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Behavioral Screening and Chiral Bioanalysis of Emerging Stimulant-Type Drugs in Rats

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By

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ABBREVIATIONS

5-HT	serotonin (5-hydroxytryptamine)
ANOVA	analysis of variance (statistical test)
α-PHP	alpha-pyrrolidinohexanophenone
CDC	U.S. Center for Disease Control and Prevention
DA	dopamine
DAT	dopamine transporter
DEA	U.S. Drug Enforcement Administration
FDA	U.S. Food and Drug Administration
FR	fixed ratio
GC-MS	gas chromatography-mass spectrometry (or spectrometer)
ICSS	intracranial self-stimulation
i.p.	intraperitoneal
IS	internal standard(s)
LC-MS/MS	liquid chromatography tandem mass spectrometry (or spectrometer)
MCAT	methcathinone (no enantiomeric specificity)
MCR	maximum control rate
MFB	medial forebrain bundle

NAc	nucleus accumbens
NE	norepinephrine
NET	norephinephrine transporter
NPS	novel (or new) psychoactive substance
RMCAT	R(+)methcathinone
SEM	standard error of the mean
SERT	serotonin transporter
SMCAT	S(-)methcathinone
UNODC	United Nations Office on Drugs and Crime
VMAT	vesicular monoamine transporter
VTA	ventral tegmental area

ABSTRACT

The epidemic of drug use in the United States and elsewhere in the world has resulted in tragic loss of life and substantial economic costs. Novel psychoactive substances (NPS) are one of the contributors to this problem, and the lack of information about many of these drugs compounds their risk. This dissertation proposes a strategy to use intracranial self-stimulation (ICSS) as a behavioral screening tool to assess emerging drugs of abuse for their abuse potential in order to generate a proactive threat assessment. A series of stimulant-type drugs including methcathinone, α-pyrrolidinohexanophenone (α-PHP), cocaine, and the phenyltropane analogs of cocaine WIN-35428 and RTI-31 were evaluated using ICSS, and a bioanalytical method was developed to detect methcathinone and its metabolites in blood. ICSS predicted high abuse potential for methcathinone, novel methcathinone analogs, α-PHP, cocaine, and phenyltropane analogs of cocaine. The ICSS procedure was modified to account for pharmacokinetic differences resulting in different rates of drug onset and had sufficient resolution to distinguish between the rate of onset of cocaine, WIN-35428, and RTI-31. A preliminary chiral bioanalytical method was developed to quantify methcathinone and cathinone enantiomers in blood, and to qualitatively detect ephedrine, pseudoephedrine, norpseudoephedrine, phenylpropanolamine, and 4-hydroxyephedrine. Methcathinone and cathinone were detected in the blood, with a time course that correlated with the behavioral effects observed in ICSS testing and formed a counterclockwise hysteresis loop. ICSS appears to be an effective tool for an in vivo bioassay to screen emerging drugs of abuse for abuse potential, and appropriate bioanalytical methods should be developed to monitor the effectiveness of interventions.

CHAPTER 1: INTRODUCTION

The Problem with Novel Psychoactive Substances

The United States is currently experiencing an epidemic of drug use and overdose deaths. During the 12-month period through December 2021, over 100,000 overdose deaths were reported by the U.S. Center for Disease Control and Prevention (CDC) (Ahmad et al., 2022), and overdose deaths were the leading cause of accidental death for ages 18-65, surpassing common causes of death related to automobile incidents (Centers for Disease Control and Prevention, National Center for Health Statistics, 2021). In addition to the devastating cost of human lives in the US, the drug epidemic has taken a heavy economic toll. Opioid use alone was estimated to cost over $1.0 trillion in 2017 (Luo et al., 2021). In order to mitigate the harms posed by this sustained surge of drug use, mechanisms need to be in place to proactively assess the risks of new drugs and to continuously monitor for outbreaks.

Novel (or new) psychoactive substances (NPS), sometimes called designer drugs, are some of the contributors to this drug epidemic. These drugs are emergent psychoactive compounds that are taken for recreational or non-approved-medical use but are not the traditional archetype of their pharmacological class. The United Nations Office on Drugs and Crime (UNODC) defines NPS more specifically as drugs that are "not controlled by the 1961 Single Convention on Narcotic Drugs or the 1971 Convention on Psychotropic Substances, but which may pose a public health threat" (United Nations Office on Drugs and Crime, 2013). Due to their unscheduled status, they are thus considered "legal highs." The descriptor "novel" is commonly a misnomer. NPS act on the same biological targets as traditional drugs of abuse. They are often old research compounds

that were synthesized as candidate therapeutic agents and discontinued due to their apparent abuse potential or lack of novelty.

Unlike traditional drugs of abuse such as heroin or methamphetamine, most NPS have not been subjected to rigorous scientific study. The unknown pharmacology of these compounds introduces additional risk to their consumers. Regulatory and public health entities have a need for pharmacological data in order to thoroughly understand the risk posed by emerging NPS; meanwhile, forensic and clinical laboratories are challenged by a lack of validated analytical methods and unavailable certified reference materials, and thus the ability to detect the presence of NPS lags far behind the emergence of these drugs (Morrow et al., 2019). Users of NPS are often drawn to the ambiguous legal status of these drugs as well as their ability to avoid detection by the most common screening mechanisms (for instance, in workplace drug testing) (Baumann, 2016; Huestis et al., 2017). Some NPS are marketed as having therapeutic benefits (e.g. nootropic, anxiolytic, or anti-fatigue) or sold as innocuous-sounding supplements.

Regulatory agencies require scientific data to support the scheduling decisions for the control of pharmacological agents. In the United States, the 8-factor analysis (8-FA) requires inclusion of data addressing "(1) its actual or relative potential for abuse (2) scientific evidence of its pharmacological effect, if known (3) the state of current scientific knowledge regarding the drug or other substance (4) its history and current pattern of abuse (5) the scope, duration, and significance of abuse (6) what, if any, risk there is to the public health (7) its psychic or physiological dependence liability (8) whether the substance is an immediate precursor of a substance already controlled" (Controlled Substances Act, 2015). In addition to the 8-factor analysis, the Controlled Substances Act authorizes the Attorney General to issue temporary emergency scheduling orders dependent upon the substance's current scope of abuse and the risk

to public health. Temporary scheduling is intended to accelerate the process of controlling dangerous emerging drugs compared to the full 8-FA, but there can still be considerable delays. For example, the drug α-pyrrolidinohexiophenone (α-PHP) was detected in the U.S. as early as 2015 (U.S. Drug Enforcement Administration, Diversion Control Division, 2016), but a temporary scheduling order was not issued until 2019 (United States Drug Enforcement Administration, 2019). In the European Union, there have been several decisions by the European Council to act on the emergence of NPS using a three-step process of information gathering, risk assessment, and regulatory control. Joint Action no. 97/396/JHA focused largely on information sharing and creation of an early warning system, and further decisions in 2005 and 2017 further refined the process for generating risk assessment reports and shortened time frames from 26 weeks to 13 weeks (Neicun et al., 2019; Varì et al., 2020). Unfortunately, with NPS the gathering of scientific knowledge and formation of the risk assessment usually occurs *after* wide-spread abuse is detected. By this time, agencies have a problem to address that is potentially already large in scale.

The goal of this work was to present a strategy to rapidly and efficiently screen NPS using a behavioral procedure called intracranial self-stimulation (ICSS) as a prognostic tool for risk assessment, and bioanalysis for diagnostic monitoring. Chapter 2 explores the use of an in vivo behavioral screening procedure to evaluate the degree to which an emerging cathinone-type NPS can produce abuse-related ICSS facilitation in rats. Chapter 3 expands upon this ICSS technique by introducing the consideration of the rate of drug onset to further refine the assessment of abuse potential. Chapter 4 demonstrates the sensitivity of ICSS to distinguish between the effects of closely-related compounds, including stereoisomers. Chapter 5 introduces bioanalysis including assessment of the blood time course of the drug and detection of metabolites as a final component of the NPS evaluation.

Preclinical Abuse Potential Testing

Abuse potential is the ability of a drug to elicit a pharmacological response that is predictive of a pattern of repeated non-therapeutic use, and the magnitude of this property is a large factor in a risk assessment. Self-administration and drug discrimination are two operant procedures that are the most widely adopted methods for preclinical assessment of abuse potential (Gauvin, Zimmermann, & Baird, 2018; Gauvin, Zimmermann, Code, et al., 2018; U.S. Food and Drug Administration, 2017). In self-administration procedures, animals are taught to engage with a manipulandum to self-administer a dose of drug. If the test drug functions as a reinforcer, it will increase the rate of the behavioral response (e.g. a rat depressing a lever). Drugs that are self-administered at a rate higher than that of vehicle are considered to have abuse potential. Self-administration procedures have strong face-validity in that the self-administration of a drug is inherently analogous to the self-administration behavior the procedure is designed to predict in humans. Despite this, there are several considerations and limitations that can make self-administration procedures undesirable for an initial evaluation of a new drug. Self-administration procedures are not easily amenable to routes of administration other than intravenous, and their experimental design necessitates a priori knowledge of the time course of the drug effects, making it a complicated procedure to apply to new drugs that are poorly understood. In drug discrimination procedures, the experimental manipulation is to the discriminant stimulus. Animals are presented with a choice of response, usually two levers. The animals are trained to respond upon one lever to receive a non-drug reward when vehicle is administered as a discriminative stimulus, and the animals are trained to respond upon the other lever for reward when the training drug is administered. Once the animals reliably and accurately respond for vehicle or their training drug, the training drug can then be swapped for a dose of the test drug. When presented with the

test drug as the discriminant stimulus, the animal's choice of the drug-associated lever indicates an interoceptive similarity between the test drug and the training drug. A test drug substituting for an abused training drug is suggestive of abuse potential as well as that the two drugs exert their effects through the same mechanism. One limitation of this procedure is that it cannot identify the specific interoceptive stimulus that the subject is responding to; the stimulus could be abuse-related, but the stimulus could also be benign.

Intracranial self-stimulation (ICSS) procedures are another tool to evaluate the abuse potential of drugs, and these are distinct from self-administration and discrimination assays. Rather than the drug serving as a discriminative or consequent stimulus, in ICSS the drug acts as a contextual stimulus to alter the rate of responding for electrical brain stimulation. In these procedures, electrodes are implanted into brain reward regions of test subjects, usually rats, as is the case throughout this dissertation, but prior studies have examined the effects of ICSS on both humans (Heath, 1963) and non-human primates (Rolls et al., 1980). These electrodes commonly target either the medial forebrain bundle (MFB) or ventral tegmental area (VTA) (Negus & Miller, 2014). Electrical stimulation of these regions indirectly activates dopamine neurons projecting from the VTA to the nucleus accumbens (NAc) that are implicated in the reinforcing effects of drugs and natural rewards (Di Chiara & Imperato, 1988; Wise, 1978, 1998; Wise & Rompre, 1989). In ICSS procedures, test chambers are equipped with a manipulandum such as a lever or wheel that can be activated to deliver an electrical signal from a stimulator box to the implanted electrode. At sufficient amplitudes and frequencies of electrical stimulation, this serves as a powerful and immediate reinforcer and robustly increases rates of operant responding. Once an animal has achieved a stable baseline response for ICSS, a test drug can be administered noncontingently, and changes in responding can be monitored. Both ICSS and abused drugs are

hypothesized to exert their reinforcing effects by the release of dopamine from the terminals of mesolimbic neurons, therefore non-contingent administration of a rewarding drug should have an additive effect on the responding maintained by electrical brain stimulation. This facilitation of responding is indicative of abuse potential (Kornetsky, 1979; Negus & Miller, 2014).

Several variables are critical for the reinforcing effects of ICSS. One of these parameters is the anatomical position of the electrode in the brain. For studying brain reward, the MFB and VTA are common targets, but the mechanism of neuronal activation by electrical brain stimulation is relatively non-specific. Electrodes whose tips terminate in other brain regions can trigger different effects, such as motor effects by stimulating motor neurons. Electrode placement is critical, but small deviations from the intended target site can sometimes be compensated for by increasing the amplitude of electrical brain stimulation. The amplitude of the electrical pulse determines the distance from the electrode tip that the electric pulse will travel. Increasing amplitudes will increase the radius of the electrical pulse, and therefore increase the recruitment of neurons. Another critical electrical parameter is the frequency of the pulse. The higher the frequency of electrical pulses, the higher the frequency of neuronal firing. Increases in both the amplitude and the frequency of electrical stimulation can have the downstream effect of increased dopamine release into the NAc—amplitude by the increase in the number of neurons releasing DA, and frequency by increasing the rate of input of a set of neurons, causing them to release more dopamine (temporal summation).

Several designs of ICSS procedures exist that can be used for assessment of abuse potential. Simple free operant-response procedures will evaluate the increase or decrease in rate of responding at a single amplitude and frequency of electrical stimulation by a test compound. A number of procedural variants for ICSS have emerged since the introduction of the free operant

ICSS technique, including discrete trial current intensity threshold procedures (Kornetsky, 1979) and hybrid frequency-rate procedures.

ICSS is sensitive to most major classes of abused drugs including amphetamine-like DAT substrates (Bauer et al., 2013; Bonano et al., 2014; Carey et al., 1974; Elmer et al., 2010; Olds, 1995; Robinson et al., 2012), cocaine-like DAT inhibitors (J. S. Bonano et al., 2014; Lazenka & Negus, 2017), fentanyl-like mu opioid receptor (MOR) agonists (Adams et al., 1972; Altarifi et al., 2012, 2013; Altarifi & Negus, 2011; Elmer et al., 2010; Esposito & Kornetsky, 1977; J. Olds & R. P. Travis, 1960; Koob et al., 1975; Lorens & Mitchell, 1973; Negus & Moerke, 2019), diazepam-like gamma-aminobutyric acid (GABA) A receptor positive allosteric modulators (Schwienteck et al., 2017), and nicotine-like nicotinic acetylcholine receptor agonists (Freitas et al., 2015). Conversely, many drugs that are not abused (e.g. haloperidol-like DA receptor antagonists (Lazenka et al., 2016) or fenfluramine-like (Bauer et al., 2013) serotonin transporter substrates) fail to facilitate ICSS, and overall, the predictive validity of ICSS is equivalent to that of drug self-administration procedures for preclinical abuse-potential assessment (Negus & Miller, 2014). Consequently, ICSS can be used as an agnostic behavioral screen to identify abuse liability of NPS from a broad array of pharmacological classes. Preliminary evidence for mechanism of action can then be collected by evaluating sensitivity of NPS effects to blockade by receptor-selective antagonists. For example, effects of MOR agonists on ICSS can be blocked by the opioid antagonist naltrexone (Altarifi et al., 2013; Baird et al., 2022). ICSS is usually not facilitated by two other major classes of abused drugs: marijuana-like cannabinoid receptor agonists (Kwilasz & Negus, 2012; Vlachou et al., 2005) and psilocybin-like 5-HT_{2A} receptor agonist hallucinogens (Jaster et al., 2022; Sakloth et al., 2019). However, both classes of drugs produce robust decreases in ICSS as a pharmacological signal, with these decreases manifested as downward shifts of the

frequency-rate curve and decreases in reinforcement rates maintained by high brain-stimulation frequencies. The potency, time course, and magnitude of these effects can be readily quantified, and receptor mechanisms can be determined from studies with receptor-selective antagonists such as rimonabant (Kwilasz & Negus, 2012; Vlachou et al., 2005) and volinanserin (Jaster et al., 2022; Kehne et al., 1996) for cannabinoid and 5-HT_{2A} receptors, respectively. Some drug classes (e.g. salvia-like products containing kappa opioid receptor agonists (Negus et al., 2012)) may have low overall abuse liability but nonetheless display transient or localized patterns of abuse and toxicity (González et al., 2006; U.S. Drug Enforcement Administration, 2020; Vohra et al., 2011). Again, these classes of drugs produce robust ICSS depression that permits pharmacological characterization of drug potency, time course, and receptor mechanism of action. This broad sensitivity to multiple drug classes makes ICSS advantageous as an initial evaluation procedure for a new unknown substance. Theoretically, even if an NPS with an entirely novel mechanism of action emerged, its abuse potential would be detectable by ICSS if its reinforcing effects were mediated by alterations in mesolimbic dopamine homeostasis. ICSS procedures are less definitive at assessing the abuse potential of NMDA receptor antagonists. Both phencyclidine (Carlezon & Wise, 1993; Hillhouse et al., 2014b) and ketamine (Hillhouse et al., 2014a) are scheduled drugs of abuse, but while there is evidence of abuse-related ICSS facilitation for PCP, the predominant effect of ketamine seems to be depression of ICSS rates.

MOR agonists are a pharmacological class of particular concern during the current drug-overdose crisis in the United States. Acute dosing with MOR agonists can induce motor impairment that obscures abuse-related ICSS facilitation; however, repeated dosing with MOR agonists for as little as one week can produce tolerance to ICSS rate-decreasing effects and reveal

robust ICSS facilitation consistent with the abuse liability of these compounds (Moerke & Negus, 2021; Negus & Miller, 2014).

Electrical stimulation of the lateral hypothalamus and subsequent activation of the mesolimbic dopamine pathway is one of the strongest reinforcers known (Wise & Rompre, 1989). This gives ICSS advantages over other operant procedures. The reinforcing strength of electrical brain stimulation combined with its short duration (usually less than 1 s) yields opportunities for high rates of emitted behavior. Higher rates of behavior are necessary to provide a larger window of response possibilities, especially with integer variables (e.g., number of stimulations delivered to a rat). This large window allows precise quantitative measurements that can be used to compare the relative abuse potential between drugs.

Temporal Considerations in Abuse Potential Testing

One well-established principle in behavioral economics is that the subjective value of a reward is correlated with its temporal proximity to the completion of the operant response. As the delay between the completion of a task and its consequent stimulus increases, the subjective value of the stimulus decreases, in a phenomenon known as "delay discounting" (Vanderveldt et al., 2016). The pharmacokinetics of a drug, especially the rates of absorption and distribution to the sites of action, impact the time between the drug's administration and the onset of its abuse-related effects. This has been demonstrated with cocaine in self-administration experiments in non-human primates in which the rate of drug infusion was varied, and reinforcement strength decreased with the rate of cocaine infusion (Balster & Schuster, 1973; Panlilio et al., 1998; Woolverton & Wang, 2004). A recent study in rats examined cocaine reward in a choice context and demonstrated that although cocaine is generally considered to be extremely rewarding, the pharmacokinetics of cocaine lowered its reinforcing efficacy relative to a non-drug reward considered to be of smaller

magnitude but faster onset of reward (Canchy et al., 2020) . Experiments in which the rate of cocaine infusion was varied in human subjects have shown a pattern of diminishing subjective effects as infusion rate was slowed (Abreu et al., 2001). This pattern extends to cocaine administered by different routes, in which the routes that led to faster DAT occupancy (smoked, intravenous) produced greater subjective feelings of "high" than the slower intranasal route (Volkow et al., 2000). Correlation between reinforcing efficacy and onset of drug effects has been established for other classes of drugs as well, including opioids (Ko et al., 2002) and NMDA antagonists (Winger et al., 2002).

Quantitative Bioanalysis

Quantitative bioanalysis is a critical aspect to developing a full risk assessment of NPS. Whereas the assessment of abuse potential can proactively indicate drug threats, bioanalysis is a necessity for the monitoring of current threats. Bioanalysis is an application of analytical chemistry techniques to the measurement of drug and metabolite concentrations in biological systems. There are multiple disciplines where bioanalytical data is used, including for medical diagnosis, forensics, and drug monitoring and surveillance. Bioanalytical procedures generally involve two steps: the extraction of the drugs from their biological matrix, and the qualitative or quantitative detection of those drugs. A variety of sample preparation procedures exist for the purposes of extraction, ranging from simple dilutions to multiple step extractions such as solid-phase or liquid-liquid extractions. Measurement of the drug concentrations in biological samples is achieved by one of many potential instruments. The current workhorses for these analyses in forensic settings are gas chromatography mass spectrometry (GC-MS) and liquid chromatography tandem mass spectrometry (LC-MS/MS). Forensic identifications are thorough and the quantitative values are reasonably accurate, but the process can be laborious and expensive. In

clinical settings, suspected drug overdose cases generally rely upon the results of point-of-care urine drug screenings. These screenings are often immunoassay-based urine drug screens, which give rapid results to indicate a general class of drug to help diagnose conditions and direct treatment, but the lack of specificity makes identification of the exact species of drug difficult. Confirmatory testing is not always performed or considered necessary, especially in cases in which drug use is not denied (SAMHSA, 2012; Stellpflug et al., 2020). This introduces two problems. First, immunoassays are typically based around traditional drugs of abuse or prescription drugs. Consequently, they may fail to indicate the presence of NPS and give a false-negative result. Second, because of the lack of definitive identification, outbreaks of new drugs may not be detected unless they lead to fatal overdose in which a pathologist will order a more comprehensive toxicology analysis. If sub-lethal drug overdoses are not properly monitored and reported, then a major indicator of an ongoing drug epidemic could be missed.

Bioanalysis elucidates the pharmacokinetic properties of a drug. Pharmacokinetics is the study of a drug's movement into, throughout, and out of the body, and is considered in four stages: absorption, distribution, metabolism, and excretion. Each of these parameters can affect the presence of a drug at its site of action and thus the rate and intensity of its pharmacological effect. Establishing pharmacokinetic-pharmacodynamic relationships is important for attributing physiological or behavioral effects to the presence of a particular drug. Identification of metabolites can also provide another indicator of the presence of drugs in a biological system, and analyzing for both parent drug and metabolites can extend the detection window in these analyses.

Forensic toxicology requires an understanding of the relationship between the drug concentration measured in a biological specimen and the physiological or behavioral effects exerted by that drug in order to make inferences of drug effects based upon measured drug

concentrations. This is typically applied to determinations of cause and manner of death in the process of medicolegal death investigations as well as to the role of psychoactive compounds in drug-facilitated crimes. Some drugs, especially many NPS, are incredibly potent and have strong effects at even low doses, which challenges the bioanalytical capabilities of laboratories by requiring analytical techniques that are very sensitive.

Chirality is an important molecular property of drugs. This phenomenon occurs whenever a molecule has identical atomic composition and bonds, but differ in spatial orientation. This can arise any time there is a carbon atom in a molecule that is bonded to four different groups. In molecules with a single chiral center, two isomers called enantiomers exist which are structurally non-superimposable mirror images of one another. Most of the physical and chemical properties of enantiomers are identical, complicating analytical separations, but there are two ways in which they differ. First, enantiomers in solution will differentially bend plane-polarized light to either the left or right, and are designated as *l* or *d* (i.e. levorotatory or dextrorotatory). Second, their spatial orientation enables them to interact differently with biomolecules, which can result in dissimilar binding affinity at receptors. The differences can be clinically meaningful.

Stereospecific bioanalysis adds an extra layer of complexity to standard analyses. Typical chromatographic methods of separation rely on simple molecular interactions that cannot differentiate between enantiomers, and because they are isobars they are also unable to be separated based upon mass. There are primarily two methods for the separation of stereoisomers: chiral derivatization and chiral column chromatography. Chiral derivatization involves a chemical reaction that combines the drug of interest with another chiral molecule. With the introduction of a second chiral center, enantiomeric pairs become diastereomers which can be separated on traditional chromatographic columns. Chiral derivatization methods have been used extensively

with the determination of stereoisomers of methamphetamine in forensic toxicology (Fitzgerald et al., 1988). The second method involves the use of a chiral column. Instead of the stationary phase being composed of simple hydrophobic molecules, it is instead comprised of a complex chiral molecule. The affinity for the drug with the stationary phase with these complex biomolecules is based much more on the spatial orientation of the molecule and its ability to interact through intermolecular forces such as hydrogen bonding instead of simply hydrophobic or hydrophilic interactions with simpler chromatographic columns. Although expensive, chiral column chromatography is often the more preferrable method (Almeida & Silva, 2022).

Psychostimulants as drugs of abuse

This research focuses primarily on the evaluation of drugs that are commonly classified as "stimulants" or "psychostimulants." These terms are used here as they are colloquial descriptors that are familiar in most disciplines, including forensics; however, it should be noted that the pharmacological effects of a drug are dependent upon several factors, including the behavior being measured, the time course of the drug, and the environmental context during the observation of behaviors. Stimulant-type drugs do not increase rates of all behaviors. Although most overdose deaths in the current drug epidemic are caused by opioid-induced respiratory depression from illicitly manufactured fentanyl analogs, it is important to not let this overshadow the similar increase in psychostimulant use. Deaths due to psychostimulants are increasing rapidly and represent the second most common drug class involved in overdose deaths (Centers for Disease Control and Prevention, National Center for Health Statistics, 2021). Emergency department visits due to psychostimulant overdose are also increasing dramatically, and the increases in both fatal and non-fatal overdoses are occurring in cases with and without concurrent opioid intoxication (Hoots et al., 2020; Kariisa et al., 2019; Suen et al., 2022).

Psychostimulants can include drugs such as caffeine or nicotine, but the term is primarily used to describe compounds which act upon the dopamine transporter (DAT). In normal dopamine neurotransmission, an action potential in the presynaptic neuron causes dopamine-containing vesicles in the axon terminal to fuse with the plasma membrane and release dopamine into the synapse. Dopamine molecules can then diffuse across the synapse to bind to and activate dopamine receptors embedded in the cellular membrane of the post-synaptic neuron, resulting in a signaling cascade. This signal is attenuated by the function of DAT which recycles dopamine from the synapse back into the presynaptic cell, thus decreasing synaptic dopamine concentrations and limiting the number of DA receptors occupied by DA. Some compounds, such as methamphetamine, are substrates for DAT, and ultimately act by inducing the release of dopamine from the presynaptic cell into the synapse. Other compounds, such as cocaine, are inhibitors of DAT, and act by blocking the reuptake of dopamine from the synaptic space. Both DAT substrates and inhibitors elevate extracellular dopamine concentrations, which leads to increased signaling in pathways activated by postsynaptic dopamine receptors. Figure 1-1 shows a basic mechanism of action of psychostimulants that act at DAT.

The primary mechanistic focus in this dissertation is the abuse-related modulation of DA by compounds that are either substrates or inhibitors at DAT. The monoamine neurotransmitters, DA, NE, and 5-HT, are similar in structure, and thus it would be expected for similarities to also exist in their associated transport proteins. Due to these similarities, compounds that act as inhibitors or substrates at one MAT are often at least partially active at the other two. Abuse-related effects are primarily driven by activity at DAT. The health dangers of these compounds, however, are generally due to increases in NE or 5-HT. Increases in NE cause a response by the activating sympathetic nervous system. Stimulation of the sympathetic nervous system leads to

cardiovascular effects including the increase of blood pressure due to the constriction of arteries and an increase in heart rate; these physiological effects increase the probability of adverse cardiovascular events such as cardiac arrhythmia, myocardial infarction, and stroke. Catecholaminergic stimulants frequently cause cardiac dilation and other cardiomyopathies in both acute and chronic use cases (Kim & Park, 2019; Redfern et al., 2015; Won et al., 2013). Increases in 5-HT can trigger serotonin syndrome, characterized by symptoms such as hypertension, tachycardia, hyperthermia, changes in mental status including delirium, and neuromuscular abnormalities ranging from tremor to rigidity (Boyer & Shannon, 2005; Rasimas, 2012; Warrick et al., 2012). The severity of serotonin syndrome varies from mild to severe, but it can be fatal. Another condition known as "excited delirium" presents with similar symptoms of hypertension, tachycardia, hyperthermia, delirium, and neuromuscular abnormality, as well as an aggressive and often violent behavior (Gill, 2014; Gonin et al., 2018; Mash, 2016). This condition, however, occurs in the absence of serotonergic agents, and while the exact cause is not clear, it has been attributed to hyperdopaminergia resulting from excessive psychostimulant use. Both serotonin syndrome and excited delirium diagnoses require confirmation of intoxication by either a serotonergic or dopaminergic drug, which necessitates the use of thorough toxicological analyses in cases where these syndromes are suspected.

Increases in extracellular DA in the nucleus accumbens are responsible for the reinforcing effects of drugs of abuse (Di Chiara & Imperato, 1988), but one feature that impacts abuse liability in these compounds is the selectivity for DAT over SERT. Compounds that are highly selective for DAT over SERT have high abuse liability. Conversely, compounds that are highly selective for SERT over DAT have low abuse liability. Non-selective compounds can have abuse liability somewhere between the two extremes. This has been demonstrated in ICSS procedures for both

MAT substrates as well as inhibitors (Bauer et al., 2013; Miller et al., 2015; Rosenberg et al., 2013).

These studies use several DAT inhibitors and substrates which are structural analogs to the traditional and naturally-occurring cathinone (Figure 1-2) and cocaine (Figure 1-3). Synthetic cathinones have emerged as novel stimulants that exert their effects by altering monoamine signaling in the brain (Baumann et al., 2018; Simmons et al., 2018). Cathinones, the β-ketone analogs of amphetamines, exhibit chirality. In cathinones (and amphetamines), the carbon at the alpha position is bonded to four different groups, and there are two possible enantiomers that result from their spatial arrangement, designated *R*/S or +/-. Methamphetamine is a closely related compound to methcathinone, and notably *d*-methamphetamine is a Schedule II controlled substance in the United States due to its high abuse liability and widespread recreational misuse. On the other hand, *l*-methamphetamine has negligible abuse liability at therapeutic doses, and is available as an over-the-counter nasal decongestant inhaler.

α-PHP is a cathinone-type drug first patented in the 1960s (Seeger, 1967). Structurally, it differs from cathinone by the presence of a pyrrolidine ring at the amine position, and an elongated hydrocarbon chain at the alpha position (see Figure 1-2). Experiments have shown α-PHP to be a potent reuptake inhibitor of DAT (Kolanos et al., 2015), and it is highly selective for DAT and NET over SERT (Zawilska & Wojcieszak, 2017). In self-administration assays, α-PHP maintains significant increases in the number of infusions compared to vehicle (Javadi-Paydar et al., 2018). A number of human case studies have appeared in recent years (2018-2021) that implicate α-PHP in cases of extreme aggression and agitation, impaired driving, and fatal intoxications (Grapp et al., 2022; Pieprzyca et al., 2021).

Cocaine is a psychoactive compound that is produced naturally in the leaves of the coca plant, and it has a long history of use spanning thousands of years. Originally the leaves were chewed or brewed into tea to enjoy the euphoric and stimulating effects, but in the last 150 years it has been carefully processed and extracted to refine a higher purity and more addictive product (Drake & Scott, 2018; Niemann, 1860). Cocaine is a much more complex molecule than cathinone-type stimulants (see Figure 1-3) that binds to MAT and acts as a non-selective inhibitor of reuptake by DAT, NET, and SERT (Carroll et al., 1992). Cocaine is readily self-administered (Bozarth & Wise, 1985; Fitch & Roberts, 1993) and facilitates ICSS (Bauer et al., 2014) in rats, consistent with its long history of abuse. In the process of exploring the potential therapeutic benefits of MAT inhibitors, a series of phenyltropane cocaine analogs was synthesized. Two of these were WIN-35428 and RTI-31 (Clarke et al., 1973). In these molecules, an ester linkage is removed from the cocaine molecule and a halogenated phenyl ring is bonded directly to the tropane ring (see Figure 1-3). These modifications to the cocaine structure resulted in two major effects: affinity for their receptor site on MATs was substantially increased (Boja et al., 1990; Carroll et al., 1992), and slower pharmacokinetics resulted in a slower rate of binding in-vivo (Stathis et al., 1995; Wee et al., 2006). One consequence of the slower rate of binding, and thus a slower rate of onset of drug effects, is that cocaine, WIN-35428, and RTI-31 have different reinforcing efficacies as determined by progressive ratio breakpoints in a self-administration procedure in rhesus monkeys (Wee et al., 2006).

Methcathinone is the simplest of the synthetic cathinones, differing from the naturally-occurring cathinone by the replacement of a single hydrogen in the amino group with a methylation (see Figure 1-2). Methcathinone was first synthesized in the early 20th century (Eberhard, 1915) and patented in 1957 by Parke, Davis & Company for use as an analeptic (L'Italien & Rebstock,

1957). In the 1980s widespread abuse began occurring in the Soviet Union (Emerson & Cisek, 1993; Glennon et al., 1995). In the United States, methcathinone was scheduled in 1994 as it emerged as one of the first NPS. Similar to cathinone, methcathinone is a strong monoamine releaser (Davies et al., 2020) and stimulant-type drug. One reason for the selection of methcathinone as a drug of interest is the recent reports emerging from wastewater monitoring projects in which methcathinone was identified as the most prevalent NPS detected (EMCDDA, 2020; González-Mariño et al., 2016; Liu et al., 2021; O'Rourke & Subedi, 2020; Salgueiro-González et al., 2022). Subsequent findings have suggested that the presence of methcathinone in wastewater is an artifact caused by chemical conversion from other compounds such as methamphetamine or ephedrine (Huang et al., 2022; Simpson et al., 2022). Nonetheless, as the progenitor of the class of synthetic cathinone stimulants, methcathinone is an interesting drug as a case study. The two primary routes of metabolism for methcathinone are demethylation of the amine functional group and reduction of the ketone functional group to a hydroxyl (Figure 1-4).

Goals

The major goal of this work was to demonstrate how ICSS could be used as a threat assessment tool for emerging drugs of abuse, and to test this using several stimulant-type drugs. More specifically, a protocol for using abuse potential testing with ICSS procedures as a threat assessment tool needs to be able to rank potential threats relative to one another, to assess common pharmacological properties such as potency, efficacy, and time course, and to be sensitive enough to distinguish between drugs of substantial similarity in molecular structure. A second goal of this work was to develop a method for bioanalytical monitoring of methcathinone and its metabolites in order to relate measured blood concentrations to the corresponding observed behavioral effects.

Appropriate monitoring of drug use with bioanalytical procedures can assess the effectiveness of the threat assessment tool and the interventions made as a result of that assessment.

Tables and Figures

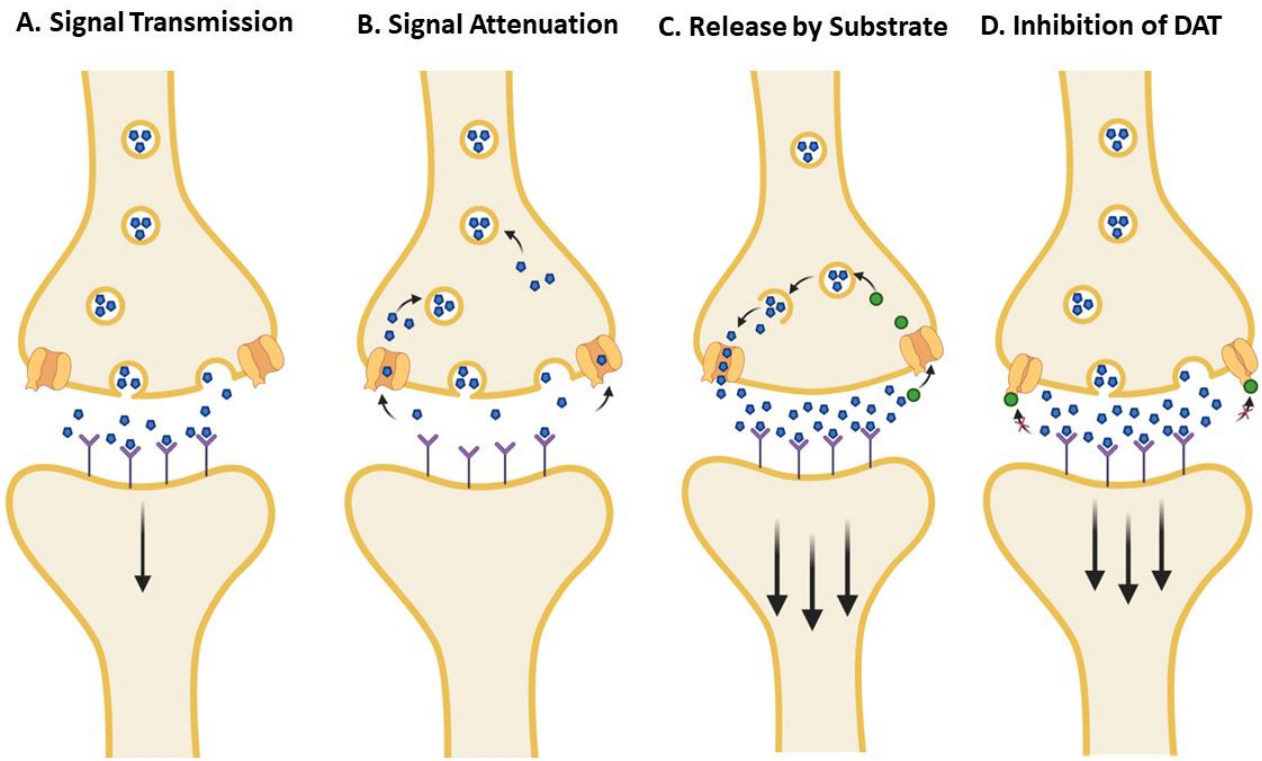

Figure 1-1: Basic Mechanism of Action of Psychostimulants acting at Monoamine Transporters

DAT substrates and inhibitors alter normal dopamine signaling. *Panel A* demonstrates typical vesicular release of dopamine following an action potential, diffusion of DA across the synapse, binding to the dopamine receptor, and activation of the postsynaptic neuron. *Panel B* indicates normal attenuation of the signal transmission as synaptic dopamine is transported back into the presynaptic cell and repackaged into vesicles. *Panel C* shows a DAT substrate passing into the presynaptic cell and inducing intracellular release of dopamine and reverse transport out of the cell through DAT, increasing synaptic dopamine concentrations. *Panel D* shows an inhibitor of DAT binding and preventing dopamine reuptake into the presynaptic cell. Figure generated using *Biorender.com*.

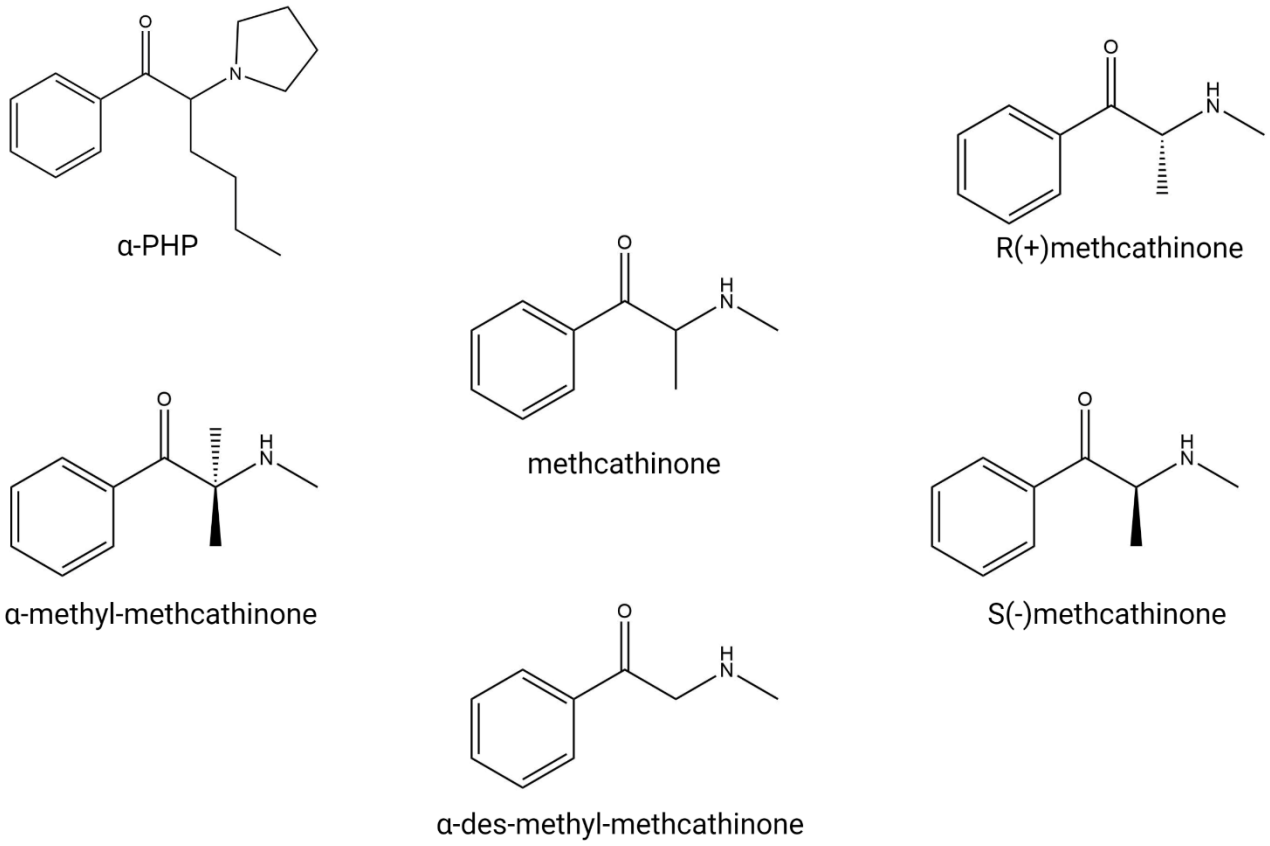

Figure 1-2: Cathinone-type drugs

Methcathinone (center) is one of the simplest synthetic cathinone molecules, differing from natural cathinone by the presence of a single methylation on the amine group. Despite all of these molecules being generally classified as synthetic cathinones and psychostimulants, the mechanism of α-PHP differs from the other molecules here in that it is a DAT uptake inhibitor, and the others act as DA releasers.

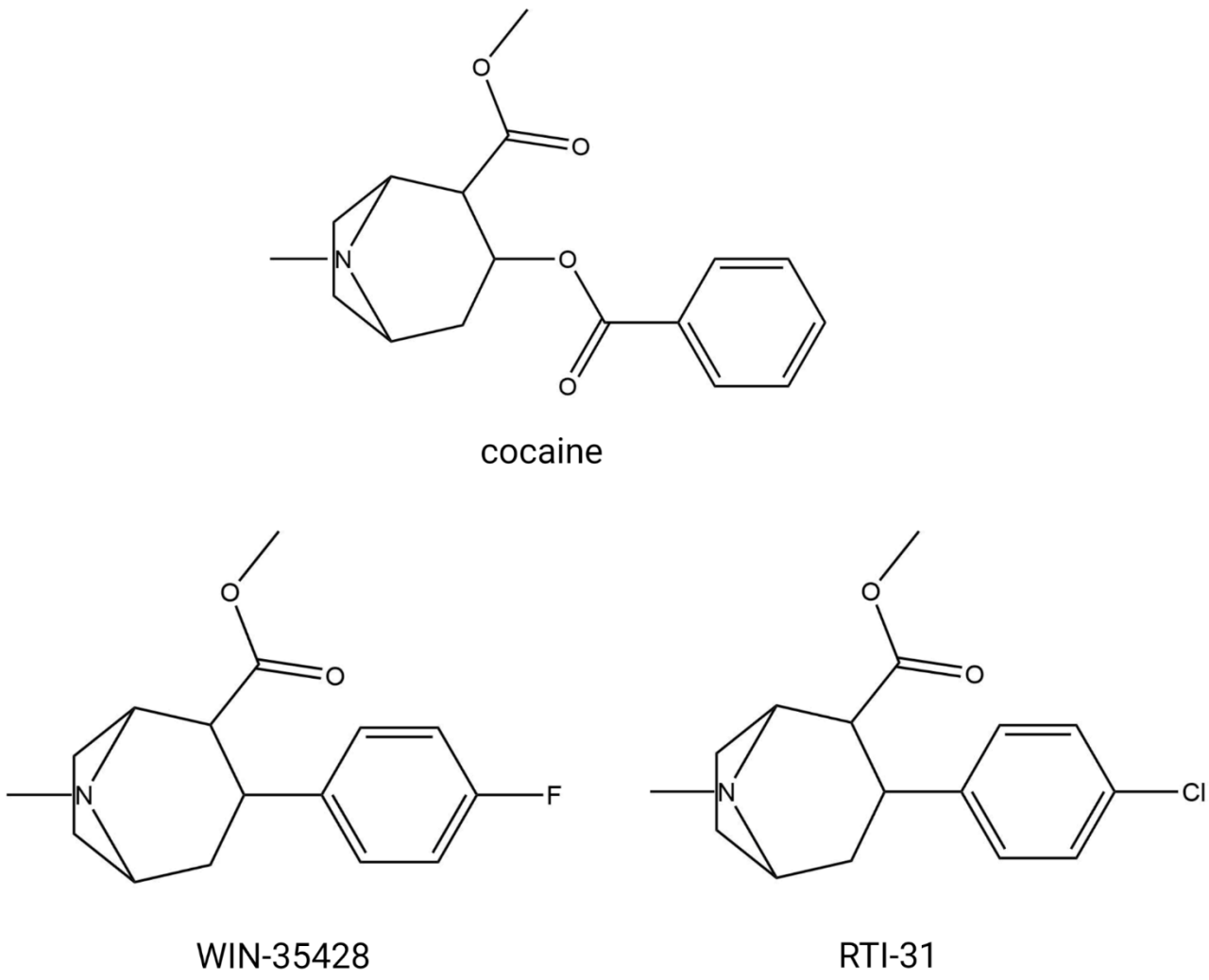

Figure 1-3: Cocaine and two phenyltropane analogs

Cocaine is a traditional drug of abuse. WIN-35428 and RTI-31 are synthetic phenyltropane analogs of cocaine that act similarly as inhibitors of DAT but with different potency and rates of onset.

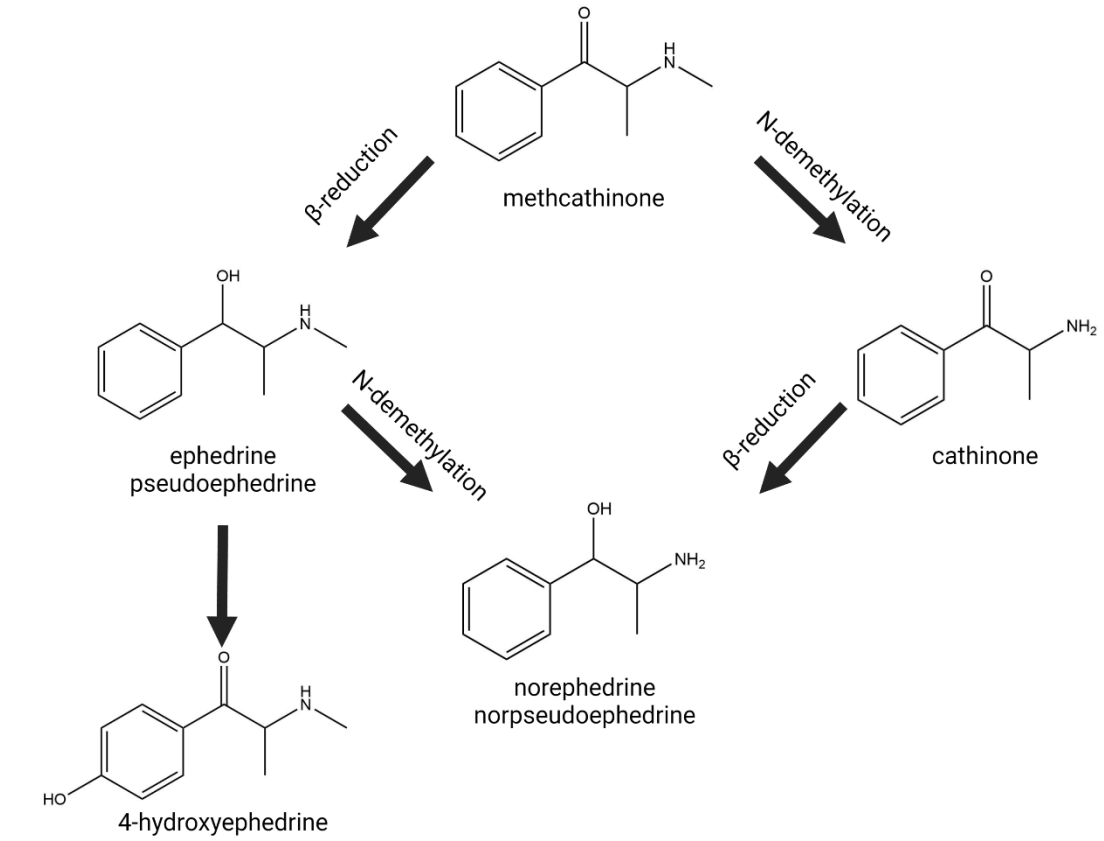

Figure 1-4: Metabolic pathways for methcathinone

Methcathinone has two likely metabolic pathways: N-demethylation to cathinone and reduction of the beta-ketone to form ephedrine or pseudoephedrine. The next step of reactions may form norephedrine or norpseudoephedrine through the same demethylation or reduction reactions. Hydroxylation of the para-position on the phenyl ring has also been shown to occur in rats with ephedrine.

CHAPTER 2: BEHAVIORAL SCREEN OF α-PYRROLIDINOHEXANOPHENONE BY ICSS

Introduction

The goal of this chapter was to illustrate the potential of ICSS for NPS assessment by testing the synthetic cathinone α-pyrrolidinohexanophenone (α-PHP) and comparing it to the more well-known abused stimulant cocaine. Although α-PHP was identified as an NPS in the United States as early as 2015 (U.S. Drug Enforcement Administration, Diversion Control Division, 2016, 2019), it was uncontrolled in the United States until its placement in Schedule I as of July 2019 (United States Drug Enforcement Administration, 2019).

Given the extensive timeframe between its initial appearance and the scheduling action, α-PHP was chosen as a candidate for abuse potential assessment using ICSS to see if early assessment could have successfully predicted its abuse liability and informed a timelier scheduling decision. The magnitude, potency, and time course of α-PHP effects on ICSS were determined and compared to saline vehicle and to cocaine as negative and positive controls, respectively. Additionally, the experimental design in this study with α-PHP was used as the foundation for a procedure that could be used to evaluate any NPS.

Methods

Subjects

Subjects were six (6) adult male Sprague-Dawley rats (Envigo, Indianapolis, IN, USA) weighing between 318 and 365 g at the time of surgery. Prior to the present study, these rats were used in studies of methcathinone and two methcathinone analogs (see Chapter 4) (Davies et al.,

2020); however, there was a three-week drug-free washout period between completion of these prior studies and initiation of the present study. The rats were housed individually in cages with free access to food and water except during ICSS sessions, and were exposed to a 24-hour light/dark cycle with lights on from 6:00 am to 6:00 pm. Animals resided in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). Animal care and research complied with the National Institutes of Health guidelines for the care and use of animal subjects in research, and all protocols were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee.

Surgical Procedure

Surgical and experimental procedures have been described previously (Bauer et al., 2013; Bauer et al., 2014; Kolanos et al., 2015). Anesthesia was induced by inhalation of 2.5-3.0% isoflurane (Zoetis Inc., Kalamazoo, MI, USA) in oxygen until the rat was unresponsive to toe pinch, and this level of anesthesia was maintained throughout surgery. A stainless steel, bipolar electrode (Plastics One, Roanoke, VA, USA) was implanted via stereotaxic surgery by inserting the cathode into the left medial forebrain bundle at the lateral hypothalamus (2.8 mm posterior to bregma, 1.7 mm lateral to the midsagittal suture, and 8.8 mm ventral to the exterior surface of the skull). The anode was grounded by coiling around one of three screws anchored into the dorsal surface of the skull. The electrode, screws, and grounding wire were permanently affixed to the skull using dental acrylic resin. Ketoprofen (5 mg/kg) was administered for post-operative analgesia immediately and 24 h after surgery, and rats were allowed to recover for at least 7 days prior to commencing ICSS training.

Apparatus

Behavioral sessions were conducted in modular operant test chambers (29.2 x 30.5 x 24.1 cm) (Med Associates, St. Albans, VT, USA) constructed of stainless steel and clear polycarbonate with a grid floor and housed inside ventilated sound-attenuating cubicles. The test chamber contained a response lever located 3 cm above the floor and 7.6 cm below three stimulation lights (red, yellow, and green), a 2 W house light, and an ICSS stimulator (see Figure 1A). A bipolar cable and swivel commutator (Model SL2C; Plastics One, Roanoke, VA, USA) connected the ICSS stimulator to the electrode. Data collection and ICSS programming were accomplished using a computer system running Med-PC IV software (Med Associates, St. Albans, VT, USA).

Training

Following initial shaping of lever-press responding, rats were trained under a fixed-ratio 1 (FR 1) schedule of brain stimulation. 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 until ICSS rates > 30 stimulations/min were observed. This intensity was then held constant, 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. The chamber remained dark until the response period, during which the house light would

illuminate as a discriminative stimulus that electrical brain stimulation could be received contingent upon pressing the response lever. The stimulation intensity was again adjusted to yield baseline frequency-rate curves that had high rates of reinforcement for the upper frequencies and minimal responding at the remaining frequencies (see Figure 1-4B). This intensity (120-340 μA across rats) was then held constant throughout the study. Training continued until frequency-rate curves were not statistically different over three days of training as indicated by lack of a significant effect of "day" in a two-way analysis of variance (ANOVA) with frequency and day as the two variables (see data analysis).

Testing

ICSS testing was performed on α-PHP at doses of 0.32, 1.0, and 3.2 mg/kg. These doses were selected following pilot studies that gradually increased dosage in 0.5 log units in individual rats until a behaviorally active dose was identified. ICSS testing was also performed using a 10 mg/kg dose of cocaine and saline as positive and negative controls, respectively. Test sessions consisted of three consecutive baseline components followed by intraperitoneal (i.p.) drug injection and then by pairs of consecutive test components beginning after 10, 30, 100, and 300 min. For 3.2 mg/kg α-PHP, ICSS testing was also performed after 1440 min (24 hr). Treatments were counterbalanced across rats using a Latin-Square design. Testing was generally performed on Tuesdays and Fridays, and three-component training sessions were conducted on other weekdays. Data from training sessions were monitored to ensure a return to ICSS rates consistent with pre-drug levels to confirm drug wash-out. All testing was performed during the light phase of the light/dark cycle.

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 for that rat on that day. 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 each pair of 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 $P < 0.05$. Frequency-rate curves for vehicle (saline), the negative control, were compared to baseline frequency-rate curves, with the expectation of no significant difference. All drug treatments were compared to vehicle. Cocaine was expected to significantly facilitate ICSS responding compared to vehicle (Bauer et al., 2014; Bonano et al., 2014).

To provide a secondary summary measure of drug effects, the total number of stimulations delivered across all 10 frequency-trials of each component was determined for each drug dose at each time point. Mean number of stimulations per component for baseline and vehicle test components at all time points were compared to evaluate the negative control using a repeated measures ANOVA, with significance criterion set at $P < 0.05$. Summary test data from each day were then normalized to individual baseline data from that day using the equation % baseline total stimulations per component = (mean total stimulations per test component) / (mean total

stimulations per baseline component) x 100. Data were then averaged across rats and plotted as a function of time for each drug dose. Results were compared using a repeated measures two-way ANOVA using time as one factor and drug dose as the second factor. A significant ANOVA was followed by the Holm-Sidak *post hoc* test, with the criterion for significance set at $P < 0.05$. Statistical analyses were performed using Prism 8.1.2 (GraphPad Software, San Diego, CA, USA).

Drugs

α-PHP oxalate was synthesized as previously reported (Kolanos et al., 2015) and (−)-cocaine HCl was supplied by the National Institute on Drug Abuse Drug Supply Program (Bethesda, MD, USA). Drugs were dissolved in sterile saline (Hospira Inc., Lake Forest, IL, USA) and doses are expressed as the salt form of the drug.

Results

Across all rats in the study, the mean ± standard error of the mean (SEM) MCR was 53.1 ± 3.6 stimulations per trial, and the mean ± SEM total stimulations earned per baseline component was 200 ± 14.0 stimulations per component. Under baseline conditions, electrical brain stimulation maintained a frequency-dependent increase in ICSS rates. Two-way ANOVA of baselines for the five experimental days indicated stable baseline responding with no significant main effect of day ($F(1.373, 6.864) = 1.637$, $P=0.2534$) and no significant day x frequency interaction ($F(3.470, 17.35) = 1.274$, $P=0.3166$), but a significant main effect of frequency ($F(1.890, 9.448) = 130.3$, $P<0.0001$). Baseline ICSS rates were minimal for low frequencies of electrical stimulation (56-79 Hz), increased at intermediate frequencies (89-112 Hz), and reached a maximum rate at high frequencies (126-158 Hz).

Figure 2-2 shows the effects of our negative control, saline, on ICSS performance. Figure 2-2A compares frequency-rate curves for baseline components and test components after injection of saline with a 10-minute pretreatment time. Statistical analysis of these curves shows no significant effect of saline treatment ($F(1, 5) = 0.6856$, $P=0.4454$) and no significant frequency x treatment interaction ($F(2.928, 14.64) = 0.2439$, $P=0.8601$). Figure 2-2B shows the summary measure of ICSS from 10 to 300 min after saline injection. A one-way ANOVA comparing mean total number of stimulations per component indicates no significant difference between baseline ICSS responding and ICSS responding after saline injection for all four experimental time-points ($F(2.307, 11.53) = 2.068$, $P=0.1675$).

Figure 2-3 compares the effects of saline with the effects of our positive control, 10 mg/kg cocaine, on ICSS performance. Figure 2-3A compares frequency-rate curves determined 10 min after saline or cocaine injection. Statistical analysis of these curves shows a significant effect of cocaine treatment ($F(1, 5) = 185.4$, $P<0.0001$) as well as a significant cocaine x frequency interaction ($F(9, 45) = 9.333$, $P<0.0001$). These data indicate significant facilitation of ICSS responding maintained by low and intermediate brain-stimulation frequencies after cocaine administration compared to saline vehicle. Figure 2-3B compares the time course of effects produced by saline and cocaine on the summary measure of ICSS. A two-way ANOVA comparing cocaine and saline shows a significant effect of time point ($F(1, 5) = 85.69$, $P=0.0002$) and significant time point x treatment interaction ($F(3, 15) = 39.79$, $P<0.0001$). *Post hoc* tests indicate total stimulation rates for cocaine treated rats were significantly elevated compared to vehicle at the 10-, 30-, and 100-min time points ($P < 0.05$). At the 300-min time point, total stimulation rates for cocaine treated rats were not significantly different from saline vehicle. These results show

that cocaine, an established drug of abuse and positive control, produced robust ICSS facilitation with rapid onset and a duration of at least 100 min.

Figure 2-4 compares the effects of three doses of α-PHP to the effects of saline on ICSS performance. Figure 2-4A compares the frequency-rate curves of 0.32, 1.0, and 3.2 mg/kg α-PHP against vehicle determined 10 min after α-PHP or saline injection. A two-way ANOVA shows a significant effect of dose ($F(1.772, 8.859) = 26.64$, $P=0.0002$) as well as a significant dose x frequency interaction ($F(3.866, 19.33) = 5.391$, $P=0.0046$). *Post hoc* tests indicate a lack of facilitation compared to saline at any individual frequency for a dose of 0.32 mg/kg α-PHP, but robust facilitation of ICSS maintained by low- and intermediate-frequencies of brain stimulation at a dose of 3.2 mg/kg. Additionally, no dose attenuated responding for high-frequency brain stimulation. Figure 2-4B compares summary measures of time courses of effects for 0.32, 1.0, and 3.2 mg/kg doses of α-PHP and saline. A two-way ANOVA comparing drug doses and the negative control over time shows significant main effects of drug dose ($F(2.816, 14.08) = 27.05$, $P<0.0001$) and time point ($F(1.455, 7.275) = 23.77$, $P=0.0010$), as well as a significant dose x time interaction ($F(2.602, 13.01) = 11.44$, $P=0.0008$). *Post hoc* tests indicated that the dose of 1.0 mg/kg α-PHP was only significantly different from saline at the 10-min time point, whereas for 3.2 mg/kg α-PHP, robust ICSS facilitation compared to baseline was significant to the 300-min time point ($P<0.05$). Peak facilitation occurred during the 10-min test components. An additional pair of ICSS test components were collected for the 3.2 mg/kg dose of α-PHP at 1440 min (24 hr), and the rates of ICSS seem to return to baseline by that time; however, statistical analysis was not performed as rats treated with saline were not tested at that time point. Overall, α-PHP exhibited a profile of strong ICSS facilitation manifested as a leftward shift of the frequency-rate curve with a similarly rapid onset to that of cocaine. The ICSS profile of rats treated with 3.2 mg/kg α-PHP

is similar to that of rats treated with 10 mg/kg cocaine. However, cocaine and α-PHP at these doses differ in their duration of action, with α-PHP causing significant facilitation of ICSS at least to 300 min.

Discussion

This study used an ICSS procedure in rats to evaluate the abuse liability of the synthetic cathinone α-PHP. ICSS is a preclinical behavioral procedure in which laboratory animals (e.g. rats) are trained to press a response lever to receive pulses of electrical brain stimulation, and drugs of abuse produce characteristic increases in ICSS responding, a phenomenon called "ICSS facilitation" (Negus & Miller, 2014). Here we used a hybrid frequency-rate method which is quantifiable and sensitive to both abuse-related facilitation of responding maintained by low-frequency electrical stimulation as well as abuse-limiting depression of responding maintained by high-frequency electrical stimulation. Our data show α-PHP produced a potent, robust, and sustained ICSS facilitation similar to that produced by cocaine and consistent with the recent DEA decision to emergency-schedule α-PHP. This study also illustrated the potential of ICSS for use in rapid abuse liability testing of NPSs.

Our results showed baseline responding was stable over the experimental period, and the baseline frequency-rate data ranged from an absence of responding at low frequencies to maximal responding at high frequencies of electrical brain stimulation. This spectrum of baseline response rates allows the experimental procedure to detect both rate-increasing and rate-decreasing effects of test drugs. Although these rats were not drug naïve, they underwent a lengthy wash-out period of three weeks between test compounds. Furthermore, ICSS procedures are resistant to sensitization by dopaminergic drugs (Riday et al., 2012), and we have unpublished data also indicating that ICSS facilitation by dopamine transporter blockers and substrates is consistent with

repeated dosing and in drug-naïve versus drug-experienced animals. Saline was assessed as a negative control in this experiment, and it did not alter rates of responding relative to baseline. Cocaine (10 mg/kg) was assessed as the positive control in this experiment, and as expected, it robustly facilitated ICSS response rates. This is consistent with the effects of cocaine on ICSS shown in previous experiments (Bauer et al., 2014; Bonano et al., 2014). The controls clearly demonstrated the ability of this procedure to differentiate between a substance known to have high abuse potential and a negative vehicle control.

The main goal of this study was to evaluate the degree to which α-PHP might produce a cocaine-like facilitation of ICSS. This study examined three characteristics of α-PHP relevant to abuse potential: the potency, time course, and magnitude of effects on ICSS. A comparison of our results to a previous study examining multiple cocaine doses (Bonano et al., 2014) suggests that α-PHP was approximately three times more potent than cocaine at facilitating ICSS. It exhibited a rapid onset, similar to cocaine, and a longer duration of action. Lastly, both α-PHP (3.2 mg/kg) and cocaine (10 mg/kg) produced similar magnitudes of effect, approximately doubling the overall rates of ICSS facilitation. The high potency, rapid onset of effects, and, most importantly, the substantial magnitude of ICSS facilitation of α-PHP are all indicative of high abuse potential. The abuse-related effects and long duration of action of α-PHP are supported by findings from Gatch et al. (2017), which demonstrated that α-PHP fully substituted for the discriminative stimulus effects of both methamphetamine and cocaine in rats, and had a long duration of action in locomotor studies (5-8 hr) in mice. Furthermore, recent reports of α-PHP involvement in hospitalizations illustrate the abuse potential indicated by these results (Beck et al., 2018). These data are also consistent with the ICSS profile exhibited by other controlled DAT inhibitors including methylphenidate (Lazenka & Negus, 2017), 3,4-methylenedioxypyrovalerone (MDPV)

(Bonano et al., 2014), and the closely related analog α-pyrrolidinopentiophenone (α-PVP) (Watterson et al., 2015). Another DAT-selective substituted cathinone, bupropion, is a medication currently prescribed as an antidepressant and smoking cessation aid. ICSS testing of bupropion demonstrates a similar facilitation of ICSS, but at a 10-fold higher dose (32 mg/kg) and a longer pre-treatment time (30 min) (Rosenberg et al., 2013). Although bupropion is not scheduled under the Controlled Substances Act, it is regulated as prescription-only, and cases of abuse have been documented (Stall et al., 2014). Overall, the profile of ICSS and other behavioral effects exhibited by α-PHP is consistent with the mechanism of action of a DAT-selective inhibitor as indicated by Kolanos et al. (Kolanos et al., 2015). The evidence for high abuse potential of α-PHP determined by this study support the U.S. Drug Enforcement Administration's emergency classification of α-PHP as a Schedule I substance in 2019, and subsequent permanent scheduling action (United States Drug Enforcement Administration, 2019).

Tables and Figures

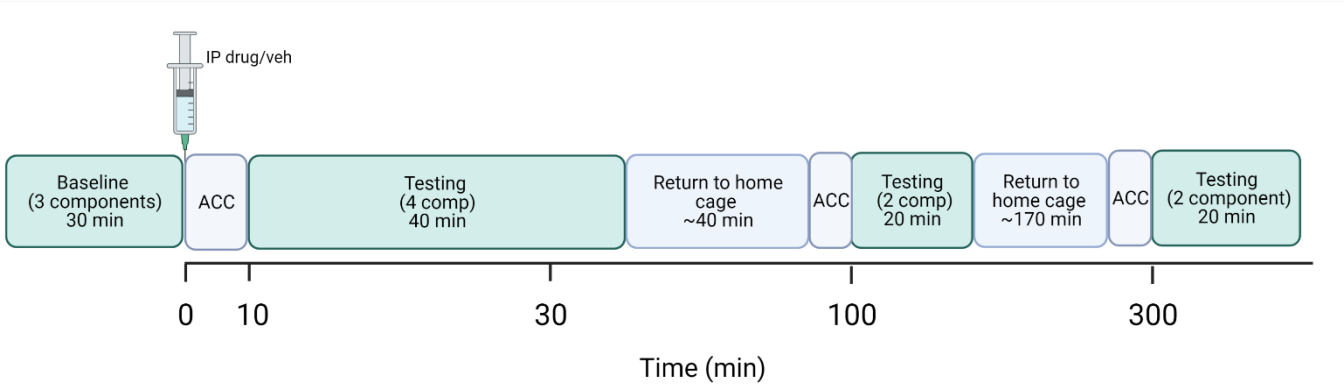

Figure 2-1: Experimental Design for ICSS testing of α-PHP and Cocaine

Following a 3-component baseline session, rats were administered IP vehicle or drug and placed into the ICSS chamber to acclimate for ten minutes following the injection. Test components were completed in three blocks between which animals were returned to their home cage. Animals were returned to the ICSS chambers approximately ten minutes prior to ICSS test sessions and allowed to acclimate before testing began.

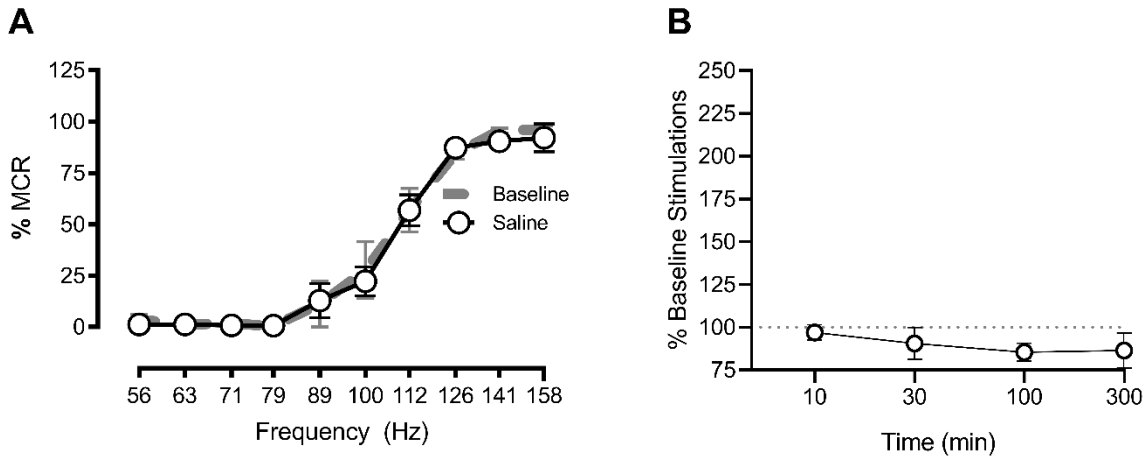

Figure 2-2: Saline injection does not affect ICSS responding.

Panel A shows a lack of significant effect of i.p. saline injection compared to baseline ICSS responding at 10-min post-injection. *Abscissa:* Frequency of electrical brain stimulation in Hz. *Ordinate:* Percent maximal control reinforcement rate (%MCR) during each 1-min frequency trial. Panel B shows a summary measure of the lack of effect of saline on ICSS responding over time. *Abscissa:* Time post-administration of saline injection. *Ordinate:* Percent baseline number of stimulations (% Baseline Stimulations) obtained across all frequencies during each 10-min component. All data are expressed as mean ± SEM for six rats (n=6).

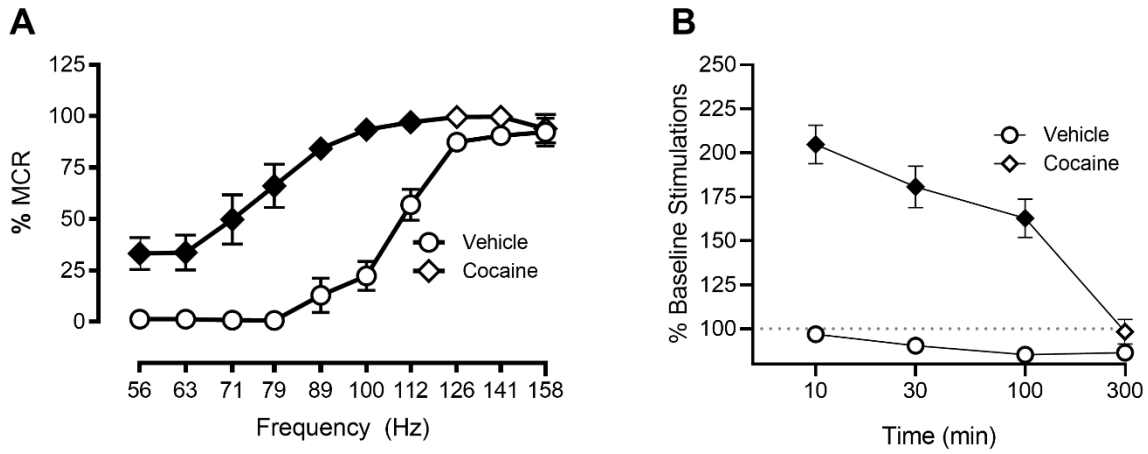

Figure 2-3: Cocaine (10 mg/kg) facilitates ICSS

Panel A shows significant facilitation of ICSS responding at low and intermediate frequencies of electrical brain stimulation after i.p. cocaine injection (10 mg/kg) compared to vehicle at 10 minutes post-injection. *Abscissa:* Frequency of electrical brain stimulation in Hz. *Ordinate:* Percent maximal control reinforcement rate (%MCR). Panel B shows a summary measure of the effect of 10 mg/kg cocaine on ICSS responding over time. *Abscissa:* Time post-administration of injection. *Ordinate:* Percent baseline number of stimulations delivered per component. For both panels, data are expressed as mean ± SEM for six rats (n=6). Filled points denote a significant difference compared to vehicle by a two-way ANOVA followed by a Holm-Sidak post hoc test ($P < 0.05$).

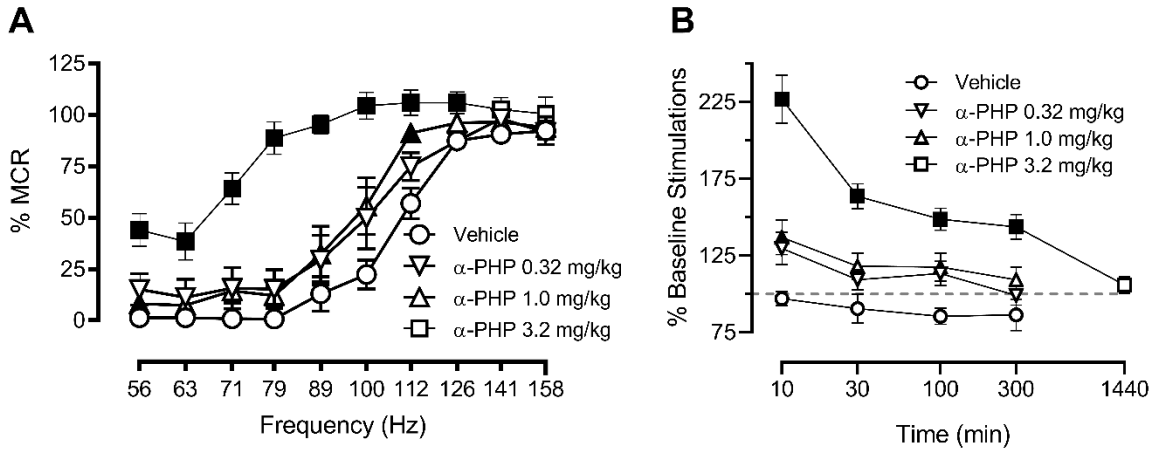

Figure 2-4: α-PHP (1.0 and 3.2 mg/kg) facilitates ICSS.

Panel A shows significant facilitation of ICSS responding at low and intermediate frequencies of electrical brain stimulation after i.p. α-PHP injection (3.2 mg/kg) compared to vehicle at ten 10 minutes post-injection, and modest facilitation of ICSS at a dose 1.0 mg/kg. Abscissa: Frequency of electrical brain stimulation in Hz. Ordinate: Percent maximal control reinforcement rate (%MCR). Panel B displays shows a summary measure of the effect of 0.32, 1.0, and 3.2 mg/kg α-PHP on ICSS responding over time. Abscissa: Time post-administration of injection. Ordinate: Percent baseline number of stimulations delivered per component. For both panels, data are expressed as mean ± SEM for six rats (n=6). Filled points denote a significant difference compared to vehicle by a two-way ANOVA followed by a Holm-Sidak post hoc test ($P < 0.05$).

CHAPTER 3: RATE OF ONSET AS A DETERMINANT OF ABUSE POTENTIAL

Introduction

Preclinical testing plays a key role in predicting abuse potential and guiding regulatory control of new psychoactive substances (U.S. Food and Drug Administration, 2017). Drug self-administration is the most widely used family of procedures for preclinical abuse-potential testing, and positive reinforcing effects indicative of abuse potential depend on factors that include both the pharmacodynamic mechanism of drug action and the time course of drug effect (Ator & Griffiths, 2003; Carter & Griffiths, 2009; O'Connor et al., 2011). The phenomenon of delay discounting in behavioral economics relates the temporal proximity of an action and its consequent stimulus and its subjective reward value. The subjective value of the reward determines the reinforcing efficacy, and thus the rate of the behavior. Applied to the use of drugs, this principle would manifest such that drugs with a shorter delay between the action of taking the drug and the consequent stimulus of experiencing the psychoactive effects of the drug have a higher abuse potential than those with a delay.

ICSS has similar predictive validity relative to drug self-administration across a wide range of pharmacodynamic mechanisms of action (Negus & Miller, 2014); however, the role of time course has not been systematically examined as a determinant of abuse-related ICSS facilitation indicative of abuse potential. In ICSS procedures, the drug effects act as a contextual stimulus; the operant contingency is electrical brain stimulation immediately following manipulation of a lever. Due to this procedural feature, we hypothesized that the maximal magnitude of facilitation of ICSS, our typical metric of abuse potential, would not be sensitive to changes in rate of onset of a drug. To test this hypothesis, we compared cocaine and two phenyltropane cocaine analogs

with staggered rates of onset. Cocaine was reported to have the most rapid onset, followed by WIN-35428 with an intermediate onset, and RTI-31 with the latest onset.

Methods

Apparatus

ICSS studies were conducted as described previously (Baird et al., 2021; Bonano et al., 2014; Davies et al., 2020; Johnson et al., 2018). Behavioral sessions occurred inside modular operant test chambers (29.2 x 30.5 x 24.1 cm; Med Associates, St. Albans, VT, USA) made of stainless steel and clear polycarbonate with a grid floor. Test chambers were contained within opaque sound-attenuating cabinets. The test chamber contained a response lever positioned 3 cm above the floor and 7.6 cm below three stimulation lights (red, yellow, and green), a 2 W house light, and an ICSS stimulator. A bipolar cable and swivel commutator (Model SL2C; Plastics One, Roanoke, VA, USA) connected the ICSS stimulator to the electrode. Data collection and ICSS programming were controlled using a computer system running Med-PC IV software (Med Associates, St. Albans, VT, USA).

Training

Rats were trained to depress a lever under a fixed-ratio 1 (FR 1) schedule to receive electrical brain stimulation consisting of a 0.5-s train of 0.1 ms electrical pulses. Electrical stimulation was accompanied by the simultaneous illumination of the stimulus lights above the lever. Additional lever presses during each 0.5-s period of electrical stimulation had no scheduled consequences. Initial training consisted of 30- to 60-minute sessions, during which the frequency of electrical stimulation was held constant at 126 Hz and the amplitude was adjusted to a magnitude sufficient to maintain responding in excess of 30 stimulations per minute. Once sufficient rates of

responding were acquired, rats entered the second phase of training in which frequency manipulations were introduced. These sessions consisted of 3 to 6 consecutive 10-minute components. Each component was divided into ten one-minute frequency trials, and the frequency of stimulation decreased across trials from 158 to 56 Hz in 0.05 log increments. Each frequency trial began with a 10-s time-out period, during which five pulses of electrical stimulation at the frequency for that trial were presented noncontingently, and was followed by a 50-s response period, during which lever presses resulted in contingent electrical stimulation under the FR1 schedule. Amplitudes were adjusted until individual rats responded at high rates for the highest 3 to 4 frequencies and at low rates for the remaining frequencies. Once responding was stable during 3-component training sessions for at least three consecutive days, amplitudes were fixed for the remainder of the study (130-310 µA).

Testing

The goal of the study was to evaluate and compare the time courses of three different DAT inhibitors: cocaine, WIN-35428, and RTI-31. Each DAT inhibitor was evaluated in in a separate cohort of rats (n = 6-7; 3-4 males and 3 females). Test sessions consisted of three daily baseline components followed first by IP injection of a dose of the test drug and then by 12 successive 10-min test components for the first 120 min (see Figure 3-1). Rats were placed back into their home cage following these initial 12 test components and returned to the testing chamber for additional test components beginning 300 and 1440 min post-injection (WIN-35428 and RTI-31 only). Within each group, dose order was randomized across rats with a Latin-square design. Doses tested were as follows: cocaine, 1.0-10 mg/kg; WIN-35428, 0.10-0.32 mg/kg; RTI-31, 0.032-0.10 mg/kg. Doses for each drug were based on published and preliminary studies (Baird et al., 2021; Johnson et al., 2018; Wee et al., 2006), which indicated that these doses ranges were associated

with dose-dependent ICSS facilitation, and that higher doses were associated with evidence for behavioral toxicity such as severe stereotypies and a decline from peak levels of ICSS facilitation. Testing was performed on Tuesdays and Fridays, and three-component training sessions were conducted on all other weekdays.

Data Analysis

The first baseline component of each session was considered an acclimation component, and data were discarded. The primary dependent measure for this study was the total number of stimulations delivered across all 10 frequencies during each 10-min component. To normalize these raw data, data for each test component in each rat were expressed as a percentage of the mean total stimulations per component earned during the second and third baseline components for that rat on that day (%BL). Thus, % BL was calculated as (total stimulations during a 10-min test component/mean total stimulations during baseline components) × 100. During the initial 120 min of each test session, when test components were presented continuously, we observed a modest but significant decline in responding over time after saline vehicle administration. To adjust for this changing baseline after vehicle administration and isolate drug-induced changes in ICSS performance, data were further transformed into a "Difference Score," calculated as the $\%BL_{drug}$ - $\%BL_{vehicle\ mean}$ at the corresponding time point.

To provide a more granular analysis of ICSS data at selected times, we also evaluated the total number of stimulations during each 1-min frequency trial. To normalize these raw data, 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 for that rat on that day. Thus, % MCR = (rate during a frequency trial/MCR) × 100. Normalized ICSS rates at each frequency were used to generate a 'frequency-rate' curve

for each experimental manipulation, and frequency-rate curves are shown at times of approximate peak effect for each drug.

Data were averaged across rats and analyzed by repeated measures one or two-way ANOVA as appropriate. For data sets with any missing values, data were evaluated using a mixed-effects analysis using the restricted maximum likelihood method, and conditions with missing values are specified in the figure legends. For all analyses, the Geisser-Greenhouse correction was used to adjust for unequal standard deviations. A significant ANOVA was followed by Dunnett's post hoc test, and the criterion for significance was set at $p < 0.05$. Data relating time on the X axis to the "Difference Score" ($\%BL_{drug}$ - $\%BL_{vehicle\ mean}$) on the Y axis were used to calculate four additional values for each drug: (1) the maximum effect (E_{max}; calculated by averaging the maximum Difference Scores obtained at the highest dose for any time point in each individual rat), (2) time of maximum effect (T_{max}), calculated by averaging the log-transformed time of peak Difference Score in each rat for the highest dose tested), (3) time to 50% of maximum effect (T_{50}), calculated by interpolation of log-linear regression of ascending Difference Score time course, and (4) potency estimated as the ED_{75} (calculated by log-linear regression of E_{max} values for each dose as the effective dose to achieve a Difference Score of 75). E_{max}, T_{max}, T_{50}, and ED_{75} values were considered to be different across drugs if 95% confidence limits did not overlap. All statistical analyses were performed using GraphPad Prism 9.3.1 (GraphPad Software, San Diego, CA, USA).

Results

Under baseline conditions, electrical brain stimulation maintained frequency-dependent increases in ICSS rates. Across all rats, the mean MCR ± SEM was 53.5 ± 1.9 stimulations per trial and the mean ± SEM total number of stimulations per component was 214.2 ± 11.9. To maximize temporal resolution of drug-effect onsets, test sessions began with 12 consecutive 10-

min ICSS components to permit continuous assessment of drug effects, and Figure 3-2 shows a time-dependent decline in ICSS rates per component over these initial 12 components after saline vehicle administration. After a three-hour break between test sessions, response rates returned to initial levels. To control for this shifting vehicle baseline and to isolate drug-induced changes in behavior, drug data were normalized to vehicle data using the Difference Score.

Figure 3-3 shows the time course of drug effects on ICSS expressed as Difference Scores for cocaine (1-10 mg/kg), WIN-35428 (0.1-0.32 mg/kg), and RTI-31 (0.032-0.1 mg/kg). Table 3-1 shows E_{max}, T_{50}, and T_{max} values for the highest dose of each drug. All three drugs produced dose- and time-dependent ICSS facilitation with similar E_{max} values; however, time courses differed. In general, cocaine had the fastest onset and shortest duration, whereas RTI-31 had the slowest onset and longest duration. T_{max} values for both cocaine and WIN-35428 were significantly faster than for RTI-31. T_{50} values had the same rank-order as T_{max}, and T_{50} for WIN-35428 was significantly faster than for RTI-31; however, cocaine-induced facilitation of ICSS was too rapid to determine T_{50}.

Although these ICSS studies included both male and female rats, the experiments were not designed for the purpose of detecting sex differences. Due to the low N, this study is underpowered to detect sex differences, and therefore any differences, or lack thereof, should be interpreted with caution. Despite this, data from male and female rats were analyzed separately to look for any overt sex differences. The drug effects between male and female rats were qualitatively similar, with little apparent difference between sexes. However, there may be a trend for higher potency and E_{max} in female rats compared to male rats.

Figure 3-4 shows dose-effect data for all three test drugs at the approximate T_{max} time of peak effect. Regression analysis showed a significant linear dose-effect relationship for each drug,

and ED_{75} values are shown in Table 3-1. WIN-35428 and RTI-31 were approximately 38 and 110 times more potent than cocaine, respectively, to facilitate ICSS. Each drug produced qualitatively similar dose-dependent leftward shifts in ICSS frequency-rate curves.

Discussion

In contrast to their different break points in a progressive-ratio drug self-administration procedure in monkeys (Wee et al., 2006), cocaine, WIN35428, and RTI-31 produced similar magnitudes of peak facilitation in the rat ICSS procedure. Thus, for these compounds with a shared mechanism of action but different time courses, peak magnitudes of ICSS facilitation did not correspond to peak break points in the drug self-administration procedure. One implication of this finding is that peak ICSS facilitation may be insufficient as a metric to predict the magnitude of reinforcing effects in drug self-administration procedures or the magnitude of abuse potential in humans. In particular, peak ICSS facilitation may serve as an exaggerated metric of abuse potential for slow-onset drugs.

Although peak ICSS facilitation may be insufficient alone as a reliable metric of abuse potential, it is relevant to note that ICSS readily permits assessment of drug time course as well as peak drug effect (Negus & Miller, 2014). For example, the procedure used here determined ICSS frequency-rate curves every 10 min and provided sufficient temporal resolution to differentiate the rates of onset and durations of action of the three drugs tested. Moreover, time courses for these drugs determined from ICSS studies correspond well with time courses assessed in rats and mice using other dependent measures such as locomotor activity (de Saint Hilaire et al., 1995; Hemby et al., 1995; Tolliver & Carney, 1995) and DAT binding (Wee et al., 2006). Thus, ICSS procedures permit assessment of both the time course and magnitude of abuse-related ICSS facilitation, and both time course and peak effect should be jointly considered in the use of ICSS data to make

predictions of abuse potential. High abuse potential is indicated not only by a high magnitude of ICSS facilitation, but also by rapid onset of that facilitation.

Tables and Figures

Table 3-1. T_{max}, E_{max}, and ED_{75} of difference score data

Drug	**T_{max} (min)**	**E_{max} (%BL Drug – %BL Veh)**	**ED_{75} (mg/kg)**
Cocaine	13.0 (7.1-23.8)	122.6 (79.2-166.0)	5.4 (3.5-7.9)
WIN35428	24.7 (16.6-36.8)	160.2 (113.5- 207.0)	0.14 (0.11-0.17)
RTI-31	83.0 (75.0-91.7)	132.4 (107.0-157.8)	0.049 (0.039-0.058)

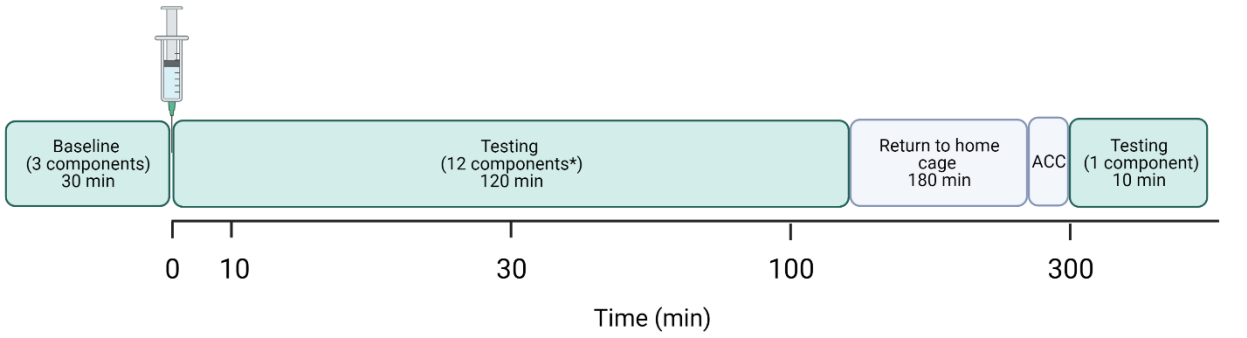

Figure 3-1: Experimental Design of DAT Inhibitor ICSS Time Course

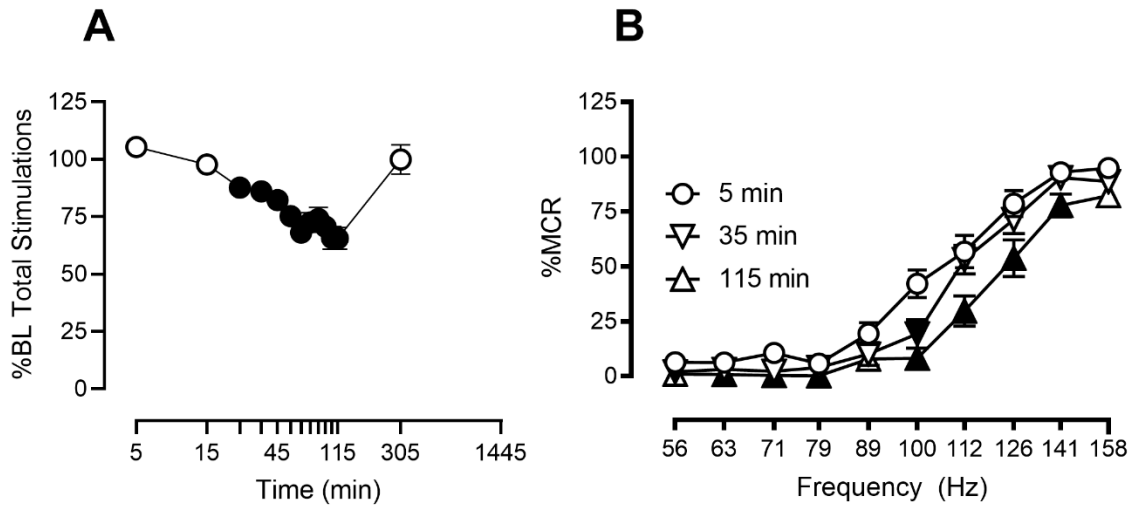

Figure 3-2. Time-dependent decrease of ICSS rates following vehicle injection

Time-dependent decrease of ICSS rates following vehicle injection. Panel A shows the time course of ICSS following injection of saline summarized as percent baseline number of stimulations per component delivered across all stimulation frequencies. Abscissa: Time post-injection of i.p. saline. Ordinate: Percent baseline number of stimulations per component. Each data point represents the mean ± SEM of 19 rats, and filled points indicate a significant difference from the 5 min component determined using a repeated measures mixed-effects analysis followed by Dunnett's post hoc test ($P < 0.05$). Mixed-effects analysis indicated a significant difference between time points ($F(3.764, 65.87) = 22.36$; $P < 0.0001$). Panel B shows selected frequency-rate curves from points along the declining segment of the vehicle %BL time course. Abscissa: Frequency of electrical brain stimulation in Hz (log scale). Ordinate: Number of stimulations per trial expressed as a percentage of daily maximum control rate (MCR). Each data point represents the mean ± SEM of 19 rats, and filled points represent frequencies at which ICSS rates were different compared to the 5 min component as determined by a repeated-measures two-way

ANOVA followed by a Dunnett post hoc test ($P<0.05$). Two-way ANOVA indicated a significant frequency x time interaction ($F(6.142, 110.6) = 2.801$; $P=0.0135$).

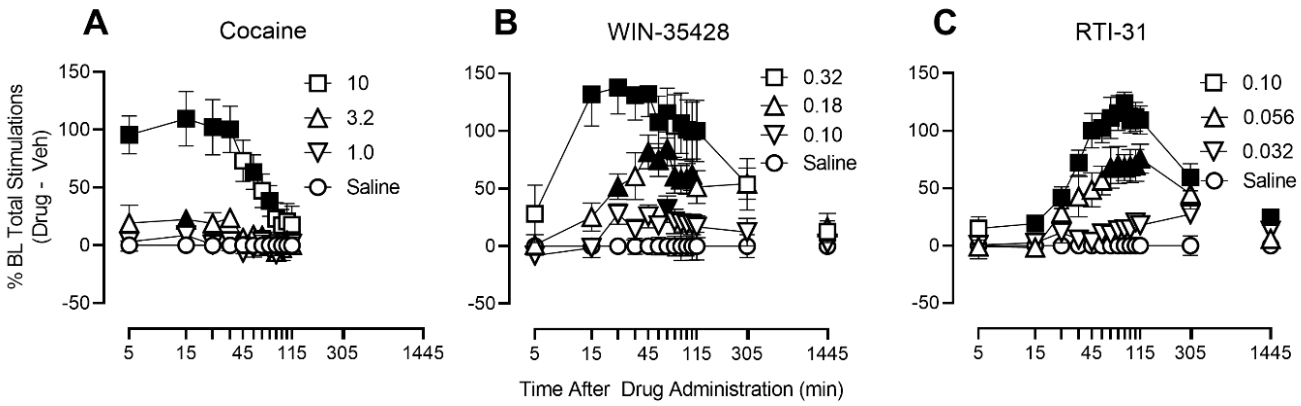

Figure 3-3: Time course of ICSS difference scores for cocaine, WIN-35428, and RTI-31

Abscissae: Time in min (log scale) after administration of IP saline or test drug, with numerals indicating the midpoint of each 10-min component (i.e. the number "5" indicates the midpoint of the component conducted from 0-10 min after injection). Ordinates: Number of stimulations per component expressed as Difference Scores (%BL Drug - %BL Vehicle). Each data point represents the mean ± SEM of 6-7 rats, and filled points indicate a significant difference from saline vehicle at that time point using a repeated-measures two-way ANOVA (cocaine, RTI-31) or mixed-effects analysis (WIN-34528) followed by a Dunnett post-hoc test ($p < 0.05$). Statistics for significant dose x time interactions were as follows: cocaine ($F(3.155, 15.78) = 7.244$; $p = 0.0026$), WIN-35428 ($F(3.062, 15.15) = 5.600$; $p = 0.0084$), RTI-31 ($F(4.123, 24.74) = 9.908$; $p < 0.0001$). A mixed-effects analysis was used for WIN-34528 because there was missing data for one rat at the 1445-min time point for doses of 0.10 and 0.18 mg/kg.

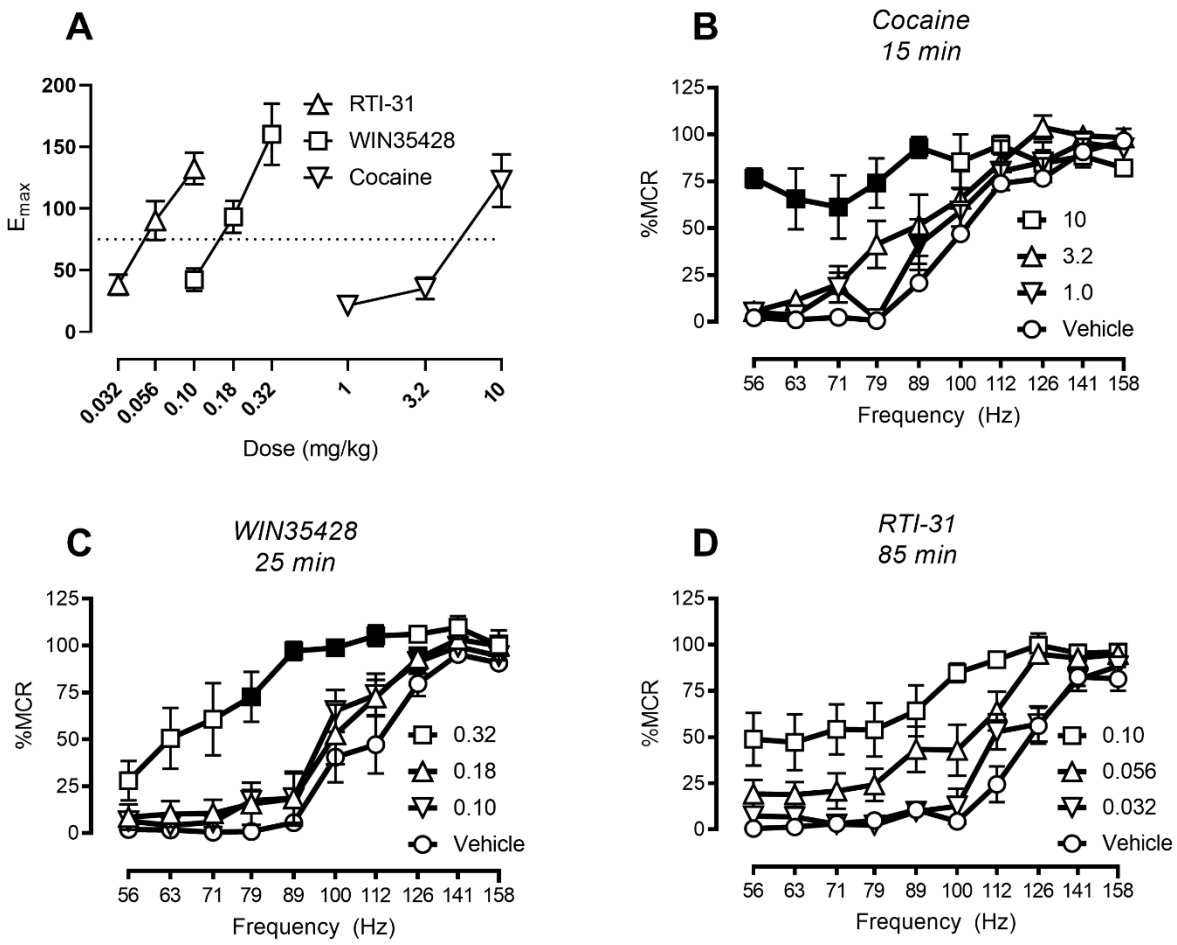

Figure 3-4. Dose-effect comparisons for cocaine, WIN-35428, and RTI-31.

Panel A shows the maximum effect of each drug dose across all time points. Dotted line indicates the difference score used for relative potency comparisons (ED75-). Abscissa: Drug dose in mg/kg. Ordinate: Emax for each drug dose. Each point represents the mean ± SEM of 6-7 rats. Statistics for linear regressions are as follows: cocaine ($p=0.0035$; $r^2=0.5901$), WIN35428 ($p=0.0001$; $r^2= 0.6118$), RTI-31 ($p<0.0001$; $r^2=0.6012$). Panels B-D show frequency-rate curves for cocaine, WIN35428, and RTI-31 during the component closest to their calculated Tmax. Abscissae: Frequency of electrical brain stimulation in Hz (log scale). Ordinates: Number of stimulations per trial expressed as a percentage of daily maximum control rate (MCR). Each data

point represents the mean ± SEM from 6-7 rats, and filled points represent frequencies at which ICSS rates were different compared to saline as determined by a repeated-measures two-way ANOVA followed by a Dunnett post-hoc test ($P<0.05$). Two-way ANOVA for frequency x dose interactions were as follows: cocaine ($F(4.028, 20.14) = 5.873$; $P=0.0026$), WIN35428 ($F(3.355, 16.77) = 3.922$; $P=0.0242$), RTI-31 ($F(3.888, 23.33) = 2.651$; $P=0.0600$). Although the frequency x dose interaction was not significant for RTI-31, there was a significant main effect of dose ($F(1.553, 9.316) = 40.88$; $P<0.0001$).

CHAPTER 4: EVALUATION OF METHCATHINONE ENANTIOMERS AND RELATED NOVEL COMPOUNDS USING ICSS

Introduction

One common feature of NPS is the gradual evolution of these compounds by small structural changes in order to avoid detection, circumvent scheduling laws, and/or enhance desired pharmacological effects. In order to effectively screen NPS, ICSS procedures have to be sensitive enough to detect and quantify differences in abuse potential resulting from these minor structural modifications. Methcathinone has a stereocenter at the α carbon, which generates two enantiomers: R and S. Locomotor and drug discrimination procedures have indicated that S(-)methcathinone is approximately 3 times more potent than R(+)methcathinone (Glennon et al., 1995). The sensitivity of our ICSS procedure was tested against racemic methcathinone and its two enantiomers. Additionally, minor structural modifications were made by adding or removing a methyl group from the α-carbon, generating the achiral analogs α-methyl- and α-desmethyl-methcathinone. For these five total drug preparations, dose-effect and time-course data was collected and their effects were compared to estimate potency, efficacy, and temporal differences.

Methods

Subjects

Subjects consisted of 13 adult Sprague-Dawley rats (Envigo, Indianapolis, IN, USA) weighing from 317 to 365 g at the time of surgery. The rats were individually housed in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) and maintained on a 24-hour light/dark cycle (lights on from 6:00 am to 6:00 pm).

Rats had free access to food and water except during behavioral sessions. Animal care and research complied with the National Institutes of Health guidelines for the care and use of animal subjects in research, and all protocols were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee.

Surgical Procedure

Surgical and experimental procedures were similar to those described previously. Anesthesia was induced by inhalation of 2.5-3.0% isoflurane (Zoetis Inc., Kalamazoo, MI, USA) in oxygen until the rat was unresponsive to toe pinch, and this level of anesthesia was maintained throughout surgery. A stainless steel, bipolar electrode (Plastics One, Roanoke, VA, USA) was implanted via stereotaxic surgery by inserting the cathode into the left medial forebrain bundle at the lateral hypothalamus (2.8 mm posterior to bregma, 1.7 mm lateral to the midsagittal suture, and 8.8 mm ventral to the exterior surface of the skull). The anode was grounded by coiling around one of three screws anchored into the dorsal surface of the skull. The electrode, screws, and grounding wire were permanently affixed to the skull using dental acrylic resin. Ketoprofen (5 mg/kg) was administered for post-operative analgesia immediately and 24 h after surgery, and rats were allowed to recover for at least 7 days prior to commencing ICSS training.

Apparatus

Behavioral sessions were conducted in modular operant test chambers (29.2 x 30.5 x 24.1 cm) (Med Associates, St. Albans, VT, USA) constructed of stainless steel and clear polycarbonate with a grid floor and housed inside ventilated sound-attenuating cubicles. The test chamber contained a response lever located 3 cm above the floor and 7.6 cm below three stimulation lights (red, yellow, and green), a 2 W house light, and an ICSS stimulator. A bipolar cable and swivel

commutator (Model SL2C; Plastics One, Roanoke, VA, USA) connected the ICSS stimulator to the electrode. Data collection and ICSS programming were mediated by a computer system running Med-PC IV software (Med Associates, St. Albans, VT, USA).

Following initial shaping of lever-press responding, rats were trained under a fixed-ratio 1 (FR 1) schedule of brain stimulation. 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 increased for each rat until it sustained ICSS rates > 30 stimulations/min. This intensity was then held constant, 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. The stimulation intensity was adjusted up or down to yield baseline frequency-rate curves that had high rates of reinforcement for the upper frequencies and minimal responding at the remaining frequencies. This intensity (110-340 μA across rats) was then held constant throughout the study. Training continued until frequency-rate curves were not statistically different over three days of training as indicated by lack of a significant effect of "day" in a two-way analysis of variance (ANOVA) with frequency and day as the two variables (see data analysis).

Each drug was evaluated with dose-effect and time-course testing procedures. For dose-effect studies, test sessions consisted of three sequential "baseline" components followed first by a time-out period and then by two sequential "test" components. A single dose of test drug was administered at the beginning of the time-out period, and dose-effects studies with all drugs were conducted with 10-min time-out periods and associated 10-min pretreatment times. *R*(+)-Methcathinone was also tested in dose-effect studies with 30-min time-out periods and 30-min pretreatment times because time-course studies indicated maximal ICSS facilitation at 30 min (see Results). Time-course test sessions consisted of three consecutive baseline components followed first by drug injection and then by pairs of consecutive test components beginning after 10, 30, 100 and 300 min. Five drugs were tested: racemic MCAT (0.10 – 1.0 mg/kg), *S(-)*MCAT (0.10-1.0 mg/kg), *R(+)*MCAT (0.10-3.2 mg/kg), **α**-methyl MCAT (0.32-3.2 mg/kg), and **α**-*des*methyl-MCAT (1.0-32 mg/kg). For dose-effect studies, drug doses were counterbalanced across rats using a Latin-Square design. The maximally effective dose of each drug was tested in time-course studies. Rats were tested in groups of six (n=6) except for the time course on *S(-)*MCAT, which was tested with five animals due to the spontaneous detachment of the electrode in one animal. Testing was generally performed on Tuesdays and Fridays. All testing was performed during the light phase of the light/dark cycle.

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 for that rat on that day. 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 each pair of 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 $p < 0.05$.

To provide a secondary summary measure of drug effects, the total number of stimulations delivered across all 10 frequency-trials of each component was determined for each drug dose or time point. These summary data were normalized to individual baseline data using the equation % baseline total stimulations per component = (mean total stimulations per test component) / (mean total stimulations per baseline component) x 100. Data were then averaged across rats and plotted as a function of drug dose or time. Dose-effect functions were used to calculate measures of drug potency and efficacy. For potency, linear regression of logarithm-transformed doses was used to identify the ED_{150} value, defined as the effective dose to reach 150% of baseline total stimulations. For efficacy, the maximum effect (E_{Max}) was defined as the peak % baseline total stimulations per component at any drug dose. ED_{150} and E_{max} values were considered to be significantly different across drugs if 95% confidence limits did not overlap. Statistical analyses were performed using Prism 8.1.2 (GraphPad Software, San Diego, CA, USA).

Results

Under baseline conditions, electrical brain stimulation maintained a frequency-dependent increase in ICSS rates. Across all rats in the study, the mean ± SEM MCR was 59.0 ± 2.6 stimulations per trial, and the mean ± SEM total stimulations earned per baseline component was 249.6 ± 12.8 stimulations per component.

Figure 4-1 shows that all five compounds produced dose-dependent ICSS facilitation manifested as leftward and upward shifts in ICSS frequency-rate curves. Two-way ANOVA indicated significant frequency x dose interactions for racemic MCAT [$F(3.083, 15.42) = 8.577$, $P=0.0013$], S(-)MCAT [$F(3.082, 15.41) = 9.105$, $P=0.0010$], R(+)MCAT [$F(3.379, 16.90) = 7.849$, $P=0.0013$], α-methyl MCAT (3) [$F(2.528, 12.64) = 9.465$, $P=0.0020$], and α-des-methyl MCAT (4) [$F(3.648, 18.24) = 9.864$, $P=0.0003$]. A higher 32 mg/kg dose of α-des-methyl MCAT (4) was tested in two rats and produced robust stereotypies (e.g. excessive sniffing) without a further increase in ICSS frequency-rate curves (data not shown); therefore, further testing of this dose was discontinued for 4, and data were not included in statistical analysis.

Table 4-1 compares the potencies (ED_{150} values) and efficacies (E_{max} values) of drugs to facilitate ICSS. The S(-)-enantiomer of MCAT was significantly more potent than its R(+)-enantiomer by 2.4-fold as indicated by non-overlapping 95% confidence intervals. Both racemic MCAT and α-methyl MCAT (3) displayed potencies that were intermediate between potencies of the MCAT isomers, and statistically indistinguishable from either each other or from the potencies of the MCAT optical isomers. In contrast, α-des-methyl MCAT (4) was significantly less potent than all other compounds. Emax values were statistically indistinguishable across all compounds.

Figure 4-2 shows the time course for the highest E_{max} dose of each compound. In general, all agents produced peak ICSS facilitation after 10 to 30 min, and effects were no longer significant after 300 min. Two-way ANOVA indicated significant frequency x time interactions for racemic MCAT [1.0 mg/kg; $F(3.168, 15.84) = 8.265$, $P=0.0014$], S(-)MCAT [1.0 mg/kg; $F(2.742, 10.97) = 6.455$, $P=0.0098$], R(+)MCAT [3.2 mg/kg; $F(3.357, 16.78) = 11.72$, $P=0.0002$], α-methyl MCAT [1.0 mg/kg; $F(3.007, 15.04) = 4.363$, $P=0.0212$], and α-des-methyl-MCAT [10 mg/kg; $F(3.035, 15.17) = 10.00$, $P=0.0007$].

Discussion

This study evaluated racemic methcathinone, its two individual enantiomers, and two novel achiral analogs of methcathinone. All five of these compounds had doses that were sufficient to facilitate ICSS, and facilitation increased in a dose-dependent manner. Only methcathinone and its enantiomers have been previously studied. These results are in accord with the results from the current study. A previous study on ICSS facilitation by racemic methcathinone was consistent with the ICSS facilitation observed in the present study (Bonano et al., 2014). Glennon et al. (1995) found an approximate 3-fold potency difference in their assays of locomotor stimulation and drug discrimination. A recent study confirmed the greater potency of the S enantiomer in cathinone compounds (Mayer et al., 2022). In this study, our ICSS procedure had sufficient sensitivity to discriminate a 2.4-fold difference in potency. Addition of a methyl group to form α-methyl-methcathinone had no effect on the drug's potency or efficacy compared to methcathinone. However, the removal of a methyl group to form α-desmethyl-methcathinone resulted in a substantial and significant decrease in potency, approximately 16-fold. The overall efficacy of these compounds to facilitate ICSS is similar, indicating similar abuse potential.

Tables and Figures

Table 4-1. Potency (ED_{150}) and efficacy (E_{max}) of methcathinone isomers and analogs to facilitate ICSS in rats

Drug	**ED_{150} (95% CI) (mg/kg)**	**E_{max} (95% CI) (% BL)**
R(+)methcathinone	0.60 (0.37-0.98)	207 (166-248)
S(-)methcathinone	0.25 (0.17-0.34)	189 (159-219)
(±)methcathinone	0.38 (0.25-0.59)	180 (141-220)
α-methyl-methcathinone	0.36 (0.22-0.61)	189 (151-226)
α-desmethyl-methcathinone	6.3 (3.6-26.2)	164 (119-210)

95% confidence limits are shown in parentheses (n=5-6 animals per dose), and values are considered significantly different if 95% CI values do not overlap.

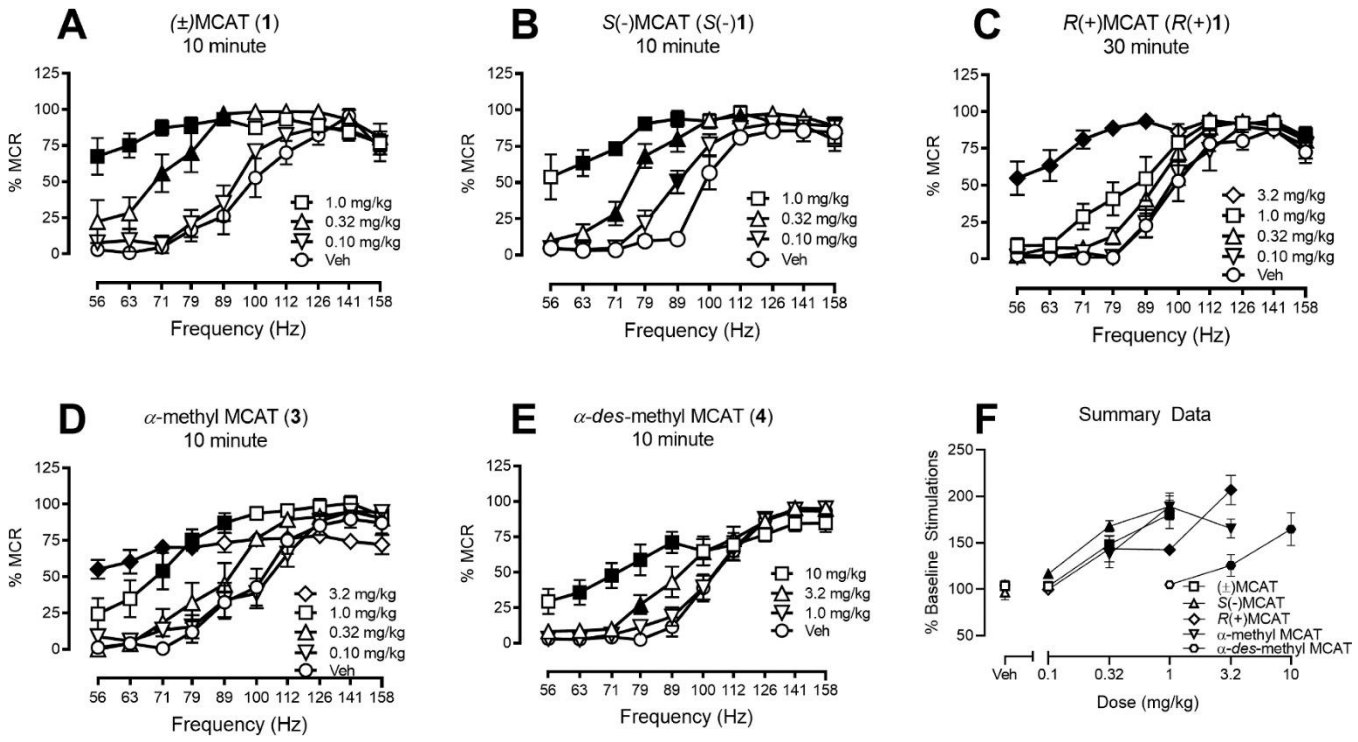

Figure 4-1. Dose effect of methcathinone enantiomers and novel compounds on ICSS

Dose-dependent ICSS facilitation by racemic MCAT, S(−)MCAT, R(+)MCAT, α-methyl MCAT, and α-des-methyl MCAT. Panels A−E show drug effects by dose on full ICSS frequency-rate curves. Abscissas: Frequency of electrical brain stimulation in Hz (log scale). Ordinates: Percent maximal control reinforcement rate (%MCR). Legends indicate the drug name and doses. Filled points represent frequencies at which the ICSS rate was statistically different from vehicle as determined by two-way repeated-measures ANOVA followed by a Holm-Sidak post hoc test, $p < 0.05$. Panel F shows summary ICSS data expressed as percent baseline number of stimulations per component delivered across all brain-stimulation frequencies for all drugs. Abscissa: Drug dose in mg/kg (log scale). Ordinate: Percent baseline number of stimulations per component. Filled points represent significant difference compared to vehicle using a repeated-measures one-way ANOVA for each drug followed by a Holm-Sidak post hoc test, $p < 0.05$. All points show mean ± SEM for six rats ($n = 6$).

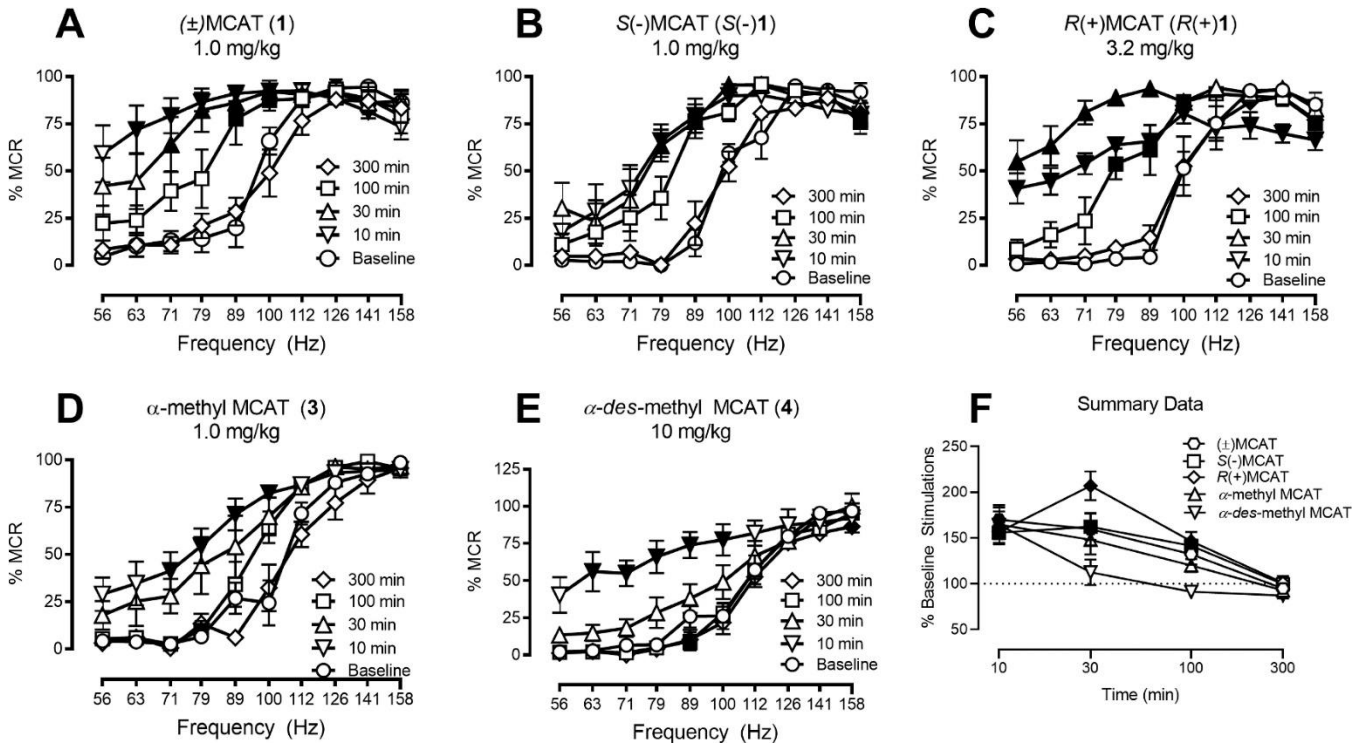

Figure 4-2. Time course of methcathinone enantiomers and novel compounds on ICSS

Time course of ICSS facilitation by racemic MCAT, S(−)MCAT, R(+)MCAT, α-methyl MCAT, and α-des-methyl MCAT. Panels A−E show full frequency-rate curves determined 10, 30, 100, and 300 min after drug injection. Abscissas: Frequency of electrical brain stimulation in Hz (log scale). Ordinates: Percent maximal control reinforcement rate (%MCR). Filled points represent frequencies at which the ICSS rate was statistically different from baseline as determined by two-way repeated-measures ANOVA followed by a Holm-Sidak post hoc test, $p < 0.05$. Panel F shows summary data for total stimulations per component compared to baseline for all drugs. Abscissa: Time after injection in minutes (log scale). Ordinate: Percent baseline number of stimulations per component. Filled points represent significant difference compared to baseline using a repeated-measures one-way ANOVA followed by a Holm-Sidak post hoc test, $p < 0.05$. All points mean ± SEM for six rats (n = 6) with the exception of S(−)MCAT, which had five subjects (n = 5).

CHAPTER 5: PHARMACOKINETIC EVALUATION OF METHCATHINONE ENANTIOMERS AND PK/PD ANALYSIS

Introduction

The effects of methcathinone on ICSS were determined in the previous chapter where it was shown that facilitation of ICSS by methcathinone is dose-dependent. Pharmacokinetic studies help gain a more proximal understanding of the relationship between the drug and the effects by measuring the concentration of the drug and potentially active metabolites that are in systemic circulation. Some drugs, such as mitragynine, the main constituent of the herbal preparation kratom, act as prodrugs. Prodrugs are inactive or less active than their metabolite(s) which drive the pharmacological effect. One method to explore this phenomenon is to conduct PK/PD analysis. A hysteresis plot can be constructed by measuring the blood concentrations of methcathinone and probable metabolites and comparing those against the magnitude of behavioral effects. The direction of the hysteresis loop can indicate a number of potential pharmacokinetic or pharmacodynamic effects, such as the presence of active metabolites, drug distribution, or the development of tolerance. The goal of this study was to develop a method to separate, identify, and quantitate the various chiral isomers of methcathinone and its metabolites using liquid chromatography tandem mass spectrometry. Two primary routes of metabolism were hypothesized: reduction of the β-ketone to a hydroxyl group, and N-demethylation of the amine group (see Figure 1-4).

Methods

Analytical Standards

R(+)methcathinone, S(-)methcathinone, (+/-)methcathinone-D3, R(+)cathinone, S(-)cathinone, (1R,2S)(-)ephedrine, (1S,2R)(+)ephedrine, (1S, 2R)(+)ephedrine-D3, (1R,2R)(-)pseudoephedrine, (1S,2S)(+)pseudoephedrine, (1S,2S)(+)pseudoephedrine-D3, (+/-)phenylpropanolamine, and cathine were purchased as certified reference standards from Cerilliant, Inc. (Round Rock, TX) and 4-hydroxyephedrine was purchased as reference material from Toronto Research Chemicals (Toronto, ON, Canada) and diluted to 1 mg/mL in methanol.

Sample Preparation

Specimens were thawed and vortex mixed prior to sampling 100 µL aliquots into a 13 x 100 mm borosilicate disposable glass tubes. 100 µL of internal standard solution (100 ng/mL of each R(+) and S(-)methcathinone-D3, (+)ephedrine, and (+)pseudoephedrine) in methanol was added to each test tube. Calibrators were fortified in a volume of 100 µL of methanol added to 100 µL of drug-free rat blood. 100 µL of methanol was added to all non-calibrator tubes. 200 µL of freezer-temperature acetonitrile (-20 °C) was added to each tube while vortex-mixing for 20 seconds. Tubes were then centrifuged at 3800 rpm for 10 minutes, and the supernatant was transferred to fresh 13 x 100 mm tubes; the remaining pellet and tube were discarded. The supernatant was evaporated using a LabConCo RapidVap at 40 °C at a pressure of approximately 10 psi. Dry samples were then reconstituted in 50 µL of 70:30 water:methanol and transferred into autosampler vials with 300 µL low-volume inserts for analysis.

Bioanalytical Method Validation

Calibrators were prepared for each analyte at (1, 3, 10, 30, 100, 300, and 1000 ng/mL), and the peak area ratio of the analyte's quantitative ion transition to the internal standard's quantitative ion transition was plotted against the known concentration. Quantitative measures were

interpolated based upon a linear regression of the calibrators' response ratios, with each term weighted by 1/x. Low, mid, and high controls were prepared at 3, 50, and 800 ng/mL in drug-free rat blood and and stored at -20 °C until analysis. Limit of detection (LOD) was defined as the lowest concentration calibration standard at which peak shape and ion transition ratios were acceptable (+/- 20% of the mean of all calibration standards) for all 5 validation runs. Quantitative bias and precision were calculated for methcathinone and cathinone by analyzing control samples prepared at 3 ng/mL, 50 ng/mL, and 800 ng/mL each in triplicate over 5 runs. Bias was calculated using the formula $\frac{nominal\ concentration - control\ grand\ mean}{nominal\ concentration}$ for each concentration (n=15 per concentration), and expressed as a percent. Between-run precision was calculated as the percent coefficient of variation (%CV; $\frac{standard\ deviation}{mean}$) of all 15 control samples. Within-run precision was calculated as the %CV of the three control samples in each run. For all analytes other than methcathinone and cathinone, only a qualitative method validation was performed to determine the LOD, as there were no peaks in any of the experimental samples drawn from rats that corresponded to any of these metabolites. Carryover was assessed by analyzing a matrix blank fortified with internal standard immediately following the highest calibrator. The fortified matrix blank was also used to analyze for interferences from stable-isotope internal standards. A lack of carryover or interference from internal standards was determined as a peak area less than 10% of the lowest calibration standard for each analyte.

To quantify the degree of ionization variability due to the effects of the sample matrix, one set of samples was prepared as neat standards of analytes reconstituted in 70:30 water:methanol at concentrations of 10 ng/mL and 300 ng/mL, and separately the deuterated internal standards at a concentration of 100 ng/mL. A separate set of samples (n=3 per concentration) was prepared by fortifying the supernatant of protein-precipitated blank matrix, evaporating, and reconstituting in

70:30 water:methanol. Neat standards were injected 6 times each under both the methcathinone and the metabolite methods, and each prepared vial of fortified matrix was injected twice each, for a total of 6 injections per concentration. The degree of ionization suppression or enhancement is calculated as $\left(\frac{mean\ area\ fortified-matrix\ samples}{mean\ area\ neat\ standards}\right) - 1$ expressed as a percentage.

Instrumental Analysis

Instrumental analyses were conducted on a Shimadzu LCMS-8050 liquid chromatograph tandem mass spectrometer controlled by LabSolutions software (Shimadzu Corporation, Kyoto, Japan). Quantitative analysis by LC-MS/MS was conducted in two stages. In the first stage, prepared samples are placed onto the instrument and analyzed for both enantiomers of methcathinone. Those samples remained in the refrigerated (5 °C) autosampler until the LC column could be exchanged the following day.

Methcathinone Separation and Quantitation by LC-MS/MS

The separation of R(+) and S(-)methcathinone was achieved using an InfinityLab Poroshell 120 Chiral-V LC column with dimensions 4.6 x 100mm and a particle size of 2.7 µm (Agilent Inc., Santa Clara, CA; P/N 685975-604). Elution occurred with an isocratic mobile phase system of 5% 20 mM ammonium formate in water and 95% methanol at a flow rate of 0.6 mL/min. The total run time of the method was 10 min. Additional settings for the liquid chromatograph system and mass spectrometer are presented in Table 5-1 and Table 5-2, respectively. Analyte-specific parameters, such as ion transitions, are presented in Table 5-3.

Metabolite Separation and Detection by LC-MS/MS

The separation of (+)cathinone, (-)cathinone, (+)ephedrine, (-)ephedrine, (+)pseudoephedrine, (-)pseudoephedrine, (+)phenylpropanolamine, (-)phenylpropanolamine, cathine ((+)norpseudoephedrine) was achieved using a Phenomenex Lux® AMP LC column with dimensions 3.0 mm x 150 mm and a particle size of 3.0 µm (Phenomenex Inc., Torrance, CA; P/N 00F-4751-Y0). Mobile Phase A was 5 mM ammonium bicarbonate in water adjusted to approximately pH 11 using ammonium hydroxide (2%) and Mobile Phase B was 95% methanol at a flow rate of 0.4 mL/min. The initial gradient started at 32.5% B and held for 7 minutes, then increased linearly to 45% B at 15 min. The gradient increased to 95% B for two min to wash the column, and returned to 32.5% B for 2 min at the end of the method. The total run time of the method was 20 min. Additional settings for the liquid chromatograph system are presented in Table 5-4 and general mass spectrometer parameters were the same as the methcathinone method above (Table 5-2). Analyte-specific parameters, such as ion transitions, are presented in Table 5-3.

Subjects

Subjects consisted of 4 adult Sprague-Dawley rats (Envigo, Indianapolis, IN, USA). The rats were individually housed in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) and maintained on a 24-hour light/dark cycle (lights on from 6:00 am to 6:00 pm). Rats had free access to food and water. Animal care and research complied with the National Institutes of Health guidelines for the care and use of animal subjects in research, and all protocols were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee.

Surgical Procedure

The rats were equipped with indwelling intravenous jugular catheters by Dr. Drew Townsend using a method described in depth elsewhere (Townsend et al., 2021). Briefly, under isoflurane anesthesia (2-3% in oxygen), the proximal end of a polyurethane catheter (1.0 mm OD, 0.6 mm ID) was inserted approximately 3 cm (1 cm/100 g rat weight) into the right jugular vein and secured in place. The distal end was routed subcutaneously to a vascular access port (VABR1B/22, Instech Laboratories, Plymouth Meeting, PA) implanted just posterior to the scapulae along the midline of the back. Catheters were flushed with 0.1 mL of 4 mg/mL gentamicin and 0.1 mL of heparinized saline (30 U/mL) following surgery, and daily afterward to maintain catheter patency. Ketoprofen (5 mg/kg) was administered subcutaneously for post-operative analgesia immediately and 24 h following surgery. Rats were allowed at least one week to recover from surgery before any tests were performed.

Drugs

R(+) and S(-)methcathinone hydrochloride were generously provided by Dr. Dukat and Dr. Glennon of the Virginia Commonwealth University Department of Medicinal Chemistry. Methcathinone solutions were prepared daily by dissolving the salt in bacteriostatic saline (Hospira Inc). Drugs were administered via intraperitoneal injection at a fixed volume of 1 mL/kg for 1.0 mg/kg S(-)methcathinone and 3.2 mg/kg R(+)methcathinone. Doses were selected based upon peak behavioral response in the ICSS procedure discussed in the previous chapter (see Figure 4-2).

Sample Collection

Blood was drawn using the indwelling intravenous jugular catheter. The volume of each catheter lumen was approximately 10 µL. Blood draws were preceded by the administration of

approximately 20 µL of heparinized saline through the jugular catheter using a 1 mL insulin syringe outfitted with the PNP3M injector (Instech Laboratories, Plymouth Meeting, PA) to adapt to the vascular access port. Next, 200-250 µL of blood was collected using a new syringe and adapter. Blood specimens were dispensed into green-topped lithium-heparin BD Microtainers™ (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), capped, and immediately inverted ten times. The addition of heparin prior to collection prevented blood clotting within the catheter or the syringe adapter during sample collection and storage, and the minimal volume prevented excessive sample dilution. Following sample collection, an equal amount of saline was administered via the catheter to replace lost fluid in the rats as well as to flush the catheter of any accumulated debris. A blood sample was collected on each test day prior to drug administration to verify the drug-free status of the rat as well as to confirm catheter patency prior to initiating the experiment. Once this pre-session sample (t = 0 min) was collected, rats (N=4) were dosed with either R(+) or S(-)methcathinone via IP injection. All rats received both treatments with a minimum of one week between experiments. Samples were drawn 3, 10, 30, 100, and 300 minutes following drug administration. Blood samples were drawn over a period of approximately 1 min, and the rats remained in their home cage between samplings. All samples were stored immediately on ice and transferred to a freezer at -20 °C as soon as possible, where they remained until analysis.

Results

The bioanalytical method was able to resolve enantiomers of methcathinone and cathinone as well as the diastereomers of phenylpropanolamine/cathine. The diastereomers of ephedrine/pseudoephedrine were separated but only achieved partial resolution, precluding accurate quantitation. Individual enantiomers of 4-hydroxyephedrine were not separated. Representative chromatography can be seen in Figure 5-1 (blank), Figure 5-2 (methcathinone),

Figure 5-3 (cathinone), Figure 5-4 (ephedrine and pseudoephedrine), Figure 5-5 (phenylpropanolamine and cathine), and Figure 5-6 (4-hydroxyephedrine). None of the chromatograms indicated the presence of interferences from the matrix or from stable isotope labeled internal standards. There was a strong signal in the methcathinone-D3 chromatogram due to the presence of ephedrine and pseudoephedrine that was resolved in the course of method development. Carryover was not detected for any analyte in samples following concentrations of 1000 ng./mL. Two pairs of enantiomers, methcathinone and cathinone, were able to be quantified with a reasonable degree of accuracy and precision. The lower limit of quantitation (10 ng/mL) for both enantiomers of cathinone were above the lowest control, and therefore only the mid and high controls were included in the precision and bias calculations for those analytes. The greatest magnitude of bias observed was -26 % for the low S(-)methcathinone control. Between-run precision was worst for R(+)cathinone with a CV of 19% for the high control. Within-run precision was worst for R(+)cathinone with a CV of 32% for one run. Overall, all four analytes showed a negative bias for all controls, with the greatest inaccuracy for the low methcathinone controls. Quantitative analysis of both cathinone enantiomers had poor precision across the five days. A full tabulation of the quantitative validation results are contained in Table 5-6. Ionization efficiency was greatly impacted by the matrix, with enhancement as high as 89% for S(-)methcathinone and suppression as low as -98% for 4-hydroxyephedrine. Limits of detection were 1 ng/mL for methcathinone and 4-hydroxyephedrine, 3 ng/mL for ephedrine and pseudoephedrine, and 10 ng/mL for cathinone, phenylpropanolamine, and cathine.

Blood sampled from four rats at 0, 3, 10, 30, 100, and 300 min was analyzed for the presence of these compounds. The 0-min blood samples drawn prior to the administration of methcathinone were negative for all compounds. None of the blood samples at any of the time

points had concentrations above the limit of detection for ephedrine, pseudoephedrine, 4-hydroxyephedrine, phenylpropanolamine, or cathine.

Figure 5-7 shows measured blood concentrations of methcathinone and cathinone over the full time course. The following quantitative values are expressed as the arithmethic mean (± SD). Samples collected after the i.p. administration of R(+)methcathinone had peak concentrations of R(+)methcathinone of 266 (± 124) ng/mL at 3 min, and peak concentrations of S(-)methcathinone of 165 (± 66) ng/mL at 3 min. By the 300 min time point, R(+)methcathinone concentrations diminished to 0 and S(-)methcathinone concentrations were negligible at 1.1 (± 0.9) ng/mL. These samples also contained the metabolite R(+)cathinone, which peaked at a concentration of 54 (± 24) ng/mL at 30 min. S(-)cathinone was measured above the limit of detection in only one rat. Samples collected after the i.p. administration of S(-)methcathinone had peak concentrations of S(-) methcathinone of 87 (± 17) ng/mL at 3 min, and peak concentrations of R(+)methcathinone of 53 (± 11) ng/mL at 3 min. Concentrations of both enantiomers of methcathinone were negligible below LOD at 300 min. No cathinone was detected in these samples. Using a semilogarithmic plot and linear regression of the concentration at points 3 through 100 min, the half-life for R(+)methcathinone was 32 min (95% CI: 26-41 min) and the half-life for S(-)methcathinone was 40 min (95% CI: 35-46 min). Figure 5-8 shows counterclockwise hysteresis loops for both R(+) and S(-)methcathinone.

Discussion

The developed method demonstrated the ability to separate enantiomers of the chiral molecules associated with methcathinone and its metabolism. Detection limits were likely impacted substantially by the robust matrix effects on ionization efficiency, and the desired 1 ng/mL LOD was not achieved for most of the analytes. Although cathinone was the only detected

metabolite, the high LOD for the method precludes definitively stating that they are fully absent. Quantitative bias and precision exceeded the desired limits of ± 20% for methcathinone and cathinone, but were sufficient for reasonable estimation. These parameters were likely affected by matrix impacts to ionization efficiency. The method was able to quantify R(+) and S(-)methcathinone in authentic samples drawn from rats dosed with R(+) or S(-)methcathinone, and the time course of those effects was roughly correlated with the behavioral effects observed in Chapter 4.

Tables and Figures

Table 5-1: Methcathinone chromatographic settings

Parameter	Value
Mobile Phase A	20 mM ammonium formate in water
Mobile Phase B	Methanol
Injection Volume	1 µL
Flow Rate	0.60 mL/min
Isocratic separation	5% A / 95% B
Needle Wash	90% methanol in water
Total method time	10 min
Column	Agilent PoroShell 120 Chiral-V 100 x 4.6 mm, 2.7 µm
Column Oven Temp.	40 °C

Table 5-2. Mass spectrometer method parameters

Parameter	Value
Interface Temperature	300 °C
Desolvation Line Temperature	200 °C
Heat Block Temperature	500 °C
Nebulizing Gas Flow	3 L/min
Heating Gas Flow	10 L/min
Drying Gas Flow	10 L/min

Table 5-3. Ion transitions and analyte-specific parameters

Compound	MS Mode (+/-)	Dwell Time (ms)	Precursor Ion (m/z)	Product Ion (m/z)	Q1 Pre-Bias (V)	Collision Energy (V)	Q3 Pre-Bias (V)
Methcathinone	+	40	164.0	146.0	-12	-17	-25
	+	40	164.0	131.0	-12	-21	-23
Methcathinone-D3	+	40	167.0	149.0	-12	-17	-14
	+	40	167.0	131.0	-12	-22	-22
Cathinone	+	40	150.0	117.0	-11	-25	-18
	+	40	150.0	132.0	-11	-14	-24
Pseudoephedrine/Ephedrine	+	40	166.0	148.0	-12	-16	-27
	+	40	166.0	133.0	-12	-22	-22
Phenylpropanolamine/Cathine	+	40	152.0	134.1	-10	-14	-23
	+	40	152.0	117.0	-10	-21	-21
4-hydroxyephedrine	+	40	182.2	164.2	-16	-14	-30

Table 5-4: Metabolite chromatographic settings

Parameter	Value	
Mobile Phase A	5 mM ammonium bicarbonate in water, pH 11	
Mobile Phase B	4:2:1 methanol:acetonitrile:isopropanol	
Injection Volume	5 µL	
Flow Rate	0.35 mL/min	
Strong Wash	4:2:1 methanol:acetonitrile:isopropanol	
Final Wash	90% methanol in water	
Total method time	20 min	
Column	Phenomenex Lux® AMP LC Column, 150 x 3.0mm, 3 µm	
Gradient Elution	0 min	67.5% A; 32.5% B
	7 min	67.5% A; 32.5% B
	15 min	55% A; 45% B
	15.5 min	5% A; 95% B
	17.5 min	5%A; 95% B
	18.0 min	67.5% A; 32.5% B

Table 5-5. Quantitative Bioanalytical Validation Results

The percent bias and precision for the methcathinone and cathinone quantitative validation are shown below. Between-run precision is the % CV across all controls on all days (n=15). Within-run precision is presented as the %CV range of per-run precision (n=3) over the five validation runs.

	Low (3 ng/mL)			Mid (50 ng/mL)			High (800 ng/mL)		
	Bias (%)	Precision Within-run (%)	Precision Between-run (%)	Bias (%)	Precision Within-run (%)	Precision Between-run (%)	Bias (%)	Precision Within-run (%)	Precision Between-run (%)
RMCAT	-22	2-6	14	-16	2-5	11	-8	2-5	11
SMCAT	-26	1-5	11	-15	2-4	8	-11	1-4	6
RCAT	--	--	--	-5	1-32	16	-9	1-28	19
SCAT	--	--	--	-6	1-26	14	-7	1-24	15

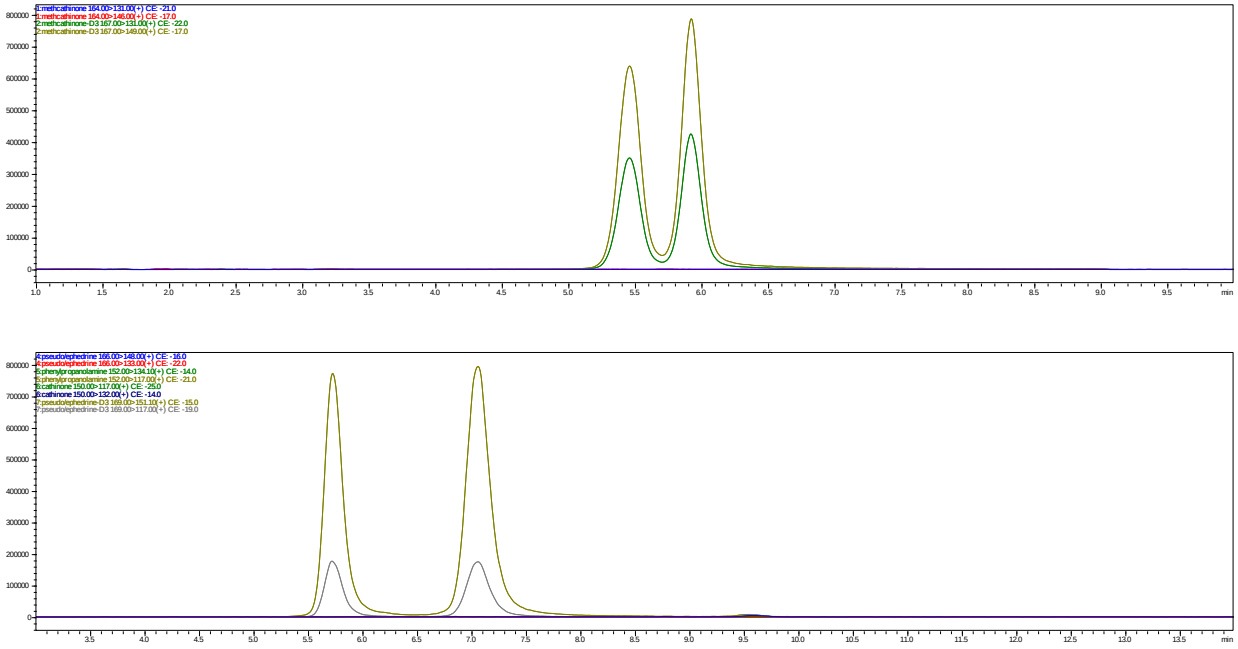

Figure 5-1. Representative Chromatography – Blank

Pictured are blank chromatograms of IS-fortified matrix for the methcathinone analysis (top) showing R(+) and S(-)methcathinone-D3 and the metabolite analysis (bottom) showing R(+)ephedrine-D3 and R(+)pseudoephedrine-D3.

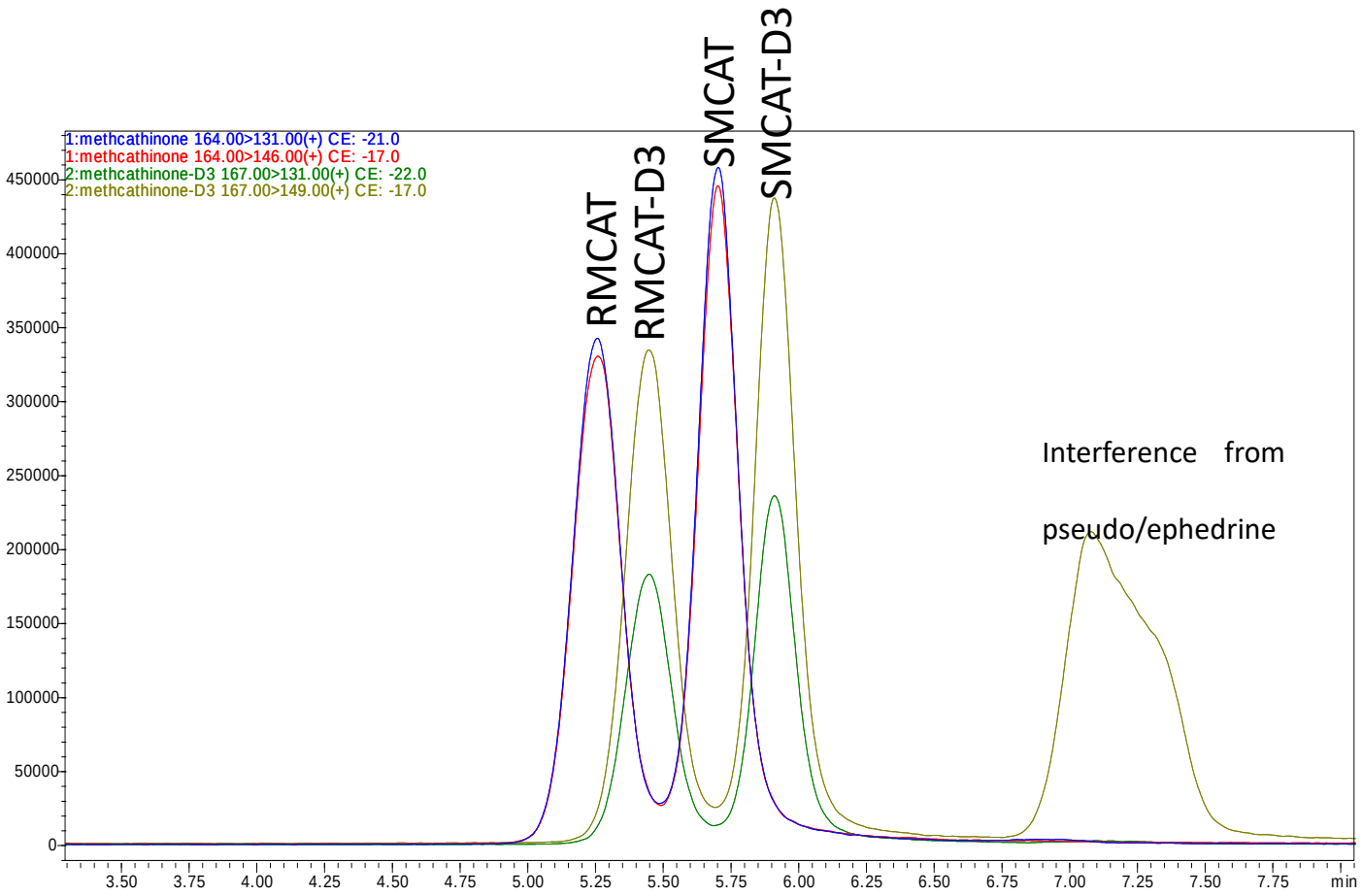

Figure 5-2. Representative Chromatography – Methcathinone

Chromatographic separation was achieved for R(+)methcathinone and S(-)methcathinone (blue + red) as well as for R(+)methcathinone-D3 and S(-)methcathinone-D3 (yellow + green). Shown is a 100 ng/mL calibration standard extracted from blood.

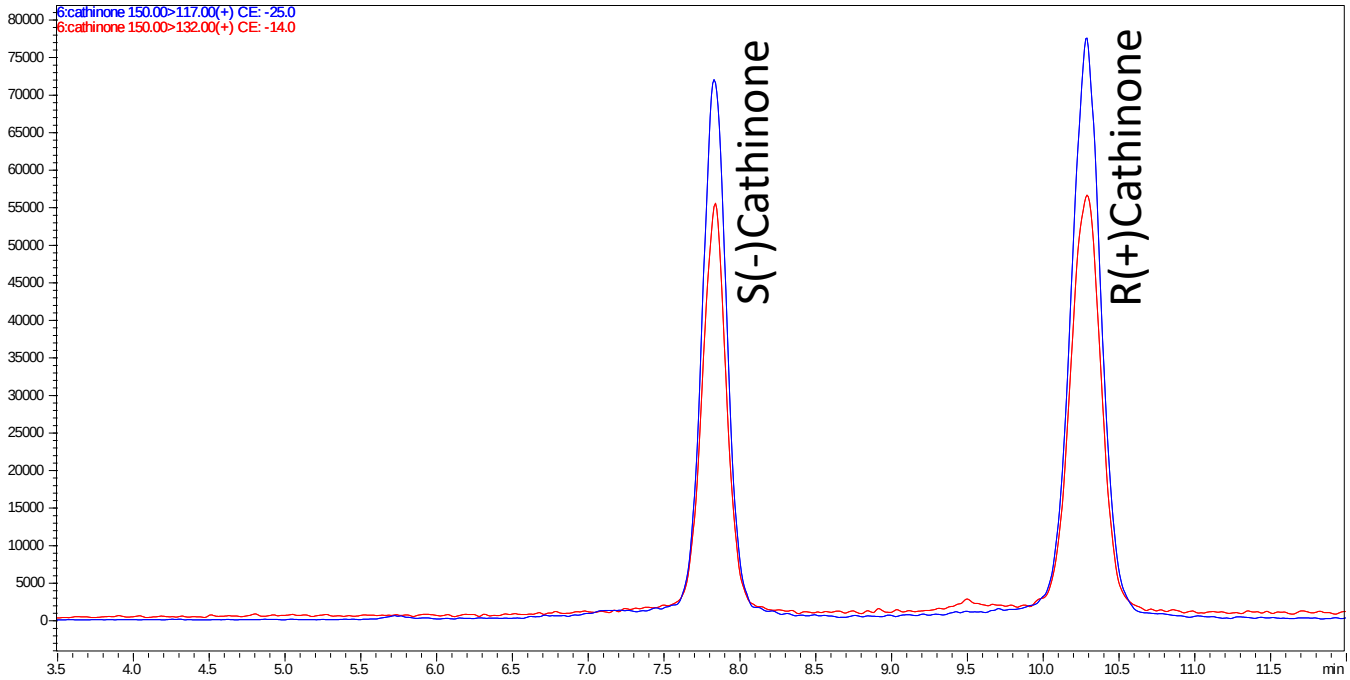

Figure 5-3. Representative Chromatography – Cathinone

Full baseline chromatographic separation was achieved for S(-) and R(+) cathinone. Shown is a 100 ng/mL calibration standard extracted from blood.

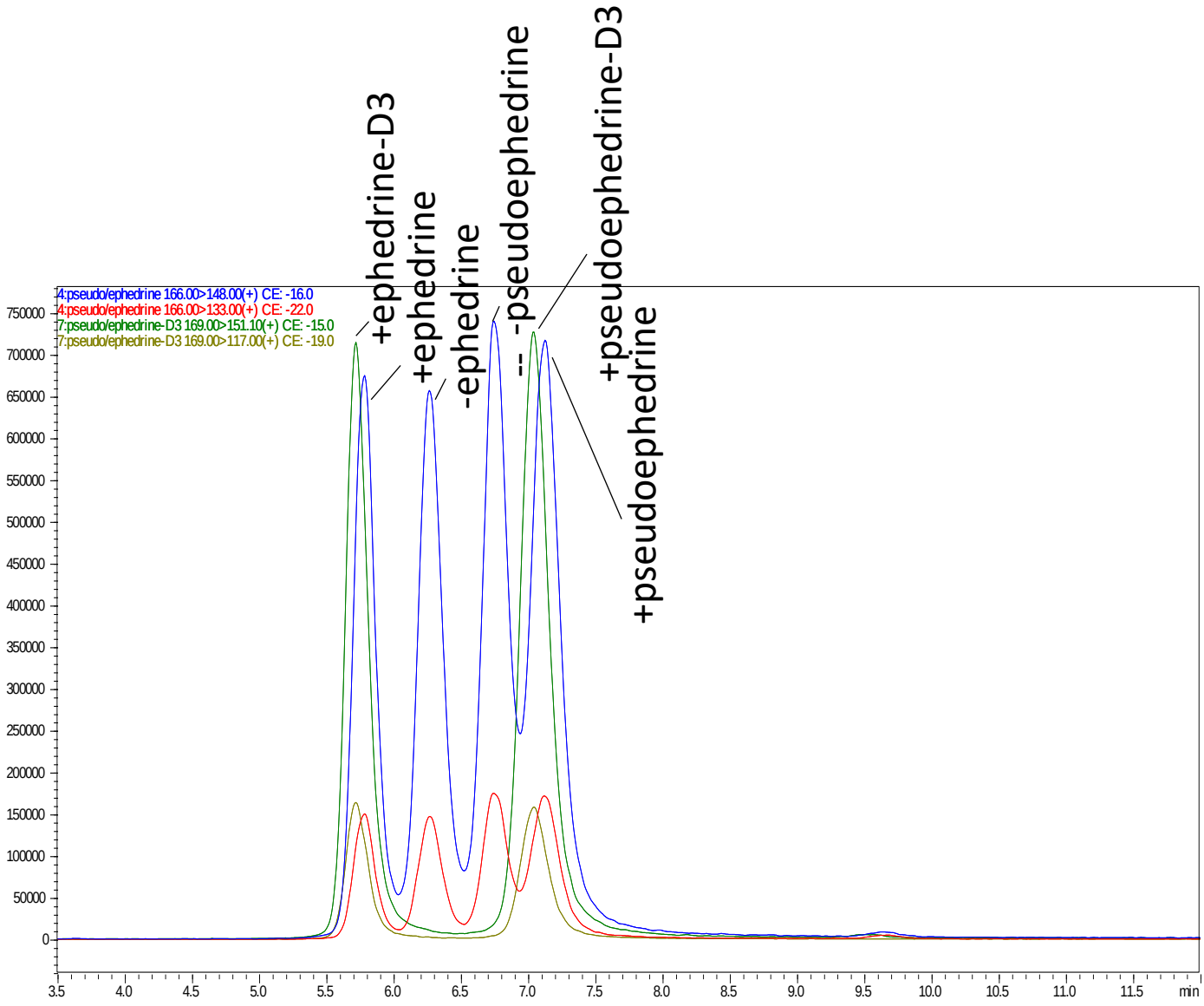

Figure 5-4. Representative Chromatography – Ephedrine and Pseudoephedrine

Chromatographic separation was achieved for (+)ephedrine and (-)ephedrine. (-)pseudoephedrine and (+)pseudoephedrine were partially resolved. (+)ephedrine-D3 and (+)pseudoephedrine-D3 are fully resolved. Shown is a 100 ng/mL calibration standard extracted from blood.

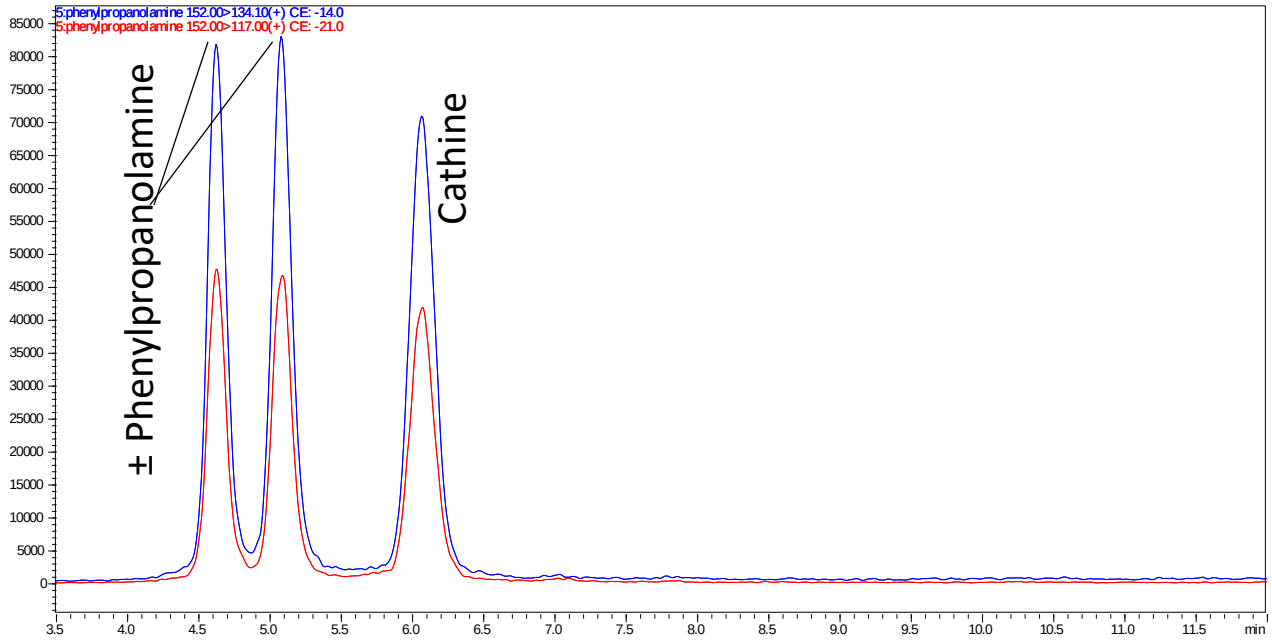

Figure 5-5. Representative Chromatography – Phenylpropanolamine and Cathine

Chromatographic separation for the individual enantiomers of phenylpropanolamine as well as cathine was achieved. Shown is a 100 ng/mL calibration standard extracted from blood.

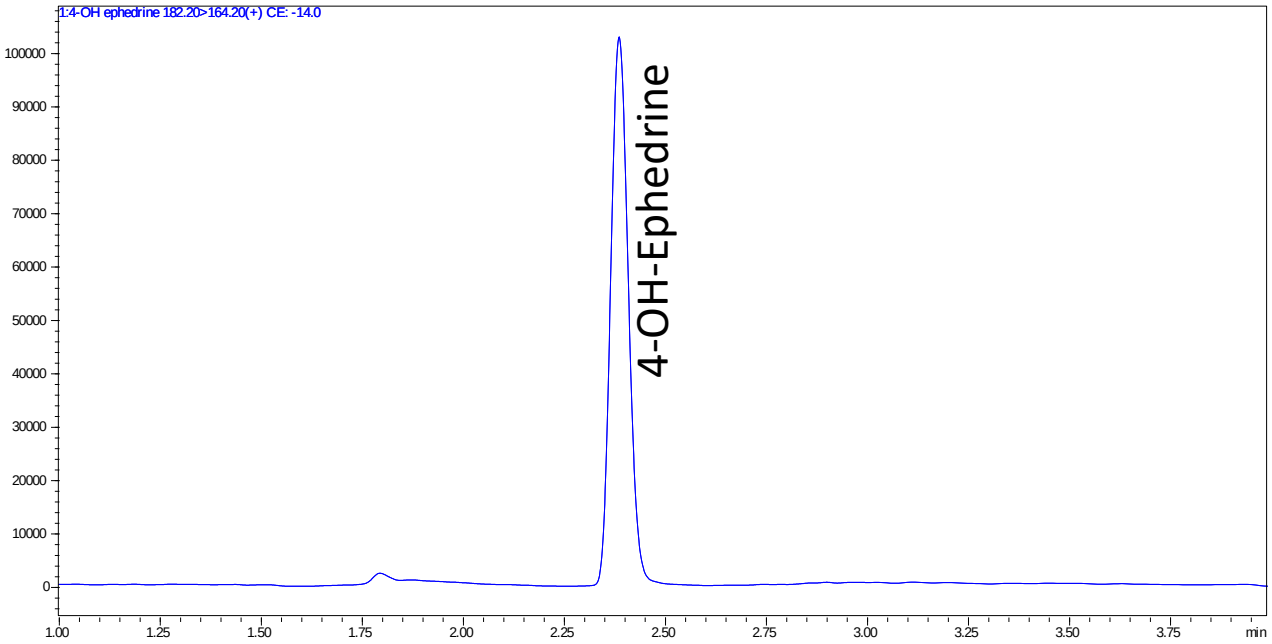

Figure 5-6. Representative Chromatography – 4-hydroxyephedrine

Individual enantiomers of 4-hydroxyephedrine were not able to be separated. Shown is a single ion transition of a 100 ng/mL calibration standard extracted from blood.

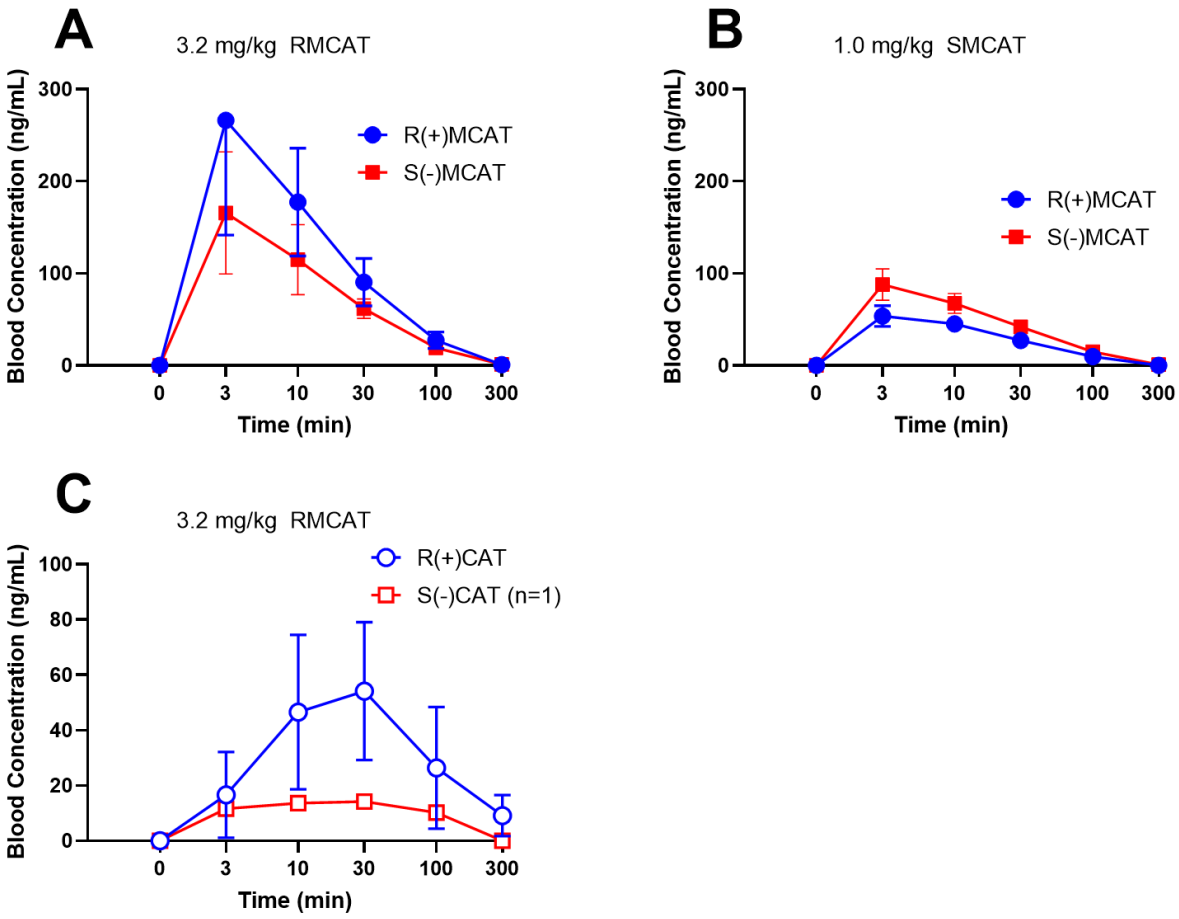

Figure 5-7. Pharmacokinetic Time Course of Methcathinone Enantiomers in Blood

Panel A shows the time course of R(+) and S(-)methcathinone concentrations in blood following 3.2 mg/kg R(+)methcathinone administered i.p. Panel B shows the time course of R(+) and S(-)methcathinone concentrations in blood following 1.0 mg/kg S(-)methcathinone administered i.p. Panel C shows the time course of R(+) and S(-)cathinone concentrations in blood following 3.2 mg/kg R(+)methcathinone administered i.p. Only one of four rats had S(-)cathinone concentrations above the limit of detection. Data points represent the mean (± SD) of the concentration at each sample time.

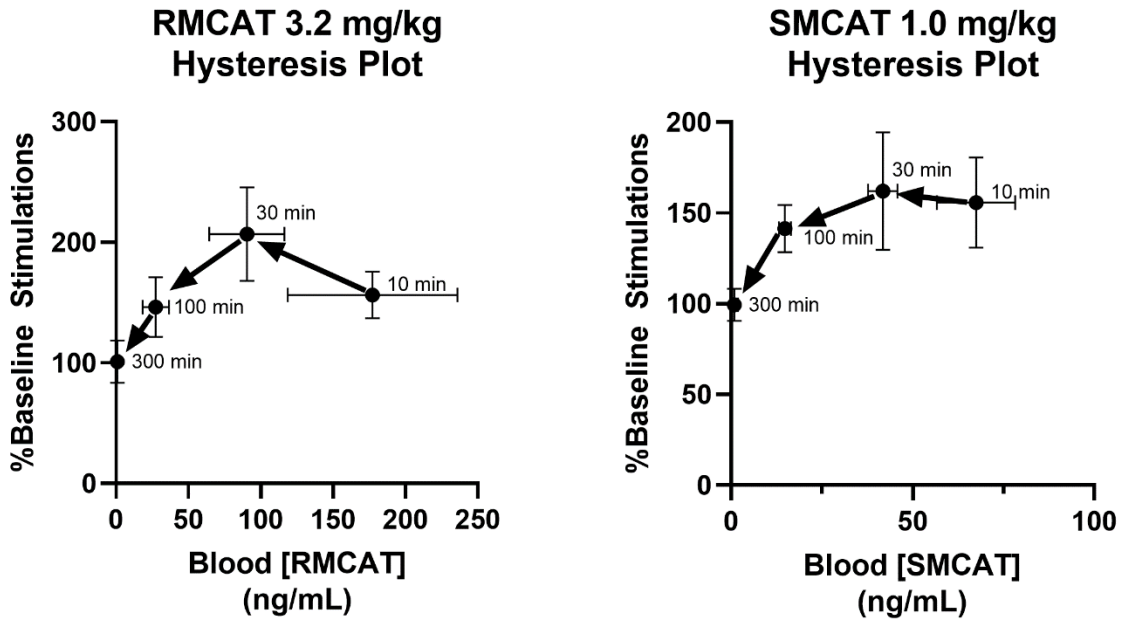

Figure 5-8. Counterclockwise hysteresis plots of RMCAT and SMCAT

Each data point represents the blood concentration of the drug and the facilitation of ICSS per component compared to baseline at each time point (10, 30, 100, and 300 min). Horizontal error bars represent the SD of the concentration at each time point. Vertical error bars represent the SD of the %BL ICSS facilitation at each time point.

CHAPTER 6: DISCUSSION

Summary

The overall aim of this research was to develop mechanisms for the threat assessment of NPS. ICSS was explored as an in vivo screening tool in rats to conduct preliminary threat assessments on emerging stimulant drugs of abuse, and a bioanalytical method was developed to monitor for the enantiomers of methcathinone and its metabolites. Chapter 2 proposed using ICSS as a screening tool for NPS and exercised it using the novel synthetic cathinone α-PHP, finding abuse potential consistent with its pattern of use in human population. Chapter 3 broadened the assessment to systematically examine the effect of drug time-course on responding for ICSS. Chapter 4 investigated the sensitivity of ICSS to detect small molecular changes to methcathinone including discriminating between enantiomers. Chapter 5 examined the pharmacokinetics of the individual enantiomers of methcathinone and its likely metabolites using liquid chromatography mass spectrometry to relate the blood concentrations of methcathinone and its metabolites to the behavioral effects observed in ICSS.

Refinement of a model of abuse potential to prioritize emerging drugs

The proliferation of NPS is taxing capabilities for detection and analysis, and forensic testing would benefit from data-driven strategies to prioritize compounds and guide efficient allocation of resources. Given the sensitivity of ICSS to abused drugs from a broad range of pharmacological classes, we propose the algorithm shown in Figure 6-1 as an initial behavioral screen to prioritize NPS for further forensic analysis. NPS from seized samples, purified samples, or newly synthesized samples would be administered to rats in an ICSS procedure. If the drug

produces ICSS facilitation as a signal of high abuse liability, then the drug should be considered a significant threat and be categorized as Priority 1 for further pharmacological study as well as the development of CRM and analytical methods. If a drug fails to produce ICSS facilitation but does produce ICSS depression, then the sensitivity of that drug effect to cannabinoid and 5-HT_{2A} receptor antagonists should be assessed to test for cannabinoid- or hallucinogen-like mechanisms of action. A positive result here would warrant assignment to Priority 2 status. If a drug produces ICSS depression resistant to cannabinoid or 5-HT_{2A} receptor antagonists, then it is unlikely to have long-term abuse potential, but it may still pose a threat of sporadic abuse and toxicity (e.g. as with salvia-containing products), and it should be assigned to Priority 3 status. Lastly, if a drug fails to alter ICSS, then it is unlikely to be a threat for either significant abuse or toxicity, and it should be assigned to Priority 4 status.

The robust facilitation of ICSS and application of the general algorithm presented in Figure 6-1, classifies cocaine, WIN-35428, and RTI-31 as compounds with high abuse potential, and would be categorized into Priority 1. Further ranking of these within the category would rank the three compounds in order of their rate of onset, cocaine > WIN-35428 > RTI-31. Despite this, cocaine is the only drug of the three that has emerged as an abused drug. This exemplifies the complex nature of a full abuse liability assessment, where non-pharmacological factors also influence the probability of a drug proliferating. In this case, both WIN-35428 and RTI-31 use cocaine as a starting material in their synthesis. The synthetic structural modifications to cocaine delays the rate of onset and potentially make WIN-35428 and RTI-31 a less efficient reinforcer, as suggested by Wee et al. (2006). WIN-35428 and RTI-31 also fail to avoid scheduling laws due to the Federal Analog Act which controls compounds structurally similar to Schedule I or II drugs. While the Analog Act is often considered to be vague and difficult to enforce, the direct synthesis

of WIN-35428 and RTI-31 from cocaine strongly indicates their structural similarity. Scheduling actions have been demonstrated to decrease the availability of NPS; however, new compounds tend to emerge to take their place (Papsun et al., 2022; Vandeputte, Krotulski, et al., 2022; Weedn et al., 2021).

Both enantiomers of methcathinone and the two achiral analogs robustly facilitated ICSS, with rapid onset and equivalent E_{max} values. The equivalent E_{max} values and indistinguishable rates of onset makes it difficult to provide relative ranks of abuse potential among these compounds. There are two considerations that may impact this determination. First, the α-des-methyl analog is less potent than the other compounds. The potency is a small contributor to differences in abuse potential, as the user can simply increase their dose to reach the ideal pharmacological effect. Low potency can contribute to some non-pharmacological factors that may impact abuse liability. Solubility can become problematic for some low potency drugs, complicating the drug delivery process. Low potency compounds also will require a greater volume of drug for the desired dose, and this can make the illicit transport of some of these drugs more difficult as larger volumes are more difficult to conceal. The second consideration among this series of drugs is the rate-decreasing effect that is apparent at high frequencies of electrical brain stimulation for the highest dose of R(+)methcathinone. This could be indicative of behavioral toxicity, and may make R(+)methcathinone less desirable.

Limitations and alternatives to threat assessment using ICSS

Although the ICSS procedures described here can provide a robust set of pharmacological information about NPS, there are several practical aspects to consider. In addition to the facilities and resources necessary for the housing and care of laboratory animals, ICSS requires specialized equipment such as electrical stimulators, operant chambers, and the computer interface, all of

which can be obtained from commercial suppliers. Additionally, the procedure requires infrastructure and expertise in stereotaxic surgery for implantation of electrodes, and there is some attrition (~25%) due to missed electrode placements or poor operant learning. Initial time investments in behavioral training can be extensive, but once reliable responding for electrical brain stimulation has been established, individual rats can perform at stable levels for months. This procedure's efficiency as a behavioral screening tool is dependent upon maintaining a population of trained rats. Finally, it must be recognized that pharmacological reward measured by ICSS is only one of several factors that may contribute to the incidence of abuse for a specific drug, and the predictive validity of this assay will depend at least in part upon those non-pharmacological factors.

In vitro assays can be used to study a drug's binding affinity and functional activity at biological receptors. In vitro methods of testing have the advantage of higher throughput and easily quantifiable data without the burden of experimentation on living subjects. Recent studies on novel synthetic opioids have demonstrated the ability of functional assays to compare relative potency and efficacy of a compound to activate the mu opioid receptor (Vandeputte et al., 2020; Vandeputte, Vasudevan, et al., 2022). This is valuable to a risk assessment: opioids with a high degree of intrinsic efficacy at the mu opioid receptor would be at greater risk of causing opioid-mediated respiratory depression and overdose death. Screening procedures could be calibrated using systematic probing of efficacy requirements to cause undesirable behavioral effects with the addition of agonist/antagonist mixtures to characterize the assay (Selley et al., 2021).

A third strategy for the assessment of NPS is to rely on structural similarities and to base predictions upon structure-activity relationship (SAR) experiments that have been performed on the molecular scaffolds that are common to many of these drugs. SAR studies have been

performed on many drugs, including two of the largest contributors to the NPS epidemic: synthetic cathinones and fentanyl analogues (Glennon & Dukat, 2016; Vardanyan & Hruby, 2014). Along with data from SAR studies, "in silico" methods can also be used for the prediction of drug effects. Modern technology has enabled the structural elucidation of many proteins, including receptors and transport proteins that mediate the effects of the drugs. Software now exists that can model the three-dimensional structures of molecules and their potential protein targets. One example of this is the FDA's PHASE (Public Health Assessment via Structural Evaluation) methodology which evaluates a compound's similarity to existing controlled substances, identifies likely receptor targets, and predicts the binding affinity at those targets (Ellis et al., 2019).

In vitro and in silico methods of pharmacological assessment have great advantage in their speed and cost effectiveness compared to in vivo procedures such as ICSS, but they ultimately lack the ability of procedures such as ICSS to fully integrate both the pharmacokinetic and pharmacodynamic properties of a drug into a single experiment. In vitro assays will not determine a time course of drug effects, distinguish between drugs that are centrally active or peripherally restricted, indicate the presence of active metabolites, or account for differences in routes of administration. All of these methods have roles to play in the risk assessment of NPS, but ICSS generates the most robust set of data that is relevant at the organism level.

Bioanalysis

This method of chiral bioanalysis of methcathinone and its metabolites was fraught with challenges. While the results from the method validation were less than ideal, it serves as a starting point for future refinement of the method. The extreme variability in ionization efficiency for the analytes in this method had a strong impact on the limits of detection and the precision of quantitative results. Suppression or enhancement of ionization in a sample due to a substance co-

eluting with the analyte and competing for or donating free hydrogen adducts (Panuwet et al., 2016; Van Eeckhaut et al., 2009). This is a common phenomenon in systems that use electrospray ionization to generate charged molecules. The method developed in Chapter 5 was subject to large variations in ionization efficiency, and this likely impacted the LOD, bias, and precision of the analytical method, especially for those compounds without a homologous deuterated internal standard. Deuterated internal standards have deuterium atoms, or "heavy hydrogen", in place of normal hydrogen atoms. This leads to a compound with almost identical properties, but it is easily distinguished from the analyte of interest by the mass difference. As deuterated internal standards co-elute with their homologous analyte, changes in ionization efficiency impact both equally. Quantitation in this LC-MS/MS procedure relies on the ratio of analyte to internal standard, and that ratio is not affected if the ionization enhancement or suppression is of equal magnitude for both the analyte of interest and its corresponding internal standard. One major improvement to this method would be the addition of deuterated internal standards for all analytes, if available.

One unexpected result was the presence of both enantiomers of methcathinone in the samples from rats that received injections of enantiopure R- or S-methcathinone. It was considered that this conversion could be due to in vivo biotransformation, but in that situation one would expect the time course of the converted enantiomer to lag behind that of the parent drug. In this case, both enantiomers seemed to occur in the same ratio at every time point. The injectable solutions were prepared fresh daily to ensure enantiopurity, as previous experiments demonstrated spontaneous racemization of methcathinone when in solution for extended periods of time. Eventually, enantiopure methcathinone solutions injected directly onto the LCMS were compared with enantiopure methcathinone solutions put through the sample preparation procedure prior to injection, and a substantial amount of racemization was observed in samples that underwent the

protein precipitation, evaporation, and reconstitution process. The chiral instability of cathinones has been noted by others performing chiral separations and attributed to keto-enol tautomerization (Mohr et al., 2012). Unfortunately, the enantiospecific quantitation of methcathinone and cathinone in these samples is suspect. Nonetheless, the time course of the drug in blood corresponded well with the time course of the behavioral effects in ICSS. Hysteresis plots for R and S methcathinone both indicated a counterclockwise orientation; that is, the magnitude of the behavioral effects diminished more slowly than the concentration of the drug in blood. One possible—and likely—reason for this is the presence of an active metabolite, but other possibilities exist, including acute receptor sensitization. Cathinone is a known active metabolite of methcathinone with abuse potential. Due to the complications with the analytical method, no strong conclusions can be drawn.

Several improvements need to be made to the sample preparation procedure. First, the gradual downward trend of concentrations of the positive controls suggests a lack of stability. Cathinones are notoriously unstable, especially in biological matrices, and additional steps should be taken to mitigate that instability. First, the sample tubes should contain preservative to prevent the growth of bacteria in the sample. The most widely suggested preservative in forensic toxicology laboratories is 2% sodium fluoride / potassium oxalate (grey top tubes) (Toennes & Kauert, 2001). Second, samples were stored at -20 °C. In the future, storage at -80 °C may be preferrable.

Improved clean-up of the sample matrix is necessary in the case of these drugs. While a simple protein precipitation procedure was selected as the most efficient starting point during the development of this method, it is now apparent that more rigorous clean-up is necessary. Several extraction techniques can be explored, including solid phase extraction, supported liquid

extraction, and liquid-liquid extraction. Each of these can clean up interfering substances such as phospholipids, which are known to cause ion suppression, from the biological matrix.

This method evaluated potential metabolites by a targeted method, which may not detect metabolites which are unexpected. Non-targeted screening approaches would be preferrable in future analyses of emerging drugs. Further, this bioanalytical procedure was conducted in rats, specifically Sprague-Dawley rats. While this is preferrable for comparison to the behavioral results collected in the same species and strain, it may not translate as well to human metabolism. Future analyses would benefit from non-targeted screening for compounds following drug incubation in rat and human microsomal preparations.

Future Directions

This research examined two steps of a three-step approach to combatting the drug epidemic. The first step is introducing newly obtained drugs into the ICSS procedure as soon as possible and developing a risk assessment. Second, the results of ICSS testing should inform an action, which could include the allocation of resources to research, method development, harm reduction policies, drug scheduling, or any other proactive step. Third, bioanalytical methods that are developed should be used diagnostically to monitor the effectiveness of the steps taken to prevent drug proliferation. With this three-step iterative process, strategies can be continuously refined to maximize the predictive capabilities of the screening mechanism and the effectiveness of interventions.

Another area of deficiency in drug abuse research is the effect of drug mixtures on abuse potential. While some attention has been given to obvious choices, such as heroin/cocaine ("speedballs") or fentanyl/methamphetamine ("goofballs"), little has been done to probe some other common adulterants in street drugs. Two frequently observed drug mixtures are cocaine

with levamisole and fentanyl or other opioids with xylazine. Current research is lacking on the effects of levamisole and xylazine in these combinations. ICSS is an ideal procedure to systematically examine the contribution of adulterants in street drugs to abuse potential.

The ICSS behavioral screen is valuable and informative as an initial screening tool. Additional information would help to improve the threat assessment, and the threat assessment need not be a static tool. A continually evolving machine learning approach could take advantage of multiple sources of information to cover the gaps in abuse liability assessment that ICSS is not designed to evaluate. For instance, data from drug intelligence sources could be input into a machine learning approach, assessing variables such as the difficulty of the drug synthesis, the availability of precursor materials, the regulatory status of the drug and precursor materials, the cost to produce, and the availability of alternative drugs of the same class. Bioanalytical surveillance can be used to check and improve the accuracy of the machine learning algorithm. Other additions could increase the specificity of risk assessment measures by including data on drug trends in specific localities, or common drug distribution routes. A degree of regional specificity could further help focus the limited resources of communities toward problems with the greatest local impact.

Figures and Tables

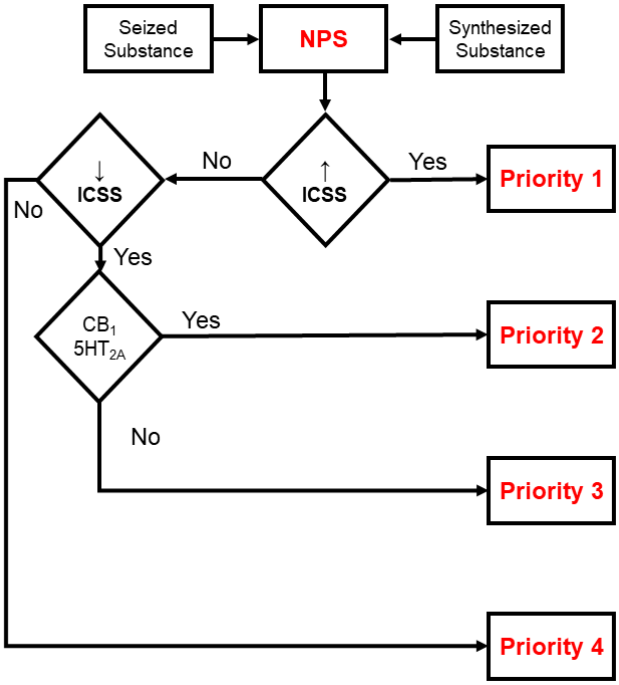

Figure 6-1. An algorithm to prioritize emerging drugs of abuse for further analysis

ICSS can be used as a screening tool to rapidly prioritize NPS for further analysis. Either seized or newly synthesized NPS can be tested in an ICSS procedure and classified according to four priority categories.

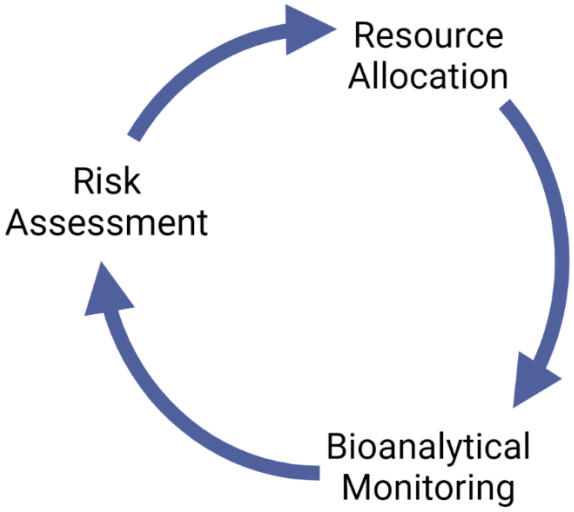

Figure 6-2. Refinement of the predictive model

ICSS Risk assessment can be used to direct resources toward the most likely problematic drugs, before they proliferate. Bioanalytical monitoring can be used diagnostically to monitor the predictive validity of the risk assessment as well as the effectiveness of resource allocation. The information can then be used to refine the risk assessment.

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VITA

Tyson Richard Baird is from Wichita, Kansas. He earned his Bachelor of Science degree in the Forensic Science program at Wichita State University. Following the receipt of his undergraduate degree, Tyson moved to Richmond, Virginia to pursue his Master of Science in Forensic Science degree at Virginia Commonwealth University with a concentration in drug analysis and toxicology. Tyson's research under Dr. Michelle Peace focused on developing methods to evaluate the capability of electronic cigarettes to aerosolize and deliver drugs to users. Tyson returned to Wichita, Kansas to the Regional Forensic Science Center where he worked in the toxicology section for three years doing post-mortem analyses and method development. Tyson eventually returned to VCU in 2018 for his doctorate in the Integrative Life Sciences program, where he worked in the laboratories of Dr. Michelle Peace and Dr. Steve Negus. Tyson's doctoral dissertation focused on the bioanalysis and behavioral effects of novel psychoactive substances, primarily synthetic cathinones, in rats. Tyson's research activities have led to multiple peer-reviewed publications and presentations at national and international scientific conferences.