [F(34,136)= 14.58, P<0.0001] and 5-HT [F(34,136)= 4.503, P<0.0001] in Nac. DA levels reached a maximum of 559% of baseline, and 5-HT levels increased to 1,026% of baseline. As with the other halogenated MCAT analogs shown in Figure IV.2, 4-Br MCAT-induced 5-HT increases exhibited a rapid onset and offset, while increases in DA levels exhibited a slower onset and were sustained for a longer duration. 4-OCH₃ MCAT (3.2-32mg/kg) increased DA concentrations maximally to 384% of baseline, and it was the least potent para-substituted MCAT analog. However, 4-OCH₃ MCAT was the most efficacious compound for elevating accumbal 5-HT and increased extracellular 5-HT levels to 2,428% of baseline [F(51,204)= 6.280, P<0.0001].
Correlational Analyses

ED_{250} values to increase DA and 5-HT levels, and in vivo DA/5-HT selectivity values, are summarized for each drug in Table IV.1. Correlational analysis indicated significant correlations between in vivo DA/5-HT selectivity and both (1) in vitro DA/5-HT selectivity determined from rat brain synaptosome preparations (R=0.95, P<0.01; Fig. IV.5A), and (2) maximal facilitation of ICSS in behavioral studies (R=0.89, P=0.02; Fig. IV.5B). Additionally, linear regression analysis revealed a significant negative correlation between steric volume of the para substituent and in vivo neurochemical selectivity to produce increases in NAc DA vs. 5-HT (R= -0.85, P=0.03; Fig. IV.5C), such that para substituents of lower volume (i.e. smaller; less steric bulk) are more selective for increasing NAc DA relative to 5-HT, and neurochemical selectivity to increase accumbal DA vs. 5-HT decreases as volume of the para substituent increases.

Summary

MCAT and all five para-substituted analogs increased accumbal DA and/or 5-HT levels. With regard to efficacy, 4-Cl MCAT produced the greatest increase in NAc DA levels (4-Cl > 4-F > MCAT = 4-Br = 4-CH_{3} > 4-OCH_{3}) and 4-OCH_{3} MCAT produced the greatest increase in NAc 5-HT levels (4-OCH_{3} > 4-Cl > 4-F > 4-CH_{3} = 4-Br >MCAT). In vivo selectivity to elevate DA vs. 5-HT levels to 250% of baseline varied across compounds, such that MCAT was the most DA selective and 4-OCH_{3} MCAT was the most 5-HT selective (DA/5-HT selectivity: MCAT> 4-F > 4-Cl > 4-Br > 4-CH_{3} > 4-OCH_{3}). Furthermore, in vivo DA vs. 5-HT selectivity correlated with both in vitro
DAT-vs.-SERT selectivity in rat-brain synaptosomes (R=0.95, P<0.01) and in vivo facilitation of intracranial self-stimulation in rats (R=0.89, P=0.02). Overall, these results suggest that in vivo selectivity of para-substituted MCAT analogs to elevate NAc extracellular DA vs. 5-HT levels may be a key neurochemical determinant of abuse-related behavioral effects.
### Table IV.1

<table>
<thead>
<tr>
<th>Drug</th>
<th>DA ED$_{250}$ (mg/kg)</th>
<th>5-HT ED$_{250}$ (mg/kg)</th>
<th>DA/5-HT Selectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCAT</td>
<td>0.37</td>
<td>4.65</td>
<td>12.56</td>
</tr>
<tr>
<td>4-F MCAT</td>
<td>0.86</td>
<td>1.07</td>
<td>1.24</td>
</tr>
<tr>
<td>4-Cl MCAT</td>
<td>0.93</td>
<td>1.15</td>
<td>1.23</td>
</tr>
<tr>
<td>4-CH$_3$ MCAT</td>
<td>1.82</td>
<td>1.12</td>
<td>0.62</td>
</tr>
<tr>
<td>4-Br MCAT</td>
<td>1.46</td>
<td>1.30</td>
<td>0.89</td>
</tr>
<tr>
<td>4-OCH$_3$ MCAT</td>
<td>11.12</td>
<td>3.61</td>
<td>0.32</td>
</tr>
</tbody>
</table>

In vivo selectivity of MCAT and its para-substituted analogs to produce increases in nucleus accumbens DA vs. 5-HT of wakeful rats. Drug doses (mg/kg) to produce 250% increases in baseline neurotransmitter levels (ED$_{250}$ values) were calculated by linear regression analysis of plots of maximum % baseline neurotransmitter vs. drug dose.
Figure IV.1

Effects of d-amphetamine (AMPH; 0.1-1.0 mg/kg, i.p.) and (±)-fenfluramine (FEN; 1.0-3.2 mg/kg, i.p.) on DA and 5-HT levels in the nucleus accumbens of wakeful rats (N=8, AMPH; N=7, FEN) expressed as a percentage of baseline neurotransmitter levels (0.975 ± 0.044pg DA; 0.533 ± 0.028pg 5-HT). Left panels indicate temporal changes in % baseline DA, while right panels indicate changes in % baseline 5-HT. Arrow indicates time of drug administration.Filled symbols indicate statistical significance (P<0.05) compared to vehicle conditions within a given time point.
Figure IV.2

Effects of MCAT, 4-F MCAT (flephedrone) and 4-Cl MCAT on NAc DA and 5-HT levels in wakeful rats (N=12, MCAT; N=10, 4-F MCAT; N=8, 4-Cl MCAT) expressed as a percentage of baseline neurotransmitter levels (0.686 ± 0.011pg DA; 0.618 ± 0.041pg 5H-T). Filled symbols indicate statistical significance (P<0.05) compared to vehicle conditions within a given time point.
Figure IV.3

Effects of 4-CH₃ MCAT (mephedrone), 4-Br MCAT, and 4-OCH₃ MCAT (methedrone) on NAc DA and 5-HT levels in wakeful rats (N=7, 4-CH₃ MCAT; N=11, 4-Br MCAT; N=12, 4-OCH₃ MCAT) expressed as a percentage of baseline neurotransmitter levels (0.987 ± 0.061pg DA; 1.216 ± 0.056pg 5-HT). Otherwise, details same as Figure IV.1.
**Figure IV.4**

**Group mean peak effects on % baseline neurotransmitter produced by a 1.5-log range of doses of MCAT and each para-substituted MCAT analog. Error bars are omitted for clarity.**
Correlational analysis across experimental endpoints and steric volume. Panel A shows the correlation between in vivo selectivity (abscissa) and in vitro selectivity for DAT-vs.-SERT-mediated monoamine release (ordinate). Panel B shows the correlation between in vivo selectivity (abscissa) and in vivo ICSS facilitation (ordinate). Panel C shows the correlation between volume (abscissa) and in vivo selectivity to increase accumbal DA vs. 5-HT levels (ordinate).
Figure IV.6

Guide cannula placements for the 61 rats used in microdialysis experiments.
Chapter V: Discussion

Summary

Overall, the studies described in this dissertation investigated neurochemical and behavioral effects produced by MCAT and a series of para-substituted MCAT analogs. The main findings are reiterated here. First, as described in Chapter II, an ICSS procedure was used to evaluate abuse-related behavioral effects produced by MCAT and three recently scheduled cathinone derivatives (mephedrone, methylone and MDPV) in rats. Although all compounds produced abuse-related ICSS effects consistent with their emergence as drugs of abuse, the profile of effects produced by MCAT differed from that produced by mephedrone despite differing only by substitution of a methyl group (-CH\(_3\)) at the para position on the phenyl ring of the MCAT scaffold. Accordingly, the remainder of my dissertation focused on the use of QSAR analysis to investigate molecular determinants of abuse-related neurochemical and behavioral effects of a novel series of systematically manipulated, para-substituted MCAT analogs.

All MCAT analogs functioned as substrates at both DAT and SERT, and in vitro DAT-vs.-SERT selectivity varied >4000-fold across compounds. Furthermore, all para-substituted MCAT analogs dose-dependently altered ICSS, and efficacy to produce abuse-related facilitation of ICSS also varied across compounds. Notably, in vitro DAT-vs.-SERT selectivity correlated with in vivo efficacy to facilitate ICSS (R=0.92, P=0.003), lending support to the hypothesis that ICSS is a sensitive behavioral tool for discerning DA-mediated abuse-related effects from 5-HT-mediated abuse-limiting effects. Third, all para-substituted MCAT analogs produced
increases in accumbal DA and/or 5-HT levels, and *in vivo* selectivity to elevate DA vs. 5-HT levels to 250% of baseline varied across compounds. *In vivo* neurochemical selectivity to increase NAc DA vs. 5-HT correlated with *in vitro* DAT-vs.-SERT selectivity (R=0.95, P<0.01) and *in vivo* facilitation of ICSS (R=0.89, P=0.02). Lastly, QSAR analysis identified a significant correlation between steric volume of the *para* substituents and *in vitro* DAT-vs.-SERT-selectivity (R= -0.97, P<0.01), *in vivo* facilitation of ICSS (R= -0.92, P<0.01), and *in vivo* selectivity to elevate accumbal DA vs. 5-HT (R=-0.85, P=0.03). The correlations between steric volume and each experimental endpoint are outlined in Figure V.1 below. Together, these results suggest that steric volume of the *para* substituent of the MCAT scaffold is a key determinant of *in vitro* selectivity to produce monoamine release via DAT-vs.-SERT, *in vivo* selectivity to increase extracellular DA vs. 5-HT levels in NAc, and *in vivo* expression of abuse-related behavioral effects in ICSS.
Results of QSAR and correlational analyses indicate significant, negative correlations between steric volume of the para substituent and each experimental endpoint investigated (i.e. in vitro selectivity for monoamine release mediated by DAT vs. SERT, in vivo selectivity to increase extracellular DA vs. 5-HT concentrations in NAc, maximal facilitation of ICSS), as well as significant, positive correlations between each experimental endpoint. These data suggest that MCAT analogs containing para substituents of lower steric volume exhibit higher DAT selectivity, both in vitro and in vivo, and this selectivity for DAT vs. SERT is a critical determinant of abuse-related facilitation of ICSS.
Implications of Chapter II: MCAT vs. 4-CH₃ MCAT

The study described in Chapter II was the first to report MCAT effects on ICSS, and the potency, efficacy and time course of MCAT to facilitate ICSS directly parallel effects of its amphetamine counterpart, methamphetamine (Bauer et al. 2013). Previous studies demonstrated that MCAT functions as a monoamine releaser with approximately 200-fold selectivity for promoting \textit{in vitro} release of DA versus 5-HT (Cozzi et al. 1999, 2013). Accordingly, the results in Chapter II are consistent with previous evidence to suggest that the maximal degree of ICSS facilitation produced by monoamine releasers correlates with pharmacological selectivity to release DA vs. 5-HT (Bauer et al. 2013). Additionally, the ICSS effects of MCAT presented in Chapter II agree with other measures of psychostimulant effects and abuse liability. For example, MCAT substituted for amphetamine in rats trained to discriminate amphetamine from saline (Glennon et al. 1986), and reciprocally, rats trained to discriminate MCAT exhibited stimulus generalization to amphetamine, methamphetamine and cocaine (Young and Glennon 1998). MCAT also produced a dose-related increase in spontaneous locomotor activity in rats (Glennon et al. 1986) and maintained dose-dependent self-administration in baboons with rates comparable to those maintained by cocaine (Kaminski and Griffiths 1994). Taken together, results from Chapter II support previous data in suggesting that MCAT is a DA-selective psychostimulant with significant potential for abuse, consistent with its Schedule I classification by the DEA.

In contrast to MCAT, methylone and mephedrone function as monoamine releasers with little selectivity between their \textit{in vitro} potencies to release DA and 5-
HT (Cozzi et al., 1999, 2013; Baumann et al., 2012; Rosenauer et al., 2013). We showed previously that selectivity of monoamine releasers to promote DA vs. 5-HT release correlated with efficacy to facilitate ICSS (Bauer et al., 2013b), and in agreement with this relationship, both methylone and mephedrone produced mixed effects that included abuse-related facilitation of low ICSS rates and abuse-limiting depression of high ICSS rates. This is the first study to evaluate methylone effects on ICSS; however, methylone is the β-ketone analog of MDMA, and methylone effects in the present study were similar to effects reported previously in this assay for MDMA (Bauer et al., 2013b). The present results with mephedrone in rats agree with a previous study that reported facilitation of low ICSS rates and depression of high ICSS rates by mephedrone in mice (Robinson et al., 2012). Results outlined in Chapter II also complement previous data on stimulant effects and abuse liability of methylone and mephedrone. For example, both drugs produce significant ambulatory hyperactivity in rodents (López-Arnau et al., 2012; Marusich et al., 2012; Shortall et al., 2012), and mephedrone has been shown to support intravenous self-administration in rats (Hadlock et al., 2011; Aarde et al., 2013a; Motbey et al., 2013).

The data included in Chapter II added to the growing “bath salts” literature by suggesting that mephedrone has relatively low efficacy to facilitate ICSS. To the degree that methylone and mephedrone display similar in vitro selectivities to release DA vs. 5-HT, the greater ICSS depressant effects of mephedrone suggest that factors other than 5-HT release may also contribute to abuse-limiting ICSS depressant effects. As one possibility, methylone and mephedrone also block DA and
5-HT reuptake, and one previous study suggests that mephedrone has lower selectivity than methylone to block reuptake of DA vs. 5-HT (Rosenauer et al., 2013). A second possibility is that mephedrone may act directly on 5-HT receptors (López-Arnau et al., 2012; Simmler et al., 2013). Regardless of mechanism, Chapter II results are consistent with the conclusion that mephedrone has lower abuse liability than MCAT or the other recently scheduled bath salts. One implication of this finding is that mephedrone abuse might be expected to decline over time not only because of recently enacted legal constraints but also because of its pharmacological profile. Interestingly, data from the DEA National Forensic Laboratory Information System (NFLIS) support this conclusion and indicate that, as a percentage of all synthetic cathinones reported to NFLIS from 2010 to 2012, reports of mephedrone experienced a steady decline over the years, falling from approximately 40% in 2010 to <5% in 2012 (see Figure V.2).
Figure V.2

Synthetic cathinone prevalence data supplied by the National Forensic Laboratory Information System (NFLIS) of the DEA Surveillance Network. Following emergency scheduling of mephedrone, methylone and MDPV in October 2011, mephedrone reports, as a percentage of all synthetic cathinones, dropped off more quickly than reports of MDPV.

MDPV, the primary constituent of “bath salts” in the United States prior to its emergency scheduling in October 2011 (Kyle et al., 2011; Spiller et al., 2011), is distinct among the synthetic cathinones because it functions as a monoamine uptake inhibitor rather than as a releaser (Baumann et al., 2013; Cameron et al., 2013b). Nonetheless, it displays high in vitro selectivity to block DA vs. 5-HT uptake (Baumann et al., 2013) and, like cocaine and other more DA-selective uptake
inhibitors, such as RTI-113 (Esposito et al., 1978; Rosenberg et al., 2013), MDPV produced robust facilitation of ICSS with an efficacy similar to that of MCAT. This agrees with an earlier report that MDPV facilitated ICSS in rats responding under a discrete-trial current-threshold ICSS procedure (Watterson et al., 2012b). Moreover, this evidence from our ICSS studies agrees with other evidence of psychostimulant and abuse-related effects of MDPV. For example, MDPV maintained intravenous self-administration in rats (Watterson et al., 2012b) and produced both stimulant-like discriminative stimulus effects and locomotor-activating effects in mice (Marusich et al., 2012; Fantegrossi et al., 2013). Taken together, these data converge in suggesting that MDPV has high, stimulant-like abuse liability.

A distinguishing feature of the behavioral effects of MDPV in this study was its long duration of action. MDPV maintained significant facilitation of ICSS beyond 300 min (see Figure II.2), with facilitation still apparent as long as 24 h after drug administration. While the present study provides the first in vivo data to suggest a long duration of action for MDPV, previous in vitro studies also support an extended time course profile for MDPV. For example, electrophysiological data show that MDPV blocked DAT longer and was more resistant to washout than cocaine (Cameron et al., 2013b). Consequently, the long duration of ICSS effects observed with MDPV may be explained by its high potency at and slow dissociation from DAT.

One major goal of the study described in Chapter II was to stratify the relative efficacies of MCAT, MDPV, methylone and mephedrone to facilitate ICSS. This was accomplished using two approaches that have been described and validated previously with amphetamine and a series of 10 other monoamine
releasers: calculation of % baseline total stimulations and rate-dependency analysis (Bauer et al., 2013a, 2013b). In those previous studies, both approaches yielded metrics of efficacy that correlated with both (a) in vitro selectivity of compounds to release DA vs. 5-HT and (b) in vivo efficacy to maintain self-administration in nonhuman primates responding under a progressive-ratio procedure. Moreover, in the present study, both approaches yielded similar rank-ordering of efficacies to facilitate ICSS. These approaches differ from more conventional approaches to ICSS data analysis, which often focus on calculating “threshold” intensities or frequencies of brain stimulation to maintain ICSS (Miliaressis et al. 1986; Carlezon and Chartoff 2007). As discussed previously (Bauer et al., 2013b), threshold measures have proven useful for dissociating hedonic from motor effects of experimental manipulations on brain reward substrates. However, the abuse liability of drugs likely reflects an integration of hedonic and motor effects, and the approaches used here provide analytical strategies for quantifying that integration.

Despite the general agreement in results from ICSS and drug self-administration approaches to abuse liability assessment, results with mephedrone suggest a potential disconnect. Mephedrone produces a mixed profile of ICSS facilitation and depression in both rats and mice (present study; Robinson et al., 2012) that is generally associated with significant but relatively weak self-administration (Bauer et al., 2013b). However, recent studies in rats have reported robust mephedrone self-administration (Hadlock et al., 2011; Aarde et al., 2013a; Motbey et al., 2013). Determinants of this apparent discrepancy and implications for human abuse liability will require further research.
In addition to being one of the first studies to compare preclinical, behavioral effects of MCAT and the three primary bath salts constituents mephedrone, methylone and MDPV using ICSS, the results of Chapter II were critical to this dissertation by setting the stage to conduct QSAR analysis of para-substituted MCAT analogs. By elucidating major differences in the behavioral pharmacological profiles of MCAT and mephedrone (4-\text{CH}_3 \text{MCAT}), which differ structurally only by the substitution of –H with –\text{CH}_3 at the para position on the benzene ring, these preliminary studies suggested the importance of the para substituent of the MCAT scaffold to the pharmacology of synthetic cathinones and drove the remainder of the studies described in Chapters III and IV.

**Implications of Chapter III: *In vitro* Selectivity of and ICSS Facilitation by para-substituted MCAT analogs**

Results from Chapter III confirm and extend previous research showing that MCAT and its para-substituted analogs act as substrates at DAT and SERT but differ in their DAT-vs.-SERT selectivity. Thus, results with MCAT in this study were similar to results reported previously using the same *in vitro* procedures with rat brain homogenates (DAT EC\textsubscript{50} = 20±3 nM; SERT EC\textsubscript{50}=4±1 \mu M; DAT-vs.-SERT selectivity = 400; Cozzi et al., 2013). Results here are also consistent with a previous study using HEK cells expressing human transporters, which found higher DAT-vs.-SERT selectivity for MCAT than for 4-F MCAT (Eshleman *et al.*, 2013). 4-\text{CH}_3 \text{MCAT} was slightly more DAT-vs.-SERT selective than 4-F MCAT in the study with human transporters expressed in HEK cells (Eshleman *et al.*, 2013), which contrasts with
results in rat brain homogenates (Baumann et al., 2012; present study) and suggests some potential for species differences in 4-CH$_3$ MCAT effects at human vs. rat transporters. Finally, the SERT-selectivity of 4-CF$_3$ MCAT agrees with the SERT-vs.-DAT selectivity of fenfluramine (Rothman et al., 2001), which has a 3-trifluoromethyl substituent on the phenyl ring of a phenethylamine scaffold closely related to the MCAT scaffold used here.

The study described in Chapter III also extends these previous results by including 4-OCH$_3$ MCAT and two halogenated MCAT analogs, 4-Cl MCAT and 4-Br MCAT. Like 4-CH$_3$ MCAT, all three compounds had similar potencies for promoting monoamine release through DAT and SERT, and all three compounds had lower DAT-vs.-SERT selectivity than 4-F MCAT. Consistent with the present results with halogenated MCAT analogs, previous studies with halogenated amphetamine analogs also showed that para-fluoroamphetamine had greater DAT-vs.-SERT selectivity and more robust amphetamine-like behavioral effects than para-choroamphetamine (Marona-Lewicka et al., 1995; Baumann et al., 2011).

ICSS results described in Chapter III confirm and extend previous research showing that MCAT and many of its analogs modulate ICSS but differ in the degree to which they facilitate low rates of ICSS and/or depress high rates of ICSS. We showed previously that MCAT, which has high DAT-vs.-SERT selectivity, produced exclusive facilitation of ICSS across a broad range of doses (Bonano et al., 2014b; see Chapter II), and the present study found that 4-F MCAT, which also has moderate DAT-vs.-SERT selectivity, produced a similar profile of effects. MCAT and 4-F MCAT also produced similar locomotor activating effects in mice (Marusič et al., 2012).
and fully substituted for the discriminative stimulus effects of stimulant drugs of abuse, such as cocaine and methamphetamine, in rats (Gatch et al., 2013). Conversely, 4-CF₃ MCAT, a SERT-vs-DAT selective compound, produced exclusive depression of ICSS, a profile of effects which parallels the effects previously reported for fenfluramine, another 5-HT-selective releaser (Bauer et al., 2013b). Lastly, 4-CH₃ MCAT, which is a relatively nonselective monoamine releaser, has been reported to produce mixed effects on ICSS consisting of both facilitation of low ICSS rates and depression of high ICSS rates (Robinson et al., 2012; Bonano et al., 2014b). In the present study, similar profiles of mixed ICSS effects were also produced by the other MCAT analogs 4-OCH₃ MCAT, 4-Cl MCAT and 4-Br MCAT, which also have low DAT-vs-SERT selectivity. These effects on ICSS are consistent with the relatively weak magnitude of locomotor stimulation reported in rodents for 4-OCH₃ MCAT (Marusich et al., 2012) and 4-Br MCAT (Foley and Cozzi, 2003).

Correlational analysis confirmed a significant positive correlation between in vitro DAT-vs-SERT selectivity and ICSS facilitation (R=0.92, P=0.003). Insofar as magnitude of ICSS facilitation is predictive of abuse potential (Negus and Miller, 2014), these results suggest that DAT-vs-SERT selectivity is a key determinant of abuse potential for a wide range of monoamine releasers. Furthermore, to explore molecular mechanisms that contribute to monoamine releaser abuse potential, this study employed QSAR analysis to evaluate structural determinants of DAT-vs-SERT selectivity and abuse-related ICSS effects. Of the physicochemical parameters evaluated (steric bulk, Eₛ; electron-withdrawing capacity, σₛ; lipophilicity, πₛ), only Eₛ correlated with both DAT-vs-SERT selectivity and ICSS facilitation, suggesting
that steric attributes of the \textit{para} substituent play a key role in determining selectivity of releasers for DAT vs. SERT. Large \(E_s\) values indicate low functional steric bulk of the substituent, and increasingly negative \(E_s\) values indicate progressively greater magnitudes of steric hindrance. Thus, these results suggest that small \textit{para} substituents that produce little steric hindrance promote selectivity for DAT, whereas larger substituents that produce greater steric hindrance promote selectivity for SERT (see Figure V.3).
Effects of steric volume on monoamine transporter selectivity, DA vs. 5-HT release, and ICSS. Small para substituents with low volume promote selectivity for DAT, produce greater increases in DA release, and facilitate ICSS. Para substituents of intermediate volume are non-selective for DAT vs. SERT, produce increases in release of both DA and 5-HT, and yield mixed ICSS effects. Large para substituents with high volume promote selectivity for SERT, produce greater increases in 5-HT release, and depress ICSS.
In contrast to the significant correlations obtained for $E_s$ values, neither lipophilic ($\pi_p$) nor electronic ($\sigma_p$) parameters of the para substituent correlated with \textit{in vitro} or \textit{in vivo} drug effects. The nonsignificant correlations of these values with DAT-vs.-SERT selectivity or with ICSS facilitation suggest that, within the ranges studied here, these parameters are less important than steric hindrance as determinants of abuse-related neurochemical and behavioral effects of MCAT analogs. However, more extreme lipophilic and/or electronic values beyond these ranges might influence neurochemical and behavioral effects, and lipophilic and electronic parameters might also influence other aspects of pharmacology, such as pharmacokinetics.

As a follow-up to preliminary QSAR analyses, which indicated an important role for steric bulk ($E_s$), additional aspects of steric hindrance underlying DAT-vs.-SERT selectivity and ICSS behavioral effects were considered in a companion manuscript (Sakloth et al., 2014). In these supplemental studies, steric bulk was broken down further to determine which specific aspects of bulk were playing a role in DAT-vs.-SERT selectivity. Steric volume, length, maximum width and minimum width of the para substituents of MCAT analogs were correlated with \textit{in vitro} DAT-vs.-SERT selectivity and \textit{in vivo} modulation of ICSS. These additional studies not only identified volume as the primary component of steric bulk influencing monoamine transporter binding ($R=-0.97, P<0.01$) and facilitation of ICSS ($R=-0.92, P<0.01$), but also contributed to the generation of molecular models, which help explore structural interactions that may account for interactions between MCAT analogs and both DAT and SERT. Homology models of DAT and SERT were generated using the
3.0 Å X-ray crystal structure of *Drosophila melanogaster* DAT (dDAT) as a template. Using sequence alignment software (ClustalX 2.1) along with modeling software (MODELLER 9.10), the amino acid sequences of hDAT and hSERT (shown below in Figure V.4) were aligned with dDAT, and a population of 100 models was generated for both hDAT and hSERT. The molecular structures of compounds to be docked (i.e. *para*-substituted MCAT analogs) were sketched in SYBYL-X 2.1 and energy-minimized using the Tripos Force Field prior to docking (using GOLDSuite 5.2) of the S-isomer of each substrate into each of the 100 hDAT and hSERT models. Docking stimulations were then run without constraints, and common and energetically favorable transporter-substrate complexes were identified for all substrates at both hDAT and hSERT. The molecular models generated using this procedure are depicted in Figure V.5. A major conclusion of this analysis was that different residues located at homologous sites in transmembrane domain III of hDAT and hSERT (S149 in hDAT, A169 in hSERT) may contribute to the different preferences of these transporters for small (hDAT) vs. bulky (hSERT) substituents at the *para* position of the MCAT scaffold.
Amino acid alignment for complete sequences of hDAT, hNET and hSERT. Amino acid residues of interest (hDAT S149 and hSERT A169) are noted in both (a) the full amino acid sequences and (b) the schematic of transmembrane domain III where these amino acid residues are located within the 12 transmembrane monoamine transporter proteins. Image adapted from Torres et al., 2003.
Superimposed positions of MCAT analogs in the putative binding site of hDAT (A), and hSERT (B). Unfavorable interactions of hDAT S149 with para substituents (shown for 4-OCH$_3$ MCAT; C) are not seen in hSERT due to the smaller side chain of A169 (again, shown for 4-OCH$_3$ MCAT; D). The Connolly surface (grey density in C and D) represents the channels in both transporters. Image from Sakloth et al., 2014.
Implications of Chapter IV: *In vivo* Selectivity of *para*-substituted MCAT analogs

The study described in Chapter IV investigated abuse-related *in vivo* neurochemical effects produced by MCAT and five *para*-substituted analogues. There were three main findings. First, across a 1.0 log range of doses, all MCAT analogs increased extracellular levels of DA and/or 5-HT in rat NAc, albeit with different potencies. Second, *in vivo* selectivity to elevate DA vs. 5-HT levels to 250% of baseline (ED$_{250}$) varied across compounds, with MCAT as the most DA-selective and 4-OCH$_3$ MCAT as the most 5-HT-selective. Third, *in vivo* DA vs. 5-HT selectivity correlated with *in vitro* selectivity for DAT vs. SERT in a rat-brain synaptosome preparation (R=0.95, P<0.01), and with *in vivo* facilitation of intracranial self-stimulation in rats (R=0.89, P=0.02). Together, these results suggest that *in vivo* selectivity of *para*-substituted MCAT analogs to elevate extracellular DA vs. 5-HT levels in NAc may be a key neurochemical determinant of abuse-related behavioral effects.

The results from Chapter IV confirm and extend previous results showing differences in neurochemical selectivity of various monoamine releasers, including the DA-selective releasers amphetamine and MCAT and the 5-HT-selective releaser fenfluramine. Similar to results with amphetamine in this study, early microdialysis studies in male Sprague-Dawley rats reported amphetamine-induced increases in accumbal DA release by a factor of 10 (~1000% increase) at a dose of 1 mg/kg, s.c. (Di Chiara and Imperato, 1988). Results presented here are also consistent with a previous study investigating effects of MCAT on striatal extracellular DA
concentrations in male Sprague-Dawley rats, in which administration of 1 mg/kg, s.c. increased DA levels to 419% of control values (Gygi et al., 1997). Furthermore, the present results agree with a prior study that investigated concurrent changes in DA and 5-HT levels in the NAc of male Sprague-Dawley rats and showed that administration of 1 mg/kg, i.p. fenfluramine produced significant increases in 5-HT levels to ~300% of baseline without producing any significant changes in extracellular DA concentrations (Baumann et al., 2000).

Results from the present study also agree with previous research showing mixed neurochemical effects (i.e. non-selective increases in extracellular levels of both DA and 5-HT) with compounds like 4-CH$_3$ MCAT. One previous study, which investigated neurochemical effects of 10 mg/kg, s.c. 4-CH$_3$ MCAT in the NAc shell of male Wistar rats, reported increases in DA and 5-HT levels to approximately 22-fold and 9-fold of control levels, respectively (Wright et al., 2012). Similarly, another study showed that administration of 1 mg/kg, i.v. 4-CH$_3$ MCAT in male Sprague-Dawley rats produced peak increases in accumbal DA levels to 2.9-fold above baseline and in 5-HT levels to 11.1-fold above baseline (Baumann et al., 2012). Microdialysis studies have also been conducted on the de-carbonylated, amphetamine analog of 4-F MCAT (i.e. 4-fluoroamphetamine, PAL-303; Baumann et al., 2011). Results from this study show that i.v. injection of 3 mg/kg 4-fluoroamphetamine produces similar increases in NAc DA and 5-HT levels, to ~1200% and 1400% of baseline, respectively. These data suggest that 4-fluoroamphetamine non-selectively increases DA and 5-HT concentrations in NAc, similar to our present results for the N-methyl, beta-ketone analog 4-F MCAT.
The present study extends these previous results by including 4-OCH₃ MCAT and three halogenated MCAT analogs, 4-F MCAT, 4-Cl MCAT and 4-Br MCAT. Like 4-CH₃ MCAT, these para-substituted MCAT analogs function as non-selective monoamine releasers. 4-F MCAT and 4-Cl MCAT had similar potencies for increasing DA and 5-HT levels to 250% of baseline, in addition to having similar DA/5-HT selectivities. Consistent with these results, previous studies with halogenated amphetamine analogs showed that para-fluoroamphetamine had greater DAT selectivity and more robust, amphetamine-like behavioral effects than para-chloroamphetamine (Marona-Lewicka et al., 1995; Baumann et al., 2011). 4-Br MCAT, the other halogenated MCAT analog, exhibited similar potency to increase 5-HT, but was less potent at increasing NAc DA levels and thus had reduced DA/5-HT selectivity when compared to its 4-F and 4-Cl counterparts. Of all tested compounds, 4-OCH₃ MCAT was the least potent analog to increase accumbal DA and 5-HT levels, and it had the lowest selectivity for DA/5-HT.

As described in Chapter III, QSAR analysis was used to explore molecular mechanisms contributing to monoamine releaser abuse potential by evaluating structural determinants of selectivity for in vitro monoamine release via DAT-vs.-SERT, and of abuse-related ICSS effects (Bonano et al., 2014a; Sakloth et al., 2014). Results of QSAR analysis suggested that steric characteristics of the para substituent play a critical role in determining in vitro selectivity for DAT-vs.-SERT, and that in vitro DAT-vs.-SERT selectivity is a key determinant of abuse-related ICSS effects. Results presented in Chapter IV extend on these findings by also demonstrating a
significant correlation between in vivo DA/5-HT selectivity and ICSS facilitation (R=0.89, P=0.02).

Although the correlation between in vivo DA/5-HT selectivity and ICSS facilitation provides support for the conclusion that DA is a neurochemical driver of abuse potential and that 5-HT is an abuse-limiting neurotransmitter, it does not entirely explain the mechanism by which para-substituted MCAT analogs are producing abuse-related effects. While our results, taken together, suggest that selectivity to increase DA vs. 5-HT levels is one of the most important determinants of a compound’s abuse potential, rather than a compound’s capacity to increase concentrations of either neurotransmitter individually, the results do not provide direct answers to questions like “how much DA release is “enough” to produce abuse?” or “how much 5-HT release is sufficient to hinder DA’s abuse-related effects and prevent a compound from having abuse potential?”

Altogether, the results from the microdialysis studies presented in Chapter IV provide evidence for a key role of relative DA/5-HT selectivity in determining the abuse potential of para-substituted MCAT analogs. Compounds with higher selectivity to increase accumbal DA maintain higher rates of abuse-related ICSS, while compounds with higher selectivity to increase 5-HT exhibit a reduction in their capacity to facilitate low rates of ICSS as well as a concomitant depression of high ICSS rates.
Drawing Conclusions

Structural features of drug molecules are critical to determining pharmacological activity. In this dissertation, we explored QSARs for para-substituted MCAT analogs, but other aspects of the MCAT scaffold are certainly important and also play a role in the neurochemical and behavioral effects produced by synthetic cathinones. While we focused on differential pharmacological effects based on structural variations of the para substituent on the phenyl ring of the MCAT scaffold (based on the notable differences in preliminary behavioral studies of MCAT and mephedrone), other substituents on the MCAT backbone can also be manipulated and would almost certainly influence pharmacological profiles.

An example of structural manipulations at sites other than the para position on the benzene ring (see Figure V.6 below) that yield differential effects on synthetic cathinone pharmacology is described in a recently published manuscript by Baumann et al. (2015), in which two analogs of mephedrone (4-methyl-N-methylcathinone) with extensions to the N-alkyl chain were investigated. These compounds, 4-methyl-N-ethylcathinone (4-MEC) and 4-methyl-N-pyrrolidinopropiophenone (4-MePPP), were studied to determine effects of each drug at monoamine transporters and to compare these effects with those produced by the parent compound mephedrone. In contrast to mephedrone, which functions as a monoamine transporter substrate (i.e. releaser) at both DAT and SERT, 4-MEC displayed unique activity by acting as an uptake blocker at DAT but as a substrate at SERT. This finding suggests that subtle extension of the N-alkyl chain of cathinones, from methyl to ethyl, is sufficient to convert activity at DAT from release
(mephedrone) to uptake inhibition (4-MEC). However, this minor change in $N$-alkyl chain length was not sufficient to alter release activity at SERT, as mephedrone and 4-MEC displayed similar potency (EC$_{50}$ ~100nM) to evoke release at SERT. Interestingly, 4-MePPP did not function as a substrate at either DAT or SERT but rather as a selective DAT blocker with little activity at SERT. Thus, further extension of the alkyl chain to form a pyrrolidine ring converted the compound from a non-selective monoamine releaser/uptake inhibitor to a DAT-selective blocker. The molecular pharmacology of 4-MePPP closely resembles that of other pyrrolidinophenone compounds like pyrovalerone and MDPV, which function as potent blockers at DAT and NET with little action at SERT (Meltzer et al., 2006; Baumann et al., 2013; Cameron et al., 2013b; Kolanos et al., 2013; Marusich et al., 2014).
Figure V.6

$R_1$ indicates the site of the para substituent, which was manipulated in these dissertation studies. $R_2$ indicates the site of the alkyl amine chain. At the para position on the benzene ring ($R_1$), increased steric volume reduces potency for DAT-mediated release, increases potency for SERT-mediated release, reduces DAT-vs.-SERT selectivity, and reduces abuse-related behavioral effects. For the alkyl amine ($R_2$), the addition of substituents can convert compounds from monoamine transporter substrates to inhibitors, and as the length of the chain increases, potency for DAT uptake inhibition increases, potency for SERT uptake inhibition decreases, and DAT-vs.-SERT selectivity increases.

Limitations

Although the main conclusion from this dissertation is that para-substituted MCAT analogs yield abuse-related behavioral effects by producing effects on monoaminergic systems, specifically by increasing selectivity for DAT-vs.-SERT in the mesolimbic pathway, drug effects on other systems may also exert an important influence on behavioral outcomes following drug administration. Three ways in
which this series of MCAT analogs may influence abuse-related behavior other than by exerting effects on DAT and SERT, which manifest as increases in extracellular levels of NAc DA and 5-HT, include: 1) acting on brain regions other than the mesolimbic DA pathway; 2) acting at monoamine transporters other than DAT and SERT; and 3) binding and directly activating other pre- and/or post-synaptic receptors. First, monoaminergic neurotransmitter systems project diffusely throughout the brain, and involvement of brain regions other than VTA-NAc may play an important role in producing abuse-related behaviors. For example, studies have found roles for non-dopaminergic mechanisms of the supramamillar, rostromedial tegmental, and midbrain raphe nuclei in reward, including GABAergic and glutamatergic mechanisms (Ikemoto, 2010; Ikemoto 2005; Miliaressis et al. 1975). Second, drug effects on abuse-related behavior may also be influenced by drug activity at other monoamine transporters (i.e. NET). Various studies have demonstrated that MCAT and its analogs function as monoamine releasers that promote release of NE as well as DA (Kalix and Glennon, 1986; Wagner et al. 1982; Nielsen and Schechter 1985; Cozzi et al. 1999, 2013), suggesting a role for noradrenergic neurotransmission in the rewarding effects produced by these compounds. Lastly, drug effects on other receptor targets may influence behavioral endpoints by directly activating receptors located both pre- and post-synaptically. For example, one recent study investigating monoamine transporter and receptor interaction profiles of a series of amphetamine and cathinone derivatives demonstrated affinity of some compounds for the 5-HT$_2A$ receptor (Rickli et al. 2015). Other studies have shown that 5-HT acts on 5-HT$_2A$ receptors located in
midbrain to increase firing of DA neurons in the VTA (Pessia et al. 1994; Prisco et al. 1994); thus, direct activation of 5-HT$_{2A}$ receptors by test drugs may play a role in producing abuse-related behaviors.

**Future Directions**

QSAR analysis is a useful tool, not only for predicting the pharmacological effects of novel compounds, but also for generating molecular models to better understand the mechanisms underlying drug effects. Future QSAR studies exploring other molecular aspects of the MCAT scaffold, such as the impact of terminal $N$-substitutions or ortho- and meta- substitutions on the benzene ring, would be helpful in understanding precisely how the structure of cathinone analogs influences molecular function at monoamine transporters, thereby driving or limiting abuse potential. Additionally, as the library of structural MCAT analogs continues to grow and the understanding of neuropharmacological effects improves, more accurate models of drug interaction with human DAT and SERT proteins will be developed.
Chapter VI: References


Matecka, D., Rothman, R.B., Radesca, L., Costa, B.R. de, Dersch, C.M., Partilla, J.S., et al. (1996). Development of novel, potent, and selective dopamine reuptake inhibitors through alteration of the piperazine ring of 1-[2-(diphenylmethoxy)ethyl]-and 1-[2-


