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Following widespread scheduling, many synthetic cathinone compounds have been diverted from "bath salts" to "Ecstasy" tablets or "Molly" powder formulations in addition to or *in lieu* of 3,4-methylenedioxymethamphetamine (MDMA). The current study aimed to assess the mechanism and reinforcing effects of three under-researched synthetic cathinone analogs of MDMA frequently used as adulterants in "Ecstasy" formulations: methylone, butylone, and pentylone. To assess the mechanism of these compounds *in vitro*, we utilized whole-cell patch clamp electrophysiology on HEK293 cells expressing the serotonin transporter (SERT). The abuse-related, *in vivo* mechanisms were determined using a drug discrimination assay with rats trained to discriminate methamphetamine, the hallucinogenic phenethylamine 2,5-dimethoxy-4-methylamphetamine (DOM), or MDMA from vehicle, and drugs that substituted were tested with the D1-like receptor antagonist SCH23390 to assess relative differences in dopaminergic signaling. The reinforcing effects were assessed in an intravenous self-administration assay using continuous and progressive ratio schedules of reinforcement. Methylone and butylone, like MDMA, produced inward currents at SERT, indicative of a substrate-like mechanism. Each test compound fully substituted for the discriminative stimulus effects of methamphetamine. MDMA, methylone, and butylone substituted partially for DOM, and methylone and butylone substituted fully for MDMA. Pentylone, conversely, substituted partially for MDMA, but failed to substitute for DOM. SCH23390 fully and dose-dependently attenuated methamphetamine-appropriate responding, with pentylone being least sensitive to these antagonistic effects, but failed to attenuate MDMA-like responding against MDMA, methylone, and butylone. Each test

compound maintained robust self-administration under a continuous schedule of reinforcement, but pentylone was the most reinforcing test compound under a progressive ratio. These data indicate that methylone and butylone produce complex discriminative stimulus effects, similar to MDMA, that are mediated by both dopamine and serotonin, whereas pentylone is predominately dopaminergic. The underlying differences in relative dopaminergic and serotonergic mechanisms likely influence the relative abuse liability, with pentylone's predominately dopaminergic mechanism conferring a greater reinforcing efficacy relative to the more serotonergic methylone and butylone. In conclusion, incorporation of these compounds into "Ecstasy" formulations, especially pentylone, may lead to compulsive, uncontrolled use of "Ecstasy".

“Ecstasy” to addiction: Mechanistic and reinforcing effects of synthetic cathinone analogs of MDMA

DISSERTATION

Presented to the Graduate Council of the

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For the Degree of

DOCTOR OF PHILOSOPHY

by

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INTRODUCTION

Novel Psychoactive Substances: Prevalence and Concern

Over the past decade, there has been a shift in the prevalence of recreational drug use from traditional drugs of abuse to novel psychoactive substances, also known as "designer drugs" and "legal highs." (UNODC, 2013; 2014; EMCDDA, 2015). As of 2009, novel psychoactive substances on the market and in use outnumber the drugs currently under international control (UNODC, 2014). Although difficult to estimate the exact prevalence of these substances, surveys have indicated a steady increase in the use of novel psychoactive substances with 8% of the European population admitting using them in 2014, up from 5% in 2011 (EMCDDA, 2014); however, the incidence is much higher amongst individuals who frequent dance clubs, with self-reported incidence as high as 40% (Prosser & Nelson, 2012). These substances are readily available online and in novelty shops and are often marketed as "spice", "bath salts" or other innocuous brand names designed to circumvent drug regulations (Nelson et al., 2014; German et al., 2014). Novel psychoactive substances contain a vast array of structurally and functionally diverse compounds that produce effects similar to those of well-known drugs of abuse (UNODC, 2013; EMCDDA, 2015; Liechti, 2015; Nelson et al., 2014). These compounds have become a significant public health concern given the difficulty associated with controlling these compounds and the numerous adverse health risks associated

with their use, including, but not limited to: seizure, tachycardia, stroke, hyperthermia, psychosis, agitation, and death (Prosser & Nelson, 2012; Nelson et al., 2014).

The most prevalent of these compounds are the synthetic cannabinoids, comprising 28% of the designer drug market, followed by synthetic cathinones, which constitute a quarter of this market (UNODC, 2014). The synthetic cannabinoids, as their name implies, produce cannabis-like effects and are largely used as alternatives to cannabis (Nelson et al., 2014). The synthetic cathinones, conversely, produce a broad range of subjective effects that users report as similar to cocaine, methamphetamine, hallucinogenic compounds such as lysergic acid diethylamide (LSD), and/or 3,4-methylenedioxymethamphetamine (MDMA; German et al., 2014; Liechti, 2015). This wide range of effects has garnered attention from the scientific community regarding differential patterns of use. Cocaine and methamphetamine-like synthetic cathinones, such as methylenedioxypyrovalerone (MDPV), α-pyrrolidinopentiophenone (α-PVP), and cathinone (Gatch et al., 2013; 2015b; Young & Glennon, 1998), have demonstrated substantial potential for compulsive use and risk for addictive behaviors (Aarde et al., 2013; 2015; Gosnell et al., 1996). Putative entactogen-, or MDMA-like cathinone analogs, such as mephedrone and methylone, have demonstrated reduced potential for compulsive abuse (Vandewater et al., 2015; Watterson et al., 2014; Schindler et al., 2015). Consequently, less attention has been paid to this class of compounds. However, given the rising popularity of Ecstasy use and the increased presence of synthetic cathinone analogs in Ecstasy formulations (UNODC, 2014), further investigation into the abuse liability of these compounds is warranted.

MDMA: History, mechanism of action, and abuse liability

Ecstasy or "Molly", which are illicit drug formulations in tablet- or powder-form, respectively, taken recreationally for their euphoric effects, have been widely used at dance

clubs, rave parties, and music festivals since their introduction to the black market in the 1980s (Pentney, 2001; Palamar et al., 2016a). Although the synthetic cathinones are becoming regular active components of Ecstasy formulations, MDMA has historically been the primary pharmacological mediator of Ecstasy's effects. Thus, in order to understand the putative entactogenic effects in the context of Ecstasy use, some discussion on MDMA is required.

MDMA is a methylenedioxy-ring-substituted phenethylamine known for producing euphoria, increased energy, and sexual arousal that is largely used in dance clubs and rave parties (Cohen, 1995). Because of its unique subjective effects, MDMA serves as the prototypical compound in a class of drugs known as "entactogens," etymologically derived from Latin and Greek and roughly translating to "the touch within" (Nichols, 1986). The pro-social, introspective, and empathy-producing effects of MDMA made it an attractive experimental drug for use as an adjuvant to psychotherapy in the 1970s; however, numerous adverse effects, including cardiotoxicity, hyperthermia, dehydration, tremor, serotonin syndrome, and, in rare cases, death, occurred concurrently with the rise in recreational use of MDMA in the 1970s and '80s, leading the DEA to classify MDMA as a schedule I compound in 1988 (Pentney, 2001; de la Torre et al., 2004; Shifano, 2004).

Like other amphetamine derivatives, MDMA functions as a substrate for the monoamine transporters with greatest potency at the norepinephrine transporter (NET), followed by the serotonin transporter (SERT), then the dopamine transporter (DAT; Eshleman et al., 2013; Simmler et al., 2013). The substrate activity of amphetamine derivatives is unique from psychostimulants like cocaine in that they are transported into the cytoplasm of the neuron and produce impulse-independent, non-vesicular neurotransmitter release through a reversal of directionality of transmitter flow through the transporter (Miller, 2011). *In vivo* microdialysis

studies indicate that MDMA increases synaptic serotonin to a greater extent than dopamine (Baumann et al., 2012; Kehr et al., 2011). In addition to increasing synaptic concentrations of monoamines, MDMA also acts directly as a 5-$HT_{1A/2A}$ agonist and substrate for the vesicular monoamine transporter 2 (VMAT2; Eshleman et al., 2013; Simmler et al., 2013). Altogether, these mechanisms contribute to MDMA's unique effects and the production of sympathomimetic, stimulant, and pro-social effects.

The unique phenotype of MDMA is perhaps best illustrated by its complex discriminative stimulus properties, which are mediated both by serotonin and dopamine. Early studies with pigeons indicated that MDMA fully substituted for the discriminative stimulus effects of *d*-amphetamine, but at doses that suppressed responding (Evans & Johnson, 1986). Other studies in rats have indicated symmetrical cross-substitution of methamphetamine and MDMA, in which MDMA fully substituted for the discriminative stimulus effects of methamphetamine, and vice versa, but in both cases fully substituting doses produced decrements in response rates (Gatch et al., 2009). Other studies have indicated a lack of cross-substitution between MDMA and the indirect dopamine agonists *d*-amphetamine and methylphenidate (Schechter, 1988; Mori et al., 2014). These studies indicate a dopaminergic component to MDMA's discriminative stimulus effects, but the reduced response rates and discrepant results suggest an additional component preventing rapid cross-substitution between MDMA and primarily dopaminergic compounds.

Studies addressing other neurotransmitter systems have demonstrated a strong serotonergic component to the discriminative stimulus effects of MDMA. Early experiments addressing MDMA's discriminative stimulus effects in rats trained on various dopaminergic and serotonergic compounds demonstrated full substitution in animals trained to discriminate fenfluramine and cathinone, a selective serotonin releasing agent and selective dopamine

releasing agent, respectively, but not apomorphine, a non-selective dopamine receptor agonist (Schechter, 1986). A later study using MDMA as the training drug demonstrated substitution of norfenfluramine, but not cathinone, for the discriminative stimulus effects of MDMA (Schechter, 1988). Further probing into the stereoselectivity has demonstrated full substitution of amphetamine in mice trained to discriminate (+)-MDMA from saline, but not in (–)-MDMA-trained mice, and, conversely, the hallucinogenic amphetamine 2C-T-7 fully substituted in (–)-MDMA-trained mice, but not for (+)-MDMA (Murnane et al., 2009). Interestingly, in the same study, cocaine, a non-selective monoamine reuptake inhibitor, and *N,N*-dipropyltryptamine (DPT), a selective serotonin reuptake inhibitor, substitute for both (+)- and (–)-MDMA, with cocaine being more potent and efficacious in (+)-MDMA-trained mice and DPT being more potent in (–)-MDMA-trained mice (Murnane et al., 2009). Another study has indicated time-dependent dopaminergic and serotonergic contributions to the discriminative stimulus effects wherein haloperidol pre-treatment produced minimal attenuation of MDMA's discriminative stimulus effects in rats trained on MDMA with a 20-minute pretreatment time, but full antagonism in rats trained with a 105-minute pretreatment time, whereas pirenperone attenuated MDMA's discriminative stimulus effects at both pretreatment times (Schechter, 1988). The author concluded this time-dependent difference in antagonist effects was indicative of a late-acting, or biphasic, dopaminergic component of MDMA's discriminative stimulus effects (Schechter, 1988).

The aforementioned studies each utilized two-lever drug discrimination studies in which the animal discriminates between the training drug and its vehicle, providing quantal information regarding the effects of the drug in an "all-or-none" fashion. A more complex methodology, in which rats are trained with three levers to discriminate between two training drugs and vehicle,

allows for more elegant and precise determination of *in vivo* pharmacology of non-selective compounds. In one such experiment, in which rats were trained to discriminate LSD and MDMA from vehicle, administration of fenfluramine with *d*-amphetamine dose-dependently increased MDMA-lever responding with no LSD-appropriate responding (Goodwin et al., 2003). In similar study using rats trained to discriminate MDMA and amphetamine from vehicle, fenfluramine dose-dependently produced MDMA-like responding whereas cocaine produced amphetamine-like responding (Goodwin & Baker, 2000). In this same study, administration of pirenperone, a 5-HT_2 receptor antagonist, in combination with 1.5 mg/kg MDMA dose-dependently reduced MDMA-appropriate responding while increasing amphetamine-appropriate responding (Goodwin & Baker, 2000). These results further suggest a complex dopamine- and serotonin-dependent discriminative stimulus effects for MDMA. In accordance with the results from two-lever methodology discussed above, a three-lever assay with pigeons trained to discriminate fenfluramine and amphetamine from vehicle demonstrated fenfluramine responding with MDMA administration up to 3 mg/kg, after which pigeons responded on the amphetamine lever (Evans et al., 1990). These results were confirmed in rats trained to discriminate MDMA and amphetamine from vehicle, in which MDMA produced MDMA-like responding up to the training dose (1.5 mg/kg), after which rats responded more on the amphetamine lever, indicating that MDMA adopts a more dopaminergic phenotype at higher doses (Harper et al., 2014). Altogether, the data from both two- and three-lever methodologies indicate dose- and time-dependent complex discriminative stimulus effects mediated by both dopamine and serotonin.

In addition to its unique subjective and mechanistic effects, MDMA differs from other amphetamine derivatives in terms of its abuse liability. MDMA is typically used episodically at dance parties and clubs, whereas cocaine and methamphetamine tend to be used in a more

compulsive, consistent manner indicated by decreased prevalence of MDMA relative to cocaine/methamphetamine lifetime use and regular use reported by users of the different drugs (SAMHSA, 2014). Although the overall incidence of compulsive use of MDMA is lower than traditional psychostimulants, a subset of "heavy users" administer binge-like dosing regimens of MDMA fairly regularly, indicative of problematic drug use (Soar et al., 2006; McCambridge et al., 2005). This limited potential for compulsive use is also evident in the preclinical literature. Studies comparing cocaine, methamphetamine, and MDMA self-administration indicated that MDMA (racemic mixture and (+)/(–) enantiomers) was significantly less reinforcing than either cocaine or methamphetamine (Wang & Woolverton, 2007; Fantegrossi et al., 2002). Similarly, another early study using a large ratio requirement (FR 160) found considerably less self-administration of MDMA relative to cocaine in baboons (Lamb & Griffiths, 1987). A trend of MDMA self-administration at levels above saline, but less than well-established drugs of abuse is a common theme in studies utilizing both non-human primates (Fantegrossi et al., 2004; Fantegrossi, 2008) and rodents (Ratzenboeck et al., 2001; Schenk et al., 2003; 2007).

It has been suggested that the reduced reinforcing effects of MDMA results from its greater relative potency for SERT over DAT (Schenk, 2009). A study using SERT-knockout rats provides the most direct evidence for 5-HT's limiting role in the self-administration of MDMA in which the SERT knockout rats acquire MDMA self-administration much faster and self-administer more MDMA with both fixed (FR 1, 2, 5) and progressive ratios than their wild-type counterparts (Oakly et al., 2014). This is further evidenced in studies using enantiomers of MDMA in which the (+) enantiomer was administered to a greater extent than the (–) enantiomer, the latter of which demonstrates greater SERT selectivity over DAT than the former (Wang & Woolverton, 2007). The inverse relationship between serotonergic activity and

reinforcing efficacy has been demonstrated in compounds with differential monoamine selectivity profiles (Wee et al., 2005; Rothman & Baumann, 2006) or when DAT and SERT selective agents are combined (Howell & Byrd, 1995; Wee & Woolverton, 2006). Altogether, these data suggest that MDMA's limited reinforcing capabilities result from its SERT selectivity relative to DAT, wherein the serotonin release limits the reinforcing capabilities typically demonstrated with dopamine release, and furthermore, that compounds with a higher DAT/SERT ratio will be less reinforcing than DAT-selective compounds.

In summary, MDMA has a fairly long history of use as a recreational drug, produces unique subjective and discriminative stimulus effects mediated by both dopamine and serotonin, and possesses fairly limited abuse liability relative to other stimulant-like drugs of abuse.

Synthetic Cathinones

The synthetic cathinones are a structurally-diverse class of synthetic derivatives of cathinone, a naturally occurring, β-keto analog of amphetamine. Cathinone, as a component of the *khat* plant, has a long history of use in cultural practices in eastern Africa and the Middle East and the earliest synthetic cathinone derivative, methcathinone, was synthesized in 1928 (German et al., 2014). Although use of cathinone and its synthetic derivatives has been occurring for several decades, only during the past five years has it has just become a significant global health concern (German et al., 2014; Nelson et al., 2014; UNODC, 2014). These compounds have been primarily sold as "legal highs" online and in smoke shops as quasi-legal alternatives to conventional recreational drugs such as methamphetamine and MDMA.

Among the most prevalent synthetic cathinones incorporated into these formulations are 3,4-methylenedioxypyrovalerone (MDPV), 4-methylmethcathinone (mephedrone), and 3,4-methylenedioxymethcathinone (methylone), although numerous others have been reported

(Leffler et al., 2014; Nelson et al., 2014; UNODC, 2014). Despite scheduling of these compounds in the United States and much of Europe starting in 2010, MDPV and mephedrone are still popular drugs on the black market, and methylone has been increasingly used in "Ecstasy" formulations (UNODC, 2014; Palamar, 2016c). With the increased recreational use of synthetic cathinones, there has been a concurrent rise in the incidence of adverse health effects associated with overdose including tachycardia, hyperthermia, psychotic episodes, serotonin syndrome, and, in rare cases, death (Kesha et al., 2013; German et al., 2014). The adverse effects and rapid rise in popularity of these compounds served as the impetus for investigation into these compounds and, consequently, much data has been generated in the past five years regarding their mechanism and abuse liability.

Early experiments characterizing the behavioral effects of the synthetic cathinones demonstrated that these compounds produce cocaine- and methamphetamine-like locomotor stimulant and discriminative stimulus effects (Gatch et al., 2013; 2015a; 2015b). Mechanistic investigations of these compounds *in vitro* determined that these compounds exert their effects primarily on monoaminergic transporters with varying degrees of selectivity between DAT, NET, and SERT (Eshleman et al., 2013; 2016; Baumann et al., 2012; Simmler et al., 2013; 2014). Furthermore, these studies have indicated that some compounds produce cocaine-like inhibition of reuptake, others produce amphetamine-like substrate activity, and another class has mixed or "hybrid" pharmacodynamics displaying dopamine reuptake inhibition and serotonin release. These data have demonstrated mechanistic and behavioral similarities to well-characterized, highly addictive drugs of abuse indicating a strong potential for abuse.

Investigations into the reinforcing effects of synthetic cathinones using intravenous self-administration techniques have demonstrated different degrees of compulsive abuse liability

amongst the most popular of these compounds. Cathinone is self-administered similarly to cocaine, albeit at lower doses (Gosnell et al., 1996). MDPV and α-PVP, two highly selective dopamine reuptake inhibitors (Marusich et al., 2014), are both robustly self-administered at levels comparable to or exceeding cocaine and methamphetamine (Watterson et al., 2014; Aarde et al., 2013; 2015; Schindler et al., 2015). Mephedrone, which acts as a substrate for both DAT and SERT with a slightly higher affinity for DAT (Eshlemann et al., 2013), produces self-administration at levels slightly greater than MDMA (Vandewater et al., 2015; Aarde et al., 2013), but to a lesser degree than methamphetamine (Motbey et al., 2013; Aarde et al., 2013). The β-keto analog of MDMA, methylone, which possesses MDMA-like SERT-selective substrate pharmacodynamics (Eshleman et al., 2013), has produced mixed results in the self-administration assay with one study indicating fairly robust self-administration (Watterson et al., 2012), whereas others have indicated weak self-administration comparable to, or to a lesser degree than, MDMA (Schindler et al., 2015; Vandewater et al., 2015). Altogether, these data suggest that different cathinone derivatives engender different self-administration patterns that mirror findings discussed above that increased SERT selectivity produces less robust self-administration (Wee et al., 2005; Rothman & Baumann, 2006).

Objectives of Dissertation

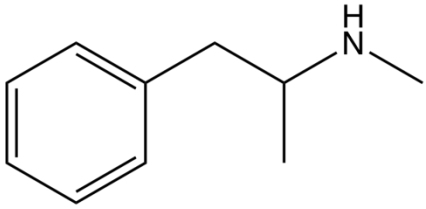
The increased prevalence of synthetic cathinones in Ecstasy formulations and user-reported entactogenic subjective effects of a subset of these compounds serve as the rationale for the current series of studies which aims to investigate three synthetic cathinone congeners of MDMA commonly reported in "Ecstasy" formulations: methylone, butylone, and pentylone (Figure 1).

Methylone's behavioral effects have been fairly well-characterized; however, data regarding the abuse-related behavioral effects of butylone and pentylone are sparse. Thus, the butylamine and pentlyamine methylone congeners butylone and pentylone will be assessed in parallel with methylone to assess how structural changes correspond to mechanism and abuse liability. Previous assessments *in vitro* have demonstrated side-chain dependent structure-activity relations across multiple factors. Medicinal-chemistry investigations on structural substitutions of MDPV have demonstrated that side-chain length is positively associated with DAT affinity (Kolanos et al., 2013). Furthermore, several studies investigating release and reuptake of radiolabeled monoaminergic transmitters have provided evidence that longer side-chains confer serotonin-reuptake-inhibition properties to a compound, whereas synthetic cathinones with shorter side-chains are likely to function as serotonin-releasing agents (Eshleman et al., 2013; 2016; Simmler et al., 2013; 2014). Because serotonin release confers unique subjective effects to psychostimulants and has been negatively associated with reinforcing efficacy (Wee et al., 2005), we hypothesized that a positive relation exists between side-chain length, serotonergic discriminative stimulus, and reinforcing efficacy.

Although butylone has been found in "bath salt" preparations (Leffler et al., 2014) and "Ecstasy" formulations (Palamar, 2016c; Warrick et al., 2011), few studies have characterized its effects and it has not maintained the same popularity as compounds like MDPV and mephedrone. Even less attention has been given to pentylone. Behavioral studies have demonstrated that both butylone and pentylone produce locomotor stimulation and substitute for the discriminative stimulus effects of methamphetamine and cocaine, indicating a dopaminergic component to their *in vivo* effects (Gatch et al., 2013; 2015a). Mechanistic studies assessing the *in vitro* effects of these compounds have produced somewhat inconsistent results. In one study,

butylone was demonstrated to possess cocaine-like uptake inhibition properties at DAT with limited releasing properties at SERT (Eshleman et al., 2013), whereas another indicated clear hybrid uptake inhibition/release properties with reuptake inhibition occurring at DAT and strong release at SERT (Simmler et al., 2013). In the same two studies, methylone demonstrated different degrees of 5-HT and dopamine release, and yet another study demonstrated much more robust monoamine-releasing properties (Baumann et al., 2012). Pentylone's *in vitro* mechanism has only been assessed in two published studies indicating DAT inhibition and weak SERT substrate efficacy (Simmler et al., 2014; Kolanos et al., 2013). Although pentylone's uptake inhibition at DAT has been fairly well-established (Simmler et al., 2014; Kolanos et al., 2013; Eshleman et al., 2016), its exact mechanism at SERT is still equivocal with one study suggesting weak release (Simmler et al., 2014) and another indicating uptake inhibition (Eshleman et al., 2016).

The current study aims to address the mechanistic discrepancies reported *in vitro* for methylone and butylone. Furthermore, we aim to expand on previous studies regarding the discriminative stimulus effects of these compounds by addressing their dopaminergic and serotonergic effects in methamphetamine-, MDMA-, and DOM-trained rats in tests for substitution. We aim to further probe their mechanism using the D1-like receptor selective antagonist SCH23390 against the test drugs that produce full substitution in the drug discrimination assay. Lastly, the reinforcing effects of these compounds will be assessed using a self-administration assay utilizing both fixed- and progressive-ratio schedules of reinforcement.



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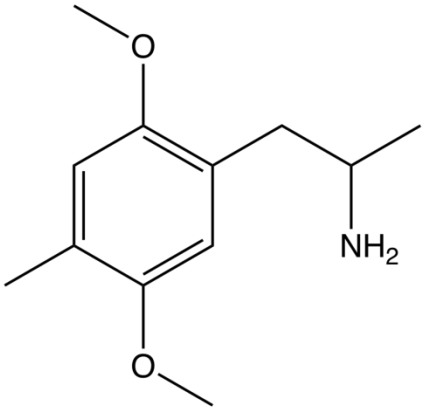

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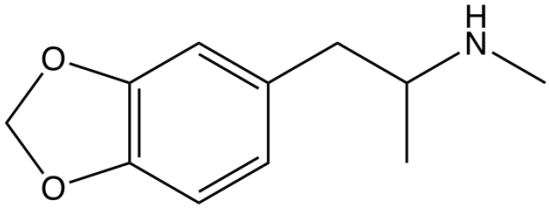
(DOM)



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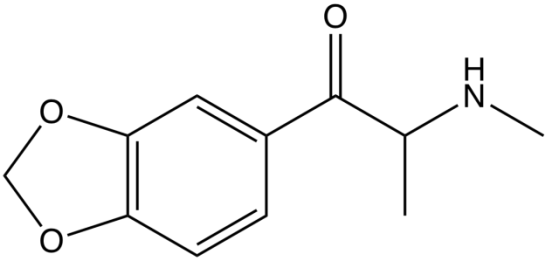

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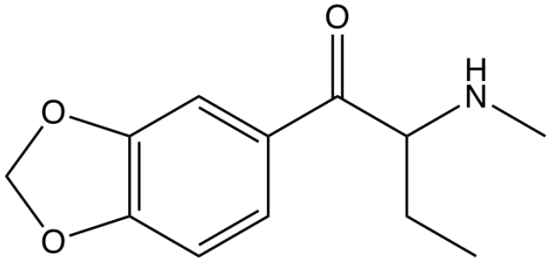
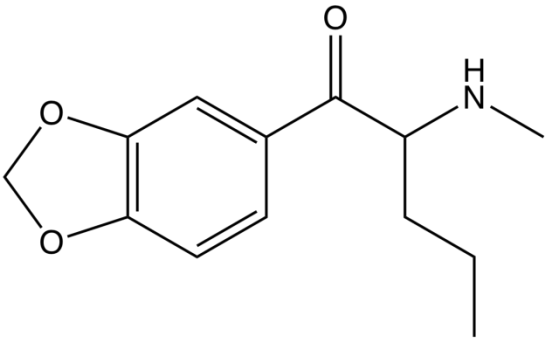
(Methylone)



β-keto-*N*-methylbenzodioxolylbutanamine
(Butylone)



β-keto-*N*-methylbenzodioxolylpentanamine
(Pentylone)

Figure 1: Chemical structures of training and test compounds

CHAPTER 2

SEROTONERGIC AND DOPAMINERGIC MECHANISM OF SYNTHETIC CATHINONE ANALOGS OF MDMA

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Running title page

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D) List of nonstandard abbreviations: 5HT, 5-hydroxytryptamine, serotonin; DAT, dopamine transporter; DAR, drug-appropriate responding; DOM, 2,5-dimethoxy-4-methylamphetamine; GFP, green fluorescent protein; HEK, human embryonic kidney; MDMA, 3,4-methylenedioxymethamphetamine; SCH23390, 7-chloro-3-methyl-1-phenyl-1,2,4,5-tetrahydro-3-benzazepin-8-ol; SERT, serotonin transporter

E) Recommended section assignment: Behavioral pharmacology

Abstract

Despite increased regulation, novel psychoactive substances remain a prominent component of global drug culture, with synthetic cathinones comprising a large portion of the market. Many synthetic cathinones are purchased legally online to be used alone; however, numerous "first generation" synthetic cathinones have been diverted into "Ecstasy" formulations *in lieu* of MDMA. The current study aimed to assess the dopaminergic and serotonergic mechanisms of three under-researched synthetic cathinone analogs of MDMA frequently encountered in "Ecstasy" formulations: methylone, butylone, and pentylone. The *in vitro* mechanism of each of the test compound was tested by measuring drug-induced current with voltage-clamp electrophysiology on cells expressing SERT. The *in vivo* mechanisms were determined using drug discrimination with rats trained to discriminate either methamphetamine, DOM, or MDMA. The D1-selective antagonist SCH23390 was tested against test compounds that substituted for the training drug. MDMA, methylone, and butylone each produced inward currents at SERT, indicative of substrate activity at SERT. Each test compound fully substituted for the discriminative stimulus effects of methamphetamine, methylone and butylone substituted fully for MDMA but partially for DOM, and pentylone substituted partially for MDMA but not DOM. SCH23390 fully attenuated methamphetamine-appropriate responding, with pentylone being the least sensitive to the antagonistic effects. Conversely, SCH23390 partially attenuated MDMA-appropriate responding for methylone and had no effect against butylone. These data indicate complex, dopamine- and serotonin-mediated mechanistic effects for methylone and butylone, but a predominately dopaminergic mechanism of pentylone. Consequently, methylone and butylone, like MDMA, may have limited potential for compulsive abuse, whereas pentylone may pose a significant risk for abuse, like primarily-dopaminergic psychostimulants.

Introduction

In recent years, the club drug "Molly", which is typically sold in powdered form and popularly considered and branded as pure MDMA, has surged in popularity and is considered a staple in modern-day dance, rave, and music festival culture (Palamar, 2017). However, much like the tablet-based "Ecstasy", powdered "Molly" formulations are regularly adulterated with other psychoactive substances in addition to and *in lieu* of MDMA (UNODC, 2014; Ecstasydata.org, 2017; Palamar et al., 2016c). The compounds most commonly used as adulterants in these "Molly" formulations are the synthetic cathinones, which present with numerous adverse effects, including serotonin syndrome, agitation, and death (Palamar et al., 2016b; Elliott & Evans, 2014; German et al., 2014; Warrick et al., 2012). The current study aimed to determine the mechanism of action for three synthetic cathinone analogs of MDMA: methylone, butylone, and pentylone (Figure 1).

MDMA is known and preferred for its unique subjective effects including improved sociability, an overall sense of trust or empathy, and enhanced appreciation of music in addition to the increased energy associated with other stimulant-type drugs (Pentney, 2001). Although MDMA reverses the direction of presynaptic monoamine transport in a manner similar to methamphetamine, it differs in its selectivity profile, with a slightly greater affinity for the serotonin transporter (SERT) over the dopamine transporter (DAT), whereas methamphetamine is roughly 10- to 100-fold more selective for DAT than SERT (Simmler et al., 2013; 2014). These DAT- and SERT-mediated effects are also apparent in MDMA's *in vivo* discriminative stimulus effects. Several studies using two- and three-choice drug discrimination procedures have indicated a complex discriminative stimulus of MDMA mediated by both dopaminergic and serotonergic signaling (Goodwin & Baker, 2000; Goodwin et al., 2003; Schechter, 1986). The

relative contributions of dopamine and serotonin to MDMA's discriminative stimulus effects appear to be dose- and time-dependent (Harper et al., 2014; Webster et al., 2016; Schechter, 1988). Furthermore, there is a stereoselective component to the discriminative stimulus effects of MDMA wherein (+)-MDMA is predominately dopaminergic, while still retaining a serotonergic mechanism, and (–)-MDMA is exclusively serotonergic (Murnane et al., 2009). The complex nature of MDMA's discriminative stimulus may provide the unique subjective effects that have made MDMA, "Ecstasy", and "Molly" so popular amongst club-goers.

The synthetic cathinones are a structurally-diverse class of compounds that exist along a mechanistic spectrum from amphetamine-like monoamine releasing agents to cocaine-like uptake inhibitors, all with varying selectivity profiles (Simmler et al., 2013; 2014; Eshleman et al., 2013; 2016). Pentylone is considered to act as a cocaine-like uptake inhibitor with equal potency at DAT and SERT (Eshleman et al., 2016). Butylone has a "hybrid" profile acting as an uptake inhibitor at DAT and a releasing agent at SERT (Simmler et al., 2013; Eshleman et al., 2013). The data for methylone, on the other hand, have been somewhat discrepant with two studies indicating MDMA-like monoamine release (Eshleman et al., 2013; Bauman et al., 2012), and another suggesting cocaine-like uptake inhibition (Simmler et al., 2013). The high volume of compounds available on the market has made it difficult to characterize many of these compounds *in vivo* in a timely manner. Of the three cathinone derivatives of MDMA, the discriminative stimulus effects of methylone have been most thoroughly characterized, with reports indicating substitution for cocaine and methamphetamine (Gatch et al., 2013) and MDMA, but not DOM (Dal Cason et al., 1997). Butylone and pentylone both substitute for methamphetamine and cocaine (Gatch et al., 2013; 2015). Together, these data implicate

dopamine in the discriminative stimulus effects of these cathinone analogs of MDMA, but provide limited, if any, information regarding their serotonergic contributions.

The current study aims to clarify the *in vitro* mechanism of methylone at SERT using an alternative, electrophysiological methodology than the radiolabeled transmitter uptake and release assays generally employed, and to evaluate the relative dopaminergic and serotonergic contributions to the discriminative stimulus effects of these compounds using rats trained to discriminative methamphetamine, DOM, or MDMA from vehicle.

Materials and Methods

Electrophysiology Experiments

Cells and Transporters

Human embryonic kidney (HEK) 293 cells were used in all electrophysiological experiments. Cells were transiently transfected using PolyJet™ transfection reagent (SignaGen, Rockville, MD) transfection system with 0.5 μg GFP-tagged human serotonin-transporter (SERT; Origene Technologies, Rockville, MD). Expression was confirmed with fluorescent microscopy (Nikon Eclipse TS100 and Nikon Imaging Software, Nikon Instruments, Tokyo, Japan) and testing occurred 48 hours following transfection.

Electrophysiology

Whole-cell patch-clamp electrophysiology was used to assess dopamine-, 5-hydroxytramptamine- (serotonin, 5HT), MDMA-, methylone-, butylone-induced Na^+ currents. All experiments were conducted at room temperature (22-25°C) on a minimum of four cells with membrane potential clamped at -70 mV. Patch pipettes of borosilicate glass (1B150F; World Precision Instruments, Inc., Sarasota, FL) were pulled (Flaming/Brown, P-87/PC; Sutter

Instrument Company, Novato, CA) to a tip resistance of 4 to 6 MΩ. Patch pipettes were filled with a solution consisting of 140 mM K-gluconate, 10 mM Na_4-EGTA, 10 mM HEPES, 4 mM Na_2-ATP, 4 mM MgCl, and 0.4 mM Na_3-GTP, pH 7.3. Coverslips containing cultured cells were placed in the recording chamber on the stage of an inverted light microscope (Olympus IMT-2; Olympus, Tokyo, Japan) and superfused continuously with an external solution consisting of 130 mM NaCl, 10 mM HEPES, 1.5 mM $CaCl_2$, 1.3 mM KCl, 0.5 mM $MgCl_2$, and 34 mM glucose, pH 7.3. Agonist-induced Na^+ currents were obtained with an PC-505B amplifier (Warner Instruments, Hamden, CT). Currents were low-pass filtered at 5 kHz, monitored simultaneously on an oscilloscope and a chart recorder (Gould TA240; Gould Instrument Systems Inc., Cleveland, OH), and stored on a computer using an on-line data acquisition system (pCLAMP 6.0; Axon Instruments) for subsequent off-line analysis.

To qualitatively assess directionality of current, MDMA, methylone, or butylone was applied for 10 secs at a concentration of 100 μM. 5HT, at a concentration of 1 or 10 μM was applied for 10 secs prior to and after drug application as a baseline control. Drugs were applied for 10 secs at 60 sec intervals.

Data Analysis

Raw currents of methylone and butylone were normalized to the 5HT current preceding drug application for analysis. The normalized currents were compared using an independent samples *t* test comparing drug. Only methylone and butylone were assessed quantitatively as MDMA was tested with a different 5HT control (1 μM) and therefore incomparable.

Drug Discrimination

Animals

Male Sprague-Dawley rats were obtained from Envigo (Indianapolis, IN). All rats were housed individually and were maintained on a 12:12 light/dark cycle (lights on at 7:00 AM). All experiments were run during the light cycle. Body weights were maintained at 320-350 g by limiting food to 15 g/day. Water was readily available. All housing and procedures were in accordance with Guidelines for the Care and Use of Laboratory Animals (National Research Council, 2011) and were approved by the University of North Texas Health Science Center Animal Care and Use Committee.

Discrimination Procedures

Standard behavior-testing chambers (Coulbourn Instruments, Allentown, PA) were connected to IBM-PC compatible computers via LVB interfaces (Med Associates, East Fairfield, VT). The computers were programmed in Med-PC for Windows, version IV (Med Associates, East Fairfield, VT) for the operation of the chambers and collection of data.

Using a two-lever choice methodology, a pool of rats previously trained to discriminate either methamphetamine (1 mg/kg), MDMA (1.5 mg/kg) or DOM (0.5 mg/kg) from saline as previously described (Gatch et al., 2016) were tested. Rats received an injection of either saline or drug and were subsequently placed in the behavior-testing chambers, where food (45 mg food pellets; Bio-Serv, Frenchtown, NJ) was available as a reinforcer for every ten responses on a designated injection-appropriate lever. The pretreatment time was 10 min for methamphetamine, 15 min for MDMA, and 30 min for DOM. Each training session lasted a maximum of 10 min, and the rats could earn up to 20 food pellets. The rats received approximately 60 of these sessions before they were used in tests for substitution of the experimental compounds. Rats

were used in testing once they had achieved 4 consecutive sessions at 85% injection-appropriate responding for both the first reinforcer and total session. The training sessions occurred on separate days in a double alternating fashion (drug-drug-saline-saline-drug; etc.) until the training phase was complete, after which substitution tests were introduced into the training schedule such that at least one saline and one drug session occurred between each test (drug-saline-test-saline-drug-test-drug; etc.). The substitution tests occurred only if the rats had achieved 85% injection-appropriate responding on the two prior training sessions.

Test sessions lasted for a maximum of 20 min. In contrast with training sessions, both levers were active, such that 10 consecutive responses on either lever led to reinforcement. Data were collected until 20 reinforcers were obtained, or for a maximum of 20 min. Each compound was tested in groups of six to eight rats. The dose effect of each compound was tested from no effect to full effect or rate suppression (<20% of vehicle control) or adverse effects. Doses were tested in no particular order. For substitution experiments, MDMA, methylone, butylone, pentylone, or their vehicle were administered intraperitoneally (1 mL/kg) 15 minutes before the start of the session. For antagonism studies, SCH23390 was administered subcutaneously 30 min before the start of the session. Dose-response studies of SCH23390 were administered in conjunction with the lowest substituting dose of the test compound that fully substituted for the training drug. A repeated-measures design was used, such that each rat was tested at all doses of a given drug.

Drugs

(+)-methamphetamine HCl, (±)-3,4-methylenedioxymethamphetamine HCl (MDMA), (-)-2,5-dimethyoxy-4-methylamphetamine HCl (DOM), methylone HCl, butylone HCl, and pentylone HCl were provided by the National Institute on Drug Abuse Drug Supply Program and

were dissolved in 0.9% saline. SCH23390 HCl was purchased from Cayman Chemical (Ann Arbor, MI) and dissolved in 0.9% saline. All drugs were injected intraperitoneally at an injection volume of 1 mL/kg, except SCH23390, which was injected subcutaneously.

Data Analysis

Drug discrimination data are expressed as the mean percentage of drug-appropriate responses occurring in each test period. Rates of responding were expressed as a function of the number of responses made divided by the total session time. Graphs for percent drug-appropriate responding and response rate were plotted as a function of dose of test compound (log scale). Percent drug-appropriate responding was shown only if at least 3 rats completed the first fixed ratio. Full substitution was defined as >80% drug-appropriate responding and not statistically different from the training drug. Full antagonism was defined as <20% drug-appropriate responding and not significantly different than the vehicle control.

Rates of responding were expressed as a function of the number of responses made divided by the total session time. Response rate data was analyzed by one-way repeated measures analysis of variance for studies assessing multiple doses, or by a paired Student's *t* test in single dose analyses. In the substitution tests, effects of individual doses were compared to the vehicle control value using a priori contrasts. In the antagonism tests, effects of individual doses were compared to the antagonist vehicle + test drug control value using a priori contrasts.

Results

Electrophysiology

Application of 5HT, MDMA, methylone, and butylone produced inward currents at SERT (Figure 2). Data are presented as the mean ± SEM of the normalized currents in Figure 3.

A concentration of 100 μM was chosen for comparative analysis as this concentration represents a transporter-saturating and likely physiological plasma concentration based on previous pharmacokinetic analyses of methylone, the only of the presently-assessed compounds to be measured in plasma, in rats (Eshleman et al., 2013; Simmler et al., 2014; Lopéz-Arnau et al., 2013; Elmore et al., 2017). Pentylone was not tested as supplies of drug were exhausted in the discrimination studies prior to electrophysiological assessment. An independent samples *t*-test determined that there was no difference between the relative currents induced by 100 μM methylone (85.3% ± 8.2) and butylone (72.4% ± 5.3; t_6=1.32, p=.235).

Drug Discrimination

Methamphetamine-trained rats

Substitution

Each test compound substituted for discriminative stimulus effects of 1 mg/kg methamphetamine (Figure 4). Methylone (5 mg/kg), butylone (10 mg/kg), and pentylone (10 mg/kg) fully substituted for the discriminative stimulus effects of 1 mg/kg methamphetamine, producing 82%, 87%, and 93% drug-appropriate responding (DAR), respectively. MDMA (2.5 mg/kg) produced 79% DAR, but was still tested as 80% DAR was exhibited in the first-reinforcer data. There were no effects on response rate by pentylone ($F_{4,28}$= 0.425, p=.79); however, MDMA reduced response rate to 26% of vehicle control following 2.5 mg/kg ($F_{3,21}$= 17.841, p<.001) and butylone reduced response rate to 68% of vehicle control following 10 mg/kg ($F_{4,28}$= 6.176, p<.001). Methylone increased response rate at 1 mg/kg ($F_{3,18}$= 4.883, p=.012).

SCH23390 antagonism

SCH23390 fully and dose-dependently attenuated DAR of each test compound (Figure 5). Methamphetamine (1 mg/kg)-induced DAR was reduced to 9% following 0.05 mg/kg SCH23390. MDMA (2.5 mg/kg)-induced DAR was reduced to 30% following 0.01 mg/kg SCH23390. Full antagonism of MDMA (<20% DAR) could not be achieved as seven of eight rats failed to receive a reinforcer following 0.025 mg/kg SCH23390. Methylone (5 mg/kg)-induced DAR was reduced to 19% following 0.01 mg/kg SCH23390 and 8% at 0.025 mg/kg SCH23390. Butylone (10 mg/kg)-induced DAR was reduced to 8% following 0.025 mg/kg SCH23390. Pentylone (10 mg/kg)-induced DAR was reduced to 17% following 0.025 mg/kg SCH23390.

SCH23390 dose-dependently attenuated response rate of each compound tested. Methamphetamine (1 mg/kg) response rate was reduced to 23% and 11% vehicle + methamphetamine control following 0.025 and 0.05 mg/kg SCH23390, respectively ($F_{4,20}$= 45.434, p<.001). MDMA (2.5 mg/kg) response rate was reduced to 1% vehicle + MDMA control following 0.025 mg/kg SCH23390 ($F_{5,35}$= 3.871, p=.007). Methylone (5 mg/kg) response rate was reduced to 43% and 20% of vehicle + methylone control following 0.01 and 0.025 mg/kg SCH23390, respectively ($F_{5,35}$= 9.455, p<.001). Butylone (10 mg/kg) response rate was reduced to 22% of vehicle + butylone control following 0.025 mg/kg SCH23390 ($F_{3,21}$= 10.647, p<.001). Pentylone (10 mg/kg) response rate was reduced to 29% and 2% vehicle + pentylone control following 0.025 and 0.05 mg/kg SCH23390, respectively ($F_{3,21}$= 32.285, p<.001).

Differences in the sensitivity to the antagonistic effects of SCH23390 on methamphetamine-DAR were determined by an analysis of variance of the slopes of the

antagonism curves among drugs ($F_{3,28}$= 12.176, p<.01). Pairwise comparisons with a Bonferroni correction revealed that pentylone's slope was significantly steeper than MDMA, methylone or butylone. At 0.01 mg/kg SCH23390, the dose of SCH23390 that was tested among each test compound and most rats tested responded, the response rate of pentylone was unaffected relative to its vehicle control and was significantly greater than the other test compounds ($F_{4,33}$= 4.984, p=.003).

DOM-trained rats

None of the compounds tested substituted for the discriminative stimulus effects of DOM (Figure 6). MDMA produced 54% DAR following 3 mg/kg, and response rate was reduced to 27% of vehicle control following this dose with two of seven rats failing to complete the first fixed ratio. Response rate was severely reduced with MDMA ($F_{4,24}$= 16.15, p<.001), impeding further testing as six of seven rats failed to complete the first fixed ratio and response rate was reduced to 1% of vehicle control following 5 mg/kg. Comparable to MDMA, methylone produced only 58% DAR following 10 mg/kg. Methylone dramatically reduced responding ($F_{4,32}$=11.065, p<.001) as 10 mg/kg reduced response rate to 33% of vehicle control with two of nine rats failing to respond. Similarly, butylone produced 45% DAR following 25 mg/kg. Response rate was significantly reduced following 25 mg/kg butylone ($F_{4,24}$= 7.25, p=.001) with three of eight rats failing to complete the first fixed ratio, precluding testing of higher doses. Pentylone substituted partially for DOM with 41.3% DAR among three rats, but reduced response rate to 17% of vehicle control and six of nine rats failed to complete the first fixed ratio ($F_{3,24}$= 22.177, p<.001).

MDMA-trained rats

Substitution

Methylone (5 mg/kg) and butylone (10 mg/kg) both fully substituted for the discriminative stimulus effects of MDMA producing 87% and 100% DAR, respectively (Figure 7). Neither methylone ($F_{3,21}$= 2.311, p=.106) nor butylone ($F_{4,28}$= 1.521, p=.223) affected response rate. Pentylone, conversely, produced only 75% DAR following 10 mg/kg, and at 25 mg/kg only one of eight rats completed the first fixed ratio. Pentylone increased response rate at 10 mg/kg, but significantly attenuated responding at 25 mg/kg ($F_{5,35}$= 45.775, p<.001).

SCH23390 antagonism

SCH23390 partially attenuated MDMA DAR for methylone and MDMA; however, the antagonism was more efficacious against MDMA, with 0.01 mg/kg SCH23390 producing 43% DAR, than methylone, against which 0.025 mg/kg SCH23390 produced 68% DAR (Figure 8). SCH23390 did not attenuate the MDMA DAR for butylone at any dose. In pentylone-treated rats, SCH23390 strongly attenuated MDMA-DAR to 25% at 0.01 mg/kg. In both MDMA- and methylone-treated rats, the highest dose of SCH23390 tested increased DAR relative to the maximal effects, with 0.025 mg/kg SCH23390 producing 81.2% DAR in MDMA-treated rats and 0.05 mg/kg SCH23390 producing 76% DAR in methylone-treated rats. SCH23390 dose-dependently reduced response rate in MDMA ($F_{4,28}$= 31.537, p<.001), methylone ($F_{5,35}$= 21.788, p<.001), and butylone ($F_{3,21}$= 18.142, p<.001), but not pentylone (t_6= -1.257, p=0256). At 0.025 mg/kg SCH23390, five of eight MDMA-treated rats failed to complete the first fixed ratio, and at 0.05 mg/kg SCH23390, four of eight methylone-treated rats and one of eight butylone-treated rats failed to complete the first fixed ratio.

Discussion

The current study aimed to determine relative contributions of dopamine and serotonin to the mechanistic effects of three synthetic cathinone analogs of MDMA: methylone, butylone, and pentylone. We found that methylone and butylone, like MDMA, produce inward currents at SERT in a whole-cell patch clamp electrophysiology assay, indicative of MDMA-like substrate activity (De Felice et al., 2014). In the drug discrimination assay, each test compound fully substituted for the discriminative stimulus effects of methamphetamine. MDMA, methylone, and butylone partially substituted for the discriminative stimulus effects of DOM and fully for MDMA, whereas pentylone produced limited DOM-appropriate responding at a dose that robustly inhibited response rate and only partially substituted (75%) for MDMA. SCH23390 fully and dose-dependently attenuated methamphetamine-appropriate responding of each test compound, with pentylone being the least sensitive to its effects, and fully attenuated pentylone-induced MDMA-DAR, but failed to antagonize MDMA-appropriate responding of MDMA, methylone, or butylone.

The substitution data indicate differences in the relative contributions of dopamine and serotonin to the discriminative stimulus effects among the compounds tested. Each of the test compounds fully substituted with similar potency for the discriminative stimulus effects of methamphetamine, which are predominately mediated by dopamine (Munzar & Goldberg, 2000) with limited noradrenergic (Munzar & Goldberg, 1999) or serotonergic influence (Munzar et al., 1999). The cathinone compounds readily substituted for methamphetamine, but MDMA significantly reduced response rate at 2.5 mg/kg. MDMA is considered to have a complex discriminative stimulus mediated by both dopamine and serotonin (Goodwin & Baker, 2000;

Schechter, 1988), and the dose that substituted may have reached a dopaminergic threshold for methamphetamine substitution with the high serotonergic signal reducing response rate.

MDMA, methylone, and butylone produced partial substitution (>40% DAR) in DOM-trained rats. Although 25 mg/kg pentylone produced 41% DAR in DOM-trained rats, only three of nine rats completed the first fixed ratio at this dose, with only one rat responding exclusively on the DOM-appropriate lever and another producing five responses on the DOM-paired lever and five on the vehicle-paired lever following the first reinforcer. The larger available sample sizes and partial substitution for DOM in MDMA-, methylone-, and butylone-treated rats suggests a serotonergic signal, either through direct $5HT_2$ activation or indirect activation through release; conversely, pentylone likely possesses a limited serotonergic mechanism given its rate reduction and small sample size at a single dose producing >20% responding. The serotonergic nature of methylone and butylone are further evidenced by their full, non-rate-inhibiting substitution for MDMA, contrasting with pentylone's partial substitution at 10 mg/kg and response diminution at 25 mg/kg. Previous studies assessing the dopaminergic and serotonergic nature of the MDMA training dose used in these experiments indicated that 1.5 mg/kg MDMA is predominately serotonergic, and compounds with primarily dopaminergic mechanisms, such as apomorphine, do not fully substitute for this training dose (Webster et al., 2016). These data preliminarily suggest a primarily dopaminergic mechanism for pentylone and complex, serotonin- and dopamine-mediated discriminative stimulus for methylone and butylone.

In order to further probe the mechanistic effects of these compounds, we utilized the D1-like selective antagonist SCH23390 against each test compound in methamphetamine- and MDMA-trained rats. SCH23390 fully and dose-dependently reduced methamphetamine-DAR in

each compound tested, which was expected given the D1-mediation of methamphetamine's discriminative stimulus (Munzar & Goldberg, 2000). MDMA-, methylone-, and butylone-induced methamphetamine-DAR was attenuated at much lower doses than those required to antagonize pentylone-induced methamphetamine-DAR. The increased sensitivity to the antagonistic effects of SCH23390 in MDMA-, methylone-, and butylone-treated rats suggests either reduced dopaminergic efficacy relative to pentylone or the involvement of an additional transmitter system in their discriminative stimulus effects. Based on the substitution profile of these compounds in DOM- and MDMA-trained rats, the latter possibility seems likely and suggests an unmasking of serotonergic effects with administration of SCH23390. This unmasking effect is further evidenced by the differential effects of SCH23390 against the test compounds in MDMA-trained rats. Previous studies using SCH23390 against MDMA have demonstrated only partial attenuation with D1-like receptor antagonism (Bubar et al., 2004), an effect that was replicated in the current study. The minimal efficacy of SCH23390 to attenuate methylone- and butylone-induced MDMA-DAR suggests that rats can still attend to the serotonergic component of the discriminative stimulus after D1-like receptor blockade. Conversely, SCH23390 attenuated pentylone-induced MDMA-DAR (28.6%) at a dose (0.01 mg/kg) that had no effect on methamphetamine-DAR, suggesting that pentylone's discriminative stimulus and *in vivo* mechanism is predominately, if not entirely, mediated by dopaminergic signaling.

By utilizing an electrophysiology approach to studying transporter pharmacology, as opposed to the traditionally-used radio-labeled neurotransmitters uptake and release assays, we provided clarifying evidence for methylone's MDMA-like substrate activity at SERT. Although this model does not directly measure transmitter release, previous studies have indicated

differential electrical currents produced by amphetamine-like substrates and cocaine-like uptake-inhibiting agents, wherein substrates produce inward currents and uptake-inhibiting agents (Hilber et al., 2005; Galli et al., 1995; De Felice et al., 2014). Thus, these data, when considered with data generated with traditional assays, provide convergent evidence for serotonin release (Eshleman et al., 2013; Bauman et al., 2012) as opposed to uptake inhibition (Simmler et al., 2013). These data indicate a molecular mechanism similar to MDMA for methylone and butylone, and suggest that the similarities among MDMA, methylone, and butylone in drug discrimination experiments may stem from their serotonin-releasing properties. Conversely, pentylone, although not directly assessed *in vitro* in this study, inhibits dopamine and serotonin uptake similarly to cocaine (Eshleman et al., 2016). Although serotonin uptake inhibition increases synaptic serotonin concentrations, there appears to be a qualitative difference in the discriminative stimulus effects of impulse-independent serotonin-releasing agents and impulse-dependent serotonin uptake-inhibitors as evidenced by the full substitution for 1.5 mg/kg MDMA by norfenfluramine and fenfluramine (Schechter, 1988; Goodwin et al., 2002), yet only partial substitution by fluoxetine (Webster et al., 2016). Studies assessing cross-substitution of MDMA and cocaine have demonstrated asymmetrical substitution with inconsistent results among studies and a primary focus on stereoselectivity of MDMA; however, there is agreement on overlapping, but distinct, mechanistic effects of the two compounds (Khorana et al., 2004; Bondareva et al., 2005; Baker et al., 1995).

Altogether, these data indicate different mechanisms and discriminative stimuli among these three synthetic cathinone analogs, with methylone and butylone having an MDMA- or entactogen-like effect and pentylone, putatively, maintaining a more stimulant-like pharmacological profile. The distinct pharmacological profiles of these compounds likely confer

differing subjective effects and suggest divergent patterns of use, with methylone and butylone being utilized as MDMA alternatives at raves or other environments where "Molly" use is popular, and pentylone being used as an alternative to traditional psychostimulants. These data also suggest that pentylone, with its greater relative dopaminergic phenotype, is more likely to engender compulsive abuse than the MDMA-like compounds, and its inclusion in "Molly" or "Ecstasy" formulations may drive increased consumption of these formulations either as compulsive re-dosing in an acute setting or increased frequency of use. Further studies investigating the reinforcing efficacy of these compounds as well as their *in vivo* neurochemistry are warranted and necessary to fully comprehend their potential for compulsive use as well as development of interventions for overdose or dependence.

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The authors have no conflicts of interest to disclose.

Authorship Contributions

Participated in research design: Dolan, Gatch, Huang

Conducted experiments: Dolan

Performed data analysis: Dolan

Wrote or contributed to the writing of the manuscript: Dolan, Gatch

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Footnotes

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Figure Legends

Figure 1: Chemical structures of test compounds

Figure 2: Sample traces of drug-induced current

Sample traces of current produced by 5HT (10 μM), MDMA (100 μM), methylone (100 μM), and butylone (100 μM) at SERT in HEK293 cells (n=4) voltage clamped to -70 mV

Figure 3: Relative efficacy of test compounds at SERT

Normalized currents (relative to 10 μM 5HT) of methylone and butylone (100 μM). N.S. $p>.05$ in two-samples *t*-test

Figure 4: Substitution in methamphetamine-trained rats

Dose-response studies of methamphetamine-appropriate responding (top) and response rate (bottom) produced by MDMA (closed squares), methylone (open squares), butylone (closed triangles), and pentylone (open triangles). Pooled values for vehicle (open circles) and methamphetamine (closed circles) controls are plotted to the left of the break. n=8 unless otherwise indicated. *$p<.05$ reduction against vehicle control

Figure 5: SCH23390 antagonism of methamphetamine DAR

Dose-response studies of SCH23390 antagonism of methamphetamine-appropriate responding produced by MDMA (2.5 mg/kg; closed squares), methylone (5 mg/kg; open squares), butylone (10 mg/kg; closed triangles), and pentylone (10 mg/kg; open triangles). Pooled values for vehicle (open circles) and test compound (closed circles) controls are plotted to the left of the break. n=8 unless otherwise indicated. *$p<.05$ reduction against SCH23390 vehicle + test drug control

Figure 6: Substitution in DOM-trained rats

Dose-response studies of DOM-appropriate responding (top) and response rate (bottom) produced by MDMA (closed squares), methylone (open squares), butylone (closed triangles),

and pentylone (open triangles). Pooled values for vehicle (open circles) and DOM (closed circles) controls are plotted to the left of the break. n=8, except in methylone and pentylone where n=9, unless otherwise indicated. *p<.05 reduction against vehicle control

Figure 7: Substitution in MDMA-trained rats

Dose-response studies of MDMA-appropriate responding (top) and response rate (bottom) produced by MDMA (closed squares), methylone (open squares), butylone (closed triangles), and pentylone (open triangles). Pooled values for vehicle (open circles) and MDMA (closed circles) controls are plotted to the left of the break. n=8 unless otherwise indicated. *p<.05 reduction against vehicle control

Figure 8: SCH23390 antagonism of MDMA DAR

Dose-response studies of SCH23390 antagonism of MDMA-appropriate responding produced by MDMA (1.5 mg/kg; closed squares), methylone (5 mg/kg; open squares), and butylone (10 mg/kg; closed triangles). Pooled values for vehicle (open circles) and test compound (closed circles) controls are plotted to the left of the break, with the drug control for pentylone plotted separately (open triangle). n=8 unless otherwise indicated *p<.05 reduction against SCH23390 vehicle + test drug control

Figures

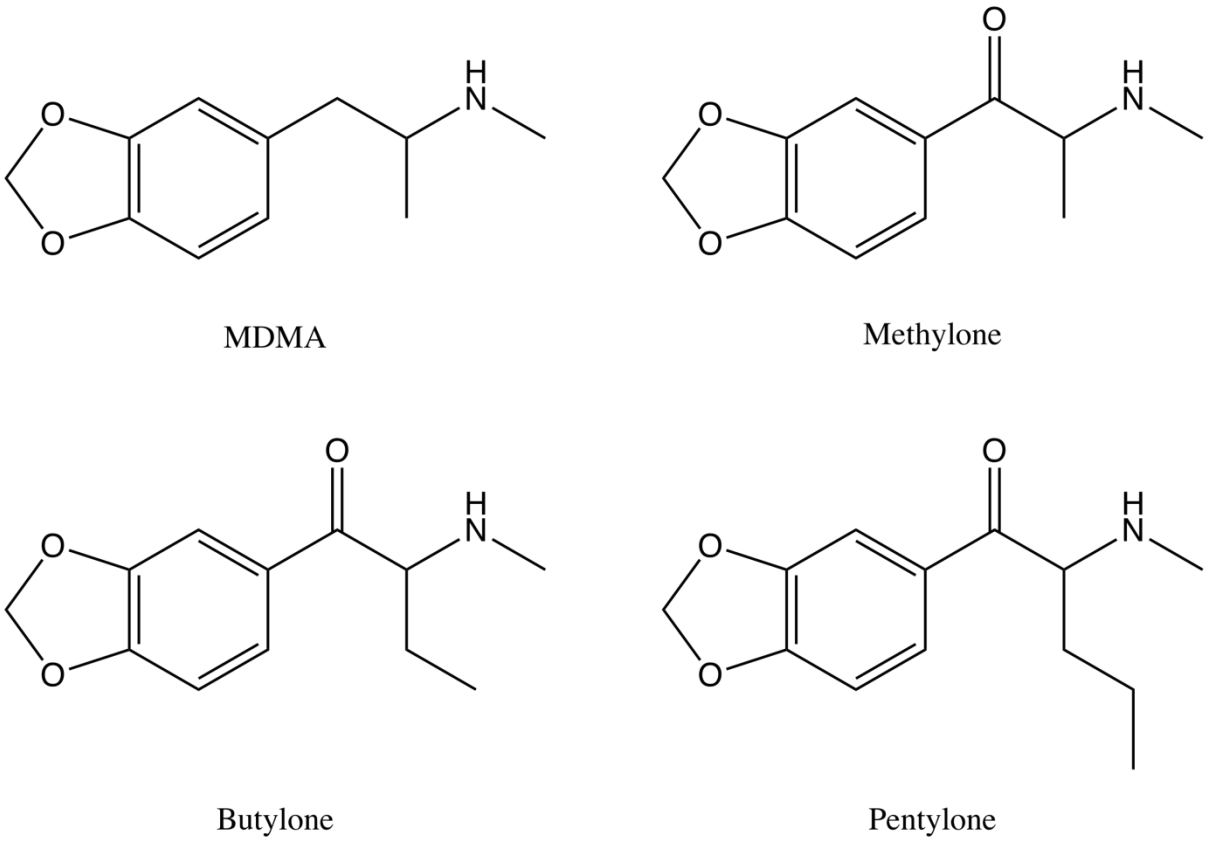

Figure 1

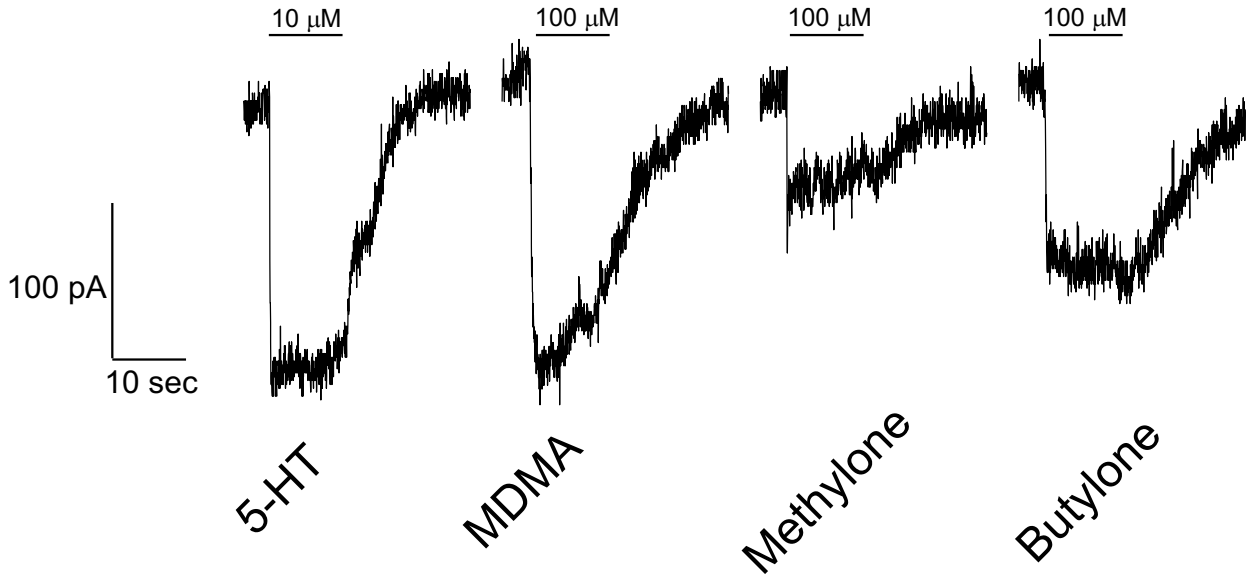
10 μM
100 μM
100 μM
100 μM
100 pA
10 sec
5-HT
MDMA
Methylone
Butylone

Figure 2

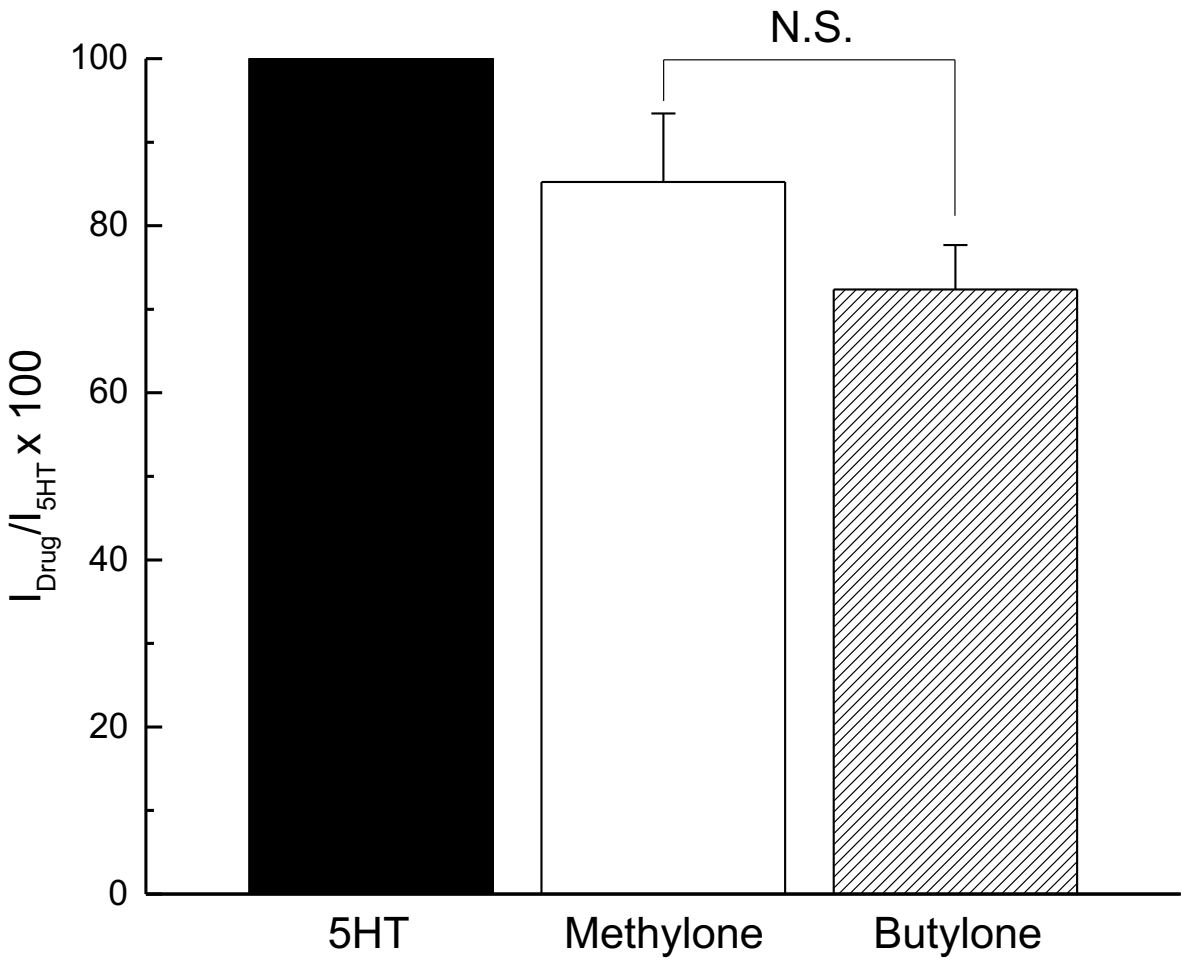

Figure 3

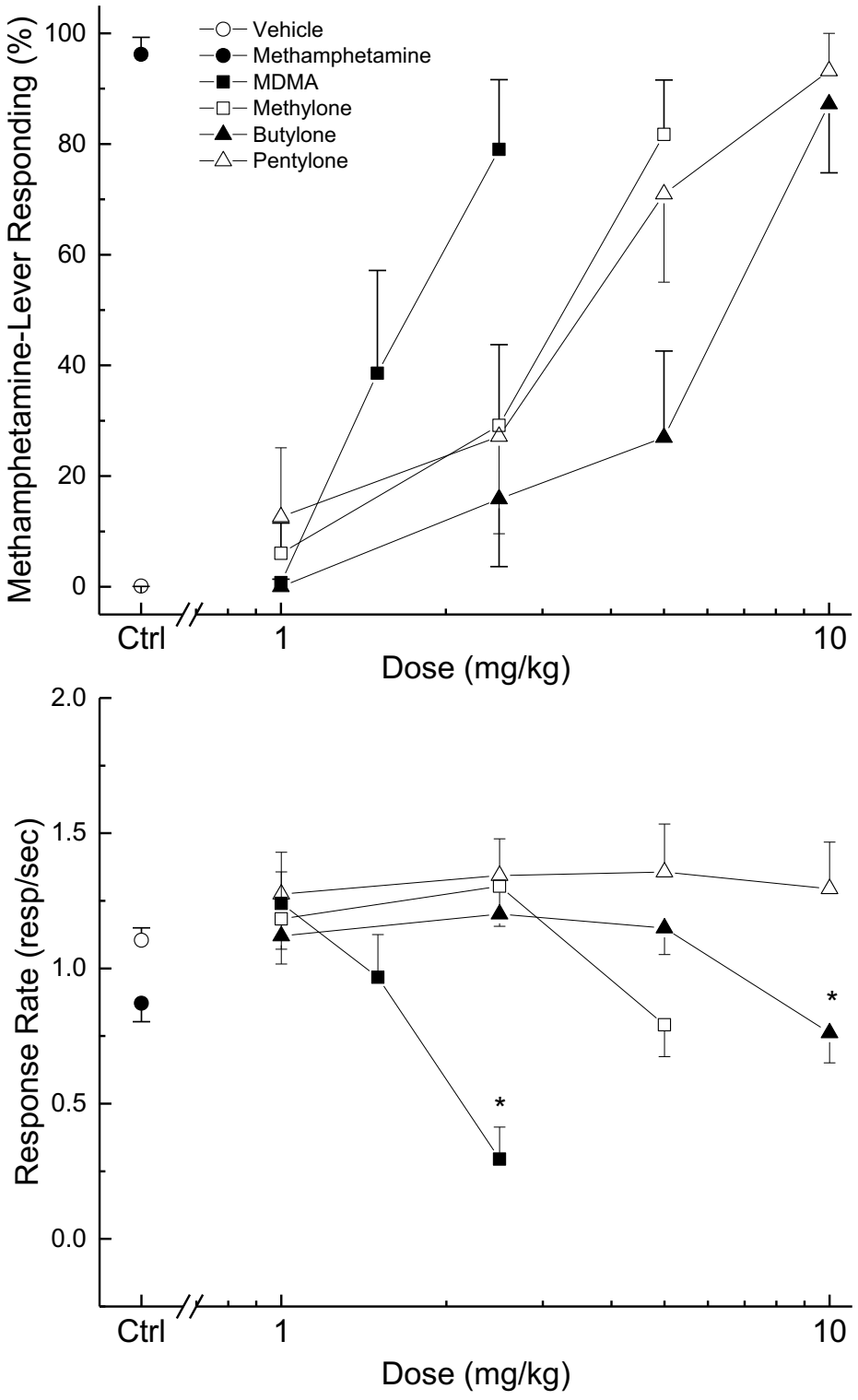
Vehicle
Methamphetamine
MDMA
Methylone
Butylone
Pentylone
Methamphetamine-Lever Responding (%)
100
80
60
40
20
0
Ctrl
1
10
Dose (mg/kg)
Response Rate (resp/sec)
2.0
1.5
1.0
0.5
0.0
*
*
Ctrl
1
10
Dose (mg/kg)

Figure 4

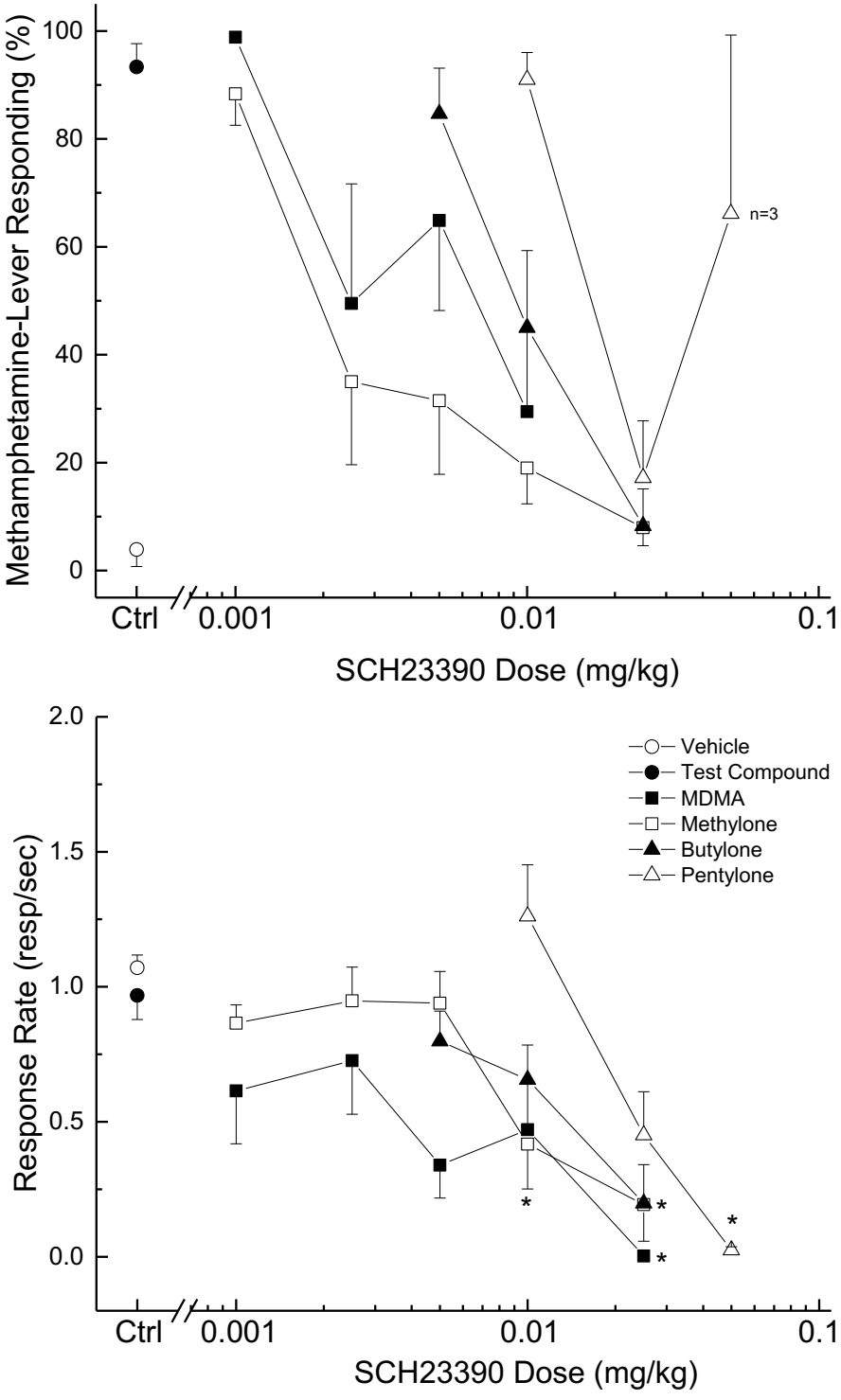
Methamphetamine-Lever Responding (%)
100
80
60
40
20
0
n=3
Ctrl
0.001
0.01
0.1
SCH23390 Dose (mg/kg)
Response Rate (resp/sec)
2.0
1.5
1.0
0.5
0.0
Vehicle
Test Compound
MDMA
Methylone
Butylone
Pentylone
*
Ctrl
0.001
0.01
0.1
SCH23390 Dose (mg/kg)

Figure 5

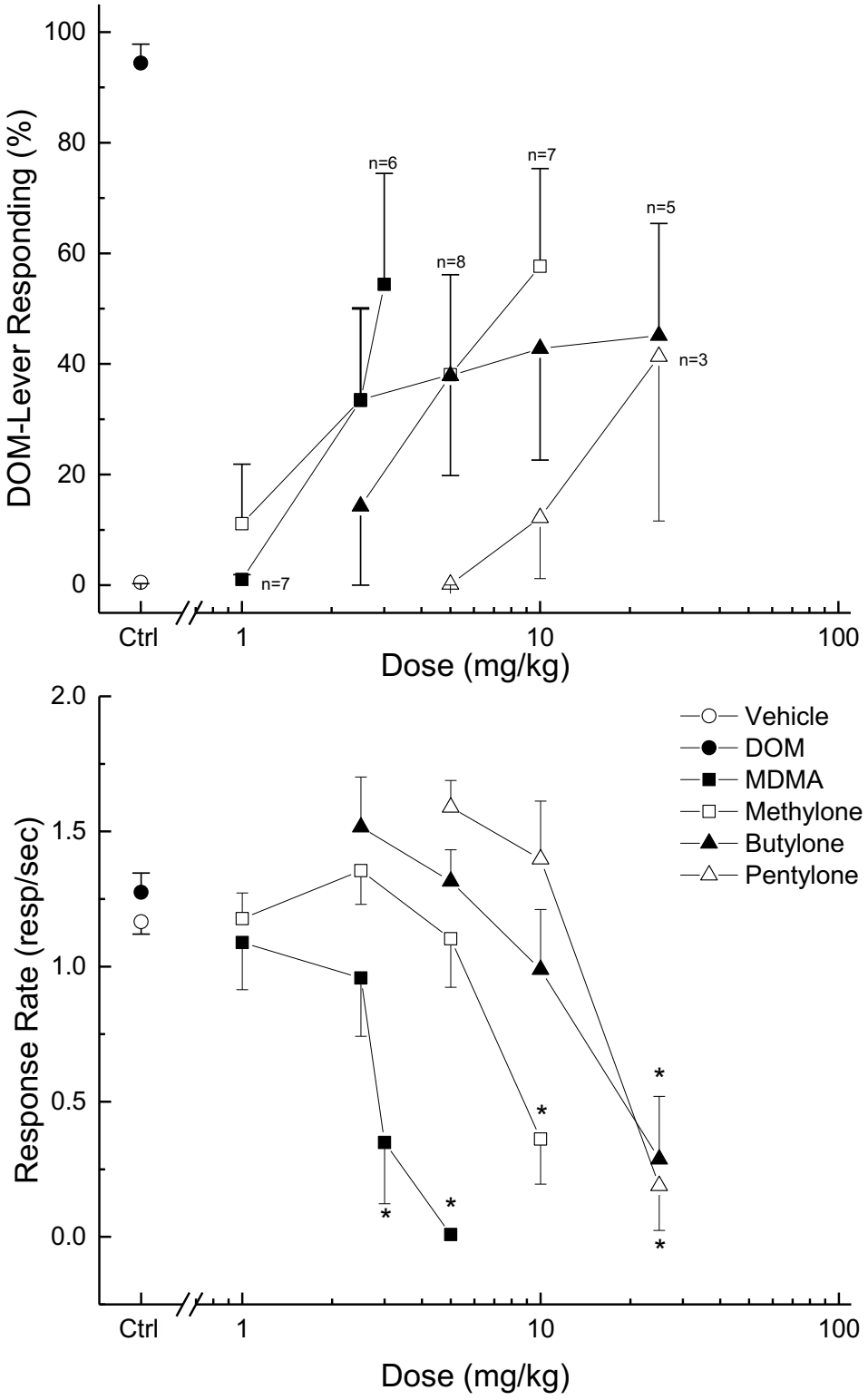
DOM-Lever Responding (%)
100
80
60
40
20
0
n=6
n=7
n=8
n=5
n=3
n=7
Ctrl
1
10
100
Dose (mg/kg)
Response Rate (resp/sec)
2.0
1.5
1.0
0.5
0.0
Vehicle
DOM
MDMA
Methylone
Butylone
Pentylone
*
Ctrl
1
10
100
Dose (mg/kg)

Figure 6

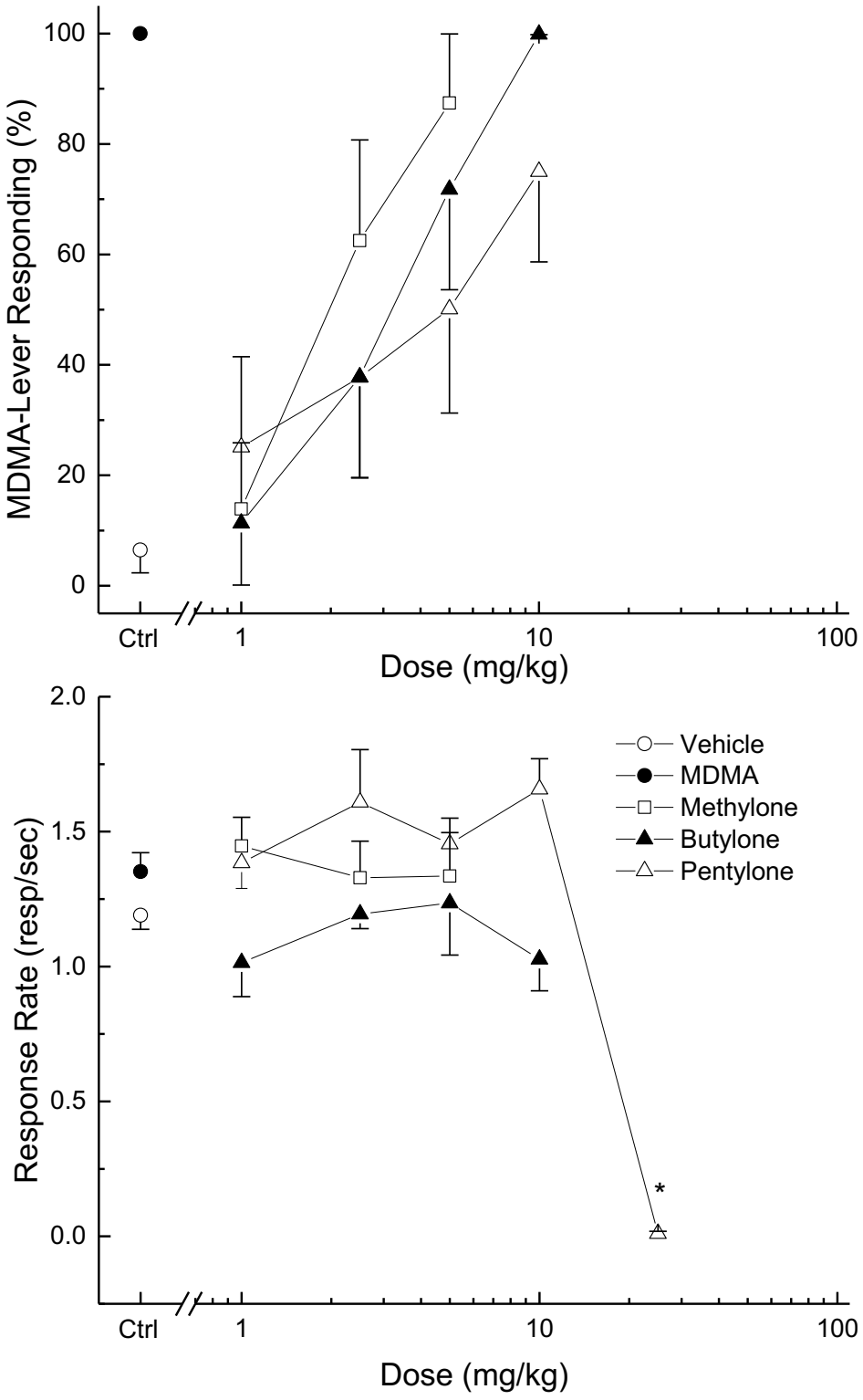
MDMA-Lever Responding (%)
100
80
60
40
20
0
Ctrl
1
10
100
Dose (mg/kg)
Response Rate (resp/sec)
2.0
1.5
1.0
0.5
0.0
Vehicle
MDMA
Methylone
Butylone
Pentylone
*
Ctrl
1
10
100
Dose (mg/kg)

Figure 7

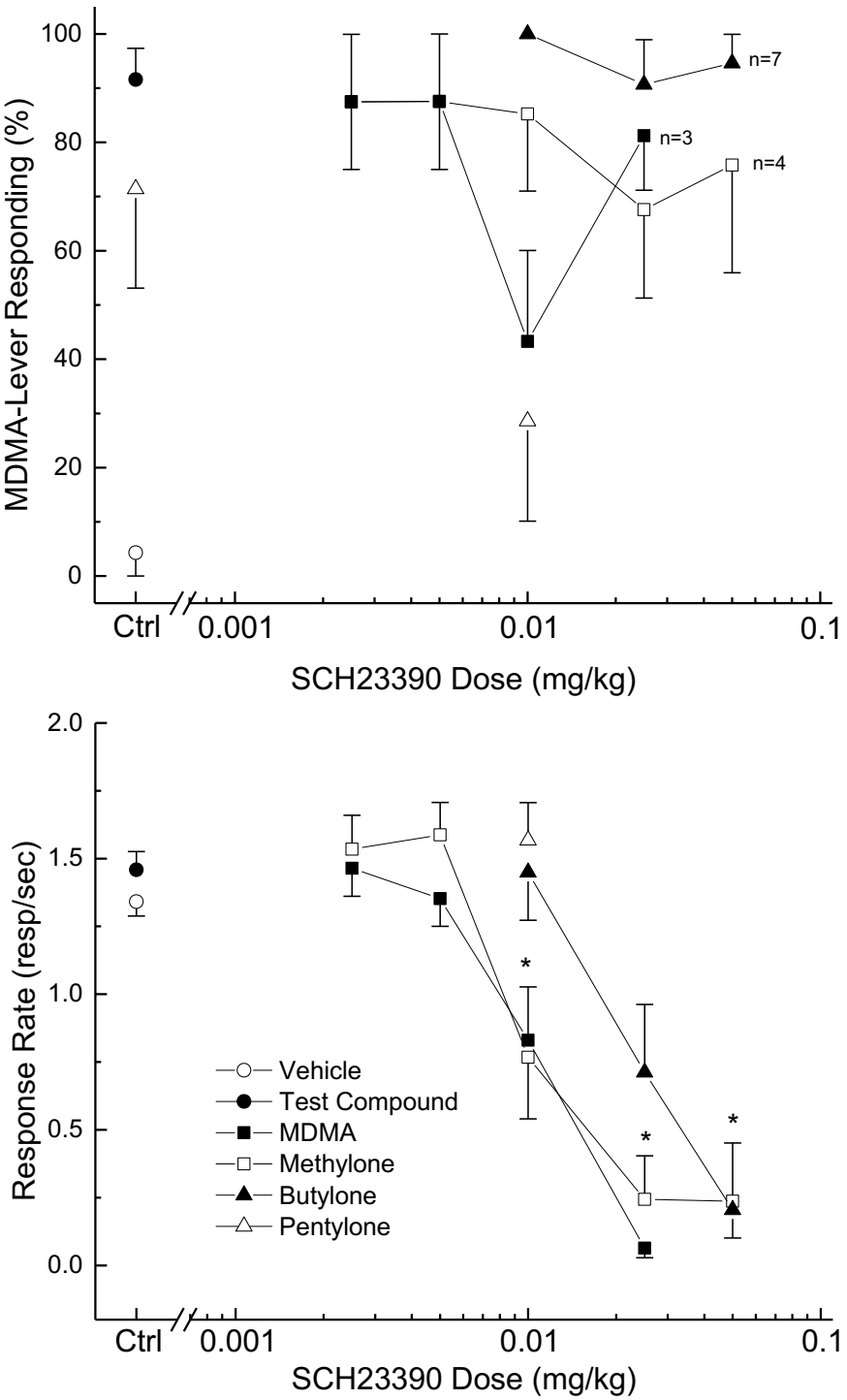
MDMA-Lever Responding (%)
100
80
60
40
20
0
n=7
n=3
n=4
Ctrl
0.001
0.01
0.1
SCH23390 Dose (mg/kg)
Response Rate (resp/sec)
2.0
1.5
1.0
0.5
0.0
*
*
*
Vehicle
Test Compound
MDMA
Methylone
Butylone
Pentylone
Ctrl
0.001
0.01
0.1
SCH23390 Dose (mg/kg)

Figure 8

CHAPTER 3

REINFORCING EFFICACY OF SYNTHETIC CATHINONE ANALOGS OF MDMA

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Abstract

Background: Synthetic analogs of cathinone remain a popular class of drugs among emerging novel psychoactive substances, and several analogs have been reported in "Ecstasy" or "Molly" formulations *in lieu* of MDMA. Although the potential for compulsive abuse of MDMA is considered relatively limited, less is known about the reinforcing effects of its synthetic cathinone counterparts.

Methods: The current study utilized intravenous self-administration to assess the reinforcing efficacy of three synthetic cathinone analogs of MDMA: methylone, butylone, and pentylone. Sprague-Dawley rats were trained to self-administer methamphetamine under either a continuous or progressive ratio schedule of reinforcement and subsequent dose-response studies for substitution were carried out with MDMA and the cathinones.

Results: Under the continuous schedule of reinforcement, all test compounds maintained methamphetamine-like responding with no differences in reinforcing efficacy among drugs. In the progressive ratio experiments, the breakpoint for each test compound increased in a dose-dependent manner; however, pentylone was more robustly self-administered than MDMA or butylone, and methylone was not significantly different from the other test compounds.

Conclusions: These data indicate that methylone and butylone are likely comparable to MDMA in terms of their potential for compulsive use, whereas pentylone may be more reinforcing than MDMA and more likely to promote compulsive use. "Ecstasy" formulations containing these compounds, especially pentylone, may pose a risk for compulsive use in addition to and exacerbating their acute toxicological effects.

Keywords: cathinones, methylone, butylone, pentylone, MDMA, self-administration

1. Introduction

Despite widespread scheduling and legal intervention, synthetic cathinone analogs continue to comprise a substantial proportion of novel psychoactive substances on the global recreational drug market (UNODC, 2014; EMCDDA, 2015). Synthetic cathinones initially gained notoriety in the United States for their inclusion in "bath salt" formulations; however, following scheduling, many synthetic cathinone derivatives have been diverted from "bath salts" into "Ecstasy" or "Molly" formulations *in lieu* of 3,4-methylenedioxymethamphetamine (MDMA) (Palamar et al., 2016a; 2016b; UNODC, 2014). Although numerous synthetic cathinone analogs have been detected in samples of "Ecstasy" (Ecstasydata.org, 2017; UNODC, 2014), methylone and butylone, two cathinone analogs of MDMA, were the adulterants most commonly detected in hair samples of self-reported "Ecstasy" or "Molly" users (Palamar et al., 2016). Given the numerous adverse effects associated with synthetic cathinones (Warrick et al., 2012; Elliot & Evans, 2014; Kesha et al., 2013), inadvertent use of synthetic cathinones in "Ecstasy" formulations may lead to a significantly increased risk for adverse health effects or lethality in "Molly" users. Furthermore, although the addictive properties and potential for compulsive use of MDMA has been thoroughly characterized in animal models and clinical populations, the data regarding reinforcing efficacy of synthetic cathinones is still slowly emerging, leaving a gap of information regarding the potential for uncontrolled or compulsive use of "Ecstasy" formulations substituted with synthetic cathinone analogs.

Although there are limited reports of MDMA dependence in a subset of users (Degenhardt et al., 2010), the relative rates of dependence are substantially less for MDMA than other stimulant-type drugs, such as cocaine or methamphetamine (SAMHSA, 2016). Furthermore, the preclinical literature regarding MDMA's potential for compulsive use

unequivocally agree that MDMA's reinforcing efficacy is minimal relative to other drugs of abuse. Classic studies of MDMA self-administration demonstrate limited rates of acquisition of self-administration (Schenk et al., 2007), reduced overall drug intake (Lamb & Griffiths, 1987; Ratzenboeck et al., 2000), and low breakpoints under progressive ratio schedules of reinforcement (Lile et al., 2005; Schenk et al., 2007) relative to other stimulant-type drugs. These data suggest that the adverse effects associated with MDMA use are largely relegated to acute toxicities occurring in overdose, as opposed to the uncontrolled and compulsive use typically associated with addiction. However, the same cannot be said for the synthetic cathinones utilized in "Ecstasy" formulations, given the limited data regarding their reinforcing effects relative to their large numbers.

The limited data regarding the reinforcing efficacy of synthetic cathinones largely results from the cat-and-mouse approach to studying novel psychoactive substances, wherein compounds are assessed thoroughly one-at-a-time as they become available on the market. Three of the most well-studied synthetic cathinone derivatives are mephedrone, methylone, and methylenedioxypyrovalerone (MDPV), which were three of the first cathinone analogs to gain popularity (Spiller et al., 2011). MDPV has been shown in preclinical models to engender robust self-administration under both fixed and progressive ratio schedules to a degree comparable to cocaine and methamphetamine (Watterson et al., 2012a; Aarde et al., 2013; 2015; Gannon et al., 2017). Although similarities in the subjective effects in humans (erowid.org) and discriminative stimulus effects in rats (Harvey & Baker, 2016) between mephedrone and MDMA have been reported, the reinforcing efficacy of mephedrone appears to be greater than MDMA and comparable to methamphetamine (Aarde et al., 2013; Motbey et al., 2013; Nguyen et al., 2016). Of the three most popular first-generation synthetic cathinones, methylone appears to serve as

the weakest reinforcer with a self-administration profile comparable to MDMA (Creehan et al., 2015; Vandewater et al., 2015) and limited reinforcing efficacy under both short- and extended-access self-administration conditions (Watterson et al., 2012b; Nguyen et al., 2016).

As novel cathinone derivatives continue to flood the market, so too does the quest to assess reinforcing efficacy in rodent models of self-administration. Novel synthetic cathinones including α-pyrrolidinopentiophenone (α-PVP; Aarde et al., 2015), α-pyrrolidinopentiothiophenone (α-PVT; Cheong et al., 2017), 4-methyl-α-pyrrolidinopropiophenone (4-MePPP), and 4-methylethcathinone (4-MEC; Huskinson et al., 2017) have demonstrated varying degrees of drug intake in self-administration models, highlighting the variability of reinforcing efficacy with minute chemical substitutions to the cathinone parent structure. The current study utilized fixed- and progressive-ratio schedules of reinforcement in a self-administration assay to determine the relative reinforcing efficacy of three synthetic cathinone analogs, alongside MDMA, reported in "Ecstasy" or "Molly" formulations: methylone, butylone, and pentylone.

2. Materials and Methods

2.1 *Animals*

Male Sprague-Dawley rats were obtained from Envigo (Indianapolis, IN). All rats were housed individually and were maintained on a 12:12 light/dark cycle (lights on at 7:00 AM). All experiments were run during the light cycle. Body weights were maintained at 320-350 g by limiting food to 15 g/day. Water was readily available. All housing and procedures were in accordance with Guidelines for the Care and Use of Laboratory Animals (National Research

Council, 2011) and were approved by the University of North Texas Health Science Center Animal Care and Use Committee.

2.2 *Apparatus*

All testing procedures occurred in standard operant chambers modified for self-administration experiments (Med Associates, St Albans, VT) containing a single lever, a cue light, and a house light at the rear of the chamber. The operant chambers were connected to IBM-PC compatible computers via LVB interfaces (Med Associates, East Fairfield, VT). The computers were programmed in Med-PC for Windows, version IV (Med Associates, East Fairfield, VT) for the operation of the chambers and collection of data.

2.3 *Food Training Procedure*

All rats were trained to respond for food reinforcers (45 mg food pellets; Bio-Serve, Frenchtown, NJ) under a FR5 schedule of reinforcement. Food sessions lasted until either 20 food reinforcers were obtained or one hour had elapsed. Rats that had completed a minimum of 5 consecutive sessions in which all 20 reinforcers were obtained then underwent surgery for intravenous self-administration testing.

2.4 *Surgical Procedure*

Rats received analgesia (Rimadyl 2 mg, p.o.; Bio-Serv, Frenchtown, NJ) 24 hrs before, immediately following, and 24 hrs after surgery. Rats were anesthetized with isofluorane (5%) and maintained under anesthesia throughout the surgery (2.5%). Polyurethane tubing (1.1 mm outer diameter, 0.6 mm inner diameter) was implanted in the right jugular vein, fixed in place with suture, passed subcutaneously, and affixed to an external guide cannula mounted

subcutaneously in the midscapular region. Catheters were flushed daily before each session with 0.9% saline and locked after each session with heparinized (50 units/mL) saline containing timentin (310 mg/mL; 0.1 mL/rat). Rats were allowed to recover for 7 days before beginning testing.

2.5 *Fixed Ratio Self-Administration Testing*

Following recovery, rats (n=4-5) were trained to self-administer methamphetamine (0.05 mg/kg/inf). Each session began with an infusion of the drug solution (0.1 mL) followed by a 15-min blackout period in which the house and cue lights were turned off. At the conclusion of the blackout period, the house and cue light were re-illuminated, indicating reinforcement was available. Responding according to a FR1 schedule resulted in an infusion (0.1 mL/1sec) of the drug solution and dimming of the cue light for 20 sec. Sessions lasted two hours and rats were tested 7 days per week. The criterion for response stability was three consecutive sessions with <20% change in number of infusions among sessions. A minimum of ten training sessions were completed prior to beginning substitution testing. Once stability criteria were reached, rats began substitution testing under the same experimental conditions as training. Test drug and dose order were assigned randomly using a Latin square. Each dose (0.03-1.0 mg/kg/inf) was tested once and all doses of a given drug were tested before beginning a new drug. Upon completing all four test compounds (MDMA, methylone, butylone, and pentylone), rats underwent extinction with 0.9% saline until a stable pattern of responding, as described above, was reached. A repeated-measures design was used, such that each rat was tested at all doses of a given drug.

2.6 *Progressive Ratio Self-Administration Testing*

Following recovery, rats (n=3-4) were trained to self-administer methamphetamine (0.05 mg/kg/inf). Responding according to a FR10 schedule resulted in an infusion (0.1 mL/1sec) of

the drug solution and dimming of the cue light for 20 sec. Sessions lasted two hours and rats were tested 7 days per week. Criteria for response stability were three consecutive sessions with <20% change in number of infusions among sessions. A minimum of ten training sessions were completed prior to beginning substitution testing. Once stability criteria were reached, rats self-administered methamphetamine (0.05 mg/kg/inf) under a progressive ratio schedule of reinforcement. Ratio requirement was determined according to the equation $Ratio=5e^{(infusion\ number*0.3)}-5$ (Richardson and Roberts, 1996). The value of 0.3 was chosen based on previous literature testing synthetic cathinones under a progressive ratio (Aarde et al., 2013) and preliminary data from our lab indicating this ratio array would lead to a breakpoint in less than 4 hours. Sessions lasted for a maximum of 4 hours or until 60 min elapsed during which the rat failed to receive an infusion, which was considered the breakpoint. Stability criteria were defined as two consecutive sessions that varied by no more than ±2 infusions. Once stability criteria with methamphetamine were reached, rats began substitution testing under the same experimental conditions. Drug and dose order were assigned randomly using a Latin square. Each dose was tested a minimum of two times until responding was stable. All doses were completed, along with saline and methamphetamine (0.05 mg/kg/inf) controls, before the next drug was tested. A repeated-measures design was used, such that each rat was tested at all doses of a given drug.

2.7 *Data Analysis*

Data are expressed as the mean number of infusions obtained and, in the progressive ratio experiments, the mean number of responses emitted. Progressive ratio data include two replicates for each rat per dose. Data were analyzed using a repeated-measures analysis of variance assessing effects of dose and drug. If a main effect of dose was revealed, individual doses were compared to the vehicle control value using a priori contrasts. If a main effect of drug was

revealed, a one-way analysis of variance was utilized to assess differences between drugs at each dose.

2.8 *Drugs*

(+)-methamphetamine HCl, (±)-3,4-methylenedioxymethamphetamine HCl (MDMA), methylone HCl, butylone HCl, and pentylone HCl, were provided by the National Institute on Drug Abuse Drug Supply Program and were dissolved in 0.9% saline.

3. Results

3.1 *Continuous Reinforcement*

As illustrated in fig. 2, each of the test compounds were readily self-administered under a continuous schedule of reinforcement in an inverted-U-shaped dose-effect with no differences among compounds. A repeated-measures analysis of variance indicated a significant main effect of dose ($F_{4,60}$=32.089, p<.001) but not drug ($F_{3,15}$=0.522, p=.674), nor was there a dose*drug interaction ($F_{12,60}$=0.757, p=.691). Main effects of dose were revealed for each test compound [MDMA ($F_{4,12}$=6.319, p=.006); methylone ($F_{4,16}$=7.133, p=.002); butylone ($F_{4,16}$=11.471, p<.001); pentylone ($F_{4,16}$=10.176, p<.001)]. Planned comparisons of dose against vehicle controls revealed significant differences at 0.1 mg/kg/inf MDMA, 0.1-1.0 mg/kg/inf methylone, and all doses of butylone and pentylone.

Similar results were obtained when analyzing the cumulative amount of drug self-administered. A repeated-measures analysis of variance indicated a significant main effect of dose ($F_{3,45}$= 100.02, p< .001) but not drug ($F_{3,15}$= 1.191, p=. 347), nor was there a dose*drug interaction ($F_{9,45}$= 1.711, p= .114). Main effects of dose were revealed for each test compound [MDMA ($F_{3,9}$= 15.507, p= .001); methylone ($F_{3,12}$= 27.272, p< .001); butylone ($F_{3,12}$= 40.504,

$p < .001$); pentylone ($F_{4,16} = 31.153$, $p < .001$)]. Post-hoc analyses in each of the test compounds, excluding MDMA, revealed a dose-dependent increase in cumulative dose, wherein each dose tested differed significantly from one another, except for 0.3 vs 1.0 mg/kg/inf. In rats self-administering MDMA, there was, additionally, no difference between 0.1 and 0.3 mg/kg/inf.

At the higher doses tested (0.3 and 1.0 mg/kg/inf), MDMA, methylone, and butylone produced adverse effects under conditions of continuous reinforcement. Salivation and exophthalmos were noted in several rats at the conclusion of the session at these doses. Lethality occurred in two rats following 1.0 mg/kg/inf butylone. In both lethal instances, rats self-administered a large number of infusions in rapid succession (43 infusions in <45 minutes and 30 infusions in <60 minutes).

3.2 *Progressive Ratio*

The number of infusions obtained and the cumulative number of responses under the progressive ratio schedule of reinforcement are illustrated in figure 3. An analysis of variance with repeated measures on number of infusions obtained revealed a main effect of dose ($F_{4,128} = 82.457$, $p <$. 001), a drug*dose interaction ($F_{12,128} = 3.255$, $p < .001$), but no main effect of drug ($F_{3,32} = 2.06$, $p = .125$). A similar effect was detected in the cumulative response data with a mean effect of dose ($F_{4,128} = 11.05$, $p < .001$) and a dose*drug interaction ($F_{12,128} = 2.82$, $p = .002$), but no main effect of drug ($F_{3,32} = 2.876$, $p = .051$).

MDMA ($F_{4,20} = 41.085$, $p < .001$) was administered to a greater degree than vehicle at 0.03, 0.3, and 1.0 mg/kg/inf. Methylone ($F_{4,44} = 39.689$, $p < .001$) was administered to a greater degree than vehicle at 0.3 and 1.0 mg/kg/inf. Butylone ($F_{4,20} = 8.867$, $p < .001$) was administered to a greater degree than vehicle at 0.1, 0.3, and 1.0 mg/kg/inf. Pentylone ($F_{4,44} = 42.452$, $p < .001$)

was administered to a greater degree than vehicle at 0.3 and 1.0 mg/kg/inf. At the highest dose tested, 1.0 mg/kg/inf, there were significant differences among drugs ($F_{3,32}$= 4.332, p= .011). Pairwise comparisons with a Bonferroni correction revealed that 1.0 mg/kg/inf pentylone was administered more than MDMA or butylone. Methylone self-administration at this dose, on the other hand, did not differ from the other test compounds.

Analyses of response data revealed similar effects as the number of infusions obtained. MDMA ($F_{4,20}$= 8.519, p< .001) produced a greater number of responses relative to vehicle at 0.3 and 1 mg/kg/inf. Methylone-induced ($F_{4,44}$= 4.506, p= .004) responding was increased following 0.3 mg/kg/inf. Butylone ($F_{4,20}$= 2.820, p= .052) did not produce a higher rate of responding relative to vehicle. Pentylone ($F_{4,44}$= 13.05, p< .001) produced a greater number of responses than vehicle at 0.3 and 1.0 mg/kg/inf. At the highest dose tested, 1.0 mg/kg/inf, there were no significant differences among drugs ($F_{3,32}$= 2.854, p= .053).

4. Discussion

The current study aimed to determine and compare the reinforcing efficacy of three synthetic cathinone analogs of MDMA under fixed- and progressive-ratio schedules of reinforcement, and is the first study to test butylone or pentylone in a self-administration assay. We found that MDMA, methylone, butylone, and pentylone are each able to maintain robust self-administration under a continuous schedule of reinforcement when substituted for methamphetamine, and there were no apparent differences in efficacy or potency under these conditions in terms of total number of infusions obtained or cumulative dose self-administered. The cumulative dose self-administered under the continuous schedule of reinforcement indicated that 0.3 and 1.0 mg/kg/inf both resulted in similar degrees of drug consumption in all test drugs.

Conversely, pentylone produced a significantly greater breakpoint at 1 mg/kg/inf than either butylone or MDMA, whereas methylone's breakpoint was not different from any other test compound at this dose.

These data indicate a significant potential for abuse of each of these compounds, given their ability to maintain high levels of responding/self-administration when substituted for methamphetamine and their dose-dependent increases in breakpoint relative to vehicle. Furthermore, these data suggest that pentylone is most likely to engender compulsive use among these compounds, as demonstrated by its high breakpoint relative to MDMA. The breakpoint produced by 1.0 mg/kg/inf butylone, conversely, did not differ from MDMA, suggesting a similar degree of reinforcing efficacy and, therefore, similarly limited potential for compulsive use. The intermediate rate of responding produced by 1.0 mg/kg/inf methylone was surprising as previous reports have indicated limited reinforcing efficacy (Nguyen et al., 2016; Aarde et al., 2013; Vandewater et al., 2015). The reasons for this discrepancy of results may stem from a variety of factors. Our sample size, with n=6 rats, was relatively limited compared to other reports and the data may reflect random variability or may have been insufficiently powered to detect a difference between methylone and pentylone. Furthermore, the rats in this study were initially trained to self-administer methamphetamine, whereas other studies employed direct acquisition of methylone self-administration without prior training. It is not unusual to find lower-efficacy compounds self-administered at higher rates if they have been previously trained to administer a high-efficacy stimulant (Schenk et al., 2003; Vandewater et al., 2015).

The mechanisms potentially underlying these differences in reinforcing efficacy have recently begun to emerge from the *in vitro* literature. Synthetic cathinones, like other stimulant-type compounds exert their pharmacological effects via disruption of normal monoamine

transporter function. Some derivatives function as amphetamine-type releasing agents (i.e. methcathinone, mephedrone), others operate as cocaine-like uptake inhibitors (i.e. MDPV, alpha-PVP), and a smaller subset possesses a "hybrid" profile with uptake-inhibition at one transporter, usually the dopamine transporter (DAT), and releasing properties at another, typically the serotonin transporter (SERT) (reviewed in Reith et al., 2015; Liechti, 2015). The three compounds assessed in the current study span these mechanistic categories.

Studies of radiolabeled transmitter uptake and release at monoamine transporters have indicated that methylone (Eshleman et al., 2013) and butylone (Eshleman et al., 2013; Simmler et al., 2013) both act as MDMA-like substrates at SERT and release serotonin. Our electrophysiological data from Chapter 2 provide further evidence for this substrate-like mechanism and our drug discrimination data indicate a strong serotonergic component to methylone's and butylone's discriminative stimulus effects *in vivo*. Pentylone, conversely, is reported to act as a cocaine-like uptake inhibitor at both DAT and SERT (Eshleman et al., 2016), which is apparent from its strong dopaminergic discriminative stimulus discussed in Chapter 2. Serotonin release in monoamine transporter-disrupting drugs is considered to limit the reinforcing efficacy associated with dopamine release (Wee et al., 2005; Wee & Woolverton, 2006). This phenomenon is further evidenced by the increased reinforcing efficacy of MDMA in SERT-knockout rats (Oakly et al., 2014) and the reduced reinforcing efficacy of (–)-MDMA, the SERT-selective enantiomer of MDMA, relative to (+)-MDMA and racemic MDMA (Fantegrossi et al., 2001; Fantegrossi, 2007; Wang & Woolverton, 2007). The release of serotonin associated with methylone and butylone may explain their limited reinforcing efficacy relative to pentylone, which, as we demonstrated in Chapter 2, produces greater dopaminergic effects *in vivo*; however, further experiments utilizing antagonists or SERT-knockout rats in the self-administration assay

are necessary to confirm the relationship of mechanism and reinforcing efficacy of these compounds.

These data are the first to demonstrate the reinforcing potential of butylone and pentylone and add to the existing literature regarding methylone's and MDMA's reinforcing efficacy. Altogether, the results indicate that "Ecstasy" or "Molly" formulations containing these synthetic cathinones, especially pentylone, may have enhanced potential for compulsive use, which is especially concerning given the lethality observed after butylone self-administration in the current study and fatalities occurring in human users (Warrick et al., 2012).

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Contributors

SBD designed the experiments, searched the literature, conducted the experiments, analyzed the data, and wrote the manuscript. MBG approved the design of the experiments and approved the final version of the manuscript.

Conflict of interest

None.

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Figure 1: Chemical structures of test compounds

Figure 2: Self-administration under a continuous schedule of reinforcement

Dose-response curves of number of infusions (top) and cumulative dose (bottom) of MDMA (closed squares), methylone (open squares), butylone (open triangles), or pentylone (closed triangles) self-administered under a continuous schedule of reinforcement. Vehicle (open circles) and methamphetamine (closed circle) controls are illustrated on left.

Figure 3: Self-administration under a progressive ratio schedule of reinforcement

Dose-response curves of MDMA (closed squares), methylone (open squares), butylone (open triangles), or pentylone (closed triangles) self-administered under a progressive ratio schedule of reinforcement. The top graph illustrates the total number of infusions received (left Y-axis) and final ratio completed (right Y-axis). The bottom graph illustrates the cumulative number of responses emitted. Vehicle (open circles) and methamphetamine (closed circle) controls are illustrated on left. *indicates $p<.05$ against vehicle control.

Figures

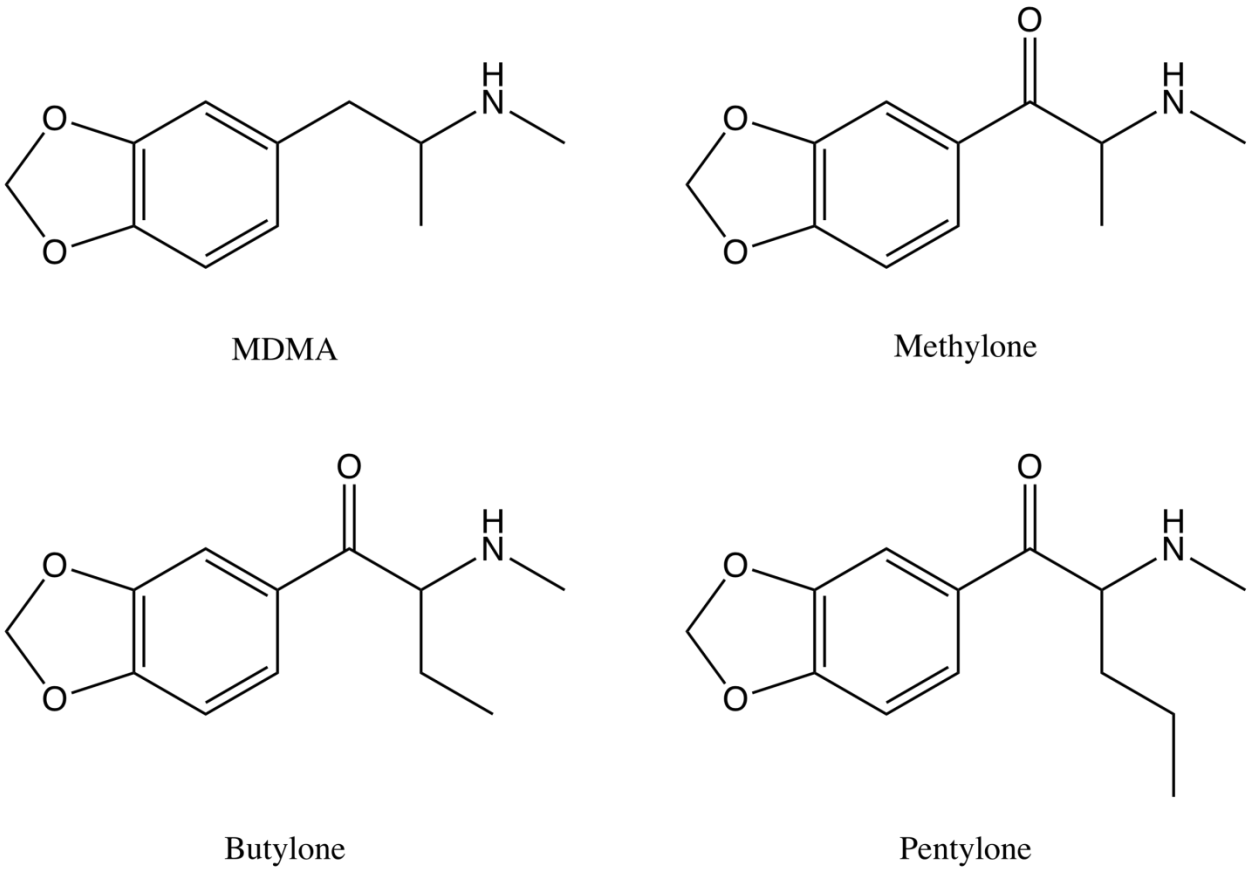
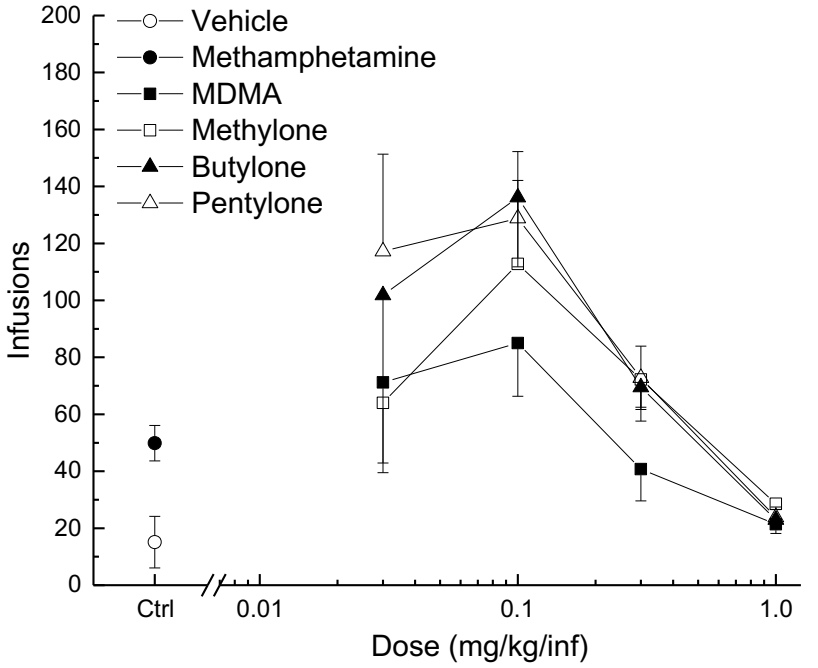


Figure 1


Vehicle
Methamphetamine
MDMA
Methylone
Butylone
Pentylone
Infusions
200
180
160
140
120
100
80
60
40
20
0
Ctrl
0.01
0.1
1.0
Dose (mg/kg/inf)

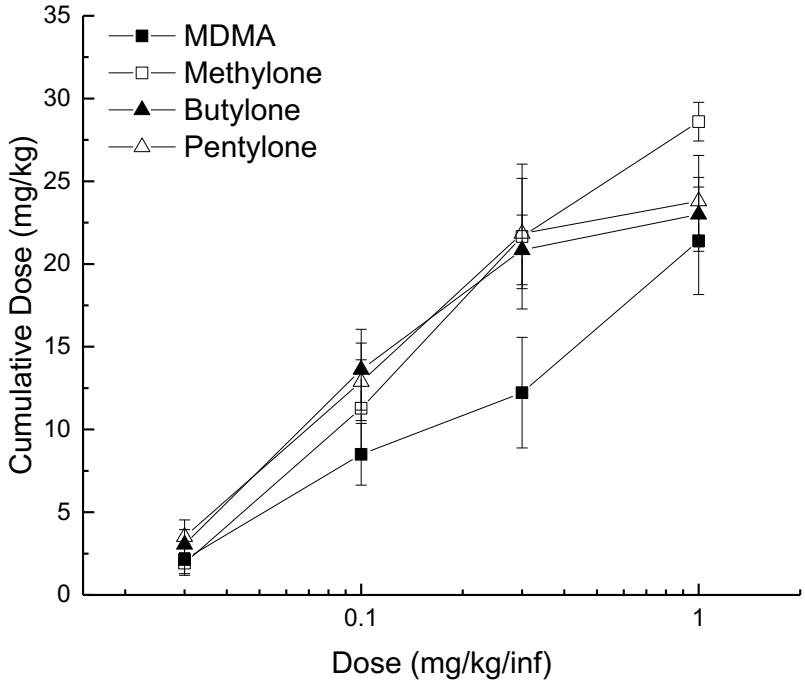

MDMA
Methylone
Butylone
Pentylone
Cumulative Dose (mg/kg)
35
30
25
20
15
10
5
0
0.1
1
Dose (mg/kg/inf)

Figure 2

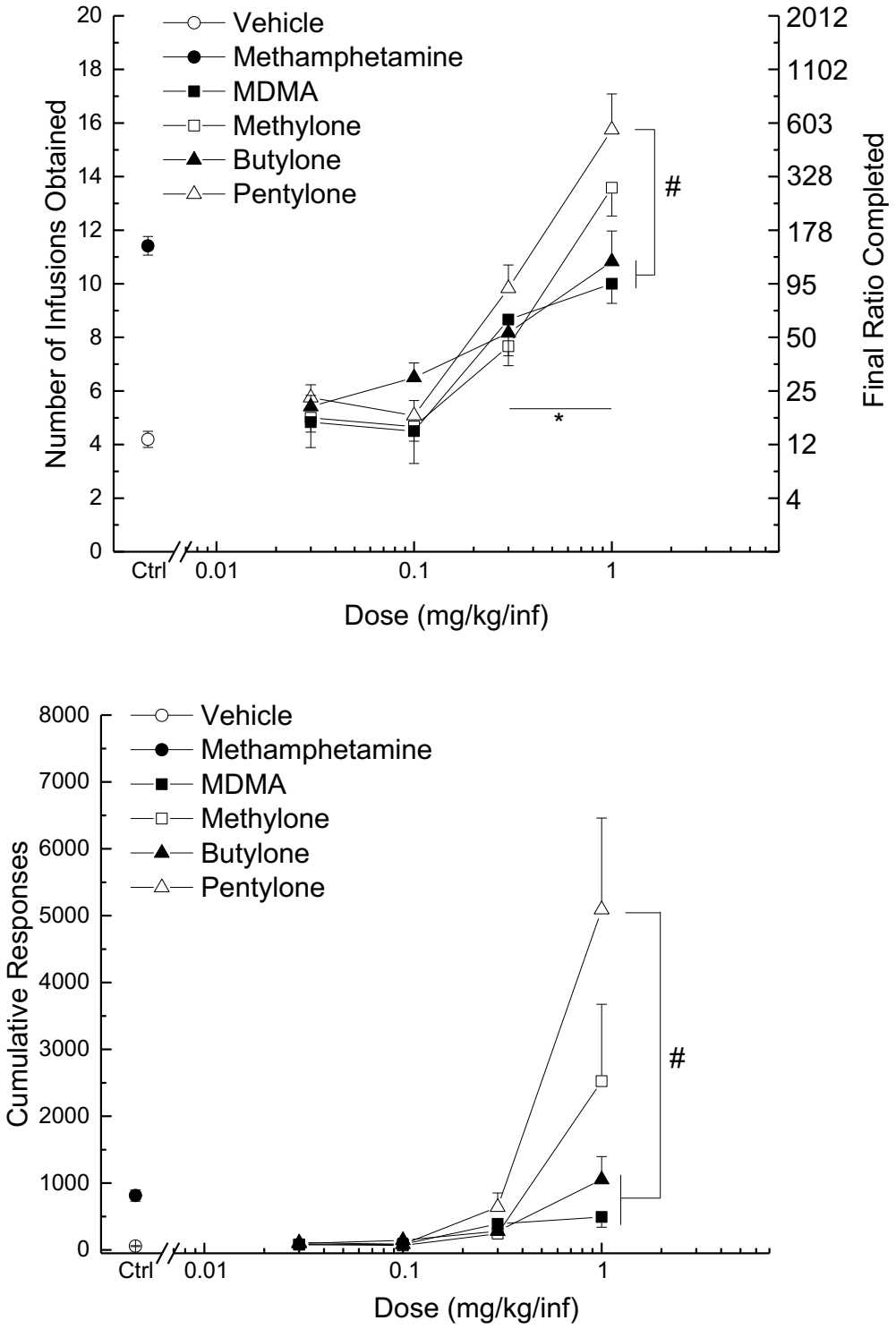

Vehicle
Methamphetamine
MDMA
Methylone
Butylone
Pentylone
Number of Infusions Obtained
Final Ratio Completed
Dose (mg/kg/inf)
Ctrl
0.01
0.1
1
#
*
Cumulative Responses
Dose (mg/kg/inf)
#

Figure 3

CHAPTER 4

SUMMARY AND DISCUSSION

Summary of Results

The current studies assessed the relative contributions of dopamine and serotonin to the pharmacological mechanism and the reinforcing efficacy of three synthetic cathinone analogs of MDMA: methylone, butylone, and pentylone.

Methylone and butylone produced inward currents at the serotonin transporter (SERT) similarly to MDMA, with no differences in efficacy between the two compounds. Each of the three test compounds substituted fully for methamphetamine in a drug discrimination assay, and the methamphetamine-like drug-appropriate responding (DAR) was fully and dose-dependently attenuated by the D1-selective antagonist SCH23390, with pentylone being least sensitive to the antagonistic effects of SCH23390. None of the compounds tested substituted for DOM, and pentylone failed to produce any DOM-DAR, with only 3 rats responding at a dose of pentylone producing 41% DAR. Methylone and butylone, but not pentylone, fully substituted for the discriminative stimulus effects of MDMA. Pretreatment with SCH23390 fully attenuated pentylone-induced MDMA-DAR, partially attenuated MDMA- and methylone-induced MDMA-DAR, but had no effect in butylone-treated rats.

In an intravenous self-administration assay, differences among compounds emerged under different schedules of reinforcement. Under a continuous schedule of reinforcement, each compound maintained methamphetamine-like responding and was robustly self-administered

according to an inverted-U dose-effect function, but there were no differences in self-administration among the test compounds. Similarly, under a progressive ratio, each test compound was robustly self-administered with a dose-dependent increase in breakpoint; however, under these conditions, the breakpoint produced by 1.0 mg/kg/inf pentylone was significantly greater than MDMA and butylone, whereas methylone fell between butylone and pentylone, but did not differ statistically from any of the compounds tested.

Mechanism

Serotonin Transporter Activity

Radioligand binding methods, generally direct-binding or displacement assays, are usually the first experiments employed when investigating the mechanism of novel drugs. Although these methods provide critical information regarding drug affinity and selectivity, they are not informative as to the efficacy or exact mechanism of the compound(s) under investigation. To this end, functional assays are required, and in the study of stimulant-type drugs, uptake inhibition or release of radiolabeled neurotransmitter at monoamine transporters are the preferred methodology. The synthetic cathinone analogs of MDMA evaluated in the current study have been previously assessed with these radioligand methods, indicating affinity of these compounds for the monoamine transporters with modest differences in selectivity. Two laboratories have independently demonstrated a "hybrid" profile for butylone, with uptake inhibition at the dopamine transporter (DAT) and SERT (Eshleman et al., 2013; Simmler et al., 2013). Functional investigations into the molecular mechanism of pentylone have definitively determined its mechanism to be uptake inhibition of dopamine (Kolanos et al., 2013; Eshleman et al., 2016; Simmler et al., 2014), and uptake inhibition (Eshleman et al., 2016) or weak release

of serotonin (Simmler et al., 2014). Methylone, on the other hand, has yielded inconsistent results with these traditional methodologies with two studies indicating MDMA-like release at both DAT and SERT (Bauman et al., 2012; Eshleman et al., 2013) but another indicating cocaine-like uptake inhibition at both transporters (Simmler et al., 2013). To clarify the mechanism of methylone at SERT, an alternative methodology was required.

As a means of circumventing the necessity of radiolabeled neurotransmitters, we took advantage of the Na^+-conducting, channel-like properties of monoamine transporters (Ingram et al., 2002; Hilber et al., 2005; Quick, 2003), and utilized whole-cell patch clamp electrophysiology techniques to assess drug action at SERT. In these experiments, we determined that methylone and butylone, like MDMA, produce inward currents at SERT. These data are the first to assess methylone and butylone using this model, and our results replicate those previously reported with MDMA (Hilber et al., 2005). Although this method does not directly assess transmitter flux, it provides evidence for the transmitter-releasing properties of these compounds.

Methylone and butylone may contribute to monoamine release through depolarization of the presynaptic neuron via Na^+ influx and Ca^{2+}-dependent vesicular release (Ingram et al., 2002). This proposed mechanism runs contrary to the typical view of amphetamine-like substrates, wherein substrates are taken up into the synapse and, through a series of intracellular intermediaries, reverse the direction of transport through the transporter proteins (reviewed in Sulzer et al., 2005). It may also serve as an ancillary mechanism for increased synaptic monoamine concentrations through impulse-independent depolarization and vesicular release (Ingram et al., 2002; reviewed in DeFelice et al., 2014); however, a strong correlation exists between substrate-induced inward currents and substrate-induced serotonin release in HEK cells,

suggesting that carrier-mediated release and the electrophysiological properties of substrate compounds are related (Sitte et al., 1998). Furthermore, the MDMA-like inward currents at SERT produced by methylone and butylone suggest these two compounds have a similar mechanism as MDMA, which is a known monoamine-releasing agent (Crespi et al., 1997). These data converge with the radiolabeled transmitter methodologies previously employed indicating direct release by these compounds (Eshleman et al., 2013; Bauman et al., 2012) rather than uptake inhibition (Simmler et al., 2013).

Dissecting the exact mechanism of serotonin release, whether transport reversal or vesicular release, would require assessments of synaptic monoamine release via inhibition of intracellular Ca^{2+} release or vesicular docking and unloading and consequential changes in synaptic serotonin concentrations with *in vivo* microdialysis studies or in *ex vivo* slices; however, the main concern of our studies was not exact physiological response to these drugs, but rather the impulse-dependence of the drug mechanism. Although monoamine uptake inhibition and release both result in increased synaptic neurotransmitter concentrations, impulse-independent release of serotonin negatively modulates the reinforcing efficacy of drugs that concurrently increase dopaminergic signaling (Wee et al., 2005; Wee & Woolverton, 2006; Oakly et al., 2014). Our electrophysiological data provide evidence that methylone and butylone, like MDMA, act as SERT substrates and likely result in serotonin release in an impulse-independent fashion. Our data clarify the discrepancies reported for methylone, but further studies using DAT under the conditions are necessary to fully characterize the mechanism of each of these test compounds.

Drug Discrimination

For the purpose of determining the relative dopaminergic and serotonergic contributions to the mechanisms of methylone, butylone, and pentylone *in vivo*, we utilized a drug discrimination assay with rats trained to discriminate methamphetamine, DOM, or MDMA from vehicle. These training drugs were chosen as they comprise a spectrum from dopaminergic to serotonergic mechanisms, with methamphetamine being primarily dopaminergic (Munzar & Goldberg, 2000), DOM acting selectively at $5HT_2$ receptors (Glennon et al., 1982; Young et al., 1980), and MDMA producing a complex discriminative mediated by both dopamine and serotonin (Goodwin et al., 2002; Schechter, 1988).

The drug discrimination data suggest that methylone and butylone have a complex, MDMA-like, dopaminergically- and serotonergically-mediated discriminative stimulus, and pentylone has a primarily dopaminergic discriminative stimulus.

Methamphetamine Discrimination

The substitution of each test compound for methamphetamine replicates previous findings from our laboratory (Gatch et al., 2013; 2015) and provides evidence for a dopaminergic component to the discriminative stimulus of each compound. These data potentially indicate a stronger dopaminergic phenotype of the methylone and pentylone relative to MDMA and butylone, given that methylone and pentylone substituted at doses that produced no rate disruption, whereas 2.5 mg/kg MDMA and 10 mg/kg butylone significantly attenuated response rate, an effect potentially mediated by the relatively greater efficacy of serotonin versus dopamine release by MDMA (Bauman et al., 2012). Although each test compound substituted for methamphetamine with roughly similar potency, differences in dopaminergic efficacy among the compounds emerged after pretreatment with the D1-selective antagonist SCH23390. At 0.01

mg/kg SCH23390, the dose of SCH23390 tested among each test compound without disruption of responding, drug-appropriate responding produced by substituting doses of MDMA (2.5 mg/kg, 30% DAR), methylone (5 mg/kg, 19% DAR), butylone (10 mg/kg, 45% DAR), and pentylone (10 mg/kg, 91% DAR) varied substantially, indicating a decreased sensitivity to the effects of SCH23390 in pentylone relative to MDMA, methylone, or butylone. Despite the steeper slope of pentylone's antagonism curve relative to that of MDMA, methylone, or butylone, the methamphetamine-DAR was reduced by lower doses of SCH23390 among the latter compounds. This reduction in sensitivity to SCH23390 is further evidenced by the relative effects of 0.01 mg/kg SCH23390 against methylone and pentylone, in which that dose had no effect on the response rate in pentylone-treated rats, but produced a significant attenuation of methylone-induced response rate.

The dose-dependent attenuation of methamphetamine-DAR in each test compound by SCH23390 was anticipated given the D1-dependent nature of methamphetamine's discriminative stimulus; however, the reasons for the differences in sensitivity to SCH23390 are not readily apparent from these studies. Given the similarities in antagonism curves among MDMA, methylone, and butylone, we hypothesized that the increased sensitivity of methylone and butylone to SCH23390 relative to pentylone was mediated by unmasking of the effects of another transmitter system, most likely serotonin. The putative contributions of serotonin to the discriminative stimulus effects of methylone and butylone are supported by our electrophysiology data indicating inward, substrate-like currents at SERT along with previous reports indicating 5HT release by these compounds (Eshleman et al., 2013; Bauman et al., 2012; Simmler et al., 2013). Blockade of D1 receptors in rats treated with methylone or butylone may have shifted the balance of serotonin and dopamine towards a more serotonergic signal, thereby

reducing the methamphetamine-like stimulus of these compounds. Conversely, a primarily, or perhaps exclusively, dopaminergic stimulus of pentylone would likely be able to surmount D1 blockade up to the point of saturation without unmasking of additional transmitter systems contributing to its mechanism and discriminative stimulus.

Although the *in vitro* data suggest a potential serotonergic mechanism of methylone and butylone, and by extension, a serotonergic contribution to the discriminative stimulus, assessment of their serotonergic effects *in vivo* are necessary to confirm the "unmasking" hypothesis presented above. To this end, we evaluated generalization of the discriminative stimulus effects of DOM and MDMA to the synthetic cathinone compounds.

DOM Discrimination

None of the compounds tested fully substituted for the discriminative stimulus effects of DOM; however, there were differences among the compounds in their ability to produce DOM-like responding. MDMA, methylone, and butylone produced partial substitution for DOM, with each test compound producing between 40-60% DOM-DAR, and limited disruption of responding at doses that fully substituted for methamphetamine. Conversely, pentylone failed to produce ≥20% DOM-DAR at any dose. At 25 mg/kg, pentylone produced 41% DOM-DAR, but only three of nine rats managed to complete the first fixed ratio, with only one completing the entire session. Consequently, these data were considered under-powered and not considered to represent a true effect, as the apparent increase in DOM-DAR occurred primarily because of drug-lever responding by a single rat. Given the substantial decrease in response rate, this effect may be merely due to loss of stimulus control. These data further suggest a serotonergic component to the discriminative stimulus effects of methylone and butylone and a predominately dopaminergic mechanism of pentylone.

Although the synthetic cathinones have been reported, in some instances, to produce hallucinogenic effects (Vazirian et al., 2015), it is likely that the lack of full substitution results from indirect serotonin agonism, given that DOM is selective for $5HT_2$ receptors. Binding and functional assays have implicated $5HT_{2A}$ receptors in the mechanism of MDMA, but similar reports for the synthetic cathinone derivatives indicate minimal affinity for any of the $5HT_{2A/C}$ receptor subtypes (Simmler et al., 2013; 2014) and reduced efficacy to activate these receptors relative to MDMA (Eshleman et al., 2013). Thus, it seems likely that any DOM-like responding produced by methylone and butylone resulted from indirect $5HT_2$ receptor stimulation.

MDMA Discrimination

Further assessments of the dopaminergic and serotonergic mechanisms of the cathinone analogs of MDMA were conducted in rats trained to discriminate MDMA from vehicle. As the discriminative stimulus effects of MDMA are mediated by both dopamine and serotonin (Goodwin et al., 2002), rats trained to discriminate MDMA provide a unique opportunity to assess potentially complex discriminative stimulus effects of novel compounds. Methylone and butylone substituted for MDMA at the same doses that substituted for methamphetamine, whereas pentylone produced only 75% MDMA-DAR. Disruption of response rate at 25 mg/kg pentylone, such that seven of eight rats failed to respond, precluded assessment of pentylone-induced MDMA-DAR at higher doses.

The training dose of MDMA (1.5 mg/kg) used in these studies is particularly sensitive to serotonergic effects of novel test compounds, but can detect dopaminergic stimuli as well, typically in the form of partial substitution (Webster et al., 2016). Given that methylone and butylone fully substituted for MDMA with no decrements in response rate, whereas pentylone substituted only partially, we can conclude that methylone and butylone produce complex

discriminative stimulus effects mediated by both dopamine and serotonin whereas pentylone is predominately dopaminergic. The limited antagonism of MDMA, methylone, and butylone, but robust antagonism of pentylone, by SCH23390 further support this conclusion, as MDMA-, methylone-, and butylone-treated rats can still attend to the serotonergic component of these compounds following D1-blockade to respond on the MDMA-appropriate lever, whereas pentylone-treated rats lose any MDMA-like discriminative stimulus effects with SCH23390 antagonism.

Our studies replicate and expand upon previous studies from our laboratory with each of these test compounds (Gatch et al., 2013; 2015). The discriminative stimulus effects of methylone have previously been assessed elsewhere (Dal Cason et al., 1997), and our substitution data replicate those findings in amphetamine- and MDMA-trained rats; however, we demonstrated partial substitution in DOM-trained rats, where they found none, an effect likely resulting from their higher (1.0 mg/kg) DOM training dose. Although a previous report has implicated serotonin in the hyperlocomotive effects of butylone (López-Arnau et al., 2012), our findings are the first to explore the serotonergic discriminative stimulus effects of butylone and pentylone. Altogether, these data indicate distinct mechanisms *in vivo* between the primarily dopaminergic pentylone and the complex, dopaminergic/serotonergic methylone and butylone.

Reinforcing Efficacy of Synthetic Cathinone Analogs of MDMA

Among the chief concerns when a novel recreational drug gains popularity is its reinforcing efficacy, often referred to in terms of abuse liability or abuse potential, which can mean the difference between a compound being a novelty or quasi-legal alternative to well-established drugs of abuse or one engendering compulsive, uncontrolled use and addiction,

creating a niche market for the compound. Although many of the synthetic cathinone derivatives were used exclusively as legal and readily-available alternatives to cocaine or MDMA (Matthews et al., 2017; Ledberg, 2015), compounds such as MDPV and mephedrone, which were among the first cathinone derivatives classified as Schedule I, are still sought after for their specific effects (Moore et al., 2013; Kriikku et al., 2014). Both MDPV and mephedrone act as strong reinforcers in the drug self-administration assay (Aarde et al., 2013a; 2013b), which is considered the "gold standard" for abuse liability testing given its strong face and predictive validity (O'Connor et al., 2011). Mephedrone, which produces complex, MDMA-like discriminative stimulus effects, but with a stronger relative dopaminergic phenotype (Harvey & Baker, 2016), demonstrates greater self-administration than MDMA under both fixed- and progressive-ratio schedules of reinforcement (Vandewater et al., 2015; Aarde et al., 2013a). MDPV exhibits robust reinforcing effects in rodents at a comparable or greater degree than the traditional psychostimulants cocaine and methamphetamine (Gannon et al., 2017; Aarde et al., 2013b). Previous studies with methylone, on the other hand, suggest a fairly-limited degree of reinforcement, comparable to MDMA (Watterson et al., 2013; Schindler et al., 2016). The currently-available data regarding the reinforcing nature of synthetic cathinone as a class demonstrate substantial variability among these compounds, illustrating the necessity for determining whether the differences in discriminative stimulus effects among the synthetic cathinone analogs of MDMA translate into differences in abuse potential.

The current study revealed that methylone, butylone, and pentylone serve as reinforcers, with pentylone producing the greatest responding under a progressive ratio. Under conditions of continuous reinforcement, there were no differences among test compounds, indicating that when drug is freely available, all compounds tested can maintain methamphetamine-like

responding. Whether this methamphetamine maintenance translates into human behavior is unclear, but these data suggest a strong reinforcing effect of these compounds, given the lack of response extinction seen with cathinone substitution of methamphetamine. The dose-dependent increases in breakpoint under a progressive ratio schedule of reinforcement demonstrated by each compound further illustrate the reinforcing efficacy of these drugs by maintaining responding under conditions of progressively increasing effort requirements. The progressive-ratio data revealed differences in breakpoint among the compounds tested wherein, at the highest dose tested, pentylone was administered to a greater degree than MDMA and butylone; however, the breakpoint induced by methylone did not differ significantly from any of the compounds tested.

These data indicate that, among the compounds tested, pentylone likely engenders the greatest potential for compulsive use, whereas the reinforcing effects and abuse liability of butylone are comparable to MDMA. The reinforcing effects of methylone lay between butylone and pentylone, but did not statistically differ from either cathinone or MDMA, making strong conclusions about its reinforcing efficacy and abuse potential difficult to draw. The ambiguous reinforcing efficacy of methylone was unexpected as previous self-administration studies have indicated limited drug intake (Schindler et al., 2016; Vandewater et al., 2015). A number of factors from our study differed from previous reports studying methylone. First, our rats were trained to self-administer methamphetamine before testing the synthetic cathinones, which may have increased overall drug intake. A previous study assessing self-administration of putative entactogens, including methylone, found that rats trained to first self-administer mephedrone administered methylone to greater degree than those initially trained to self-administer methylone or MDMA (Vandewater, et al., 2015), suggesting that training drug alters reinforcing

efficacy in studies of substitution; however, given that MDMA and butylone produced fairly-limited responding and that all rats underwent the same training, the methamphetamine training is not likely a major confound in our interpretation of relative reinforcing efficacy. Previous studies of methylone self-administration in Sprague-Dawley rats have not tested doses above 0.5 mg/kg/inf (Watterson et al., 2013; Schindler et al., 2015), and the other study assessing 1.0 mg/kg/inf methylone used Wistar rats (Vandewater et al., 2015). Considering the methylone self-administration data as a whole, there appear to be dose- and species-dependent differences in the reinforcing efficacy of methylone.

Methylone and butylone's relatively limited reinforcing effects likely arise from their SERT substrate or serotonin-releasing properties. Studies investigating monoamine transporter substrates with varying DAT/SERT selectivity have revealed drastically reduced reinforcing efficacy with compounds selective for SERT over DAT relative to their more dopaminergic counterparts in rhesus monkeys (Wee et al., 2005). Similar results were obtained in monkeys self-administering a mixture of amphetamine and fenfluramine, a selective serotonin-releasing agent, wherein increasing the relative fenfluramine concentration significantly reduced breakpoint, suggesting that serotonin release masks the reinforcing effects of amphetamine-induced dopamine release (Wee & Woolverton, 2006). The reinforcement-limiting nature of serotonin release is also apparent in MDMA self-administration. Rats lacking SERT acquire MDMA self-administration faster and exhibit a higher breakpoint under a progressive ratio schedule of reinforcement than wild-type rats (Oakly et al., 2014). Furthermore, (-)-MDMA, which is selective for SERT, is self-administered to a lesser degree than (+)-MDMA or racemic MDMA (Fantegrossi et al., 2002; Fantegrossi, 2007; Wang & Woolvertoon, 2007). Although methylone and MDMA are structurally and mechanistically similar, our results indicate an

apparent, yet not statistical, difference in reinforcing efficacy between the two compounds, with methylone producing a larger number of responses than MDMA. The beta-ketone substitution has been suggested to confer greater dopaminergic affinity to the synthetic cathinones relative to their amphetamine congeners (Kolanos et al., 2013); however, a greater sample size is required to make definitive conclusions about methylone's relative reinforcing efficacy among these compounds.

The data discussed above specifically utilized compounds that are substrates at both DAT and SERT. Butylone, on the other hand, possesses "hybrid" transporter pharmacodynamics, acting as a DAT antagonist and a SERT substrate (Simmler et al., 2013; Eshleman et al., 2013). Limited data exist regarding the reinforcing effects of these "hybrid" compounds, but MBDB, the structural and mechanistic amphetamine analog of butylone (Simmler et al., 2013), produces less self-administration in rhesus monkeys (Fantegrossi, 2007) and a weaker conditioned place preference in rats (Marona-Lewicka et al., 1996) relative to MDMA. These data suggest that serotonin release is even more efficacious at limiting the reinforcing effects of dopamine uptake inhibition than release and explain the low breakpoint observed with butylone relative to the other compounds. It has been suggested that compounds with a "hybrid" pharmacodynamic profile may serve as useful maintenance drugs for stimulant addiction (Blough et al., 2014), but the lethality and other adverse effects observed with butylone likely precludes its consideration as a pharmacotherapeutic intervention.

The discussion so far has largely focused on pharmacodynamics, arguing that between-compound differences in relative serotonergic and dopaminergic efficacy contribute to the differential reinforcing effects among compounds; however, it is worth considering that pharmacokinetic factors may also contribute to these differences. It is well-established that the

reinforcing effects of drugs of abuse are largely dependent on rapid absorption and distribution to the site of action. The rate of distribution is in-part mediated by the lipophilicity of the compound, which is measured as logP, with drugs with a higher logP being distributed at a faster rate than those with lower values. Using ChemDraw software (ChemDraw Prime 16, PerkinElmer, Waltham, MA), the logP values of MDMA, methylone, butylone, and pentylone were calculated to be 1.98, 1.06, 1.54, and 1.96, respectively. As alkyl side-chain length increases, logP, expectedly, increases correspondingly; however, the addition of the beta-ketone substitution reduces lipophilicity relative to MDMA across all cathinone compounds. Although the logP values differ among the compounds, suggesting potential differences in rate of distribution, previous assessments of locomotor activity reveal no differences among the synthetic cathinones and MDMA in terms of onset of activity *in vivo*, with each compound producing locomotor effects (either stimulation or, with MDMA, depression) within 10-minutes post-administration (Gatch et al., 2013; 2014; 2016). Similarly, the pretreatment time for each test compound in our drug discrimination studies was 15-minutes, indicating that each compound is distributed and able to serve as a discriminative stimulus rapidly after administration. Furthermore, the logP values for MDMA and pentylone are nearly identical, but pentylone produced a significantly greater breakpoint than MDMA in the self-administration assay. Given the similarities in logP values and onset of effects among compounds, it is unlikely that differences in rates of absorption and distribution contribute to the differential reinforcing efficacy among these compounds.

Metabolism of the parent compound into a more efficacious or reinforcing metabolite represents another pharmacokinetic factor which may mediate the differences in reinforcing efficacy among compounds. 3,4-methylenedioxyamphetamine (MDA), an active metabolite of

MDMA, is more reinforcing than its *N*-methylated parent compound (de la Torre, et al., 2004; Fantegrossi et al., 2007). Methylone and butylone, like MDMA, are *N*-demethylated into 3,4-methylenedioxycathinone (MDC) and beta-keto-benzodioxoylbutanamine (bk-BDB), respectively (de la Torre et al., 2004; Elmore et al., 2017; López-Arnau et al., 2013; Zaitsu et al., 2009), but no data exist regarding the reinforcing efficacy of MDC or bk-BDB. No metabolism data for pentylone currently exist. The rates of MDMA and methylone metabolism into their *N*-demethylated metabolites have been established, and the metabolites start to accumulate within 35 minutes of administration in rats; however, the concentrations of the parent compounds do not rapidly fall, suggesting that the parent compounds maintain their activity without rapidly metabolizing (de la Torre et al., 2004; Elmore et al., 2017). Given that metabolism does not occur immediately, it seems unlikely that the acute reinforcing effects of methylone and MDMA, which were of interest in this study, are mediated by active metabolites. Unfortunately, without data regarding the time-course of butylone and pentylone metabolism, it is difficult to make strong inferences regarding the role of active metabolites in the reinforcing efficacy of these compounds.

Given the lack of differences in lipophilicity, onset of effects, and, potentially, metabolism, it seems unlikely that the differences in reinforcing efficacy among these compounds are mediated by pharmacokinetic factors. Although further testing of these kinetic parameters is required for robust conclusions to be drawn, the current data suggest that the different patterns of reinforcement among these compounds are likely due to their pharmacodynamic properties. Specifically, these differences likely arise from their relative serotonergic and dopaminergic mechanisms.

Conclusions

Structure-Activity Relations

The three compounds tested in this study structurally differ only by the length of the alkyl side-chain extending from the alpha-carbon, with alpha-methyl, -ethyl, and -propyl substitutions for methylone, butylone, and pentylone, respectively. Our data suggest a difference in mechanism and reinforcing efficacy of pentylone, with the longest side-chain, relative to methylone and butylone, with shorter side-chains. The *in vitro* literature examining these compounds suggests that increasing the length of side-chain shifts the phenotype of the compound from a substrate to an uptake-inhibitor, with DAT being more sensitive to changes in size than SERT (Eshleman et al., 2013; 2016; Simmler et al., 2013; 2014). Thus, as side-chain length increases, serotonergic signaling is reduced, and the molecular "braking" mechanism exerted by serotonin release over dopamine's reinforcing effects is removed, with the subjective and reinforcing effects of the drug changing correspondingly (Wee et al., 2005). Previous assessments of structure-activity relations among these compounds have indicated a negative relation between side-chain length and thermogenicitiy, with methylone, but not butylone or pentylone, increasing core body temperature in mice (Grecco & Sprague, 2016). Other studies assessing structure-activity relations in MDPV derivatives have demonstrated side-chain length as the most important factor for DAT selectivity, with amine-alkylation state providing the second-greatest contribution (Kolanos et al., 2013; 2015).

Our findings add to these structure-activity relations by demonstrating not only a greater dopaminergic mechanism, but reduced serotonergic efficacy with increased side-chain length. The structure-activity relation of side-chain length and mechanism indicates a length threshold, in which alpha-methyl and -ethyl substitutions maintain a serotonergic effect, but addition of a

third hydrocarbon moiety shifts the mechanism to a primarily dopaminergic phenotype. Furthermore, our data indicate that side-chain length is an important factor in reinforcing efficacy, likely as a result of the dopaminergic phenotype it imparts. However, as opposed to a direct correlation between side-chain length and reinforcing efficacy, our data indicate a U-shaped or check-mark structure-activity relation with this measure, as methylone and pentylone were self-administered robustly, but butylone produced nearly identical levels of responding as MDMA. These data mirror those produced in self-administration studies with methylone's and butylone's amphetamine analogs, MDMA and MBDB, respectively, in which MBDB was self-administered to a lesser degree than MDMA (Fantegrossi, 2007). Butylone, like MBDB, has a "hybrid" receptor mechanism characterized by inhibition of dopamine uptake and release of serotonin (Simmler et al., 2013). This "hybrid" profile has been suggested as a potentially useful maintenance pharmacotherapy for stimulant addiction, as the increased dopamine would prevent withdrawal, but the serotonin release would limit the reinforcing efficacy produced by dopamine uptake inhibition, preventing compulsive use of the treatment drug (Blough et al., 2014). It is possible that the reinforcing effects of the impulse-dependent increases in dopamine from butylone are limited by concurrent impulse-independent serotonin release, an effect not seen with methylone's impulse-independent dopamine release. However, this does not fully explain methylone's putatively greater reinforcing efficacy relative to MDMA, given the similarity of their mechanism of action. Further studies assessing *in vivo* neurotransmitter changes produced by each of the test compounds are necessary to fully correlate mechanism and reinforcing efficacy among these drugs.

Future studies assessing alpha-butyl or -pentyl side-chains will be beneficial in determining if this pattern holds true with larger molecules and whether there is a limit to the

side-chain structure-activity relation. Furthermore, studies assessing the role of side-chain length in non-methylenedioxy-substituted or simply *para*-substituted cathinone derivatives are necessary for determining the relative contributions of structural constituents to mechanistic and concurrent reinforcing effects.

Differential Patterns of Abuse

The current study aimed to evaluate the relative dopaminergic and serotonergic mechanisms and the reinforcing efficacy of methylone, butylone, and pentylone, three synthetic cathinone analogs of MDMA frequently featured in "Molly" or "Ecstasy" formulations. Our data revealed differences in both mechanism and reinforcing efficacy among these compounds that may translate into different patterns of use in human drug users.

The substrate-like mechanism and predominately serotonergic discriminative stimulus effects of methylone and butylone suggest that these drugs may fall within the umbrella of the entactogens, the sub-class of stimulants of which MDMA is the prototype (Nichols, 1986). The relative abundance of methylone and butylone in "Molly" formulations further lends credence to the putative entactogenic nature of these compounds (Palamar et al., 2016). No data currently exist examining the prosocial or mood-enhancing effects of methylone or butylone in the clinical or preclinical literature; however, many of the desired effects of MDMA intoxication, such as euphoria, positive affect, and improved mood, have been linked to serotonergic signaling, especially through $5HT_{2A}$ activation, in humans (Liechti et al., 2000; van Wel et al., 2012). The partial substitution of methylone and butylone in DOM-trained rats indicate some efficacy at $5HT_{2A}$ receptors, suggesting that these compounds have the capacity to induce positive, MDMA-like serotonergic effects. This interpretation is further evidenced by the finding that the selective

serotonin-reuptake inhibitor citalopram attenuates the subjective effects of MDMA in humans (Liechti et al., 2001), which, when considered with the data obtained from our electrophysiological and MDMA drug discrimination studies, suggests that methylone's and butylone's substrate-like activity will likely translate into MDMA-like subjective effects in human drug users. Indeed, the user reports of the methylone and butylone subjective experience draw several comparisons to MDMA and tend to, overall, agree on MDMA-like phenotype of these compounds when taken alone or in combination with other drugs (e.g., Erowid.org).

In addition to similarities in subjective effects among MDMA, methylone, and butylone, our self-administration results indicate comparable reinforcing efficacy. Our findings are the first to demonstrate self-administration of butylone, and these data illustrate its capacity as a reinforcer. Furthermore, our methylone self-administration data, alongside those previously reported (Vandewater et al., 2015), indicate a similar degree of reinforcement as MDMA. Together, these preclinical data suggest an episodic, MDMA-like pattern of abuse for methylone and butylone. Preclinical studies in rodents (Bradbury et al., 2013) and monkeys (Wang & Woolverton, 2007) indicate limited reinforcing efficacy of MDMA relative to traditional psychostimulants like cocaine or methamphetamine. The episodic, as opposed to compulsive, use of MDMA is apparent in its increased lifetime versus regular use in humans (SAMHSA, 2016), although dysregulated use has been reported in a subset of heavy users (Degenhardt et al., 2010). Consequently, it seems likely that most users who encounter methylone or butylone will not compulsively consume the drugs. Although the reinforcing efficacy may be limited, any assumptions of safety should be made sparingly as consumption of combinations of methylone and butylone have previously proven lethal (Warrick et al., 2012) and methylone, under

conditions of increased ambient temperature, potentially causes MDMA-like hyperthermia and serotonergic impairments following binge use (López-Arnau et al., 2014; Kiyatkin et al., 2015).

Despite its structural similarity, pentylone stands out among the compounds tested as having a predominately dopaminergic mechanism and significantly greater reinforcing efficacy relative to MDMA. Our data suggest a departure of pentylone from an MDMA-like profile toward a more stimulant-like pattern of abuse. Dopamine has been heavily implicated in the acute reinforcing and rewarding effects of stimulant-like drugs in preclinical self-administration studies (reviewed in Koob & Volkow, 2010) and self-administration data have high predictive validity for human abuse potential (reviewed in O'Connor et al., 2011). Indeed, neuroimaging studies in humans have demonstrated a positive relation between dopamine transporter blockade and subjective "high" after cocaine administration (Volkow et al., 1997; 2000). The robust reinforcing efficacy of pentylone relative to the other compounds tested demonstrated in our data mirrors studies with MDPV, another synthetic cathinone analog of MDMA, in which MDPV produced significantly greater self-administration than methylone (Schindler et al., 2015). This same study demonstrated robust increases in brain dopamine levels for MDPV and methylone using an *in vivo* microdialysis technique; however, MDPV produced no changes in serotonergic signaling, whereas methylone increased brain serotonin concentrations nearly 1000%, roughly 2.5-fold more than its dopamine-increasing effects (Schindler et al., 2015). Although microdialysis studies are not available for pentylone, the greater relative dopaminergic effect demonstrated in our drug discrimination studies of pentylone compared to methylone suggest a similar effect as MDPV. Future studies comparing pentylone and MDPV self-administration and neurochemistry are necessary to make strong conclusions about similarities in the pharmacodynamics of the two drugs.

At this juncture, we can conclude that the drugs tested in the current study are likely to engender different patterns of use, with methylone and butylone being used episodically like MDMA, whereas pentylone would more likely lead to compulsive use, like cocaine or other stimulant-type drugs. These results are particularly concerning giving the inadvertent use of these compounds in "Molly" formulations, given that "Molly" users typically believe they are consuming pure MDMA (Palamar et al., 2016). Formulations containing methylone or butylone are less likely to lead to uncontrolled use, although their potential toxicities are not to be dismissed; however, those containing pentylone may cause compulsive use of "Molly" or "Ecstasy," either in the form of excessive re-dosing in acute settings, or more regular episodic use, potentially serving as an avenue for experimenting with more traditional and highly addictive psychostimulants.

Limitations

The current study addressed the mechanism and abuse potential of three synthetic cathinone analogs of MDMA using behavioral and electrophysiological techniques. Although the data presented above provide compelling evidence for side-chain-dependent differences in monoaminergic mechanism and reinforcing efficacy, there are limitations to the scope and breadth of conclusions that can be drawn from these studies.

Perhaps the most apparent limitation lies in the electrophysiological studies, which provide an incomplete picture of the *in vitro* mechanism of the compounds tested. First, we did not test pentylone at the serotonin transporter, limiting our ability to relate the lack of serotonergic effects of pentylone in the drug discrimination assay to its molecular mechanism. Although the mechanism of pentylone at SERT is not as starkly equivocal between publications

as methylone, there is a minor discrepancy between the reports regarding its effects, with one study indicating uptake inhibition at SERT across a wide range of concentrations (Eshleman et al., 2016) and another indicating very weak serotonin release at 1 mM (Simmler et al., 2014). Assessment via the whole-cell method may have clarified the discrepancy between reports, as was done for methylone in the current study. Based on our behavioral findings demonstrating a predominately dopaminergic mechanism and highly reinforcing effects of pentylone, it seems likely that the serotonin-uptake-inhibition properties reported by Eshleman et al. (2016) underlie pentylone's mechanism; however, we cannot definitively make a conclusion without performing the experiments. Furthermore, we did not include MDMA in the quantitative analysis of the normalized current with methylone and butylone. Assessments of MDMA were performed at an earlier junction in the experimentation against 1 μM 5HT, thus we were unable to compare the compounds without a standardized control. Because of the inefficient transfection and unreliable expression of SERT, data collection was extremely limited with few transfections providing adequate expression to generate useful data.

The electrophysiology experiments were further limited by the lack of experimentation with DAT-transfected cells. Attempts were made to assess DAT-mediated current among the test compounds; however, despite fluorescence, surface-expression of DAT was limited, as we were unable to obtain current with application of dopamine. The experiments may have been more successful had we used a cell-line stably expressing DAT or SERT, instead of the unreliable transient transfection method utilized in our studies.

Within the *in vivo* approach, the drug discrimination assay may have been better able to detect serotonergic mechanisms of these compounds with rats trained to discriminate an indirect serotonin agonist, such as fenfluramine. Although DOM provided an adequate measure of

serotonergic signaling, its mechanism is highly selective for $5HT_{2A/C}$ receptors (Young et al., 1980), consequently, the putatively indirect agonistic effects of the test compounds would have been too non-selective for accurate detection by a $5HT_{2A/C}$-selective training drug. Previous studies of the discriminative stimulus effects of MDMA have utilized fenfluramine as a training compound or in substitution experiments in animals trained to discriminate MDMA to dissociate the serotonergic and dopaminergic effects of MDMA's discriminative stimulus (Evans et al., 1990; Goodwin & Baker, 2000; Goodwin et al., 2002). Incorporation of fenfluramine-trained rats into the drug discrimination experiments would have allowed for a more robust *in vivo* assessment of the mechanisms of these synthetic cathinones; however, use of DOM allowed us to assess the mechanisms of these novel compounds against a drug with a known pattern of abuse, providing us the means to determine the abuse-related mechanisms of these drugs.

The current study included only male rats, potentially limiting its translatability to the clinical population, wherein the prevalence of "Molly" use is roughly evenly split between males and females (Palamar et al., 2016a). Although females tend to be more likely to develop drug dependence and exhibit stronger cravings than males following chronic drug use (Kennedy et al., 2013), the current study was focused on acute effects of these compounds. Previous reports have indicated no sex differences in the discriminative stimulus effects of drugs of abuse, such as cocaine and morphine (Craft & Stratmann, 1996; Craft et al., 1996). Studies utilizing intracranial self-stimulation (ICSS) methods, have demonstrated no sex-differences in ability of stimulants (Stratmann & Craft, 1997) or MDMA (Lazenka et al., 2017) to reduce the reinforcing threshold of an electrical current delivered to the nucleus accumbens following acute drug administration. Although female rats tend to acquire self-administration of methamphetamine and other stimulants more rapidly than males (Roth & Carroll, 2004; Riechel et al., 2012), no differences

between sexes are seen when comparing self-administration of entactogen-class drugs such as MDMA, mephedrone, or methylone (Vandewater et al., 2015; Creehan et al., 2015). Because the acute effects of drugs of abuse differ minimally between males and females, our data regarding the discriminative stimulus effects after acute administration and the acute reinforcing effects of these synthetic cathinone analogs likely translate into both male and female users in the clinical population. Future studies with a focus on chronic use of these compounds would benefit from the inclusion of both sexes for establishment of differences in acquisition and maintenance of cathinone self-administration.

A final limitation of the current study arises from housing the rats individually, as opposed to with a cage mate or in social housing conditions. These isolated housing and testing conditions are seemingly problematic in that MDMA and other entactogens are typically used in social environments such as raves and clubs (Palamar et al., 2016a; 2016b). Thus, testing drug self-administration in a social environment may maximize translatability. Although the translatability is important, social housing and testing would be problematic to our experiments in a number of ways. The primary reason for individual housing is to ensure that each rat eats the appropriate amount of food to maintain their weight at roughly 85% *ad libitum* weight. Previous attempts in our lab to socially house rats have resulted in asymmetrical food consumption and, consequentially, one rat being overweight and the other underweight. This asymmetrical consumption is problematic for both rat health and nutrition and motivation to work for food reinforcement in a drug discrimination assay. For the intravenous self-administration experiments, social housing, considered an enriched-housing condition, tends to reduce overall levels of drug consumption (Bardo et al., 2001; Gipson et al., 2011). Furthermore, housing conditions, whether isolated or social, would still be removed from the actual drug-taking

behaviors, as our equipment and the conditions in most self-administration studies are limited to single-rodent self-administration chambers. A few studies utilizing specialized, multi-rodent chambers for self-administration have assessed social conditions on cocaine- and methamphetamine-taking behaviors, and have demonstrated differential effects of "socializing" on drug-taking behavior depending on the exact intervention employed (Smith et al., 2016; Robinson et al., 2016). Although these studies provide much-needed data regarding the social-aspect of drug-taking and an exciting additional layer of complexity and translatability, they require highly specialized equipment. Furthermore, and perhaps most importantly, the prosocial effects and environmental factors assessing the reinforcing efficacy of these compounds are ancillary to the questions being asked in these experiments. No butylone or pentylone self-administration data currently exist elsewhere in the literature, and the primary question being asked in these experiments was whether there were differences in reinforcing efficacy among these cathinone derivatives and MDMA. Although social influences on drug-taking are important considerations for the clinical implications of our data, establishment of a reinforcement baseline under standardized self-administration conditions is necessary to put our data in the context of the global drug-taking literature and to gain insight into the reinforcing effects of the individual drugs.

Future Directions

The current study provided insight into the mechanisms and acute reinforcing effects of three novel synthetic cathinone analogs of MDMA encountered as adulterants in "Ecstasy" or "Molly" formulations: methylone, butylone, and pentylone. As is the case within any new investigations into novel compounds, these data have laid the groundwork for several new

inquires of investigation and potential expansions on the data obtained from the current experiments.

The first major set of future experiments come from the limitations of the current study. We need to complete the proposed electrophysiological studies by testing each compound, including MDMA, against a standardized control concentration of 5HT at SERT, and test each compound at DAT. Having both *in vitro* mechanisms in the same assay will allow us to make more robust inferences regarding the mechanisms *in vivo* with drug discrimination data. Furthermore, production of a full dose-response curve of these compounds under these conditions will allow for a greater understanding of potency, efficacy, and selectivity of these compounds at both transporters.

The current study assessed the discriminative stimulus effects of the test compounds via substitution for the discriminative stimulus of well-characterized training drugs. In order to expand upon the discriminative stimulus effects demonstrated in this study, we would like to train methylone, butylone, and pentylone as discriminative stimuli and determine their ability to generalize to other compounds. Many early characterizations of MDMA's discriminative stimulus effects used MDMA as the training drug and made conclusions regarding its complex discriminative stimulus based on its ability to generalize to serotonergic and dopaminergic compounds (Schechter, 1986; 1988), and more recent studies have utilized different doses of MDMA to determine dose-dependent differences in discriminative stimulus effects (Webster et al., 2016). Early studies with synthetic cathinones have taken a similar approach and have allowed for a more robust characterization of the discriminative stimulus effects of MDPV (Fantegrossi et al., 2013). Training the synthetic cathinone analogs of MDMA as discriminative stimuli and subsequent generalization to dopamine- or serotonin-selective ligands alone or in

combination would allow for more fine-tuned assessment of the serotonergic and dopaminergic mediation of their *in vivo* effects, providing expansion of the conclusions regarding complex discriminative stimulus effects of methylone and butylone and predominately dopaminergic effects of pentylone.

Following clarification of the *in vitro* mechanisms, expansion of our *in vivo* mechanistic data into an *in vivo* microdialysis assay would provide much greater insight into the underlying neurochemistry of these compounds. Although drug discrimination allows us to determine *in vivo* mechanism at postsynaptic receptors, *in vivo* microdialysis assessments provide us with the opportunity to assess the magnitude of transmitter release in an awake and moving organism and simultaneously assess multiple transmitter systems. Previous assessments of methylone and MDMA using *in vivo* microdialysis techniques have demonstrated a robust increase in synaptic serotonin concentrations relative to dopamine, an effect opposite of what is demonstrated with methamphetamine (Baumann et al., 2012). These assessments would provide valuable insight into differences in monoamine-releasing efficacy and pharmacokinetics among the compounds tested in the current study, and would provide further inferences to be made about differences among the discriminative stimulus effects of these drugs. Furthermore, these techniques would also allow for assessment of metabolism of these compounds and concurrent assessment of metabolite generation and related changes in neurochemistry.

In addition to exploring further mechanistic components of these compounds, expansion of the self-administration studies to model chronic drug use would provide an opportunity to assess the likelihood of these compounds to engender compulsive use and addiction. In the current study, we first trained rats to self-administer methamphetamine before testing the novel cathinone analogs. As mentioned above, this training procedure can potentiate the reinforcing

effects of weaker reinforcers and provide a greater degree of responding than what might be seen in drug-naïve rats. To assess the reinforcing efficacy of these compounds in rats lacking a drug-taking history, we would like to test acquisition of self-administration of these compounds. Given that each test compound produced robust self-administration under a progressive ratio at 1.0 mg/kg/inf, rats would likely have the most success at acquiring self-administration of this dose. Assessments of the rate of acquisition and total proportion of rats acquiring self-administration among these compounds would serve as a useful index of reinforcing efficacy in drug-naïve rats. Studies of MDMA self-administration typically find limited acquisition of MDMA self-administration, highlighting the limited reinforcing efficacy of MDMA relative to traditional psychostimulants (Oakly et al., 2014; Schenk et al., 2007), and comparisons of the rates of acquisition of the novel compounds to MDMA would provide further evidence of the reinforcing efficacy of the cathinone analogs relative to MDMA. Furthermore, these studies would have greater translatability to the clinical population as MDMA users are unlikely to have a strong history of methamphetamine use (Palamar et al., 2016a). Beyond acquisition, experiments modeling chronic drug use utilizing long-access self-administration techniques, extinction and reinstatement of self-administration behavior, and incubation of drug-seeking would provide valuable insight into the long-term effects of these compounds and their similarity to well-known, highly-addictive drugs of abuse, such as cocaine and methamphetamine.

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