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The Combined Neuropharmacology and Toxicology of Major 'Bath Salts' Constituents
MDPV, Mephedrone, and Methyldone

A dissertation
presented to
the faculty of the Department of Biomedical Sciences
East Tennessee State University

In partial fulfillment
of the requirements for the degree
Doctor of Philosophy in Biomedical Sciences, Pharmaceutical Science Concentration

by
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May 2018

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Keywords: synthetic cathinones, neurotoxicity, pharmacological interactions, dopamine transporter

ABSTRACT

The Combined Neuropharmacology and Toxicology of Major ‘Bath Salts’ Constituents
MDPV, Mephedrone, and Methylone

by

Serena Allen

The synthetic cathinones, 3,4- methylenedioxypropylamphetamine (MDPV), 4-methylmethcathinone (mephedrone), and 3,4- methylenedioxymethcathinone (methylone), gained worldwide notoriety as the psychoactive components of ‘bath salts,’ a marketing term used to circumvent federal drug laws and permit their legal sale. Previous studies have shown that these drugs share pharmacological characteristics with cocaine and the amphetamines, however, descriptions of their neurotoxic properties are limited. Moreover, while forensic analysis has revealed that the most frequently abused bath salts ‘brands’ contain binary and ternary mixtures of MDPV, mephedrone, and methylone, the majority of preclinical research has focused on explicating the individual effects of these drugs. Therefore, the present dissertation aimed to address this limitation and characterize the acute and chronic effects of combined synthetic cathinone exposure on dopaminergic tone in mesolimbic and nigrostriatal brain regions. To accomplish this, male Swiss-Webster mice were administered MDPV, mephedrone, and methylone, individually or concomitantly, 1 time or 7 times over the course of two weeks and the corresponding effects of each treatment on mesolimbic and nigrostriatal brain tissue levels of dopamine (DA) and DA metabolites were analyzed using a high performance liquid chromatography – electrochemical detection (HPLC-ECD) assay. Additionally, motor-stimulant activity was evaluated after both dosing regimens using locomotor activity assays, while

immunoblot and immunostaining techniques were used to evaluate the chronic effects of co-synthetic cathinone exposure on tissue levels of tyrosine hydroxylase (TH), dopamine transporter (DAT), monoamine oxidase B (MAO-B), catechol-O-methyltransferase (COMT), and glial fibrillary acidic protein (GFAP). Results from these studies provide evidence of a significant pharmacological interaction among major bath salt constituents, MDPV, mephedrone, and methyldone. This was observed acutely as enhanced DA responses and chronically as functional toxicity at the DA synapse. Furthermore, such interactions may contribute to the deleterious effects reported by bath salt users. Together, these findings have shown that the composition of bath salts preparations can significantly influence their psychostimulant and toxic effects, substantiating the importance of modeling bath salts as drug mixtures.

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CHAPTER 1

INTRODUCTION

Defining 'Bath Salts' and Related Drugs

The neuropharmacology and toxicology of synthetic compounds marketed as 'bath salts' are primary topics of this dissertation. As 'bath salts' is a blanket term for designer drugs mixes containing one or various synthetic cathinones, it is important to define the synthetic cathinones most commonly found in bath salts as they have been investigated in this dissertation.

Bath salts – Bath salts have no legitimate purpose as bath additives. This marketing term refers to designer drug products that are purchased as “legal high” alternatives to illicit psychostimulant compounds such as cocaine, methamphetamine, and 3,4- methylenedioxymethamphetamine (MDMA, ecstasy). Bath salts are primarily sold as powders or fine crystals and are given redolent names such as - “White Lightning,” “Bliss,” and “Cotton Cloud” – with labels stating “not for human consumption” or a variant in order to circumvent legislation banning the sale of these psychoactive substances (Shanks et al. 2012; Spiller et al. 2011). The main psychoactive components of bath salts are synthetic cathinones.

Amphetamine and amphetamines – Given that the synthetic cathinones are novel, chemical derivatives of amphetamine, it is helpful to understand this group of drugs. The chemical structure of amphetamine is presented in Figure 1.1. Amphetamine (AMPH), or α -methyl-phenethylamine, is chemically defined by Biel and Bopp as a compound consisting of: 1) an unsubstituted phenyl ring, 2) a two-carbon side chain (α

and β carbons) that connects the phenyl ring to nitrogen, 3) an α -methyl group, and 4) a primary amino group (Biel and Bopp 1978). Various chemical modifications and functional group substitutions to the amphetamine scaffold have yielded a large number of amphetamine derivatives. For example, methylation of the amino group and 3,4 methylenedioxy ring substitution onto the phenyl group of amphetamine produce methamphetamine (METH) and MDMA, respectively (see Fig 1.1). Amphetamine derivatives have a common phenethylamine backbone and are collectively referred to as substituted amphetamines or simply amphetamines.

Cathinone and synthetic cathinones – The word cathinone refers to the molecule referenced in Fig 1.1. Cathinone, the parent compound to synthetic cathinones, is a naturally occurring phenylalkylamine alkaloid found in the leaves of the khat plant (*Catha edulis*). The backbone of cathinone is phenethylamine with a ketone group substituted on the β carbon. Therefore, cathinones can be referred to as β -keto amphetamines. As with amphetamine, the amino group of the cathinone can be methylated (ie methcathinone, Fig 1.1), and functional groups can be added to many locations on the ring and side chain of the molecule, producing various structural analogs called substituted, or synthetic, cathinones. The compounds mephedrone, methylone, and MDPV (3,4-methylenedioxypropylone) are examples of such synthetic cathinones and will be discussed thoroughly. In this thesis, the terms cathinone(s), and synthetic cathinone(s) will refer to any specific cathinone or the entire group of cathinones.

Mephedrone – Mephedrone, 4-methylmethcathinone, consists of a methyl group attached to the amine, α carbon, and 4' carbon on the phenyl ring of the β -keto amphetamine backbone, making it similar in structure to methamphetamine (Fig 1).

Methylone – Methylone, 3,4-methylenedioxymethcathinone, is also methylated on the amine group and α carbon of the cathinone/ β -keto amphetamine backbone, but differs from mephedrone as it has a 3,4-methylenedioxy ring added to the phenyl ring. Thus, methylone is structurally similar to MDMA (Fig 1.1).

MDPV – MDPV, 3,4-methylenedioxypropylvalerone, has the largest structural divergence from cathinone when compared to mephedrone or methylone. MDPV has a 3,4 methylenedioxy ring added to the phenyl ring of the β -keto amphetamine backbone and a pyrrolidinyl ring and propyl group attached to the α carbon (Fig 1.1), making it structurally similar to propylvalerone.

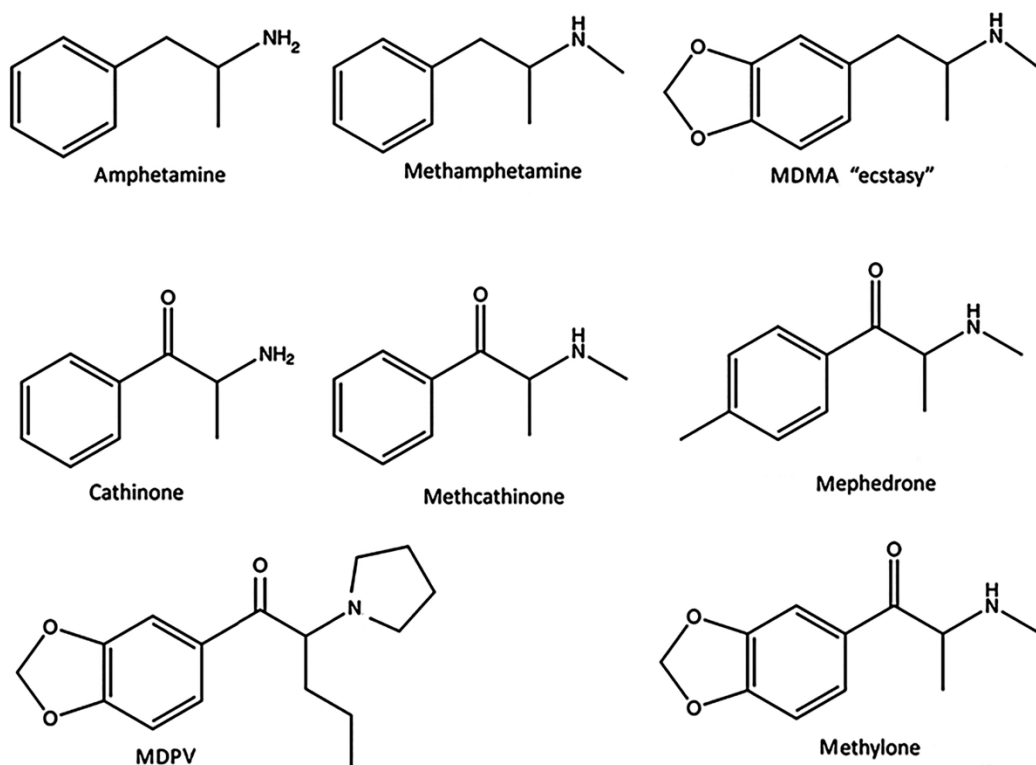


Figure 1.1 Molecular structures of amphetamine, cathinone, and related compounds

Synthetic Cathinones: History and Abuse

Catha edulis (khat) and the Discovery of Cathinone

While the first written reports of khat use date back to the 14th century, the khat shrub was not well known by Europeans until its discovery and cataloguing in the 18th century by botanist Peter Forskal (Karila et al. 2015). Later, advances in analytical chemistry allowed for the isolation of an active ingredient, identified as 'katin' ((+) norpseudo-ephedrine), from khat extracts in 1887 (Kelly 2011). However, in the 20th century the United Nations Narcotic Laboratory analyzed khat leaves and confirmed the presence of another active ingredient, cathinone (Patel 2000). A controlled study

confirmed that cathinone was the compound responsible for the amphetamine-like stimulatory effect that accompanied chewing khat leaves (Widler et al. 1994). To this day, khat shrubs continue to be cultivated in the Arabian Peninsula and specific regions of Eastern Africa where their leaves are still commonly chewed for their stimulant effects (De Felice et al. 2014). While the use of khat was localized to these regions in the past, it became a widespread problem in more recent history following advances in logistics and immigration as well as invention of the internet (Coppola and Mondola 2012b; Katz et al. 2014).

Synthesis of Cathinone Analogues

Like many designer drugs, synthetic/substituted cathinones were first synthesized by academic chemists or the pharmaceutical industry for research or medicinal purposes. Therefore, synthesis protocols and the associated effects of these compounds were available in the literature and often discovered and repurposed as drugs of abuse at later dates (German et al. 2014). The first synthetic cathinone developed was methcathinone, a methylated analogue of cathinone. It was reportedly synthesized as an antidepressant in the former Soviet Union in 1928 and separately in the western world for use as an appetite suppressant in the 1950s (Spiller et al. 2011). Despite never being marketed due its abuse potential, methcathinone was rediscovered and used as a drug of abuse in the 1990s. Development of mephedrone, MDPV, and methylone were first described in 1929, 1967, and 1996 respectively, while their abuse was not reported until the early- to mid- 2000s and came immediately prior the classification of cathinone and methcathinone as a schedule I controlled substances (German et al. 2014).

Controlled Substance Legislation and the Development of Designer Drugs

The term “designer drug” refers to the fact that these compounds are specifically designed to circumvent drug laws. The Controlled Substance Act (CSA) of 1970 established a framework for substances of abuse in the United States (USA), classifying compounds on a scale from schedule I to V based on medical use and potential for abuse (Banks et al. 2014). The CSA also permitted federal regulation of the manufacture, importation, possession, use, and distribution of controlled substances. This included the psychostimulants, cocaine and amphetamine. However, under this legislation, the government could not prosecute for the production, possession, or consumption of controlled substance analogues until those specific compounds were scheduled. Thus, after the CSA was passed, numerous synthetic designer compounds surfaced on the drug market and were abused to mimic the effects of popular illicit drugs, while eluding federal regulation (Banks et al. 2014). The first designer compounds to emerge were synthetic opioids in 1979, followed by psychostimulant analogues including methamphetamine and MDMA (German et al. 2014). As the abuse of designer drugs grew, the CSA was amended in 1986 to include compounds intended for human consumption that were “substantially similar” to schedule I and II controlled substances to be treated as schedule I substances. However, to avoid prosecution under the 1986 Controlled Substance Analogue Enforcement Act, designer drug analogues that had yet to be specifically scheduled as controlled substances began to be marketed explicitly as products that were “not for human consumption” (Seely et al. 2013; Spiller et al. 2011; Valente et al. 2014). This tactic was used to launch the

manufacturing, widespread distribution, and global abuse of synthetic cathinones without legal consequence.

Prevalence of Synthetic Cathinones and the New Designer Drug Market

Due to structural substitutions to the cathinone (β -keto amphetamine) molecule, synthetic cathinones were previously not covered by existing drug laws and could therefore be legally distributed and sold as bath salt products that were “not for human consumption” (Banks et al. 2014). MDPV, mephedrone, and methyldone were the most commonly identified synthetic cathinones in the forensic analysis of confiscated bath salts products (DEA 2011; Shanks et al. 2012; Spiller et al. 2011). Given the reported ability of these compounds to cause similar physical and neuropsychiatric effects as cocaine, MDMA, and the amphetamines, the abuse of bath salts was popularized under the notion that they were cheap and ‘legal’ alternatives to these illicit drugs. As a result, the global abuse of synthetic cathinones dramatically increased within the past decade. In 2011, the NHS reported that mephedrone was the third most commonly abused drug in the UK (German et al. 2014), while at the same time the National Forensic Laboratory Information System (NFLIS) named MDPV and methyldone the fifth and eleventh most common hallucinogens in the US (DEA 2014).

This increase in bath salts popularity has precipitated a concerning rise in the number of individuals requiring emergency medical treatment after synthetic cathinone intoxication. Data compiled from the American association of Poison Control Centers show a dramatic spike in the reports of bath salts overdose from 2009-2012, with the number of cases exceeding 7000 (Wood 2013). Another retrospective review of poison

control centers across 9 states found that 16% of reported bath salt toxicities resulted in either major medical consequences or death (Warrick et al. 2013). Furthermore, the Substance Abuse and Mental Health Services Administration (SAMHSA) reported that bath salts were involved in over 22,000 drug-related emergency department visits in 2011 alone. The alarming rise of bath salts abuse and toxicities cases led to the scheduling of the most commonly abused synthetic cathinones, MDPV, mephedrone, and methylone, as class I controlled substances in 2011 (DEA 2011). However, despite this classification, international control laws remain lacking and synthetic cathinone abuse has continued to thrive due in large part to a vast online e-commerce-based market for these compounds (Karila et al. 2015; Power 2014). In this clandestine marketplace, bath salts are synthesized, primarily in China and surrounding South East Asian countries, packaged, distributed, and sold directly to consumers (Katz et al. 2014). As such, it is reported that MDPV, mephedrone, and methylone remain available in the street drug marketplace where they continue to be abused under false notions that they are “legal” and “safe” alternatives to more regulated illicit psychostimulants such as methamphetamine, MDMA, and cocaine (DEA 2014; Seely et al. 2013). Additionally, as the phenol ring and side chain of these cathinone analogs offer many possibilities for substitutions, chemists continue to come up with new substituted cathinones and give them different names in order to replace the banned substances. Recent reports indicate that over 44 different types of synthetic cathinones have been encountered worldwide (UNODC, 2013).

Patterns of Abuse and Drug Combinations

According to numerous drug survey reports, synthetic cathinones are most popular among youths in urban environments, with adolescent males abusing them more frequently than females (DEA 2011). There is also an emergence of mid-to-late adolescents and young adult men abusing bath salts while attending techno-alternative parties (Zawilska and Wojcieszak 2013). The most common route of synthetic cathinone administration is insufflation (snorting), but they may also be ingested in the form of capsules or tablets (DEA 2011; Katz et al. 2014). Less common modalities include inhalation, intravenous injection, intramuscular injection, and rectal administration (DEA 2011; Zawilska and Wojcieszak 2013). The average amount of synthetic cathinones abused per session varies widely from 25 milligrams to 5 grams, usually achieved through repeated doses ranging from 25 to 250 milligrams (DEA 2011).

According to DEA statistics, bath salt preparations are commonly composed of MDPV, mephedrone, and methylene in combination with each other or other stimulant compounds (DEA 2014). In support of this, SAMHSA reports that over half of the registered emergency room visits associated with bath salt intoxication in 2011 involved synthetic cathinone compounds in various drug combinations (The DAWN Report, 2013). Additionally, analysis of popular 'legal high' or 'bath salts' brands, as determined by retrospective searches of records involving synthetic cathinone intoxication at poison control centers, revealed that many of the most popular bath salt 'brands' contained mixtures of MDPV, mephedrone, and methylene (Araujo et al. 2015; Spiller et al. 2011). Moreover, in an effort to keep production costs down, clandestine drug manufacturer's use these compounds to dilute the purity of MDMA and other illicit drugs (Brandt et al.

2010; Brunt et al. 2011; Winstock et al. 2011b). Thus, evidence now indicates that these compounds are frequently co-abused with each other and various other drug compounds in order to enhance their desired effects, and it appears likely that combinations of MDPV, mephedrone, methylone, or their possible analogs will continue to be found in designer drug mixtures (Coppola and Mondola 2012b; Spiller et al. 2011; Zawilska and Wojcieszak 2013).

Monoamine Neurotransmitter Systems

The pharmacological actions of synthetic cathinones include modulation of monoamine neurotransmitters, namely dopamine (DA), norepinephrine (NE), and serotonin (5-HT). The structural similarities between monoamines and psychostimulant compounds, including the cathinones, enable these drugs to interact with monoamine regulatory proteins and dysregulate signaling. Therefore, a brief overview of the anatomy and functionality of these systems is given.

Dopamine

Dopamine cell bodies are primarily found in the substantia nigra (SN) and ventral tegmental area (VTA) (Björklund and Dunnett 2007; Oades and Halliday 1987). Dopamine cell bodies in the SN send projections into the striatum (STR, caudate putamen) and globus pallidus, forming the nigrostriatal brain pathway. This pathway may also be termed the motor control pathway as it plays an important role in movement coordination and behavioral sensitization to psychostimulant drugs of abuse (Björklund and Dunnett 2007; Uhl et al. 2002). Dopamine cell bodies in the VTA send projections to the limbic system – amygdala, nucleus accumbens (NAc), hippocampus

(mesolimbic) – and to areas of the cerebral cortex (mesocortical). The mesolimbic projection pathway, also termed the reward pathway, plays a central role in the rewarding effects of naturally rewarding stimuli, such as food and sex, and reward-motivated behavior (Wise and Rompre 1989). This pathway is also implicated in the stimulant and rewarding properties of psychostimulant drugs of abuse (Volkow and Morales 2015; Wise 1996). The NAc is the primary projection site associated with rewarding stimuli and reference to the mesolimbic(Kelly et al. 2008) system in this dissertation specifically refers to the VTA to NAc pathway. Dysregulation of DA in the nigrostriatal and mesolimbic pathways is implicated in psychostimulant drug addiction.

In dopamine neurons, biosynthesis of DA begins with the cytosolic conversion of tyrosine to dihydroxyphenylalanine (L-DOPA) via the rate limiting enzyme, tyrosine hydroxylase (TH). L-DOPA is then converted to DA by aromatic L-amino acid decarboxylase (AADC). DA is sequestered from the cytosol into synaptic vesicles by vesicular monoamine transporter (VMAT) and stored in vesicles until stimulation. Firing of an action potential prompts vesicular docking and release of DA into the synaptic cleft via exocytosis. Released DA binds to and activates dopamine receptors (D1, D2, D3, D4, D5) on found both postsynaptic and presynaptic neurons (Missale et al. 1998). Activation of D1- like receptors (D1 and D5) generally produces an excitatory effect, increasing neurotransmission (Dearry et al. 1990). Moreover, enhanced D1 receptor activity in the mesolimbic and nigrostriatal pathways has been implicated in behavioral sensitization and the reinforcing and stimulant properties of psychostimulant drugs of abuse (Hu et al. 2002; Presti et al. 2003; Uhl et al. 2002). Activation of D2-like receptors (D2, D3, D4) by released DA reduces neuronal firing in the mesolimbic and nigrostriatal

brain pathways (Missale et al. 1998). Notably, presynaptic D2 receptors function as autoreceptors which negatively regulate DA signaling (Beaulieu and Gainetdinov 2011), and D2 antagonists reportedly decrease the ambulatory response but enhance the expression of sensitization of many psychostimulants (Mohd-Yusof et al. 2016). After receptor signaling, DA is taken back up into the presynaptic terminal by the dopamine transporter (DAT) where it is either recycled and repackaged into synaptic vesicles by VMAT or degraded by mitochondrial monoamine oxidase B (MAO-B) and catechol-O-methyltransferase (COMT) to produce homovanillic acid (HVA) via the intermediate metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and 3-methoxytyramine (3-MT).

Serotonin

The cell bodies of serotonin neurons are concentrated in midline areas of the midbrain and brainstem termed the raphe nuclei (Steinbusch 1981). Axonal projections are sent upward from the raphe nuclei via the medial forebrain bundle where they form widespread connections with various targets including the frontal cortex, NAc, STR, and hippocampus (Baumgarten and Grozdanovic 2000). Serotonin modulates several brain functions such as satiety, anxiety, emotion, mood, and sleep (Jacobs and Azmitia 1992; Pum et al. 2009; Ursin 2002). Serotonin biosynthesis begins via the cytosolic conversion of L-tryptophan to 5-hydroxytryptophan (5-HTP) by the rate limiting enzyme tryptophan hydroxylase (TPH). 5-HTP is then converted to serotonin, or 5-hydroxytryptamine (5-HT), by cytosolic AADC. Serotonin, 5-HT, is then packaged into vesicles by VMAT and subsequently released into the synapse via exocytosis after an action potential has fired. Once released serotonin binds to one of 14 serotonin receptor subtypes found widely expressed throughout the CNS with notable localizations of 5-

HT_{1B,2A,2C,3} in dopaminergic regions (NAc, STR, SN, VTA) (Baumgarten and Grozdanovic 2000; Egerton et al. 2008). Notably, 5-HT_{2C} receptors are expressed on inhibitory GABA (γ -aminobutyric acid) neurons in the SN and VTA (Berro et al. 2017; De Deurwaerdère et al. 2004). Thus, 5-HT activation of these receptors inhibits downstream DA neurotransmission (Olijslagers et al. 2006). Importantly, given that 5-HT_{2C} negatively regulate DA signaling, drugs which promote simultaneous increases in DA and 5-HT in the NAc (and STR) are subsequently predicted to have lower rewarding and stimulant properties (Alex and Pehek 2007; Berro et al. 2017). After receptor signaling, 5-HT is transported back into presynaptic nerve terminals via the serotonin transporter (SERT), where it is repacked into vesicles by VMAT or degraded by MAO-A to yield 5-hydroxyindolacetic acid (5-HIAA).

Norepinephrine

Noradrenergic neurons, which synthesize norepinephrine (NE), are most notably found in the locus coeruleus of the brainstem (von Bohlen und Halbach and Dermietzel 2006). These cells send projections that extend upward via the medial forebrain bundle where they form a broad range of synaptic contacts with targets in the cerebellum, cerebral cortex, and thalamic nuclei. Due to the wide distribution of projection sites, NE cells of the locus coeruleus modulate various behavioral and physiological processes including alertness, overall arousal, wakefulness and mood (Berridge and Waterhouse 2003). As NE and DA are both catecholamines, their biosynthesis occurs via the same pathway. Thus, tyrosine is converted to L-DOPA and then to DA by TH and AADC, respectively. In noradrenergic neurons DA is transported by VMAT into vesicles containing dopamine- β -hydroxylase (DBH), which subsequently converts DA to NE.

Following stimulation and exocytosis, NE is released into the synaptic cleft where it activates adrenergic receptors, which also serve as targets for adrenaline. Two subtypes of adrenergic receptors have been described: alpha (α_1 , α_2) and beta (β_1 , β_2 , and β_3) which are found throughout the CNS and on peripheral targets (Insel 1996). NE neurotransmitters are taken back up into presynaptic neurons by the norepinephrine transporter (NET) and is either recycled into synaptic vesicles by VMAT or degraded by MAO and COMT to yield 3-methoxy-4-hydroxyphenylglycol (MHPG) and vanillylmandelic acid (VMA), respectively.

Monoamine Transporters: DAT, NET, SERT

Dopamine (DAT), norepinephrine (NET), and serotonin (SERT) transporters terminate DA, NE, and 5-HT neurotransmitter signaling, respectively, via active reuptake into the presynaptic neuron (Robertson et al. 2009). As monoamine transporters are members of the solute carrier-6 (SLC6) family of secondary active transporters, the inward transport of released neurotransmitters (NTs) is driven by the sequential binding and co-transport of Na^+ and Cl^- down their electrochemical gradients (Reith 2002; Torres et al. 2003). The binding of substrate, two Na^+ ions, and one Cl^- ion triggers a conformational change in the transporter, shifting it from an “outward-facing” to an “inward-facing” conformation where the substrate, Na^+ , and Cl^- are released into the cytosol (Forrest et al. 2008; German et al. 2015). The release of the neurotransmitter substrate shifts the transporter from its inward-facing conformation to its outward-facing conformation. These plasmalemma transporters consist of 12 transmembrane domains and intracellular loop domains containing phosphorylation and other binding sites that are essential for their regulation and function (Torres et al. 2003).

Since monoamine transporters regulate the strength and duration of neurotransmitter signaling many pharmacological agents often target these molecules in order to manipulate DA, NE, and 5-HT neurotransmission and the biological processes they modulate (Robertson et al. 2009). Moreover, given the structural similarities of DAT, NET, and SERT, it is important to note that drug compounds developed to target one of these transporters may also act on any or all of the other monoamine transporters, albeit with varying affinity and potency. However, in regards to psychostimulant drugs of abuse, DAT is a major target and will thus be discussed more thoroughly in this thesis.

Neuropharmacology of Drug Abuse: DAT-Targeting Compounds

The processing of reward-related stimuli, including those affiliated with drugs of abuse, is primarily mediated by mesolimbic neuronal projections from the VTA to the NAc (Volkow and Morales 2015; Wise 1996). Amphetamine-derived drugs of abuse target DAT in the dopaminergic nerve terminal regions, NAc and STR, and produce characteristic increases in extracellular DA within these regions (Carboni et al. 1989; Jones et al. 1998; Sitte and Freissmuth 2015). Drug-induced increases in accumbal DA release mimics phasic firing of VTA neurons and provokes rapid increases in DA necessary to stimulate D1 receptors (enhanced DA neurotransmission), which corresponds to the mechanism by which reward is signaled in the brain (Fleckenstein et al. 2007; Sitte and Freissmuth 2015; Wise 1996). Thus, these drugs are said to have rewarding properties. Additionally, as the mesolimbic pathway modulates reward motivated behavior to natural reward stimuli, such as food, this also applies to rewarding stimuli from drugs of abuse and corresponds to their reinforcing properties (Koob 1992; Wise 1996). This has been directly shown by studies using the neurotoxin

6-hydroxydopamine (6-OHDA), whereby 6-OHDA-induced lesions of the NAc resulted in prolonged, significant decreases in the reinforcing effects of cocaine (self-administration) (Roberts and Koob 1982).

In addition, amphetamine-like stimulants of abuse increase locomotor activity. A specific role for extracellular DA and DAT in this behavioral response has come from studies using DAT knockout mice, whereby DAT deficient animals were unresponsive to the stimulant effects of cocaine and amphetamine and did not show DA elevations following administration of these psychostimulants (Giros et al. 1996; Tilley et al. 2009). Also, neurotoxin-induced lesions of the NAc in rats abolished the psychostimulant-induced locomotor activity of amphetamine and cocaine, specifically implicating a role for mesolimbic DA in these responses (Kelly and Iversen 1976; Kelly et al. 1975). Moreover, investigations using microdialysis have shown that the extent of drug-induced DA elevations in the NAc positively correlate with the motor-stimulant effect of abused psychostimulant compounds (Benwell and Balfour 1992; Bradberry 2002; Bradberry et al. 1991; Pierce and Kalivas 1995).

Repeated, intermittent exposure to stimulant drugs of abuse often enhances the motor-stimulant response over time, a phenomenon termed behavioral sensitization (Pierce and Kalivas 1997). This process involves activation of the mesolimbic and nigrostriatal DA systems and can be manifested as increased locomotor activity or increased stereotyped behavior (repetitive or patterned movement) (Budygin et al. 2000; Robinson and Becker 1986; Steketee and Kalivas 2011; Vanderschuren and Kalivas 2000). Amphetamine-like stimulants often display a biphasic pattern of activity, whereby these drugs produce sensitized hyperactivity at lower doses, while sensitized

stereotyped activity is reported with very high doses (Kuczenski and Segal 1997; Pierce and Kalivas 1995). It is now accepted that mesolimbic DA plays a key role in the development of behavioral sensitization. Initial evidence for this came from studies showing that the expression of behavioral sensitization could be induced by intracranial injections of amphetamine into the VTA and NAc (Carr and White 1987). Moreover, striatal DA has been primarily implicated in the induction of stereotypic activity. A specific role for the nigrostriatal system in the induction of stereotypic activity was deduced from the observations that intrastriatal injections of amphetamine and DA produced stereotypy in rodents (Kelley et al. 1988; Presti et al. 2003), while 6-OHDA-induced lesions of the substantia nigra and striatum prevented amphetamine-induced stereotypy (Joyce and Iversen 1984; Price and Fibiger 1974).

Mechanism of Transporter Interaction: Monoamine Releasers and Reuptake Inhibitors

Monoamine transporters serve as primary biological targets for many drugs of abuse and pharmaceuticals (Amara and Sonders 1998). Pharmacological agents that interact with monoamine transporters can be divided into two functionally distinct classes: 1) pure monoamine reuptake inhibitors and 2) monoamine releasers or substrates (Baumann et al. 2014; Rothman and Baumann 2003). In addition, a distinguishing property of a given monoamine reuptake inhibitor or releaser compound is its relative potency at DAT, NET, and SERT or transporter selectivity profile. Monoamine reuptake inhibitors act as competitive blockers that bind monoamine transporters and prevent transporter-mediated reuptake of synaptic DA, NE, and/or 5-HT into presynaptic terminals following their action potential –dependent release (Rothman and Baumann 2003). Cocaine is an example of a widely abused reuptake

inhibitor and its action to selectively bind DAT and block the DAT-mediated reuptake of DA from the synaptic cleft is well-documented (Boja and Kuhar 1989; Johanson and Fischman 1989; Jones 1984; Ritz et al. 1990). In contrast, monoamine releasers behave as substrates at monoamine transporters, competing with endogenous NTs for transporter-mediated uptake into the presynaptic neurons (Fleckenstein et al. 2007; Sitte et al. 1998). Once actively transported into neurons, these compounds promote the release of DA, NE, and/or 5-HT back into the synapse via reversal of monoamine transporter flux (Jones et al. 1999; Rothman and Baumann 2003). Most amphetamines, including METH and MDMA, are substrate-type releasers. Specifically, METH is a selective substrate for DAT-mediated transport into presynaptic neurons and subsequently induces DAT-mediated reverse transport of DA into the synaptic cleft (Calipari and Ferris 2013; Fleckenstein et al. 2007; Jones et al. 1999). MDMA (ecstasy) is also a transporter substrate, however, the methylenedioxy ring on MDMA results in a compound with a nearly 10-fold greater affinity for SERT as compared to METH (Eshleman et al. 2013; Rudnick and Wall 1992), while its effects at DAT are significantly weaker. Thus, MDMA is a non-selective transporter substrate and promotes the release of both 5-HT and DA, albeit with 5-HT release exceeding that of DA (Gudelsky and Nash 1996).

The mechanism by which amphetamine-like compounds promote transporter-mediated efflux involves a concerted action of these drugs to inhibit MAO and disrupt VMAT function upon neuronal entry (Schmitz et al. 2001; Sitte and Freissmuth 2015). Amphetamines are also exogenous substrates of VMAT, and thus, accumulate in synaptic vesicles (Sulzer et al. 1995). Therein, the amphetamines act as weak bases in

the acidic luminal environment of synaptic vesicles and dissipate the proton gradient. This prevents the inward transport of monoamines by VMAT and results in an elevation of monoamines in the cytosol (Gulaboski et al. 2007; Sulzer and Rayport 1990), which is further exacerbated by MAO inhibition (Sitte and Freissmuth 2015; Thomas et al. 2008). Thus, the amphetamines increase cytosolic monoamine concentrations to levels that allow for occupation of their internal binding site on plasmalemma transporters and subsequently their outward transport (Sulzer et al. 1993). Notably, elevations in cytosolic DA levels have been attributed to the neurotoxicity of METH and other amphetamine-related drugs.

Neurotoxicology of Amphetamine-Related Drugs

Dopaminergic brain regions are the primary targets of neurotoxicity induced by amphetamine-related drugs (Yamamoto et al. 2010). Neurochemical markers of this toxicity include decreased DA and DA metabolite tissue content and decreased expression levels of TH and DAT, all of which have been observed in the striatum (STR) and frontal cortex with very high single doses or repeated doses of METH and AMPH administration (Halpin et al. 2014; Moratalla et al. 2017; Wagner et al. 1980). Most importantly, METH and AMPH produce long term damage to dopamine axon terminals in the STR (Ares-Santos et al. 2014; Granado et al. 2011; Ricaurte et al. 1982), while reducing the number of TH-positive nissl stained neurons in the substantia nigra pars compacta (SNpc) (Ares-Santos et al. 2014). Additionally, the amphetamines trigger neuroinflammatory responses in the brain areas most affected by their toxicity (Thomas et al. 2004).

Biochemical Mechanisms of Neurotoxicity: Oxidative Stress and Excitotoxicity

The mechanisms underlying METH and AMPH-mediated neurotoxicity are complex and may involve several different processes. In this regard, oxidative stress is thought to be a key mechanism, given that METH and AMPH increase the production of reactive oxygen species (ROS), such as hydroxyl radicals and superoxides, and reactive nitrogen species (RNS), such as nitric oxide (NO) and peroxynitrite (Cadet and Brannock 1998; Cubells et al. 1994; Hansen et al. 2002). ROS and RNS can damage membranes, inhibit protein function, and damage DNA (Cadet and Brannock 1998). Further support for the involvement of oxidative stress in amphetamine-induced DA toxicity was provided by studies showing that methamphetamine and MDMA-induced DA depletions were attenuated by antioxidant treatment (N-acetyl-cysteine, ascorbic acid, and vitamin E) (De Vito and Wagner 1989; Fukami et al. 2004; Wagner et al. 1986) and exacerbated following glutathione depletion and inhibition of both superoxide dismutase and nitric oxide synthase (NOS) (Brown and Yamamoto 2003; Jayanthi et al. 1998). Several mechanisms contribute to the formation of ROS and RNS following exposure to amphetamine-related drugs via several mechanisms.

Reactive metabolites of DA degradation are considered a primary source of ROS in amphetamine-induced oxidative stress (Delcambre et al. 2016; Fleckenstein et al. 2007; Schmidt et al. 1985; Yamamoto and Bankson 2005). Amphetamine-related drugs increase the cytosolic pool of DA available for 1) metabolism by MAO to yield DOPAC and hydrogen peroxide and 2) direct oxidation to form DA semiquinones and superoxide radicals. Hydrogen peroxide and superoxide anions can generate highly reactive hydroxyl radicals via Fenton reactions (Kuhn et al. 2006; LaVoie and Hastings 1999).

Hydroxy radicals damage various cellular components and initiate lipid peroxidation, leading to membrane damage (necrosis), and eventually activate proteases, initiating the cell death cascade (Cadet et al. 2005). Support for the involvement of DA in METH and AMPH-induced toxicity comes from studies showing that inhibition of DA synthesis afforded neuroprotection (Ares-Santos et al. 2012; Larsen et al. 2002; Schmidt et al. 1985), while increasing cytosolic DA levels with DA precursor, l-dopa, exacerbated these toxic effects (Gibb and Kogan 1979; Schmidt et al. 1985).

Additionally, METH and AMPH induce oxidative stress by enhancing glutamate release (Mark et al. 2004; Nash and Yamamoto 1992). This process, termed excitotoxicity, culminates in increased cytosolic Ca^{2+} and leads to the formation of RNS and ROS (Forder and Tymianski 2009). Specifically, NO, which is produced subsequent activation of the Ca^{2+} -dependent nNOS, readily reacts with superoxide anions to form highly reactive peroxynitrite compounds that produce toxicity via the nitrosylation of proteins (Bruno et al. 1993). Evidence for the involvement of this process in METH and AMPH neurotoxicity has been provided by several studies demonstrating that blockade of nNOS and glutamate receptor activity provides full protection against AMPH-induced DA depletion (Chipana et al. 2008; Itzhak et al. 2000; Mark et al. 2004), and others showing that co-administration of METH with peroxynitrite scavengers partially prevented this toxic effect (Imam et al. 2000). Moreover, robust elevations in extracellular DA produced by binge doses of METH have been directly implicated in the development of excitotoxicity, whereby excessive DA release activates the nigrostriatal pathway and causes increased striatal glutamate release (Brown et al. 2005; Halpin et al. 2014; Mark et al. 2004). Another consequence of excitotoxicity is inhibition of

complex II of the mitochondrial electron transport chain by peroxynitrite, resulting in excessive leakage of ROS in addition to altered energy metabolism (Heales et al. 1999). Indeed, METH-induced striatal DA depletions were accompanied by decreased complex II activity and exacerbated by the complex II inhibitor, malonate (Brown et al. 2005). Also, inhibition of complex II activity was shown to be dependent on glutamate receptor activation and peroxynitrite formation (Brown et al. 2005). Moreover, METH-induced mitochondrial dysfunction is suggested to mediate the long-term deficits in DA and DA regulating proteins produced by the amphetamines (Yamamoto et al. 2010).

Neuroinflammation

Glial cells (astroglia and microglia) modulate the neuroinflammatory response in the CNS by releasing pro-inflammatory cytokines following brain insult (Lucas et al. 2006). Over activation of this response, as observed with various neurodegenerative conditions, causes persistent neuroinflammation and eventually contributes to the loss of neurons (Lucas et al. 2006). Thus, reactive gliosis is considered a universal reaction to injury in the CNS and is often used as a marker of neuronal damage (O'Callaghan and Sriram 2005). Biochemical markers of gliosis include the increased expression of glial fibrillary acidic protein (GFAP) and the ionized calcium-binding adaptor protein-1 (Iba1), which is indicative of astroglia and microglia activation, respectively. Notably, several studies have shown that AMPH-related compounds increase GFAP and Iba1 immunoreactivity in the STR, cortex, and SNpc; areas that are most affected by METH and AMPH-mediated neurotoxicity (Ares-Santos et al. 2013; Granado et al. 2011; Guilarte et al. 2003; Thomas et al. 2004). While the mechanism behind this response is

not well elucidated, DA quinones and glutamate receptor activation have also been shown to stimulate microglia activation (Kuhn et al. 2006).

Determining the Pharmacology of Novel Drugs

As monoamine transporters are symporter proteins, they express the electrogenic properties of ion channels (Alexander et al. 2011). Thus, molecules that interact with these proteins, including their endogenous substrates DA, NE, and 5-HT, induce transporter-mediated ionic currents that can be measured using voltage clamp techniques in cells stably expressing human monoamine transporters (Torres et al. 2003). Specifically, compounds that are translocated through transporters into neuronal cells along with sodium ions produce transporter-mediated inward (depolarizing) currents, while compounds that bind to transporters but are not translocated into cells produce outward currents (due to blockade of an endogenous leak current) (Alexander et al. 2011). Therefore, the action of a drug at monoamine transporter proteins can be determined by measuring these ionic currents in cells before and after drug exposure.

For novel drug compounds, the nature and selectivity of their interactions with monoamine transporter can be evaluated using *in vitro* radioligand uptake and release assays. In these assays, drugs are analyzed for their potencies and efficacies to (a) block the uptake of radio-labeled monoamines dispensed in the external solution (IC50 values) or (b) promote release of preloaded radiolabeled monoamine tracers (EC50 values) at specific monoamine transporters (Rothman and Baumann 2003). Pure reuptake inhibiting compounds block the uptake of radio-labeled monoamines without evoking full release of preloaded substrates. Monoamine releasing or substrate

compounds block the reuptake radiolabeled monoamines while also stimulating the full, efficacious release of preloaded substrates (Baumann et al. 2012; Rothman and Baumann 2003). Measured IC₅₀ and/or EC₅₀ values reflect the relative potency and selectivity of a given drug at DAT, NET, and SERT. However, given the complexity of biological matrices, *in vitro* analyses do not always accurately represent the *in vivo* effects of a drug (De Felice et al. 2014). Thus, in order to characterize the *in vivo* neurochemical profiles of a CNS-active drug, high performance liquid chromatography equipped with electrochemical detection (HPLC-ECD) assays are utilized to measure the impact of a given drug on endogenous monoamine levels in discrete brain tissue and cerebral spinal fluid.

The transporter selectivity profile and neurochemical effects of a given psychostimulant can be used to predict its behavioral effects and abuse potential (Baumann et al. 2014). For example, as the dopaminergic system is widely accepted to play a critical role in abuse-related effects, the potency and efficacy of a drug at DAT to enhance DA signaling, is often associated with its potential for abuse. Drug-induced elevations in mesolimbic DA are also correlated with elevations in locomotor activity responses in addition to rewarding properties. Furthermore, given that serotonin signaling can negatively regulate the release of DA, relative activity of a drug at SERT can also serve as an indicator of low abuse liability (Howell and Cunningham 2015; Howell and Kimmel 2008; Rothman and Baumann 2003).

Abuse Liability Testing for Novel Drugs

Abuse liability testing utilizes behavioral assays to investigate whether a novel drug has stimulatory, rewarding, and/or reinforcing properties that are characteristic of addictive drugs (Balster and Bigelow 2003; FDA 2016). The motor-stimulant effect of a drug is an indicator of abuse liability and is commonly assessed by monitoring for a drug's ability to induce locomotor activity and express behavioral sensitization. Additionally, a test drug may be assessed for its ability to cross-sensitize with known drugs of abuse, whereby animals repeatedly administered the novel drug display enhanced locomotor responses to a challenge dose of cocaine, METH, or MDMA. Cross-sensitization is thought to occur when two drugs have overlapping mechanisms that underlie the development of sensitization, despite having distinct transporter interactions (Steketee and Kalivas 2011). Direct abuse liability studies evaluate whether a drug has rewarding and/or reinforcing properties using the following behavioral paradigms: conditioned place preference (CPP), intracranial self-stimulation (ICSS), and self-administration (Ator and Griffiths 2003). In these assays, the degree of reward and/or reinforcement of a test drug corresponds to its efficacy and potency to produce drug side preference, facilitate low rates of self-stimulation, and induce self-administration in trained animals.

Another approach is to characterize the behavioral effects of a novel drug in comparison to those of a well-known drug of abuse (Balster and Bigelow 2003; FDA 2016). To this end, drug discrimination assays are used to determine if a test drug produces descriptive stimulus effects that are similar to those produced by established drugs of abuse (Ator and Griffiths 2003). In these tests, novel drugs are assessed for

their ability to produce appropriate responses (full substitution/generalization) in animals trained to discriminate a known drug of abuse (Berquist and Fantegrossi 2017). Moreover, compounds that can fully substitute for a specific drug of abuse are thought to have similar pharmacological activity, subjective effects, and addictive properties as the comparator drug (Balster and Bigelow 2003; Berquist and Fantegrossi 2017). In addition, specific behavioral responses elicited by a test drug (e.g. degree of motor activity and rate of self-administration) can be directly compared to those observed with known drugs of abuse. Thus, the abuse potential of a novel drug may also be defined in terms of the likeness or unlikeness of their behavioral responses relative to known drugs of abuse.

Neuropharmacology of the Synthetic Cathinones

Prior to the emergence of ‘bath salts’ abuse in 2010-2011, scientific literature regarding the pharmacology of synthetic cathinones was extremely limited. Thus, the growing prevalence of their abuse in recent years has prompted a number of preclinical investigations. Specific emphasis has been placed on elucidating the neurochemical, behavioral, and potential toxic effects of the three most commonly abused synthetic cathinones, MDPV, mephedrone, and methylone. Therefore, the terms ‘synthetic cathinone(s),’ ‘cathinone derivative(s),’ and ‘cathinone(s)’ used in the remainder of this dissertation refer to these three compounds unless otherwise noted.

Initial preclinical investigations of synthetic cathinones evaluated the *in vitro* mechanism of action and relative potency of these drugs at monoamine transporters (Baumann et al. 2012; Eshleman et al. 2013; Simmler et al. 2013). Given the novelty of

these compounds, many studies have included cocaine and various amphetamine derivatives as comparator compounds and describe the actions of the synthetic cathinones in regards to their likeness or unlikeness to these comparator drugs (Baumann et al. 2012; Baumann et al. 2013). Moreover, as discussed above, behavioral assays are also used to characterize the subjective stimulus and reinforcing properties of the synthetic cathinones in comparison to established drugs of abuse (Gregg and Rawls 2014). Therefore, the use of known comparator compounds enhances the translation of preclinical data on synthetic cathinones into clinically useful information.

MDPV

Initial preclinical investigations utilized various *in vitro* assays to determine the mechanism of action of MDPV at monoamine transporters. In these studies, MDPV displayed high affinity binding at DAT and NET, potently blocking the uptake of [3H]-DA and [3H]-NE, and evoking partial, low efficacy release of preloaded DAT and NET substrates, while producing only weak effects at SERT (100-fold greater potency at DAT and NET vs SERT) (Baumann et al. 2013; Eshleman et al. 2013). Additional *in vitro* studies reported no significant affinity of MDPV for non-transporter sites of action (Eshleman et al. 2013; Simmler et al. 2013). Thus, the action of MDPV to selectively block DAT and NET reuptake, without inducing the transporter-mediated release of DA and NE suggested that this compound was a cocaine-like, catecholamine reuptake inhibitor rather than a transporter substrate. Electrophysiology studies showing that MDPV produces outward, hyperpolarizing currents at hDAT, as seen with cocaine, confirmed this notion (Cameron et al. 2013a). However, direct comparisons with

cocaine revealed significant differences in regard to potency and selectivity for catecholamine transporters. Specifically, MDPV was 50-times more potent at the DAT and 10-times more potent at the NET than cocaine (Baumann et al. 2013). Taken together, these studies indicate that MDPV is potent and selective inhibitor of DAT and NET, and it does not significantly interact with SERT nor does it have non-transporter sites of action.

In vivo pharmacology of MDPV. In agreement with *in vitro* findings, MDPV produced significant dopaminergic effects *in vivo*. MDPV administration resulted in a dose-dependent increase in extracellular DA and not 5-HT in both rats (Baumann et al. 2013) and mice (Fantegrossi et al. 2013) in various brain regions as determined by HPLC-ECD, consistent with its selective inhibition of DA uptake. Moreover, MDPV-induced elevations in dialysate DA in the NAc were 10-fold greater than those found with cocaine and found to be positively correlated with the extent of locomotor activation (Aarde et al. 2013c; Baumann et al. 2013). Several studies have reported stimulation of locomotor activity and stereotypy following MDPV treatment (Aarde et al. 2013b; Fantegrossi et al. 2013; Glennon and Young 2016; Marusich et al. 2012), and some reported that MDPV-induced increases in ambulation and stereotypic movements were at least 10 fold greater than cocaine. Interestingly these locomotor effects and could be attenuated following pretreatment (Baumann et al. 2013; Marusich et al. 2012) with D1 receptor antagonists (Marusich et al. 2014). The psychostimulant effect of MDPV has been assessed across a range of doses (0.3 – 40 mg/kg), and the dose-response relationship for ambulatory activity is that of an inverted U-shape curve, with sensitization of ambulatory activity at lower doses (1-10 mg/kg) (Baumann et al. 2016;

Gregg and Rawls 2014) and significantly reduced ambulation at higher doses of MDPV (15-30 mg/kg) (Baumann et al. 2016; Fantegrossi et al. 2013). The high dose effect appears to be due to the manifestation of focused stereotyped behavior (Fantegrossi et al. 2013; Marusich et al. 2012). Overall, these data indicate that MDPV-mediated inhibition of DAT increases extracellular DA in mesolimbic brain regions, and that this enhanced DA neurotransmission is responsible for the stimulant effects of MDPV.

Abuse liability: MDPV. In drug discrimination studies, MDPV fully substituted for cocaine (Gatch et al. 2013), METH (Fantegrossi et al. 2013), and AMPH (Harvey et al. 2017), but only partially for MDMA (Harvey and Baker 2016), indicating that this compound produces subjective effects similar to established DA-selective drugs of abuse. Various studies have demonstrated that MDPV elicits conditioned place preference (Iversen et al. 2014; Karlsson et al. 2014; King et al. 2015), resulting in a greater preference score than amphetamine (Karlsson et al. 2014), lowers ICSS thresholds (Bonano et al. 2014), and is readily self-administered (Aarde et al. 2013b; Marusich et al. 2012; Schindler et al. 2016b). Based on these data, MDPV may have greater abuse potential than METH or cocaine.

Mephedrone and Methylone

In contrast to MDPV, mephedrone and methylone appear to be substrates at monoamine transporters. Several groups have reported that mephedrone and methylone inhibit the uptake of [3H]-DA and [3H]-NE and [3H]-5-HT (Baumann et al. 2012; Eshleman et al. 2013; Simmler et al. 2013) and stimulate full, efficacious release of preloaded substrates of DAT, NET, and SERT (Baumann et al. 2012; Eshleman et al.

2013), with a potency rank of NET>DAT>SERT for mephedrone and NET>SERT>DAT for methylone. Notably, methylone was found to be half as potent as mephedrone at all transporters (Baumann et al. 2012). These findings indicate that mephedrone and methylone induce non-selective substrate activity at all plasmalemma monoamine transporters, similar to MDMA and AMPH (Cadet et al. 2007; Kalant 2001). Definitive confirmation of their substrate activity came from studies demonstrating that mephedrone and methylone produced inward, depolarizing currents at hDAT that are indicative of a transportable substrate similar to those observed with the amphetamines (Cameron et al. 2013a; Solis 2016). In comparison studies, mephedrone's values for binding affinity, inhibition of uptake, and drug-induced release of preloaded substrates at DAT and SERT were similar to METH and MDMA values, respectively (Baumann et al. 2012; Simmler et al. 2013), while methylone displayed weaker DA effects than METH but promoted 5-HT release similar to MDMA (Baumann et al. 2012). One point of divergence from METH and MDMA was that both substrate cathinones were less potent at inhibiting uptake and inducing release of radio-labeled substrates via VMAT2 (Eshleman et al. 2013). Overall, these findings show that mephedrone and methylone are substrates for all monoamine transporters with non-selective releasing actions that are similar to MDMA and the amphetamines.

In vivo pharmacology of mephedrone and methylone. The *in vivo* neurochemical profiles of mephedrone and methylone are consistent with *in vitro* findings. Through microdialysis studies, investigators have determined that mephedrone and methylone produced dose-related concurrent increases in extracellular DA and 5-HT levels in the NAc (Aarde et al. 2013a; Baumann et al. 2012; Kehr et al. 2011; Schindler et al. 2016b),

and mephedrone was more potent than methylone (Baumann et al. 2012; López-Arnau et al. 2012). Consistent with the neurochemical effects of MDMA, the majority of HPLC analyses with mephedrone and methylone have found that the 5-HT increases are greater in magnitude than the accompanying DA increases (Baumann et al. 2012; Kehr et al. 2011; López-Arnau et al. 2012). However, while the ratio of DA: 5-HT release mimics that of MDMA, the substrate cathinones appear to have greater dopaminergic effects with potencies resembling AMPH (Kehr et al. 2011). For example, mephedrone increased dialysate DA in the NAc to levels that were significantly greater than those produced by MDMA and comparable to amphetamine DA levels (Hadlock et al. 2011; Kehr et al. 2011). A few studies have reported that mephedrone and methylone produce dose-dependent increases in locomotor activity (Baumann et al. 2012; Lisek et al. 2012; Shortall et al. 2013), with mephedrone being the more potent compound (Baumann et al. 2012; Gregg and Rawls 2014), further implicating enhanced DA neurotransmission as an effect of these drugs. Like MDPV, sensitization to high doses of mephedrone (15-30 mg/kg) is expressed as increased stereotypy (Gregg et al. 2013a), while this effect was not observed with methylone. Moreover, mephedrone-induced hyperactivity is attributed to increased striatal levels of DA and 5-HT and appears to be regulated by DA receptor signaling (Lisek et al. 2012), given that it was attenuated by D1R antagonists and enhanced by D2R antagonists. Additionally, at least one group has shown that mephedrone pretreatment cross-sensitizes with motor-stimulant effect of cocaine (Gregg et al. 2013b). However, the psychostimulant effects of mephedrone and methylone are more rapid in onset, shorter in duration, and considerably weaker than those of cocaine, MDPV, or METH (Gatch et al. 2013; Green et al. 2014; Marusich et al.

2012; Nguyen et al. 2016). Given the significant serotonergic effects of the substrate cathinones, this is most likely due to 5-HT modulation of dopaminergic effects.

Abuse liability: mephedrone and methylone. In drug discrimination studies, mephedrone and methylone fully substituted for MDMA, METH, and cocaine (Bonano et al. 2014; Gatch et al. 2013; Gregg and Rawls 2014), suggesting that these compounds produce similar subjective effects as these popular drugs of abuse and thus pose a high potential for abuse. Mephedrone and methylone induced conditioned place preference and lowered ICSS thresholds, with an ordered potency for both assays reported as MDPV > mephedrone > methylone (Bonano et al. 2014; Gregg and Rawls 2014; Karlsson et al. 2014). Moreover, mephedrone and methylone are self-administered by rodents across multiple doses (Aarde et al. 2013a; Creehan et al. 2015); however, self-administration of these substrate cathinones took longer to develop than MDPV self-administration (Schindler et al. 2016b). Overall, mephedrone and methylone produce rewarding and reinforcing effects in animals that clearly indicate their abuse potential in humans.

Neurotoxicity of Substrate Cathinones

AMPH and METH have been established to induce neurotoxic damage to DA neurons in rats and mice (Yamamoto et al. 2010). Given the close structural similarities and mechanistic overlap of mephedrone and methylone with METH, preclinical research hypothesized that these compounds may also be neurotoxic to DA nerve endings. *In vitro* cytotoxicity assays supported this notion, demonstrating that mephedrone increases the release of lactate dehydrogenase (associated with disruption of cellular

membranes) (den Hollander et al. 2014), decreases mitochondrial respiration (den Hollander et al. 2015), and dose-dependently decreases cell viability superior to that of MDMA in neuroblastoma cell lines and primary cultures of cortical neurons (Martinez-Clemente et al. 2014). However, a number of studies have established that neither mephedrone nor methylone induced blatant toxicity to DA nerve endings (Angoa-Perez et al. 2012; Angoa-Pérez et al. 2016), as indicated by a lack of persistent decreases in DA, TH expression, or DAT protein levels. Additionally, no signs of microglial or astroglial activation were observed in the striatum 2 or 7 days following binge dosing of either mephedrone or methylone (Angoa-Perez et al. 2012; Anneken et al. 2015). While one group has reported prolonged decreases in TH and DAT expression in the frontal cortex and reduced striatal D2R with repeated mephedrone treatment, doses used in this study were extremely high (50 mg/kg), and repeated daily doses of this magnitude would likely result in cardiovascular toxicity that would outweigh neurotoxic effects (Lopez-Arnau et al. 2015). Moreover, while a few studies have reported mild serotonergic toxicity with the substrate cathinones when administered at elevated ambient temperatures (López-Arnau et al. 2014; Martinez-Clemente et al. 2014), follow-up studies have been conflicting with many groups unable to reproduce this toxicity (Angoa-Pérez et al. 2014; Miner et al. 2017).

Significant Combined Effects

Interestingly, while mephedrone or methylone do not appear to induce DA toxicity on their own, they exacerbate the neurotoxic effects of METH and MDMA. When co-administered with neurotoxic doses of METH or MDMA, mephedrone and methylone significantly decreased striatal DA, DAT, and TH levels and increased GFAP expression

beyond the effects of METH or MDMA alone (Angoa-Pérez et al. 2013; Anneken et al. 2015). In contrast, MDPV provided complete protection against the toxic effects of METH on DA nerve ending markers and GFAP, most likely via its potent and competitive blockade of DAT-mediated transport (Anneken et al. 2015). While this neuroprotective effect could be predicted to extend to other DAT substrate drugs, this does not appear to be the case. Recent studies have reported that self-administration of binary mixtures of MDPV and methylone resulted in a high incident of lethality (50%) (Gannon et al. 2018), which was not observed with self-administration of either drug alone at identical doses. While the pharmacology underlying this combined toxicity has not been thoroughly investigated, drug-drug interactions at the DAT may be a contributing factor. A study utilizing *in vitro* electrophysiology techniques found that when MDPV and mephedrone were simultaneously applied to the DAT, the substrate activity of mephedrone (inward current) occurred more quickly than the blocking action of MDPV (outward current) (Cameron et al. 2013b). This suggests that co-administration of MDPV and mephedrone would first produce DA release via mephedrone and that DA reuptake would be subsequently prevented due to DAT blockade via MDPV. Another study demonstrated locomotor sensitization to mixtures of MDPV and mephedrone and found that rats pretreated with MDPV and mephedrone displayed cross-sensitization to the locomotor stimulant effects of cocaine, with activity counts greater than controls and the saline-cocaine group (Berquist et al. 2016). Additionally, prior exposure to mephedrone increased the stimulant effects of cocaine (Gregg et al. 2013b). Given the involvement of midbrain dopaminergic circuits in the

development of sensitization, these studies provide further evidence for augmented DA neurotransmission by drug combinations containing synthetic cathinones.

Summary and Rationale for the Present Study

The synthetic cathinones, MDPV, mephedrone, and methyldone gained worldwide notoriety as the psychoactive components of 'bath salts,' a marketing term used to circumvent federal drug laws and permit their legal sale. To date, the majority of preclinical investigations on MDPV, mephedrone, and methyldone have primarily focused on the individual pharmacology and abuse-related effects of these drugs. However, DEA statistics and forensic reports indicate that bath salt products generally contain various mixtures of synthetic cathinones (DEA 2011; Spiller et al. 2011). Moreover, a retrospective search of records involving synthetic cathinones at poison control centers revealed the most frequently abused bath salt 'brands,' identified by patient history reports, contained binary and ternary mixtures of MDPV, mephedrone, and methyldone (Spiller et al. 2011; Warrick et al. 2013). Therefore, the primary aim of this dissertation was to address this limitation and provide acuity into the pharmacological interactions that may occur when these compounds are abused in combination. Our central hypothesis was that pharmacological interactions at the DAT following co-exposure to MDPV, mephedrone, and methyldone will produce significant short- and long-term effects on dopaminergic tone. This hypothesis was tested in Chapters 3 and 4, which utilized a novel HPLC-ECD assay developed in our laboratory for sensitive detection of monoamines and respective metabolites in the small DA nuclei (VTA and SNpc), in addition to the larger nerve terminal areas (NAc and STR). The development and validation of this method is discussed in Chapter 2.

The individual pharmacological mechanisms of MDPV, mephedrone, and methylone have been largely determined and suggest opportunities for significant pharmacological interactions at the DAT. Similar to cocaine, MDPV acts as a highly selective reuptake inhibitor at DAT and promotes dose-dependent increases in extracellular DA levels (Baumann et al. 2013; Coppola and Mondola 2012a). Mephedrone and methylone possess similar pharmacology to METH and MDMA, acting as non-selective substrates at the DAT, inducing the transporter-mediated release of DA (Baumann et al. 2012; Simmler et al. 2013). Investigations using *in-vivo* microdialysis have indicated that these substrate-type synthetic cathinones precipitate rapid and marked elevations in dialysate DA levels that are greater than MDMA and comparable to those observed with METH (Kehr et al. 2011). Given that synergistic drug-drug interactions are often more profound when the drugs administered produce qualitatively similar effects via distinct pharmacological mechanisms (Tallarida 2011), the dichotomy of DAT interactions by MDPV (blocker) and the substrate cathinones to increase extracellular DA levels provides an avenue for accentuated dopaminergic effects when these drugs are co-abused.

Chapter 3 of this dissertation has explored the acute effect of co-synthetic cathinone exposure on DA neurotransmission in mice. This was accomplished using our novel and validated HPLC-ECD assay (Allen et al. 2017) to measure DA and DA metabolite levels in discrete brain regions of mice treated with MDPV, mephedrone, and methylone alone and in combination. In addition, locomotor activity was assessed in mice treated with the synthetic cathinones individually and in combination to determine if any combined effects extended to DA-mediated behavioral processes. We

hypothesized that the co-administration of MDPV, mephedrone, and methylone would enhance the dopaminergic effects of the individual drugs. From this study, we found a significant combined effect on both DA levels and locomotor activity that appeared to be a result of synergistic pharmacological activity. Moreover, this study was the first to report an *in vivo* synergistic effect with ternary synthetic cathinone mixtures and emphasized the importance of using drug mixtures to model bath salts in abuse in behavioral and neurotoxicity studies.

While interactions between the synthetic cathinones themselves are not well documented, evidence of a combined pharmacological effect between the substrate cathinones and neurotoxic DAT-targeting drugs has been reported (Angoa-Pérez et al. 2016). Despite structural and mechanistic similarities to well-known agents of neurotoxicity, neither mephedrone nor methylone have been shown damage DA nerve endings; however, both compounds exert the deleterious effect of enhancing METH and AMPH toxicity when co-administered with these drugs (Angoa-Perez et al. 2012; Anneken et al. 2015). Given the involvement of elevated DA levels in METH-induced neurotoxicity via degradation to reactive metabolites and enhancement of glutamate-mediated excitotoxicity, the authors of these papers proposed that the substrate cathinones exacerbated METH toxicity by increasing DA to levels that were greater than those achieved by each drug alone (Anneken et al. 2015). As noted above, in Chapter 3, we present our data which demonstrates enhanced dopaminergic activity with co-administration of MDPV, mephedrone, and methylone; thus we hypothesized that the combination of these cathinones may promote damage to DA nerve endings. Therefore, in Chapter 4 of this dissertation, we present data from studies aimed to determine if

chronic co-synthetic cathinone exposure produced significant effects on dopamine toxicity. This was accomplished using HPLC and immunoblot assays to assess the levels of DA, TH, DAT, and GFAP in mesolimbic and nigrostriatal brain tissue, and immunostaining for stereological analysis of DA neurons in the SNpc of mice chronically treated with the cathinone cocktail. We hypothesized that pharmacological interactions between MDPV, mephedrone, and methyldone following repeated co-exposure would significantly alter the toxic properties of these drugs in dopaminergic brain regions. Results from this study showed a unique combined effect that was described as a 'functional lesion,' whereby repeated co-synthetic cathinone exposure downregulated DA, TH and DAT and other key players of the dopaminergic synapse without inducing blatant reductions in neuronal numbers.

Taken together, these studies have shown that the composition of bath salts mixtures significantly influences the dopaminergic and toxic effects of these drugs. These interactions may contribute to the deleterious effects reported by users. Thus, a deepened knowledge of possible pharmacological interactions resulting from bath salts abuse will provide a platform for improved overdose and addiction treatment regimens.

CHAPTER 2

A SIMPLE AND SENSITIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY- ELECTROCHEMICAL DETECTION ASSAY FOR THE QUANTITATIVE DETERMINATION OF MONOAMINES AND RESPECTIVE METABOLITES IN SIX DISCRETE BRAIN REGIONS OF MICE

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First published on May 5, 2017,

Biomedical Chromatography, Volume 31, Issue 11. e39998.

<https://doi.org/10.1002/bmc.3998>

Keywords: monoamine, biogenic amine, HPLC-ECD, brain tissue, neurotransmitter

Abstract

A rapid, sensitive, and reproducible assay is described for the quantitative determination of the monoamine neurotransmitters dopamine, norepinephrine, and serotonin, their metabolites, and the internal standard 3,4- dihydroxybenzylamine hydro-bromide (DHBA) in mouse brain homogenate using high performance liquid chromatography with electrochemical detection. The method was validated in the following brain areas: frontal cortex, striatum, nucleus accumbens, hippocampus, substantia nigra pars compacta, and ventral tegmental area. Biogenic amines and relevant metabolites were extracted from discrete brain regions using a simple protein precipitation procedure, and the chromatography was achieved using a C18 column. The method was accurate over the linear range of 0.300 – 30 ng/mL ($r = 0.999$) for dopamine and 0.300 – 15 ng/mL ($r = 0.999$) for norepinephrine, 3,4-dihydroxyphenylacetic acid, homovanillic acid, and 5-hydroxyindolacetic acid, with detection limits of approximately 0.125 ng/mL (5 pg on column) for each of these analytes. Accuracy and linearity for serotonin was observed throughout the concentration range of 0.625 – 30 ng/mL ($r = 0.998$) with an analytical detection limit of approximately 0.300 ng/mL (12 pg on column). Relative recoveries for all analytes were approximately $\geq 90\%$ and the analytical run time was less than 10 min. The described method utilized minimal sample preparation procedures and was optimized to provide the sensitivity limits required for simultaneous monoamine and metabolite analysis in small, discrete brain tissue samples.

1. Introduction

Biogenic amines (e.g. dopamine, norepinephrine, and serotonin (5-hydroxytryptamine)) serve as major neurotransmitters in the central nervous system (CNS) and function to mediate various neurophysiological processes, including motivation, reward/ addiction, movement, attention, cognition, wakefulness, mood, hunger, temperature regulation, and hormone release (Adinoff, 2004; Carlsson, 1987; Commissiong, 1985; Cox, 1977; Jouvett, 1972; Leibowitz, 1986; Vitale & Chiochio, 1993). Disruption of dopamine (DA), norepinephrine (NE), and serotonin (5-HT) homeostasis is associated with many neuropsychiatric disorders including attention deficit hyperactivity disorder (ADHD), schizophrenia, anxiety, depression, and Parkinson's disease (Delgado, 2000; Huot, Fox, & Brotchie, 2015; Rubia, 2002). Thus, given the multitude of physiological processes effectuated by these neurotransmitters and the various neurological conditions associated with their dysregulation, a large number of pharmacological agents influence the synthesis, storage, release, uptake, and metabolism of biogenic amines. While DA, NE, and 5-HT play essential roles as signaling molecules, each regulating a distinct set of CNS functions, they all share the same major mechanism of inactivation. Of note, all of the monoaminergic neurotransmitters are regulated in a similar manner, as they are stored in vesicles via the vesicular monoamine transporter (VMAT), released from synaptic vesicles into the synaptic cleft, and their action is primarily terminated via reuptake into the presynaptic neuron. Each monoamine has a specific transporter that allows for its reuptake; for DA, it is the dopamine transporter (DAT), for NE, the norepinephrine transporter (NET), and for 5-HT, the serotonin transporter (SERT); however, given structural similarities of

these transporters, many pharmacological agents that target one of these transporters have at least some affinity for the other transporters. Additionally, all of the biogenic amines (DA, NE, and 5-HT) are metabolized by the monoamine oxidase enzyme, and the catecholamines (DA and NE) are metabolized by catechol-O-methyl transferase (COMT). Given the large number of pharmaceutical agents and drugs of abuse that affect monoaminergic systems and their tendency to affect more than one of the monoamines, there is a need for accurate methods that allow for fast, sensitive, and simultaneous determination of monoamines and their respective metabolite concentrations within specific brain regions known to send or receive monoaminergic input.

Numerous analytical methods employing high performance liquid chromatography (HPLC) coupled to a variety of detectors including ultra-violet (UV), fluorescence, electrochemical (ECD), and mass spectrometry (MS), have been developed to determine monoamine neurotransmitters and relevant metabolite concentrations in biological samples. HPLC-ECD remains the most commonly used system for these analyses due to its higher sensitivity compared to other detection methods (Tsunoda, 2006). Although fast (< 9 min), some previously developed HPLC-ECD assays utilized for the detection and quantification of DA, NE, 5-HT, and their metabolites in brain tissue are restricted by their limited sensitivity in this matrix; the reported lower limits of quantification (LLOQ) for all analytes of interest are insufficient for analysis of monoamines and their metabolites in discrete brain regions, in particular within two major dopaminergic nuclei, the substantia nigra pars compacta (SNpc) and ventral tegmental area (VTA) (Bicker, Fortuna, Alves, & Falcão, 2013; Farthing,

Halquist, & Sweet, 2015; Parrot, Neuzeret, & Denoroy, 2011). A recent HPLC-ECD methodology has reported quantification limits adequate for discrete brain region analysis of DA, DOPAC, and 5-HIAA levels, however, determination of DA turnover ($[\text{DOPAC}+\text{HVA}]/[\text{DA}]$) within the SNpc and VTA regions would be problematic given the reported calibration range and LLOQ for HVA (approx. 2.8 ng/mL after 5 μL sample volume injection) (A. T. Nguyen, Aerts, Van Dam, & De Deyn, 2010). Moreover, methods which report very sensitive limits of detection (fmol-pmol range) are often found accompanied by the use of damaging high electrode potentials (> 700 mV) (Bidel et al., 2016; Parrot et al., 2011; Vaarmann, Kask, & Mäeorg, 2002), the inability to measure all monoamines and metabolites simultaneously (Heidbreder et al., 2001; Hubbard et al., 2010), or long analytical run times (> 25 min) (Kumarathasan & Vincent, 2003; A. T. Nguyen et al., 2010; Unceta et al., 2001; Van Dam, Vermeiren, Aerts, & De Deyn, 2014). Shorter length columns and ultra-high pressure liquid chromatography (UHPLC) systems are commonly employed to decrease analysis time (Farthing et al., 2015; Parrot et al., 2011; Reinhoud, Brouwer, van Heerwaarden, & Korte-Bouws, 2013), however it has been reported that chromatographic efficiency in complex matrices, particularly during isocratic elution using electrochemical detection, is often reduced as column length decreases (Bicker et al., 2013; Mutton, 1998; D. T. Nguyen, Guillarme, Rudaz, & Veuthey, 2006). Moreover, while HPLC –ECD methods allowing for rapid, simultaneous, and sensitive detection of monoamine and metabolites have been developed and validated for use in brain dialysates using classical HPLC (Duine, Floch, Cann-Moisan, Mialon, & Caroff, 1998; Ferry, Gifu, Sandu, Denoroy, & Parrot, 2014; Reinhoud et al., 2013; Sarre et al., 1992), high background noise due to matrix effects

and loss of resolution is often observed when applied to brain tissue samples. Thus, very few methods exist for the rapid and sensitive measurement of monoamine and metabolites within distinct brain regions. Of these previously developed HPLC-ECD methodologies, measurements were achieved in larger brain regions, including the frontal cortex (FCtx), hippocampus (HIP), striatum (STR), and nucleus accumbens (NAc) but were not assessed in smaller regions, such as the dopaminergic SNpc and VTA, and LLOQs (S/N ratio ≥ 10) were not reported for these assays. In addition, these methodologies require ultrafiltration of prepared samples prior to analysis and are thus susceptible to analyte recovery issues (Bidel et al., 2016; Donzanti & Yamamoto, 1988; Saito, Murai, Abe, Masuda, & Itoh, 1992).

Therefore, to address the stated limitations, this article reports the development of a simple and reproducible HPLC-ECD assay for rapid, sensitive, and simultaneous determination of DA and its metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), 5-HT and its metabolite 5-hydroxyindolacetic acid (5-HIAA), and NE concentrations in the following mouse brain regions: FCtx, HIP, STR, NAc, SNpc, and VTA. The proposed methodology has several advantages over earlier methods including a simple sample preparation procedure that does not require ultrafiltration of prepared extracts and highly sensitive limits of detection for all six endogenous compounds that were achieved within an analytical run time of about 9 min without the use of a UHPLC system.

2. Experimental

2.1 Chemicals and Reagents

Analytical grade DA, DOPAC, HVA, NE, 5-HIAA, and the internal standard, 3,4-dihydroxybenzylamine hydro-bromide (DHBA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). For preparation of the mobile phase, certified ACS reagent grade sodium acetate, citric acid (anhydrous), ethylenediamine tetraacetic acid, disodium salt (EDTA), glacial acetic acid, and HPLC grade acetonitrile, methanol and polished water were purchased from Thermo Fisher Scientific (Fair Lawn, NJ, USA). The ion pairing agent, sodium octyl sulfate (SOS), was purchased from Acros organics (Geel, Belgium) while 2.0 N perchloric acid was purchased from RICCA chemical (Arlington, TX, USA).

2.2 Apparatus and Operating Conditions

The chromatography was achieved using C₁₈ MD- 150 mm x 3.2 mm, 3 µm column (Thermo Fisher Scientific). The mobile phase, which consisted of 100 mM sodium acetate, 20 mM citric acid, 0.38 mM SOS, 0.15 mM EDTA dissolved in 950 mL polished water and 50 mL acetonitrile, was adjusted to pH 3.3 using glacial acetic acid and filtered through a 0.22 µm filter (Supelco, Bellefonte, PA, USA). Mobile phase was delivered to the HPLC system at a flow rate of 0.6 mL/min using an ESA Model 584 pump (ESA Laboratories, Inc., Chelmsford, MA, USA) and prominence degasser (model DGU-20A5). Electrochemical detection was achieved using a Coulochem® III detector (ESA Laboratories, Inc.) equipped with 5100A dual electrode. Working potentials of -220 mV and +375 mV were set E1 and E2, respectively. Prepared samples were injected into the system in 40 µL aliquots using an ESA model 542 autosampler and all

analytes were collected in E2 in isocratic elution mode. Data were collected on-line and exported to an Agilent EZChrome Elite software system (Santa Clara, CA, USA) for peak area integration and analysis.

2.3 Tissue Collection and Extraction Procedure

Brain tissue used for method validation was obtained from 54 day-old male Swiss-Webster mice. Mice were sacrificed via decapitation and brains were collected and placed in ice cold saline for five minutes. After cooling, microdissections were performed using a mouse brain matrix (30 g Coronal, ASI Instruments, Warren, MI, USA). Brain sections containing the FCtx, NAc, STR, HIP, and SNpc were collected, halved, and corresponding brain regions were dissected and weighed from both left and right halves. The VTA was also collected and weighed from relevant brain slices. Extraction of monoamines and respective metabolites was performed via optimization of a previously described protein precipitation method (Wei et al., 2014). Brain tissue samples were homogenized on ice in five-fold excess volume of methanol for FCtx, NAc, STR, and HIP regions [volume of MeOH added (μL) = brain weight (mg) \times 5], 20 μL methanol for SNpc and VTA samples, and homogenates were centrifuged in 4 °C at 14,000 X g for 20 mins. After, either 40 μL (FCtx, NAc, STR, and HIP) or 10 μL of the supernatants (SNpc and VTA) were transferred to 1.7 mL microcentrifuge tubes containing 10 μL of internal standard solution (DHBA, 500 ng/mL prepared in methanol) and 10 μL polished water. Samples were then vortexed for five minutes and centrifuged at 3,000 X g for 10 minutes. Supernatants were quantitatively transferred to fresh 5 ml glass vials and evaporated to dryness under nitrogen gas at room temperature for approximately 15-20 minutes. Dried samples were reconstituted in 1000 μl (NAc, STR,

and HIP) or 500 μ l (FCtx, SNpc and VTA) of 0.2 N perchloric acid, sonicated for 20 minutes, and stored at -70°C.

2.4 Standard Solutions and Calibration

Standard stock solutions (1.000 mg/mL) of DA, DOPAC, HVA, NE, 5-HT, 5-HIAA, and the internal standard (IS) DHBA were prepared separately by dissolving the appropriate amount of the chemical reference in methanol and stored at -70 °C. On the day of analysis, standard stock solutions were diluted with methanol to achieve a final concentration of 1.000 μ g/mL for each analyte. Working standard solutions were freshly prepared by further diluting the 1.000 μ g/mL stock solutions of DA DOPAC, HVA, NE, 5-HT and 5-HIAA with 0.2 N perchloric acid to obtain final concentrations of 0.300, 0.625, 1.250, 2.500, 5.000, 10.000, 15.000, and 30.000 ng/mL for all analytes. DHBA was spiked into each working standard solution to achieve a final concentration of 5.000 ng/mL (NAc, STR, and HIP calibrations) or 10.000 ng/mL (FCtx, SNpc, and VTA calibrations), to compensate for variability associated with analyte extraction and sample injection. For quantitative analyses, standard curves were generated for each analyte by plotting the peak area ratio (analyte peak area / DHBA (IS) peak area) versus concentration. The least-squares linear regression method was utilized to calculate regression equations.

2.5 Linearity, Limit of Detection, and Limit of Quantification

Linearity and analytical limits of the method were evaluated over a wide range of concentrations. Using individual stock solutions, calibration solutions containing DA, DOPAC, HVA, NE, 5-HT, and 5-HIAA were prepared in 0.2 N perchloric acid at 10

concentration levels (range 0.125 ng/mL to 40 ng/mL). Calibration curves were generated via the internal standardization method and regression equations were calculated using the least-squares linear regression method. The limit of detection (LOD) was determined as the analyte concentration which gave a peak area corresponding to a signal-to-noise (s/n) ratio of 3, while the lower limit of quantification (LLOQ) was calculated as the analyte concentration that provided a peak area with an s/n ratio of 10.

2.6 Precision, Accuracy, and Recovery

Instrumental precision (percentage of the relative standard deviation, % RSD) of the HPLC-ECD system was determined in both nonmatrix (standard solutions in 0.2 N perchloric acid) and matrix (brain tissue samples) conditions. Accuracy (error percentage of the determined concentration relative to the nominal concentration, % Error) of the method was calculated from the analysis of tissue samples fortified with known quantities of standard. In non-matrix conditions, intra- and Inter- day repeatability was assessed over three days at the following calibration points: 0.300, 0.625, 1.250, 2.500, 5.000, 10.000, 15.000, and 30.000 ng/mL, using four replicates per concentration per day (n=12 at each concentration level, see Table 2.2). For method validation in matrix conditions, brain tissue homogenates were divided into two aliquots. The first was treated as a 'blank' while the second was spiked with a lower (0.800 ng/mL), medium (2.000 ng/mL and 5.000 ng/mL) and higher (10.000 ng/mL) concentration of DA, DOPAC HVA, NE, 5-HT, and 5-HIAA prior to extraction. The blank aliquot was subtracted from spiked samples to normalize for endogenous levels. Intra- and Inter-day precision and accuracy were assessed in brain matrix over three days at each

concentration level, using three replicates per concentration per day (n=9 at each concentration level, see Table 2.3). For both nonmatrix and matrix analyses, five point internal standard calibration curves were generated for DA, DOPAC, HVA, NE, 5-HT, 5-HIAA on the day of analysis. Peak area ratios were utilized for computations and analyte concentrations were calculated using interpolation of their respective standard curves. Precision was calculated at each calibration level as $\%RSD = [(A_{pa} / SD) \times 100]$, where A_{pa} represents the average peak area ratio measured by the HPLC system and SD represents the standard deviation. Accuracy was calculated at each calibration level as $\% Error = [(|C_a - C_n| / C_n) \times 100]$, where C_a = average concentration of the analyte as determined by the HPLC-ECD system and C_n = nominal concentration. For matrix conditions, A_{pa} and C_a values were determined by subtracting analyte peak area ratios and concentration values measured in 'blank' homogenate samples from those measured in their respective spiked homogenate samples.

3. Results and Discussion

3.1 Method Development

The major analytical difficulty was to determine chromatographic conditions which provided the desired sensitivity to simultaneously detect monoamines and their respective metabolites within small, discrete brain tissue samples. The complex matrix and varied chemistry between the monoamines and their respective metabolites also contributed to the challenge of optimizing chromatographic conditions. Many HPLC-ECD methods utilized for monoamine and metabolite analysis in brain tissue report LLOQs of 5 ng/mL and higher (Farthing et al., 2015; Karimi, Carl, Loftin, & Perlmutter,

2006), however a more sensitive range is required for accurate analysis in smaller brain regions such as the SNpc and VTA (0.2 – 1.5 mg tissue weights). Initially, the commercially available MD-TM mobile phase (Thermo Fisher) was utilized; however, sensitivity for discrete tissue analysis was insufficient. The final mobile phase composition was optimized from a method used for the quantitative measurement of monoamines and corresponding metabolites in rat brain dialysates (Duine et al., 1998). Sufficient separation of basic and acidic analytes was achieved by modifying both the pH (3.0-3.6) and the concentration the ion-pairing agent, SOS (0.33-0.45 mM). These optimization parameters have also been considered in previous analytical HPLC methodologies to improve both separation and sensitivity of analytes (Brodnik & Jaskiw, 2015; A. T. Nguyen et al., 2010). Increasing the pH decreased retention times of the acid metabolites; however, this resulted in an overlap of DA and 5-HIAA retention times. Moreover, increasing SOS concentration increased retention times for monoamines and also resulted in DA and 5-HIAA peak overlap, while lower SOS concentrations decreased the retention time of biogenic amines and resulted in DA and DHBA overlap. Ultimately, the best separation of all analytes was achieved at pH 3.3 and an SOS concentration of 0.38 mM (Fig. 1A). Utilization of a 3.2 mm I.D., 3 μ m particle containing column as compared to conventional HPLC methods (4.6 mm I.D., 5 μ m), allowed chromatographic separation and quantification of all analytes to occur more rapidly, resulting in an analytical run time of less than 10 min while also maintaining maximum system pressure below 170 bar.

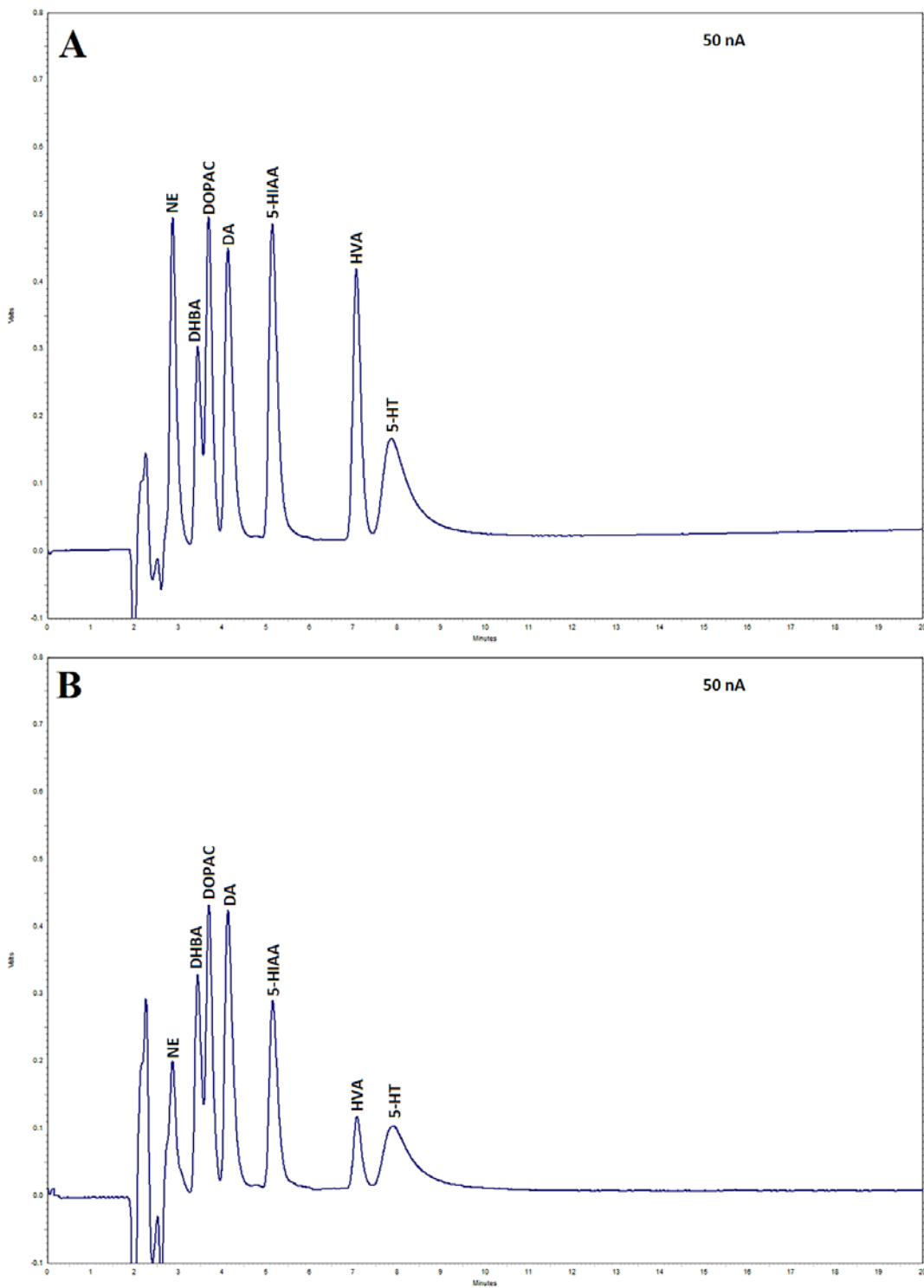


Fig. 2.1. Representative chromatograms showing separation of biogenic amines (NE, DA, and 5-HT), their respective metabolites (DOPAC, HVA, and 5-HIAA), and the internal standard DHBA in standard solution at a concentration of 2.5 ng/mL (A) and in brain homogenate (B).

3.2 Linearity and Limits of Detection and Quantification

Data for linearity assays is summarized in Table 2.1. The calibration curve was linear for DA and 5-HT throughout the concentration range of 0.300 – 30.000 ng/mL and 0.625 - 30.000 ng/mL, respectively, with a mean correlation coefficient of ≥ 0.999 (normal linear regression, n=10 runs). The calibration curve ranging from 0.300 – 15.000 ng/mL was linear for NE, DOPAC, HVA, and 5-HIAA (mean correlation coefficient of ≥ 0.998). The analytical limits of detection and quantification for DA, DOPAC, HVA, 5-HIAA, and NE were approximately 0.125 ng/mL (5 pg on column) and 0.300 ng/mL (12 pg on column), respectively. While LOD and LLOQ values for 5-HT were approximately 0.300 ng/mL (12 pg on column) and 0.625 ng/mL (25 pg on column), respectively. The stated LOD and LLOQ values were suitable for the quantitative determination of endogenous monoamine and metabolite levels in the VTA and in halved microdissections of the FCtx, STR, NAc, HIP, and SNpc (Fig. 2.2, A-F).

Table 2.1. Linearity results of the HPLC-ECD method for the quantification of monoamines (DA, NE, 5-HT) and various metabolites (DOPAC, HVA, 5-HIAA) in standard solution

	DA	DOPAC	HVA	NE	5-HT	5-HIAA
Calibration range (ng/mL)	0.300 – 30.000	0.300 – 15.000	0.300 – 15.000	0.300 – 15.000	0.625 – 30.000	0.300 – 15.000
Correlation coefficient (r^2)	0.999	0.998	0.999	0.999	0.998	0.998
Limit of detection (ng/mL)	0.125	0.125	0.125	0.125	0.300	0.125
Limit of quantification (ng/mL)	0.300	0.300	0.300	0.300	0.625	0.300

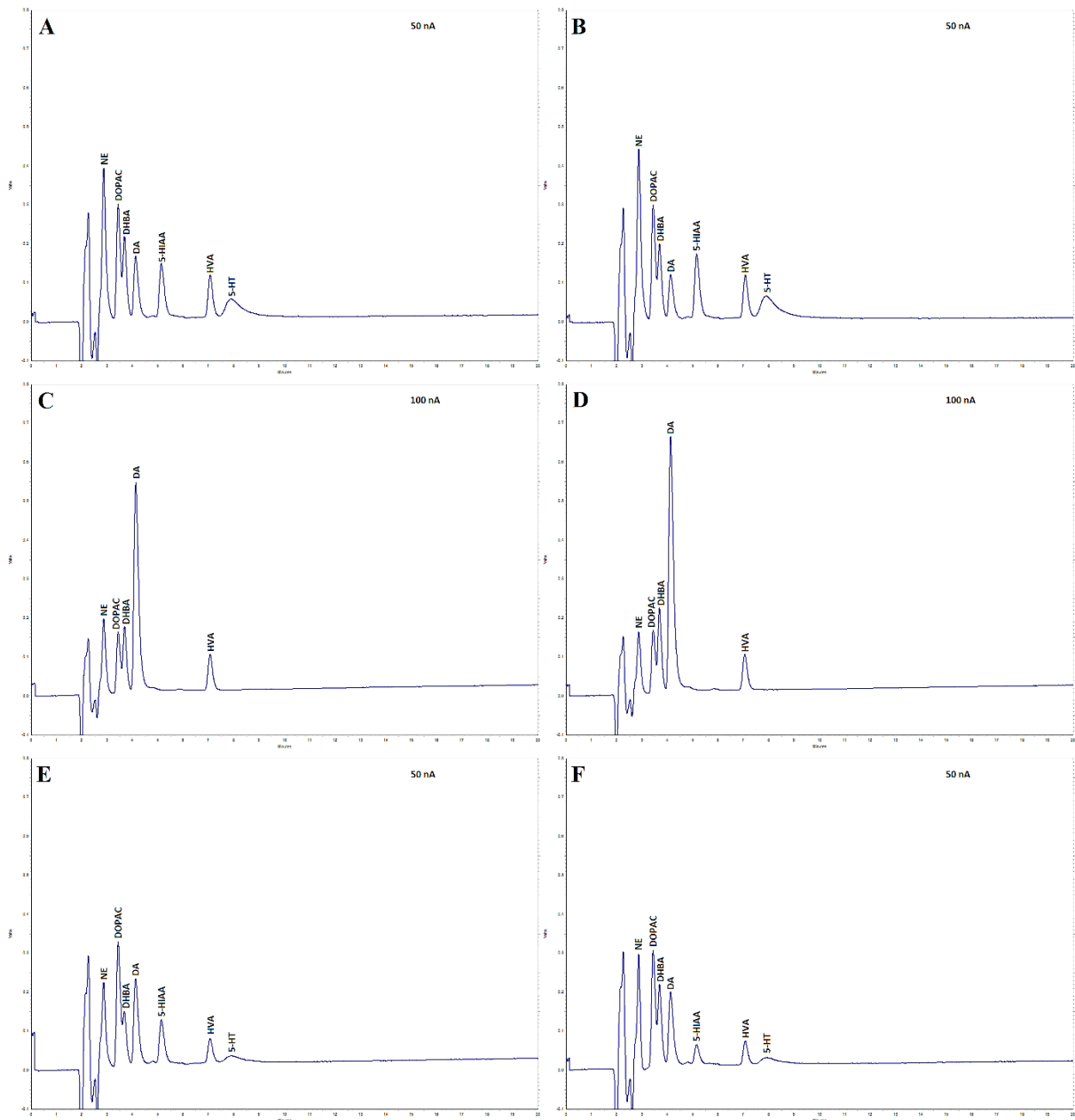


Fig. 2.2. Representative chromatograms demonstrating detection and separation of DA, DOPAC, HVA, NE, 5-HT, and 5-HIAA in the following brain regions (A) Frontal cortex; (B) Hippocampus; (C) Striatum; (D) Nucleus accumbens; (E) Substantia nigra pars compacta; (F) Ventral tegmental area.

3.3 Accuracy, Precision, and Recovery

Accuracy and precision of the assay were within the Food and Drug Administration's (FDA) requirements for bioanalytical method validation. Combined inter- and intra-day precision for DA, DOPAC, HVA, NE, 5-HIAA, and 5-HT in non-matrix conditions was within 12% RSD at each calibration point. These data are summarized in Table 2.2. Combined intra- and inter- day precision and accuracy for brain matrix conditions were < 12% RSD and < 10% error for all analytes, respectively (Table 2.3). Thus, the method demonstrated sufficient precision and accuracy for monoamines and respective metabolites using the aforementioned chromatographic conditions and sample preparation procedure. Moreover, the described method achieved chromatographic selectivity without interference from other endogenous brain matrix constituents at the retention times of each analyte of interest (Fig. 2.1B).

Table 2.2 Inter- day accuracy (% Error) and precision (%RSD) of the HPLC- ECD assay for quantification of DA, DOPAC, HVA, NE, 5-HT, and 5-HIAA in 0.2 N perchloric acid (n= 12 for each concentration)

	DA		DOPAC		HVA	
[C] ng/ml	Mean	% RSD	Mean	% RSD	Mean	% RSD
0.300	0.330	5.925	0.329	8.575	0.291	4.214
0.625	0.559	11.681	0.736	6.665	0.573	9.839
1.250	1.220	1.739	1.232	4.064	1.192	2.219
2.500	2.496	1.117	2.333	2.407	2.432	1.588
5.000	5.018	1.224	4.884	0.940	4.872	1.093
10.000	9.364	6.114	9.562	3.639	9.335	6.327
15.000	14.734	3.916	14.942	2.778	14.749	3.985
30.000	29.694	2.333	-	-	-	-
	NE		5-HT		5-HIAA	
[C] ng/ml	Mean	% RSD	Mean	% RSD	Mean	% RSD
0.300	0.288	5.345	-	-	0.325	4.310
0.625	0.594	12.305	0.661	10.643	0.583	13.344
1.250	1.265	4.056	1.163	4.075	1.226	3.072

2.500	2.543	2.336	2.514	1.735	2.603	2.274
5.000	5.048	1.569	5.068	1.565	5.236	1.814
10.000	9.762	11.567	8.823	7.155	10.917	9.139
15.000	15.011	4.065	14.321	4.221	17.017	5.300
30.000	-	-	30.078	2.153	-	-

Table 2.3: Inter-day and accuracy and precision of the HPLC-ECD assay for quantification of all analytes in brain tissue homogenate (n= 9 for each concentration).

Spiked [C] (ng/mL)	DA			DOPAC			HVA		
	Mean [C] found	%Error	%RSD	Mean [C] found	%Error	%RSD	Mean [C] found	%Error	%RSD
0.80	0.83	4.53	2.24	0.82	6.38	3.16	0.78	4.26	1.41
2.00	1.96	4.11	5.68	2.02	2.84	11.87	2.10	4.76	5.99
5.00	4.84	3.22	3.10	4.90	7.52	4.94	5.19	9.62	4.39
10.00	9.55	4.51	4.37	10.00	0.53	2.07	10.16	9.10	4.18
Spiked [C] (ng/mL)	NE			5-HT			5-HIAA		
	Mean [C] found	%Error	%RSD	Mean [C] found	%Error	%RSD	Mean [C] found	%Error	%RSD
0.80	0.88	10.27	2.41	0.78	5.62	6.02	0.86	7.06	3.49
2.00	1.92	8.99	8.54	2.19	9.68	3.68	2.13	6.31	6.53
5.00	5.36	7.12	6.28	5.28	8.58	8.65	5.52	10.38	4.51
10.00	9.68	4.81	4.88	10.45	4.60	4.63	10.73	7.27	4.34

3.4 Measurement of Monoamine and Metabolites in Discrete Brain Tissue Samples

The relative concentration levels of monoamines and metabolites measured in the FCtx, STR, NAC, HIP, SNpc, and VTA brain regions are presented in Table 2.4. These values accurately represent the degree of monoaminergic input within each brain region and are in agreement with the values previously reported for mice brain tissue (Donzanti & Yamamoto, 1988; Saito et al., 1992). Specifically, within the brain regions comprising the dopaminergic brain reward (NAc and VTA) and motor control (STR and SNpc)

pathways, tissue levels of DA and respective metabolites were determined to be significantly higher than those of the FCtx and HIP; with the major DA projection sites, NAc and STR, containing the highest levels of DA (ng/mg) as compared to all other brain regions analyzed by this method. Moreover, while the FCtx and HIP brain regions are described to be diffusely innervated by DA neurons, neuronal input from noradrenergic (NE) and serotonergic systems is more abundant. Thus, the present data showing greater concentrations of NE and 5-HT as compared to DA within in these brain areas is expected.

Table 2.4. Relative concentration levels of biogenic amines and metabolites in discrete brain regions

Brain Region	Analyte concentration levels (ng/mg wet tissue)					
	DA	DOPAC	HVA	NE	5-HT	5-HIAA
FCtx (Fig. 2A)	0.18	0.24	0.11	0.51	0.26	0.27
HIP (Fig. 2B)	0.19	0.15	0.09	0.43	0.23	0.25
NAc (Fig. 2C)	3.33	0.54	0.47	0.45	-	-
STR (Fig. 2D)	3.55	0.71	0.53	0.32	-	-
SNpc (Fig. 2E)	1.56	1.09	0.51	1.61	0.18	0.31
VTA (Fig. 2F)	1.49	0.70	0.44	2.37	0.22	0.33

4. Conclusions

DA, NE, and 5-HT are widely distributed throughout the CNS, serving as neurotransmitters and regulating a number of physiological and behavioral processes including cognitive and mnemonic processes (FCtx, HIP), reward and addiction (VTA, NAc), and movement (SNpc, STR). As dysregulation of these neurotransmitter systems is associated with numerous neuropsychiatric disorders, a growing number of pharmacological agents are utilized to either increase or reduce monoamine levels. In

order to assess the effectiveness of such agents, a sensitive analytical assay allowing for simultaneous detection of monoamine neurotransmitters and relevant metabolites within these discrete brain regions is required. This paper reports a rapid, sensitive, and reproducible HPLC-ECD method for the separation and quantification of DA, DOPAC, HVA, NE, 5-HT, and 5-HIAA in mouse brain tissue homogenate using DHBA as the internal standard. The proposed analytical method utilizes a simple sample preparation procedure that does not require the ultrafiltration of prepared tissue extracts and results in recoveries $\geq 90\%$ for all analytes. Optimization of mobile phase pH and SOS concentration resulted in effective separation of monoamines and respective metabolites on a C18 150 mm, 3 μm column, within an analytical run time of approximately 9 min. Therefore, the present method circumvents the need for dedicated UHPLC systems for rapid analysis (< 10 min) of brain tissue monoamines and relevant metabolites and is desirable for high-throughput approaches. Moreover, given the highly sensitive quantification limits reported for all analytes (0.300 - 0.625 ng/mL), this HPLC-ECD assay is suitable for quantitative analysis of these endogenous compounds within multiple areas of the brain, including the small nuclei of the SNpc and VTA (0.2 – 1.5 mg tissue weights), in mice. Thus, the proposed analytical method provides a fast, sensitive, and reliable means by which to evaluate the pre-clinical effects of pharmaceutical agents on monoaminergic tone within distinct brain regions and pathways.

5. Acknowledgements

The authors would like to thank Dr. Stacy Brown for critical reading and editing the manuscript. Additionally, the authors appreciate the Bill Gatton College of Pharmacy for

their ongoing support of student research and the Department of Pharmaceutical Sciences for funding used in the completion of this project.

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CHAPTER 3

DOPAMINERGIC EFFECTS OF 3, 4-METHYLENEDIOXYPYROVALERONE (MDPV),
MEPHEDRONE, AND METHYLONE ARE ENHANCED FOLLOWING CO-EXPOSURE

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Keywords: synthetic cathinones, dopamine, dopamine transporter, locomotor activity,
bath salts

Introduction

The synthetic cathinones include a class of designer β -ketone amphetamine compounds that are chemically related to the plant-derived stimulant, cathinone. The three most commonly abused synthetic cathinones, MDPV, 4-methylmethcathinone (mephedrone), and 3, 4-methylenedioxymethcathinone (methylone), received international popularity as psychoactive components in 'bath salts,' a marketing term used in conjunction with labels stating "not for human consumption" as a way to circumvent existing drug laws and allow for their legal distribution (DEA, 2014; Spiller et al., 2011). In recent years, the extensive abuse of bath salts as cheap and 'legal' alternatives to illicit psychostimulant compounds precipitated an alarming rise in the number of individuals requiring emergency medical treatment after synthetic cathinone intoxication (CDC, 2011; Deluca et al., 2012; SAMHSA) and led to the DEA classification of bath salt constituents, MDPV, mephedrone, and methylone as schedule I controlled substances in 2011 (DEA, 2011). However, synthetic cathinone abuse appears unabated (Mounteney et al., 2016; Palamar et al., 2015) due in large part to the internet serving as clandestine marketplace for these drugs (Karila et al., 2015; Power, 2014). The growing prevalence of MDPV, mephedrone, and methylone has prompted a number of preclinical investigations on the effects of these drugs on the central nervous system (CNS).

Pharmacokinetic studies have shown these synthetic cathinones readily cross the blood brain barrier and accumulate in rodent brain tissue, reaching peak concentrations within 15-30 min (Novellas et al., 2015; Peters et al., 2016; Štefková et al., 2017; Šíchová et al., 2017). In vitro transporter and electrophysiology assays have

revealed the individual pharmacological mechanisms of MDPV, mephedrone, and methylone. MDPV functions as a selective inhibitor of dopamine transporter (DAT) activity by blocking the reuptake of dopamine, similar to cocaine but more potent (Baumann et al., 2013; Cameron et al., 2013a). In contrast, mephedrone and methylone behave as non-selective substrates for monoamine transporters, thereby inducing transporter-mediated release of dopamine (DA), norepinephrine (NE), and serotonin (5-HT), similar to the actions of 3,4-methylenedioxymethamphetamine (MDMA, ecstasy) and other amphetamines (Baumann et al., 2012; Solis, 2016). Despite pharmacological and DAT selectivity differences, all three synthetic cathinones were found to precipitate rapid and marked elevations in extracellular DA (Baumann et al., 2012; Baumann et al., 2013; Shortall et al., 2013) and increase locomotor activity in rodents (Gregg and Rawls, 2014; Marusich et al., 2012). Moreover, the ability of MDPV and mephedrone to progressively increase locomotor activity and stereotypy, a phenomenon termed behavioral sensitization, has been demonstrated in several studies (Fantegrossi et al., 2013; Gregg et al., 2013a; Marusich et al., 2012).

While there is now a sizable body of evidence on the individual effects of MDPV, mephedrone, and methylone, forensic analysis has shown that bath salts formulations often contain various concoctions of these synthetic cathinones, with both binary and ternary mixtures of MDPV, mephedrone, and methylone detected in many of the most frequently abused bath salt ‘brands’ (Spiller et al., 2011; Warrick et al., 2013). Therefore, it is important to also evaluate the neurochemical and behavioral effects of these drugs when administered in various combinations to more accurately model bath salts abuse.

Additive, or synergistic, interactions are often more profound when drug cocktails are composed of compounds which individually produce qualitatively similar effects via distinct pharmacological mechanisms (Tallarida, 2011). Therefore, because both non-substrate (MDPV) and substrate synthetic cathinones (mephedrone and methylone) exert their psychostimulant effects by increasing synaptic DA levels, the dichotomy of their interaction with the DAT suggests an avenue for enhanced dopaminergic effects when these drugs are co-abused. While a few studies have analyzed the reinforcing (Gannon et al., 2018) and stimulant (Berquist et al., 2016) properties of different binary mixtures of the synthetic cathinones, the current study represents the first known attempt to characterize the effects of ternary mixtures of MDPV, mephedrone, and methylone on tissue DA levels and locomotor activation in comparison to each compound alone. The results presented here suggest that the dopaminergic effects of MDPV, mephedrone, and methylone are significantly enhanced when they are co-administered as a cocktail.

Materials and Methods

Animals

The experiments utilized adolescent male Swiss-Webster mice (Harlan Inc., Indianapolis, IN) weighing 20-30 g. Male adolescent mice were chosen for this study as drug survey reports suggest the main abusers of synthetic cathinones are young male adults (Karila et al., 2015; Vardakou et al., 2011). The experimental protocol was approved by the ETSU University Committee on Animal Care (UCAC) and followed the National Institute of Health Guidelines for the Care and Use of Laboratory Animals. All

drugs were dissolved in saline and injected at a volume of 0.01 mL/g body weight.

Control animals were injected with equal volumes of sterile saline.

Drugs and Reagents

Analytical grade MDPV ((±) 3, 4-methylenedioxypropylvalerone HCl), mephedrone ((±) 4-methylmethcathinone HCl), and methylone ((±)3, 4-methylenedioxymethcathinone HCl) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Certified reference standard grade DA, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 3,4-dihydroxybenzylamine hydro-bromide (DHBA) were also purchased from Sigma-Aldrich. The ion pairing agent, sodium octyl sulfate (SOS), was purchased from Acros organics (Geel, Belgium) and 2.0 N perchloric acid was purchased from RICCA chemical (Arlington, TX, USA). All other certified ACS chemicals and reagents used in this study were purchased from Thermo Fisher Scientific (Fair Lawn, NJ, USA).

Determination of Monoamine Levels

Animal dosing. Mice were separated into the following cohorts (n= 4-6 per group): 1) saline 2) 1 mg kg⁻¹ MDPV 3) 10 mg kg⁻¹ MDPV, 4) 1 mg kg⁻¹ mephedrone, 5) 10 mg kg⁻¹ mephedrone, 6) 1 mg kg⁻¹ methylone, 7) 10 mg kg⁻¹ methylone, 8) 1 mg kg⁻¹ cathinone cocktail, 9) 3.33 mg kg⁻¹ cathinone cocktail, 10) 10 mg kg⁻¹ cathinone cocktail, and appropriately dosed with a single intraperitoneal (IP) injection of saline or corresponding drug. All doses were chosen in accordance to DEA statistical reports indicating the average range of bath salt abuse per session varies widely from 25 mg- 5 g (DEA, 2011). As insufflation is the predominate modality utilized by bath salt abusers, IP injections were chosen for this study based on their analogous pharmacokinetic

profile to snorting (Bradberry, 2002). Fifteen minutes after injection, animals were deeply anesthetized with isoflurane, sacrificed by decapitation, and brains were collected immediately and placed in ice-cold saline for 5 min. This time frame was chosen based on a previous pharmacokinetic study from our laboratory indicating that peak brain concentrations of MDPV, mephedrone, and methyldone following IP injections are achieved at 15 minutes (Peters et al., 2016). Brains were sliced in 2 mm sections using a chilled stainless steel 30 g coronal mouse brain matrix (MSI) and microdissections of the nucleus accumbens (NAc), striatum (STR), substantia nigra pars compacta (SNpc), and ventral tegmental area (VTA) were performed in accord to the mouse brain atlas (Paxinos and Watson). Collected tissue was flash-frozen in liquid nitrogen and stored at -70°C until further analysis.

HPLC-ECD. DA, DOPAC, and HVA were extracted and quantified from collected brain tissue using a previously described HPLC-ECD method (Allen et al., 2017). Briefly, collected tissue samples were thawed, weighed, homogenized in methanol, spiked with the internal standard 3,4-dihydroxybenzylamine (DHBA), and centrifuged at 13,000 g. Supernatants were collected, dried under nitrogen gas, and reconstituted in 0.2 N perchloric acid. DA, DOPAC, and HVA were separated using a C₁₈ MD- 150mm x 3.2mm, 3µm column (Thermo Fisher Scientific) and detected at a potential of + 375 mV by a Coulochem® III coulometric detector (ESA Laboratories). The mobile phase, which consisted of 0.22 µm filtered 100 mM sodium acetate, 20 mM citric acid, 0.38 mM SOS, 0.15 mM EDTA and 5% v/v acetonitrile adjusted to pH 3.3 with glacial acetic acid, was delivered at 0.6 mL/min by an isocratic pump. Quantification was achieved using the

Agilent EZChrome *Elite* software system (Agilent Technologies). DA turnover was calculated as [DOPAC+HVA]/[DA].

Locomotor Activity

Animal dosing. Mice were randomly separated into four groups (n=6-8): 1) saline, 2) 10 mg kg⁻¹ MDPV (uptake inhibitor), 3) 10 mg kg⁻¹ mephedrone (substrate), and 4) 10 mg kg⁻¹ MDPV + 10 mg kg⁻¹ mephedrone + 10 mg kg⁻¹ methylone. In order to reduce animal numbers, only the high dose of each drug was utilized and only the highest potency substrate drug (mephedrone) was utilized.

Apparatus. Behavioral experiments were carried out using a locomotor arena that consisted of a square wooden box measuring 40 x 40 cm painted black. AnyMaze tracking software (ANYmaze, Stoelting Co., Wood Dale, IL) was used to superimpose grid lines on the locomotor arena and measure locomotor activity. A computer interface was used to automatically tabulate total distance traveled (m) or stereotypy (counts). Immobility episodes lasting longer than 3 s were considered as stereotypy.

Procedure. Animals were habituated to the locomotor arena for three consecutive days before drug dosing. For each habituation, mice were given IP injections of 0.9% saline 15 min prior to being placed in the locomotor area. Following habituation, mice were randomly separated into the four treatment groups listed above and correspondingly dosed in their home cages with drug or saline every other day for 14 days, a total of 7 injections. Animals were placed in the locomotor arena 15 min after each drug administration and behavior was recorded and tracked for 10 min using AnyMaze. Locomotor activity was recorded on test days 1 and 7, and testing sessions

for both habituation and treatment runs were 10 min. This seven-day dosing schedule is similar to the paradigm used by Berquist et al. (2016), with 48 h drug intervals chosen in accordance with Watterson et al. (2016).

Statistics

All statistical analyses were performed using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA, USA). Neurochemical data from HPLC-ECD analysis were evaluated using two-way analysis of variance (drug treatment x dose) followed by Bonferroni's post *hoc test*. For locomotor activity experiments, total distanced traveled (m) and the number of immobility or stereotypic episodes were summed over the course of the 10 min testing period on day 1 and day 7 and evaluated using two-way analysis of variance (drug treatment x testing day) followed by Newman-Keuls *post hoc test*. The minimum criterion for statistical significance was $P < 0.05$. The use of high and low drug doses, drug combinations, and analysis of multiple analytes in various different brain regions resulted in a very large number of planned comparisons. Thus, the outcomes from statistical tests for DA, DOPAC, HVA, and DA turnover are described minimally in Results, and full descriptions of all neurochemical statistical outcomes are presented in Tables S1-S4.

Results

Individual Effects of MDPV, Mephedrone, and Methylone on Brain Tissue Dopamine Concentration and Turnover Rate in Acutely Exposed Mice

Mice were acutely treated with MDPV, mephedrone, and methylone at either a low (1 mg kg⁻¹) or high (10 mg kg⁻¹) dose of each drug alone. Figure 3.1 shows the

results of these treatments on DA within the mesolimbic and nigrostriatal brain pathways 15 min post drug exposure. The main effects of dose and drug treatment as well as their interactions on DA concentration and turnover were statistically significant ($p < 0.05 - p < 0.0001$) in each brain region analyzed (Table S1). At the high dose (10 mg kg⁻¹), MDPV and mephedrone produced significant increases in DA over control values in all brain areas including the DA nuclei of the midbrain (SNpc and VTA) and their corresponding major projection sites (STR and NAc). Methylone (10 mg kg⁻¹) caused significant, selective increases in mesolimbic DA (NAc and VTA) in comparison to controls. Conversely, no change in DA tissue content was observed following acute, low dose (1 mg kg⁻¹) administration of MDPV, mephedrone, or methylone.

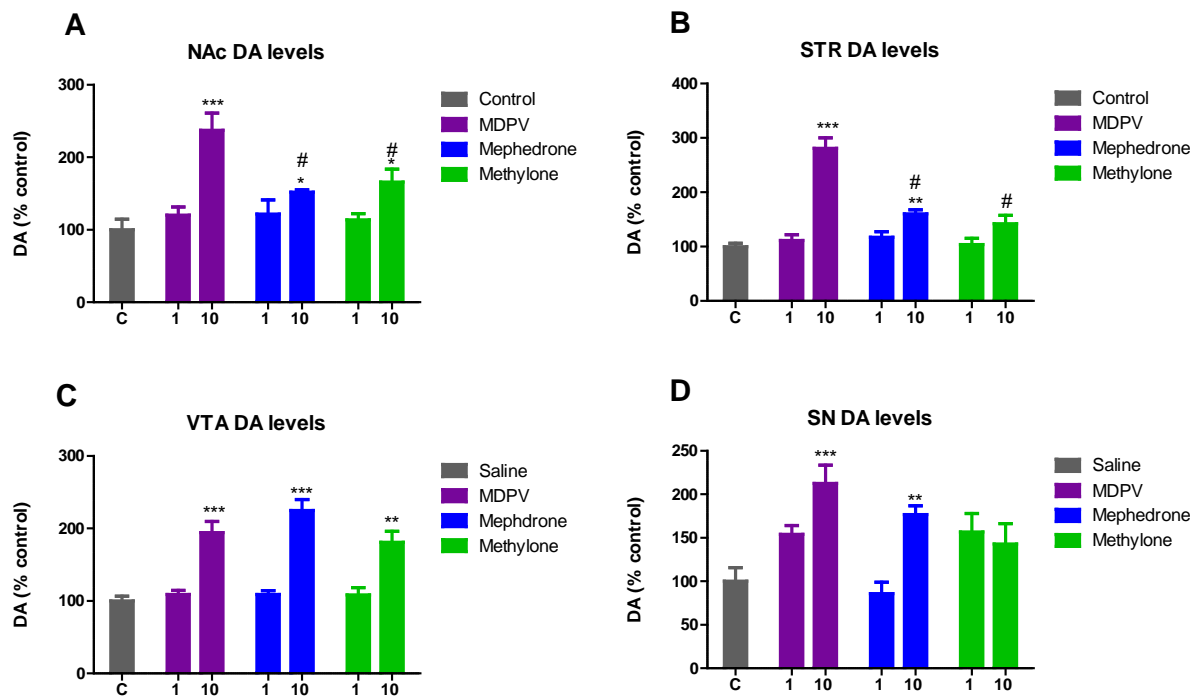


Figure 3.1 The acute effects of MDPV, mephedrone, and methylone on DA levels in the NAc (A), STR (B), VTA (C), and SNpc (F). Numerals below each indicate the treatment dose in mg kg⁻¹. DA levels are reported as % of control. Data are expressed as mean ± SEM, n = 4-6 mice per treatment group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control.

The effect of each drug on DA metabolite levels (HVA and DOPAC) was also assessed using HPLC analysis, and results are presented in Table 3.1. Significantly increased levels of HVA (NAc, STR, SN, and VTA) and decreased levels of DOPAC (SNpc and VTA) were measured in mice that received a single injection of MDPV at the high dose (10 mg kg⁻¹). The low dose of MDPV (1 mg kg⁻¹) significantly increased tissue HVA levels in the SNpc and VTA, but did not cause any significant alterations in DOPAC levels. At both treatment doses, mephedrone caused a marked increase in HVA in four brain regions. Methylnone also produced region-wide significant increases in HVA tissue levels at the higher dose, but these effects were not observed at the lower dose. Both of the substrate cathinones decreased DOPAC (NAc, STR, and SN) levels following high dose administration, while low dose exposure to neither mephedrone nor methylnone caused significant alterations in DOPAC levels. DA metabolite concentrations and their summed ratios to DA were used to evaluate the acute effect of each synthetic cathinone on DA transmitter utilization and metabolism (Fig 3.2); apparent DA turnover was evaluated in each brain region by calculating the ratio of the total concentrations of DA metabolites (HVA + DOPAC) to DA. At the high dose, MDPV, mephedrone, and methylnone each produced a marked reduction in DA turnover values as compared to control in all brain regions analyzed, while at the lower dose, each drug decreased DA turnover in the VTA only.

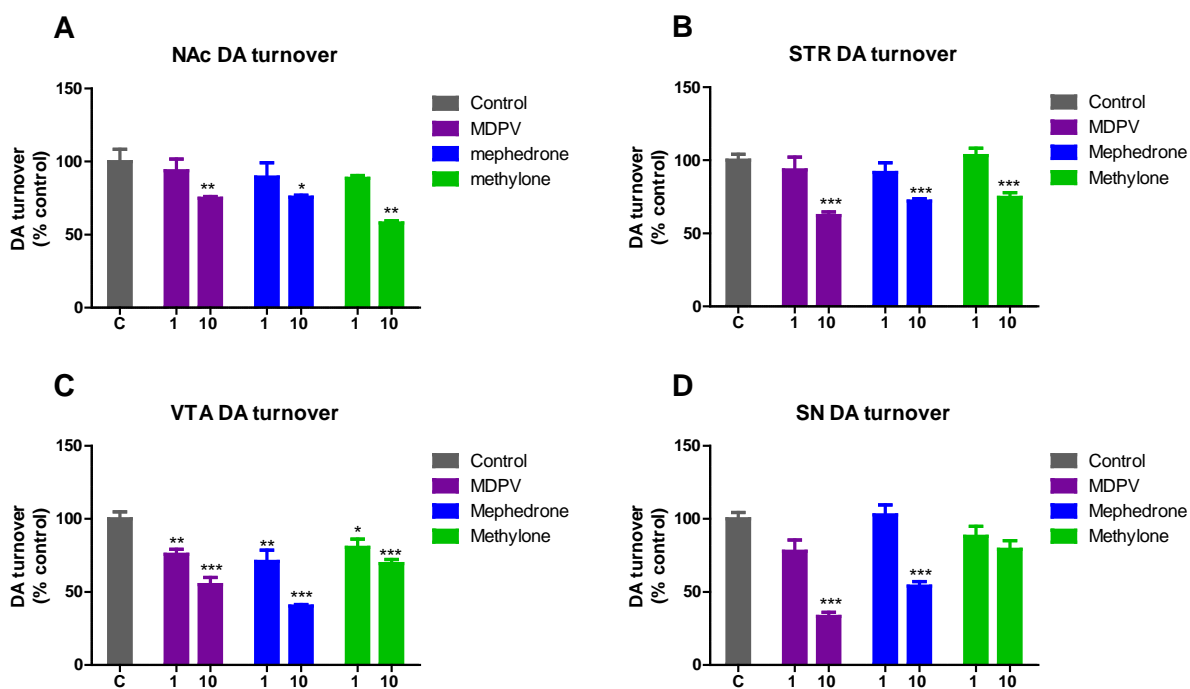


Figure 3.2 The acute effects of MDPV, mephedrone, and methylone on DA turnover in the NAc (A), STR (B), VTA (C), and SNpc (F). Numerals below each indicate the treatment dose in mg kg⁻¹. DA levels are reported as % of control. DA turnover is reported as % of control. Data are expressed as mean ± SEM, n = 4-6 mice per treatment group; * p < 0.05, ** p < 0.01, *** p < 0.001 vs. control.

Table 3.1 DA metabolite levels following individual and combined exposure to MDPV, mephedrone, and methylone. Data shows the average content (ng/mg wet tissue) of DOPAC and HVA in the NAc, STR, VTA, and SNpc 15 min post injection of drug or saline. Data are expressed as mean ± SD, n = 4-6 per group. Significance is indicated in Table S1-S2.

		Saline	Combo 1 mg kg ⁻¹	Combo 3.3 mg kg ⁻¹	MDPV 1 mg kg ⁻¹	MDPV 10 mg kg ⁻¹	Meph 1 mg kg ⁻¹	Meph 10 mg kg ⁻¹	Methy 1 mg kg ⁻¹	Methy 10 mg kg ⁻¹
NAc	DOPAC	1.0 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	0.8 ± 0.2	1.2 ± 0.3	0.9 ± 0.3	0.6 ± 0.1	0.7 ± 0.1	0.6 ± 0.1
	HVA	0.5 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.2	1.1 ± 0.2	0.6 ± 0.1	0.8 ± 0.1	0.5 ± 0.1	0.7 ± 0.1
STR	DOPAC	0.7 ± 0.1	0.6 ± 0.1	0.9 ± 0.2	0.6 ± 0.1	0.8 ± 0.1	0.7 ± 0.2	0.5 ± 0.1	0.6 ± 0.1	0.5 ± 0.0
	HVA	0.5 ± 0.1	0.7 ± 0.1	1.0 ± 0.2	0.6 ± 0.1	1.4 ± 0.2	0.8 ± 0.2	0.9 ± 0.1	0.6 ± 0.1	0.8 ± 0.2

VTA	DOPAC	0.6 ± 0.1	0.5 ± 0.2	0.3 ± 0.1	0.8 ± 0.2	0.3 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	1.0 ± 0.1	0.7 ± 0.1
	HVA	0.3 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.2
SNpc	DOPAC	0.6 ± 0.1	0.5 ± 0.1	0.3 ± 0.1	0.6 ± 0.2	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.0	0.5 ± 0.1	0.3 ± 0.1
	HVA	0.2 ± 0.0	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.3 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.3 ± 0.0

Synergistic Effects of Combined Synthetic Cathinone Exposure on Mesolimbic and Nigrostriatal DA Content

'Bath salts' products often contain various mixtures of MDPV, mephedrone, and/or methylone. Given the fact that pharmacological data presented here (Fig 3.1) and elsewhere (De Felice et al., 2014) suggest that these drugs individually function to modulate DA release to an extent; we also evaluated the acute, combined effect of MDPV, mephedrone, and methylone on brain tissue DA concentration and turnover. For combined treatment, mice received a single injection of a cathinone cocktail composed of MDPV, mephedrone, and methylone, each at a dose of 10 mg kg⁻¹ (30 mg kg⁻¹ cathinones in total), 3.3 mg kg⁻¹ (~ 10 mg/kg cathinones in total), or 1 mg kg⁻¹ (3 mg kg⁻¹ cathinone total). The overall effect of each combo dose on regional specific DA levels is presented in Fig 3.3. The results of combined treatments as compared to those of both control and dose matched individual cathinone treatment are presented in Fig 3.4 (DA levels) and Fig 3.5 (DA turnover).

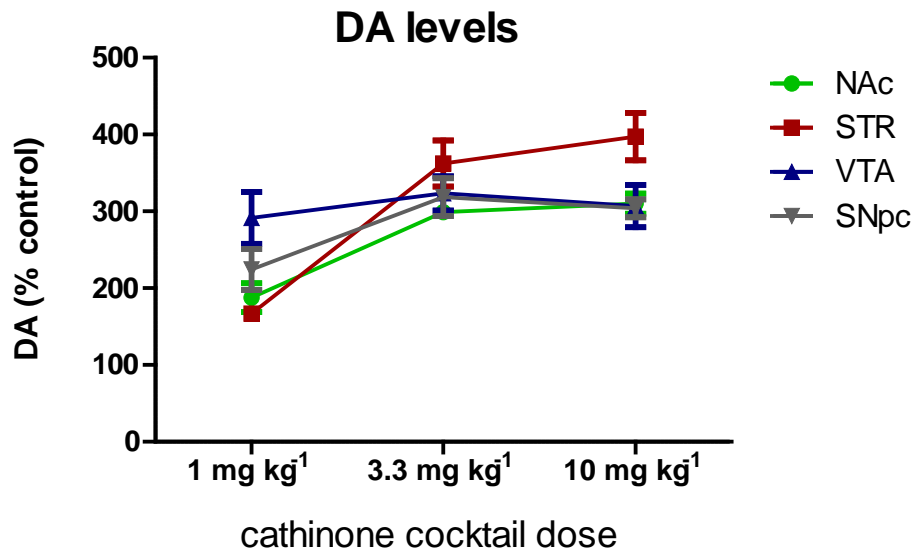


Figure 3.3 Tissue DA content corresponding to increasing doses of the cathinone cocktail. Data are expressed as % of control values. Significant increases are observed from 1 mg kg⁻¹ to 3.3 mg kg⁻¹ in all brain regions, except the VTA. Comparisons and levels of significance are reported in Tables S3-S4. DA levels appear to plateau following a single 3.3 mg kg⁻¹ combined cathinone exposure as no significant differences were observed in any brain area when compared to the 10 mg kg⁻¹ cocktail.

DA was significantly increased in all brain areas following acute exposure to the cathinone cocktail at all doses (Fig 3.3 and Table S1-S2). The DA response following co-administration of MDPV, mephedrone, and methylone appears to be due to synergistic interactions between the drugs as evidenced by two different statistical comparisons (Fig 3.4). First, while none of the synthetic cathinones significantly affected tissue DA levels over control values when individually administered at a dose of 1 mg kg⁻¹, co-administration of 1 mg kg⁻¹ MDPV + 1 mg kg⁻¹ mephedrone + 1 mg kg⁻¹ methylone resulted in DA elevations that were statistically significant across all brain regions analyzed. Second, mesolimbic and nigrostriatal DA levels were significantly greater in mice treated with the high dose cathinone cocktail (3.3 mg kg⁻¹ MDPV + 3.3

mg kg⁻¹ mephedrone + 3.3 mg kg⁻¹ methylone) as compared to mice treated with 10 mg kg⁻¹ of MDPV, mephedrone, or methylone, individually. Thus, combined 3.3 mg kg⁻¹ doses of the three drugs resulted in increased tissue DA to levels that were significantly greater than those produced by any of the individual drugs at 10 mg kg⁻¹ doses (Fig 3.4).

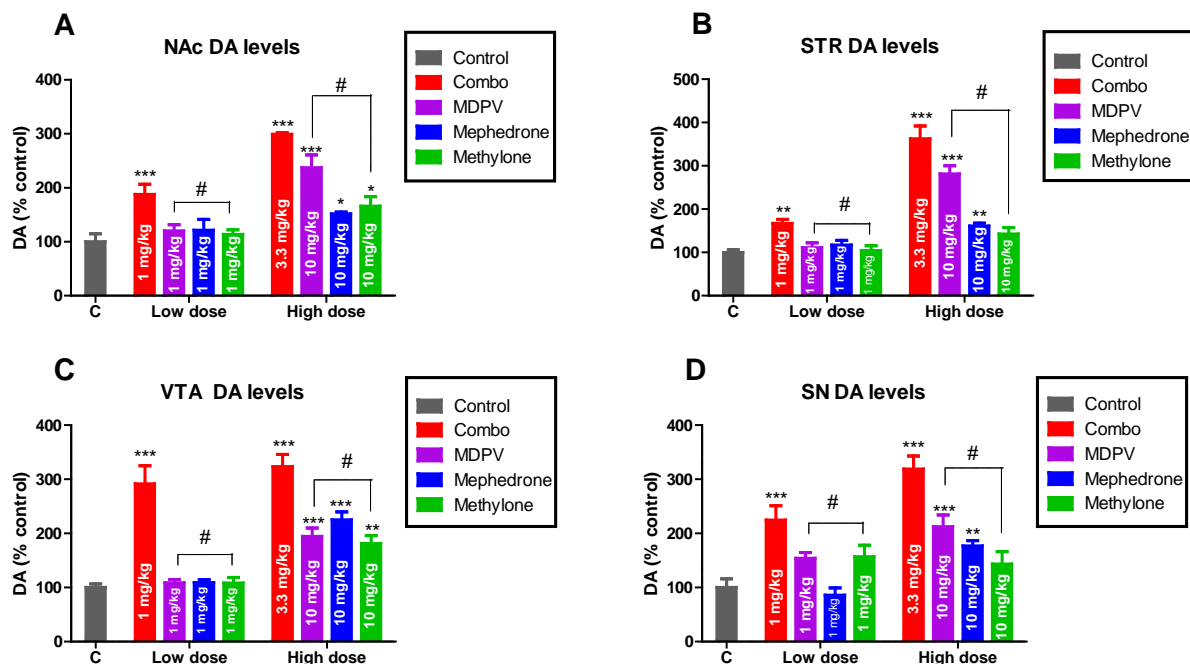


Figure 3.4 The effects of combined exposure to MDPV, mephedrone, and methylone on DA levels in the NAc (A), STR (B), VTA (C), and SNpc (D) of mice following acute dosing. Low dose values include 1 mg kg⁻¹ individual and combined drug treatments. High dose values include 10 mg kg⁻¹ individual drug treatments and 3.3 mg kg⁻¹ cathinone cocktail. Data are expressed as mean % of control \pm SEM ($n = 4-6$ mice per group); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control (c) in both the low and high dose treatment groups, while # = $p < 0.05$ vs. the corresponding cathinone cocktail.

However, DA metabolites were not as profoundly affected by the cocktail as compared to the individual drugs (see Table 3.1). DA turnover values for the 1 mg/kg cathinone cocktail were significantly reduced from those of control and the individual low

dose drug treatments in all brain areas except the VTA. While the 3.3 mg/kg cocktail significantly reduced DA turnover as compared to control and methylone (10 mg kg⁻¹) in all brain regions, these reductions were statistically different from MDPV (10 mg kg⁻¹) individual drug treatment in the NAc only (Fig 3.5).

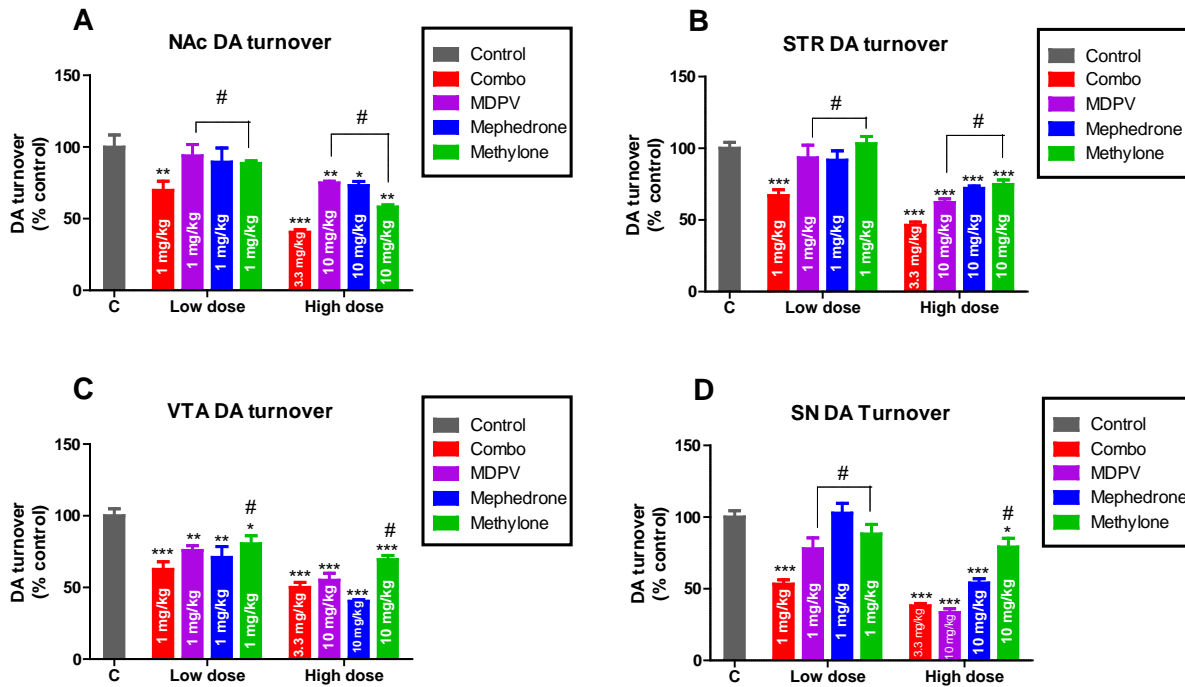


Figure 3.5 The effects of combined exposure to MDPV, mephedrone, and methylone on DA turnover in the NAc (A), STR (B), VTA (C), and SNpc (D) of mice following acute dosing. Low dose values include 1 mg kg⁻¹ individual and combined drug treatments. High dose values include 10 mg kg⁻¹ individual drug treatments and 3.3 mg kg⁻¹ cathinone cocktail. Data are expressed as mean % of control \pm SEM (n = 4-6 mice per group); * p < 0.05, ** p < 0.01, *** p < 0.001 vs. control (c) in both the low and high dose treatment groups, while # = p < 0.05 vs. the corresponding cathinone cocktail.

Effect of Individual and Combined Synthetic Cathinone Exposure on Locomotor Activity and Immobility Counts in Mice

The acute (day 1) and subchronic (day 7) effects of MDPV and mephedrone, individually and in combination with methylone, on locomotor activity are presented in Fig 3.6. Animals were administered intermittent doses of appropriate drug or saline and activity testing began 15 min after treatment. Immobility episodes of 3 s or longer were counted and used as an index of stereotypy, as animals tracked as immobile were observed to be exhibiting stereotypic behavior (Fig 3.6B). A two-way ANOVA revealed highly significant main effects of drug treatment and testing day ($p < 0.0001$). No significant changes in motor activity were observed in the mephedrone group on either the first or last day of drug treatment. However, locomotion was significantly decreased over the course of treatment in both the MDPV and combined cathinone groups, while the acute effects of these treatments differed. MDPV treatment significantly enhanced forward locomotion on day 1 (observed as a significant increase in distance traveled) and had no effect on immobility or stereotypic episodes. In contrast, the combo treatment significantly reduced motor activity and increased stereotypic counts on day 1. On the final treatment day, both drugs induced hypolocomotion and significantly increased immobility counts, with motor activity in the combo group significantly less than that observed on day 1.

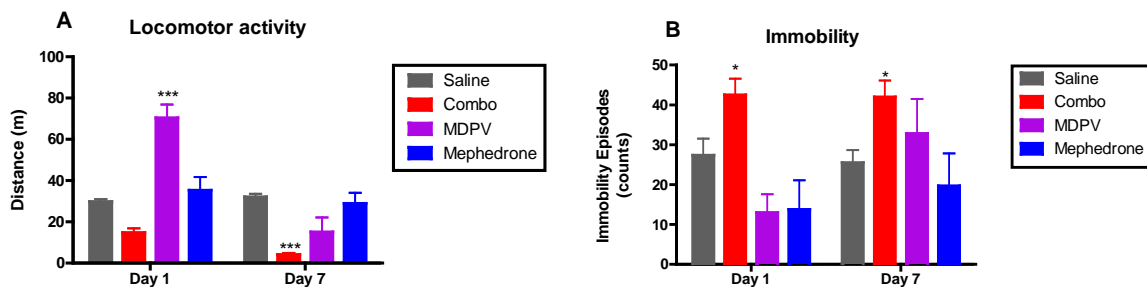


Figure 3.6 Locomotor activity levels (A) and immobility counts (B) in mice repeatedly administered 10 mg kg⁻¹ doses of MDPV, mephedrone, or the cathinone cocktail (combo). Animals were placed in the locomotor arena 15 min post-injection and locomotor activity was measured on day 1 (acute) and day 7 (subchronic) as total distance traveled or cumulative immobility episodes over a 10 min testing period. Data are expressed as group means (n = 6-8 per group) ± SEM, * p < 0.05 vs. saline.

Discussion

The primary aim of the present study was to evaluate and compare how major bath salt constituents MDPV, mephedrone, and methylone acutely influence mesolimbic and nigrostriatal DA levels and locomotor activity when administered individually and in combination. Neurochemical and behavioral assays were performed 15 min post exposure, a time point in which all three compounds are reported to reach peak concentrations in the mouse brain (Peters et al., 2016). Given that MDPV, mephedrone, and methylone primarily produce their abuse-related effects by enhancing DA signaling (Gatch et al., 2013; Karlsson et al., 2014) and that popular bath salt ‘brands’ generally contain various mixtures of these compounds (Warrick et al., 2013), investigations into their combined effects on DA neurotransmission and motor activity are clearly warranted.

In the present study, we found that all three synthetic cathinones produced significant, dose-related increases in mesolimbic DA levels, with MDPV and mephedrone also producing dose-related increases in striatal and nigral DA content. Moreover, DA elevations detected in the NAc and STR of mice treated with MDPV were significantly greater than those found in mephedrone and methylone-treated mice, suggesting that MDPV is more potent than the substrate cathinones at increasing tissue DA levels in DAT-rich brain regions. These findings are in agreement with several microdialysis studies reporting rapidly elevated dialysate DA levels following synthetic cathinone administration, with the greatest increases reported with MDPV (Baumann et al., 2013; German et al., 2014; Schindler et al., 2016). The overall effect of each drug treatment on DA metabolism was similar, with all three compounds significantly reducing DA turnover to values that were comparable between the individual drug treatment groups.

These effects can be attributed to increased synaptic DA levels and blockade of intraneuronal DA metabolism. Notably, DA elevations were accompanied by significant increases in HVA by all three cathinones, while DOPAC was either decreased or unaltered. Similar effects on DA metabolites have been described in freely moving rodents acutely treated with the synthetic cathinones at comparable doses (López-Arnau et al., 2018; López-Arnau et al., 2017). Given that the conversion of DA to HVA involves catechol-O-methyltransferase (COMT), an enzyme that is notably absent from dopaminergic terminals (Kastner et al., 1994), while the conversion of DA to DOPAC is mediated by monoamine oxidase (MAO), an enzyme found within presynaptic nerve terminals (Graves et al., 2017), these data likely reflect the DA reuptake-inhibiting

actions of the synthetic cathinones. Thus, the blockade of DA re-entry into the intracellular substrate pool prevents MAO degradation and leads to an increase in the synaptic pool of DA available for extraneuronal metabolism by COMT in glial cells (Gulley and Zahniser, 2003). Additionally, tissue levels of HVA appeared to positively correlate to DA elevations, with MDPV also producing the greatest elevations in HVA in the neuron terminal regions (NAc and STR). Interestingly, the substrate cathinones produced significantly greater reductions in accumbal and striatal DOPAC levels when compared to controls and MDPV. Given the fact that other amphetamine-like compounds have been shown to inhibit MAO (Sitte and Freissmuth, 2015), these data may reflect a similar action of mephedrone and methylone.

The combined delivery of the synthetic cathinones resulted in acute elevations in DA that were significantly greater than those observed with MDPV, mephedrone, or methylone alone in all collected brain regions. This was observed at all doses of the cathinone cocktail analyzed (1 mg kg^{-1} , 3.3 mg kg^{-1} , and 10 mg kg^{-1}). As none of the drugs significantly changed DA over control levels when individually administered at the 1 mg kg^{-1} dose, the 1 mg kg^{-1} cathinone cocktail was utilized to determine if any significantly altered effects are achieved following co-administration of the synthetic cathinones at doses that were pharmacologically insignificant when individually administered. As such, we found that DA was significantly increased in all brain regions of mice treated with the 1 mg kg^{-1} cocktail as compared to both control and 1 mg kg^{-1} individual drug treatments. In order to determine if this enhanced response was a result of synergistic drug interactions or an additive effect due to an increase in total synthetic cathinone load (e.g. 3 mg kg^{-1} total cathinones in cocktail vs. 1 mg/kg individual

synthetic cathinones), we utilized the cocktail composed of 3.3 mg kg⁻¹ MDPV + 3.3 mg kg⁻¹ mephedrone + 3.3 mg kg⁻¹ methylone (10 mg kg⁻¹ total cathinone exposure) and directly compared results of this treatment group with those obtained in both control and 10 mg kg⁻¹ individual cathinone groups. From these analyses, we found that 1) the 3.3 mg kg⁻¹ combo treatment significantly increased DA levels over control values and 2) DA elevations were significantly greater than those achieved by any of the synthetic cathinones when individually administered at 10 mg kg⁻¹. Thus, a significantly greater DA response was achieved by the drug combination, despite a 3 fold decrease in the dose of each individual cathinone, suggesting that this effect is product of drug synergism and not simply due to addition or increased total cathinone load (Tallarida, 2011). Taken together, these data show that the individual abilities of MDPV, mephedrone, and methylone, to increase mesolimbic and nigrostriatal DA levels are enhanced when they are given in conjunction one another. This, enhanced effect may be attributed to the synergistic pharmacokinetic effect demonstrated by Cameron and colleagues (2013b), whereby the substrate and DA-releasing activity of mephedrone and methylone precedes the blocking actions of MDPV, and is supported by studies demonstrating that mephedrone and binary mixtures of MDPV + mephedrone enhance the stimulant effect of DAT-blocker cocaine (Berquist et al., 2016).

Given that midbrain dopamine circuits are primarily implicated in the activation and sensitization of locomotor responses (Uhl et al., 2002), it is interesting to evaluate these effects in mice treated with the synthetic cathinones individually and in combination. Thus, to determine whether the enhanced DA response following co-administration of MDPV, mephedrone, and methylone had any measurable effects on

the psychostimulant properties of the individual drugs, locomotor activity experiments were conducted in mice treated with intermittent 10 mg kg⁻¹ doses of MDPV, mephedrone, or the cathinone cocktail. Mephedrone was chosen as the representative substrate cathinone for individual analysis as it is reported to be more potent than methylone at inducing DAT-mediated DA release and producing hyperactivity in rodents (Baumann et al., 2012; Bonano et al., 2014). In agreement with several studies (Baumann et al., 2013; Fantegrossi et al., 2013; Marusich et al., 2012), we found that MDPV induced profound forward locomotion after a single 10 mg kg⁻¹ treatment, while repeated exposure at this dose appeared to induce focused stereotypy, evidenced by a trending increase in immobility episodes. Observed stereotypies included self-licking and repetitive hindlimb jumping, which have been previously reported with MDPV (Fantegrossi et al., 2013). In the present study, mephedrone (10 mg kg⁻¹) did not influence the locomotor or stereotypic responses of mice on either treatment day, despite findings of significant dopaminergic effects. These results are in agreement with some studies (Budzynska et al., 2015; Motbey et al., 2012), but are different than others (Baumann et al., 2012; Lisek et al., 2012; Shortall et al., 2013). However, given the fact that activity testing began 15 min post injection, it is conceivable that the stimulant effects of mephedrone had already peaked and returned to near baseline by the time testing began. Although this would not be predicted from pharmacokinetic data in our lab, it is consistent with the various reports that mephedrone produces transient stimulant effects that are rapid in onset (within 10 min) and short in duration (Aarde et al., 2013; Kehr et al., 2011; Marusich et al., 2012; Šíchová et al., 2017) and coincides with the described short-lasting effects of this drug by recreational users (Dargan et al.,

2011; Winstock et al., 2011). Additionally, given the potent serotonergic effects of mephedrone (Baumann et al., 2012; Kehr et al., 2011), locomotor activation may have been dampened due to enhanced 5-HT neurotransmission in the STR and NAc.

Combined 10 mg kg⁻¹ administration of MDPV, mephedrone, and methylone induced profound evidence of stereotypic activity (high immobility counts) that was accompanied by significantly depressed forward locomotion after both acute and repeated dosing. Observed stereotypies included head bobbing and self-injurious chest biting, often resulting in skin wounding and bleeding. Similar effects have been reported following a single high dose treatment of methamphetamine (Gentry et al., 2004; Kuczenski and Segal, 1997; Segal and Kuczenski, 1987) and MDPV (30 mg kg⁻¹) (Fantegrossi et al., 2013); however, none of the individual cathinones were shown by this study or others (Gregg and Rawls, 2014; Gregg et al., 2013b; López-Arnau et al., 2012; Shortall et al., 2013) to produce acute stereotyped motor deficits at 10 mg kg⁻¹ doses. Thus, the motor-stimulant responses of the individual drugs are significantly altered when they are co-administered as a cocktail. As drug-induced increases in extracellular DA in the accumbens and, in particular, the striatum have been positively correlated to stereotypic responses (Budygin et al., 2000; Carr and White, 1987; Presti et al., 2003), these data support our neurochemical findings. Additionally, motor deficits in mice were exaggerated over time, with a greater degree of hypolocomotion on day 7 compared to day 1, while stereotypic activity was similar for both treatment days. However, because stereotypy counts corresponded to immobility episodes lasting more than 3 seconds, it is likely that these animals did, in fact, exhibit intensified stereotypic activity by remaining in each stereotypic episode for longer periods of time, resulting in

less ambulatory activity between episodes and the observed enhancement of hypolocomotion on day 7. While more precise testing parameters should be used to define this behavior, these data suggest that locomotor activity decreases with the cathinone cocktail due to an increase in stereotypic behavior.

In sum, we have shown that acute MDPV, mephedrone, and methylone induce large DA elevations within the NAc, STR, SNpc, and VTA, which are accentuated when the drugs are given in combination with one another. This effect appears to result from synergistic drug interactions, as combined 3.3 mg kg⁻¹ doses of MDPV, mephedrone, and methylone increase mesolimbic and nigrostriatal DA to levels that were significantly greater than those achieved by any of the drugs alone at 10 mg kg⁻¹ doses. In support of this, we also found that acute dosing of the cathinone cocktail induced robust stereotypic behavior and hypolocomotion that was exacerbated after repeated dosing, an effect that was not observed with the individual drugs and is consistent with enhanced DA neurotransmission. Given that excessive DA release has been implicated in methamphetamine neurotoxicity (Halpin et al., 2014) and is associated with its significant reinforcing effects (Volkow and Morales, 2015), these findings raise concerns about possible dopaminergic toxicity and addictive potential when MDPV, mephedrone, and methylone are co-abused in bath salt mixtures.

Supplementary Data

Table S1. Two-way ANOVAs for dose and treatment effects of MDPV, mephedrone, and methylone and their combination on DA, DOPAC, HVA, and DA turnover

Brain region	Dependent variable	Main effect treatment	Main effect dose	Interaction
NAc	DA	F(4,44) = 24.97 P < 0.0001	F(1,44) = 38.76 P < 0.0001	F(4,44) = 5.27 P < 0.0015
	DOPAC	F(4,43) = 7.09 P < 0.0002	F(1,43) = 0.09 P > 0.05 (ns)	F(4,43) = 3.54 P < 0.0146
	HVA	F(4,43) = 19.19 P < 0.0001	F(1,43) = 30.30 P < 0.0001	F(4,43) = 8.97 P < 0.0001
	DA turnover	F(4,43) = 19.70 P < 0.0001	F(1,43) = 36.80 P < 0.0001	F(4,43) = 2.60 P < 0.05
STR	DA	F(4,43) = 42.76 P < 0.0001	F(1,43) = 101.5 P < 0.0001	F(4,43) = 18.20 P < 0.0001
	DOPAC	F(4,43) = 5.83 P < 0.0008	F(1,43) = 2.39 P > 0.05 (ns)	F(4,43) = 8.86 P < 0.0001
	HVA	F(4,44) = 54.87 P < 0.0001	F(1,44) = 16.71 P < 0.0001	F(4,44) = 11.86 P < 0.0001
	DA turnover	F(4,43) = 22.63 P < 0.0001	F(1,43) = 42.85 P < 0.0001	F(4,43) = 3.05 P < 0.0261
VTA	DA	F(4,42) = 45.44 P < 0.0001	F(1,42) = 37.71 P < 0.0001	F(4,42) = 4.27 P < 0.0055
	DOPAC	F(4,42) = 12.14 P < 0.0001	F(1,42) = 36.38 P < 0.0001	F(4,42) = 4.46 P < 0.0044
	HVA	F(4,42) = 19.77 P < 0.0001	F(1,42) = 3.12 P > 0.05 (ns)	F(4,42) = 5.18 P < 0.0017
	DA turnover	F(4,42) = 30.20 P < 0.0001	F(1,42) = 23.26 P < 0.0001	F(4,42) = 2.90 P < 0.0331
SNpc	DA	F(4,42) = 23.74 P < 0.0001	F(1,42) = 15.10 P < 0.0004	F(4,42) = 3.55 P < 0.0138
	DOPAC	F(4,42) = 9.87 P < 0.0001	F(1,42) = 29.97 P < 0.0001	F(4,42) = 3.08 P < 0.0259
	HVA	F(4,42) = 10.64 P < 0.0001	F(1,42) = 2.68 P > 0.05 (ns)	F(4,42) = 5.49 P < 0.0012
	DA turnover	F(4,42) = 36.33 P < 0.0001	F(1,42) = 50.93 P < 0.0001	F(4,42) = 8.60 P < 0.0001

Table S2. Post-hoc statistical comparisons (Bonferroni test for multiple comparisons) for mice treated with MDPV, mephedrone, methyldone, and the cathinone cocktail

Comparison	DA	DOPAC	HVA	DA turnover
NAc				
MDPV 1 vs control	ns	ns	*	ns
MDPV 10 vs control	***	ns	***	**
Meph 1 vs control	ns	ns	ns	ns
Meph 10 vs control	*	*	***	**
Methy 1 vs control	ns	ns	ns	ns
Methy 10 vs control	*	**	**	***
Combo 1 vs control	***	ns	***	**
Combo 3.3 vs control	***	ns	**	***
MDPV 1 vs Combo 1	**	ns	ns	*
Meph 1 vs Combo 1	**	ns	*	*
Methy 1 vs Combo 1	**	ns	**	ns
MDPV 10 vs Combo 3.3	*	ns	***	***
Meph 10 vs Combo 3.3	***	*	ns	***
Methy 10 vs Combo 3.3	***	**	ns	ns
Meph 1 vs MDPV 1	ns	ns	ns	ns
Methy 1 vs MDPV 1	ns	ns	ns	ns
Meph 10 vs MDPV 10	***	***	***	ns
Methy 10 vs MDPV 10	**	***	***	ns
STR				
MDPV 1 vs control	ns	ns	ns	ns
MDPV 10 vs control	***	ns	***	***
Meph 1 vs control	ns	ns	*	ns
Meph 10 vs control	*	*	***	***
Methy 1 vs control	ns	ns	ns	ns
Methy 10 vs control	ns	**	***	**
Combo 1 vs control	**	ns	ns	***
Combo 3.3 vs control	***	*	***	***
MDPV 1 vs Combo 1	*	ns	ns	***
Meph 1 vs Combo 1	*	ns	ns	**
Methy 1 vs Combo 1	**	ns	ns	***
MDPV 10 vs Combo 3.3	***	ns	***	ns
Meph 10 vs Combo 3.3	***	***	ns	**
Methy 10 vs Combo 3.3	***	***	ns	***
Meph 1 vs MDPV 1	ns	ns	ns	ns
Methy 1 vs MDPV 1	ns	ns	ns	ns
Meph 10 vs MDPV 10	***	***	***	ns
Methy 10 vs MDPV 10	***	***	***	ns
VTA				

MDPV 1 vs control	ns	**	***	**
MDPV 10 vs control	***	*	**	***
Meph 1 vs control	ns	ns	*	***
Meph 10 vs control	***	ns	**	***
Methy 1 vs control	ns	***	*	*
Methy 10 vs control	**	ns	**	***
Combo 1 vs control	***	ns	***	***
Combo 3.3 vs control	***	**	***	***
MDPV 1 vs Combo 1	***	***	ns	ns
Meph 1 vs Combo 1	***	ns	***	ns
Methy 1 vs Combo 1	***	***	***	*
MDPV 10 vs Combo 3.3	***	ns	ns	ns
Meph 10 vs Combo 3.3	***	ns	ns	ns
Methy 10 vs Combo 3.3	***	***	ns	*
Meph 1 vs MDPV 1	ns	*	***	ns
Methy 1 vs MDPV 1	ns	ns	***	ns
Meph 10 vs MDPV 10	ns	**	ns	ns
Methy 10 vs MDPV 10	ns	ns	ns	ns
SNpc				
MDPV 1 vs control	***	ns	***	**
MDPV 10 vs control	ns	***	***	***
Meph 1 vs control	**	**	ns	ns
Meph 10 vs control	ns	***	***	***
Methy 1 vs control	ns	ns	***	ns
Methy 10 vs control	ns	***	*	*
Combo 1 vs control	***	ns	**	***
Combo 3.3 vs control	***	**	ns	***
MDPV 1 vs Combo 1	*	ns	ns	**
Meph 1 vs Combo 1	***	**	ns	***
Methy 1 vs Combo 1	*	ns	ns	***
MDPV 10 vs Combo 3.3	***	ns	*	ns
Meph 10 vs Combo 3.3	***	ns	**	ns
Methy 10 vs Combo 3.3	***	ns	ns	***
Meph 1 vs MDPV 1	*	ns	ns	**
Methy 1 vs MDPV 1	ns	***	**	ns
Meph 10 vs MDPV 10	ns	ns	ns	*
Methy 10 vs MDPV 10	*	ns	ns	***

Mice were treated with MDPV, mephedrone, methylone, or their combination at varied doses (represented by the numerals after each drug). Drug treatment induced changes in DA and HVA were increases by comparison to control. Treatment induced changes in DOPAC and DA turnover were decreases by comparison to control. Significance levels for the comparisons are indicated by * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Table S3. One-way ANOVAs for the treatment effects of the cathinone cocktail at 1 mg kg⁻¹, 3.3 mg kg⁻¹, and 10 mg kg⁻¹

Brain region	Main effect of treatment on DA
NAc	F(4) = 50.27 P < 0.0001
STR	F(4) = 42.92 P < 0.0001
VTA	F(4) = 17.84 P < 0.0001
SNpc	F(4)= 26.95 P < 0.0001

Table S4. Post-hoc statistical comparisons (Tukey's multiple comparisons) for mice treated with the cathinone cocktail at 1 mg kg⁻¹, 3.3 mg kg⁻¹, and 10 mg kg⁻¹.

Comparison	NAc (DA levels)	STR (DA levels)	VTA (DA levels)	SNpc (DA levels)
Combo 10 vs Combo 1	***	***	ns	*
Combo 10 vs Combo 3	ns	ns	ns	ns
Combo 10 vs control	***	***	***	***
Combo 3 vs Combo 1	***	***	ns	*

The effects of the cathinone cocktail at three different doses on tissue DA are presented in Fig 3.3, and levels of significance are indicated as * p < 0.05, ** p < 0.01, and *** p < 0.001 by each treatment comparison. The effects of 1 mg kg⁻¹ and 3.3 mg kg⁻¹ combo by comparison to controls are listed in Table S2 and have not been included here.

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CHAPTER 4

CHRONIC CO-EXPOSURE TO MAJOR 'BATH SALT' CONSTITUENTS MDPV, MEPHEDRONE, AND METHYLONE PRODUCES A FUNCTIONAL LESION AT DOPAMINERGIC SYNAPSES

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Keywords: neurotoxicity, dopamine, synthetic cathinones, tyrosine hydroxylase, dopamine transporter

Introduction

The synthetic cathinones, 3, 4-methylenedioxypropylamphetamine (MDPV), 4-methylmethcathinone (mephedrone), and 3, 4-methylenedioxymethcathinone (methylone), are major constituents of bath salt mixtures and continue to be one of the most prevalent classes of designer drug compounds. Despite DEA classification of MDPV, mephedrone, and methylone as schedule I controlled substances in 2011 (DEA, 2011), international control laws remain lacking and abuse continues to thrive with the internet serving as a clandestine marketplace for these drugs (Coppola and Mondola, 2012; Dybdal-Hargreaves et al., 2013; Karila et al., 2015). Bath salts are described by users to precipitate desirable psychostimulant and hallucinogenic effects; however, violent behavior, tachycardia, respiratory distress, seizures, and even death have also been reported with their abuse (DEA, 2011; Karch, 2015; Prosser and Nelson, 2012).

In recent years, great strides have been made in our understanding of the pharmacology and abuse liability of synthetic cathinones; however, descriptions of their neurotoxic properties remain limited. Moreover, while much of the preclinical data on synthetic cathinones explicates the individual effects of MDPV, mephedrone, and methylone, forensic analysis and DEA case reports have indicated that bath salt products contain various mixtures of MDPV, mephedrone, and methylone with each other or other stimulant substances (Araujo et al., 2015; DEA, 2011; Spiller et al., 2011). Also, a retrospective search of records involving synthetic cathinones at two poison control centers revealed that many of most frequently abused bath salt 'brands,' identified by patient history reports, contained mixtures of MDPV, mephedrone, and

methylone (Spiller et al., 2011; Warrick et al., 2013). Thus, to better model bath salt abuse, the effects of synthetic cathinone mixtures should be assessed.

MDPV is a catecholamine selective monoamine transporter blocker that was found to be significantly more potent than cocaine at inhibiting synaptic clearance of dopamine (DA), increasing extracellular DA, and stimulating locomotor activity (Baumann et al., 2013). Mephedrone and methylone behave as substrates at monoamine transporters and exhibit transporter-mediated DA and serotonin (5-HT)-releasing effects similar to their non-beta keto analogs, methamphetamine (METH) and 3,4 methylenedioxymethamphetamine (MDMA) (Baumann et al., 2012; López-Arnau et al., 2012). Additionally, mephedrone and methylone induce locomotion and fully substitute for METH and MDMA in drug discrimination studies (Bonano et al., 2014; Gatch et al., 2013; Harvey and Baker, 2016), further suggesting shared pharmacological activity between these drugs. However, the substrate synthetic cathinones produce unique effects with regards to DA neurotoxicity. While neither mephedrone nor methylone induce damage to DA nerve endings (Angoa-Perez et al., 2016; Angoa-Perez et al., 2012), they accentuate the neurotoxic effects of METH, amphetamine (AMPH), and MDMA when co-administered with these drugs (Angoa-Pérez et al., 2014; Angoa-Pérez et al., 2013; Anneken et al., 2015). While the underlying mechanism of the effect has not been determined, these findings suggest that the neurotoxic properties of the synthetic cathinones are enhanced when they are combined with other drugs that target the dopamine transporter (DAT). However, preclinical investigations of the combined effects of MDPV, mephedrone, and methylone on measures of neurotoxicity are extremely limited. To address this lack of experimental

data, the present study evaluated the effects of ternary mixtures of MDPV, mephedrone, and methylone on DA and major proteins of the dopaminergic synapse in mice. The effect of these drug cocktails on neuroinflammatory markers and DA neuron counts was also assessed.

Materials and Methods

Drugs and Reagents

MDPV ((±) 3, 4-methylenedioxypropylvalerone HCl), mephedrone ((±) 4-methylmethcathinone HCl), and methylone ((±)3, 4-methylenedioxymethcathinone HCl) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Certified reference standard grade dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) , and 3,4- dihydroxybenzylamine hydro-bromide (DHBA) were also purchased from Sigma-Aldrich. The ion pairing agent, sodium octyl sulfate (SOS), was purchased from Acros organics (Geel, Belgium) and 2.0 N perchloric acid was purchased from RICCA chemical (Arlington, TX, USA). All other certified ACS chemicals and buffer reagents used in this study were purchased from Thermo Fisher Scientific (Fair Lawn, NJ, USA).

Animals

The experiments utilized adolescent male Swiss-Webster mice (Harlan Inc., Indianapolis, IN) weighing 20-30 g. Male adolescent mice were chosen for this study as drug survey reports suggest the main abusers of synthetic cathinones are young male adults (Karila et al., 2015; Vardakou et al., 2011; Winstock et al., 2011). The animals were housed in a full AAALAC-accredited facility under standard laboratory conditions

($22 \pm 1^\circ\text{C}$, 12 h light/dark cycle) with food and water freely available. The experimental protocol was approved by the ETSU University Committee on Animal Care (UCAC), following the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Animal Treatment

Mice (age 40 days) were weighed and separated into the following treatment groups: 1) saline (controls), 2) MDPV (10 mg kg^{-1}), 3) mephedrone (10 mg kg^{-1}), 4) methylone (10 mg kg^{-1}), and 5) cathinone cocktail (10 mg kg^{-1} MDPV + 10 mg kg^{-1} mephedrone + 10 mg kg^{-1} methylone). The total cathinone dose used in the cocktail (30 mg kg^{-1} total cathinones) was chosen based on previous experiments reporting that 30 mg kg^{-1} injections of synthetic cathinones produced physiological responses commonly associated with psychostimulant neurotoxicity (den Hollander et al., 2013). All treatment groups were utilized for neurochemical analysis. In protein expression assays, mephedrone was chosen over methylone as the representative substrate cathinone due to its increased potency and DA-releasing capabilities at the DAT (Baumann et al., 2012; Simmler et al., 2013); thus, all treatment groups except methylone were analyzed. For chronic dosing, each animal received intraperitoneal (IP) injections according to their treatment group every other day for 14 days (7 injections total). All drugs were dissolved in saline and injected at a volume of 0.01 ml/g body weight; equal volumes of saline were used to treat control animals. This every other day dosing schedule follows the paradigm described by Pierce and Kalivas (1997), by which 48 h drug dosing allows for complete drug wash out prior to each injection, and has been traditionally used with psychostimulant studies.

Tissue Collection

For neurochemical and protein expression assays, mice were anesthetized with isoflurane and euthanized by decapitation 2 days after drug treatment. This point was chosen based on previous studies indicating that amphetamine-associated neurotoxicity reaches maximum at 2 days (Angoa-Pérez et al., 2013). Brains were collected immediately and placed in ice cold saline for 5 min. The nucleus accumbens (NAc), striatum (STR), substantia nigra pars compacta (SNpc), and ventral tegmental area (VTA) were dissected from each brain based upon coordinates in the mouse brain atlas (Paxinos and Watson) using a 30g Coronal mouse brain matrix (ASI Instruments), flash frozen, and stored at -70°C until further analysis. For immunohistochemistry, mice were deeply anesthetized with Avertin (2.5% tribromoethyl alcohol in 0.9% saline), identified via the loss of deep tendon and corneal reflexes, and transcardially perfused with ice cold 3 % paraformaldehyde. Fixed brains were then dehydrated in ethanol, incubated in xylene, and embedded in paraffin (Paraplast-X-tra™, Fisher Scientific). Brains were blocked, sectioned on the microtome at 10 µM thickness, and mounted onto Superfrost-plus slides (Fisher Scientific). Every section from the rostral hippocampus to the anterior cerebellar-midbrain junction (Bregma: -2.70 to -3.70, Paxinos and Watson) was serially-collected and mounted at five sections per slide.

Neurochemical Analysis

Concentrations of DA and relevant metabolites, DOPAC and homovanillic acid (HVA), were measured in discrete brain tissue extracts using a previously described method employing HPLC with electrochemical detection (Allen et al., 2017). Briefly, samples

were homogenized in methanol, spiked with the internal standard (3,4-Dihydroxybenzylamine (DHBA)), and centrifuged at 14,000 x g. Collected supernatants were dried under nitrogen gas, and reconstituted in 0.2 N perchloric acid. Chromatographic separations were achieved using a C18 MD- 150 mm x 3.2 mm, 3 µm column (Thermo Fisher Scientific, Sunnyvale, CA, USA), with a mobile phase consisting of 95% 50 mM sodium acetate, 10 mM citric acid, 0.38 mM SOS, 0.15 mM EDTA, and 5% acetonitrile. Electrochemical detection was performed using a Coulochem® III detector (ESA Laboratories, Inc.) equipped with 5100A dual electrode. Data were collected on-line and exported to an Agilent EZChrome Elite software system (Santa Clara, CA, USA) for peak integration and analysis. On the day of analysis, standard calibration solutions containing 0.125 - 30 ng/mL were prepared in 0.2 N perchloric acid and used to generate five-point internal standard calibration curves for each analyte. Peak area ratios were used for computations, and analyte concentrations were calculated using interpolation of their respective standard curves.

Protein Expression Assays

Tissue lysate preparation. Collected brain tissue regions were suspended in appropriate amounts of ice cold isolation buffer consisting of RIPA lysis buffer and MS-SAFE protease and phosphatase inhibitor cocktail (1:10, Sigma Aldrich). Cells in tissue samples were lysed by 30 seconds of sonication with an ultrasonic processor, and cellular debris were removed via centrifugation at 4°C at 14,000 X g for 15 min. Supernatant concentration was measured on an Epoch 2 microplate reader (BioTek, Winooski, VT, USA) using the Pierce Bradford Protein assay kit (Thermo Scientific).

Simple Western analysis. Expression levels of the dopamine transporter (DAT-1), tyrosine hydroxylase (TH), monoamine oxidase B (MAO-B), catechol-O-methyltransferase (COMT), vesicular monoamine transporter (VMAT2), glial fibrillary acidic protein (GFAP) and loading control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were measured in lysates using the capillary-based, automated Western blotting system, 'Wes' from ProteinSimple (Santa Clara, CA, USA). The primary antibodies and dilutions used for these analyses were Rabbit anti-DAT 1 (1:25, Abcam), Rabbit anti-TH (1:1000, Novus Biologicals), Rabbit anti- MAO-B (1:10, Novus Biologicals), Rabbit anti- COMT (1:10, Novus Biologicals), Rabbit biotin conjugated anti-VMAT2 (1:1000, Novus Biologicals), Rabbit anti-GFAP (1:100, Dako) and Rabbit anti-GAPDH (1:1000, Novus Biologicals). Size-based assays were performed using Wes 12-230 kDa rabbit master kit reagents according to the user manual (PS-MK14, ProteinSimple). Briefly, samples were diluted with 0.1 X sample buffer (ProteinSimple) to achieve a final concentration of 100 µg/mL (DAT-1) or 250 µg/mL (TH, MAO-B, COMT, VMAT2, GFAP), (amount of total protein content determined from Bradford assay (ug/mL)). Diluted samples were then mixed with a master mix (ProteinSimple) containing fluorescent molecular weight markers and 40 mM dithiothreitol (DTT), and heated at 95°C for 5 min. Denatured samples, blocking reagent, primary antibodies, HRP-conjugated anti-rabbit secondary antibodies (streptavidin-HRP for VMAT2), and chemiluminescent substrate were dispensed into designated wells of a manufacturer supplied microplate, pre-loaded with separation and stacking matrices. Prepared assay plates were then loaded into Wes and analyzed at room temperature using the instrument default settings. Chemiluminescent protein bands were captured by a

charge-coupled device (CCD), and the digital image was analyzed using inline Compass software (ProteinSimple). Relative band densities of all proteins were normalized to the GAPDH level for each lane.

Cell Culture and Cytotoxicity Assay

The SH-SY5Y cell line was obtained from the ATCC. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Cells were maintained at 37°C, saturated humidity, and 5% CO₂. Cells were plated onto 24-well culture plates at a density of 1×10^5 cells/well and differentiated in reduced serum media (3% FBS) supplemented with 10µM retinoic acid for six days prior to experimentation. SH-SY5Y cells were treated with vehicle, 1 mM MDPV, 1 mM mephedrone, 3 mM mephedrone, 1 mM methylone, or the cathinone cocktail (1 mM MDPV + 1 mM mephedrone + 1 mM methylone) and cytotoxicity was determined at 6, 12, 24, and 48 h after drug application by measuring the activity of the lactate dehydrogenase released into the culture media according to the manufacturer's protocol (BioVision, K311-400). Treatment doses were similar to those previously utilized with the synthetic cathinones in SH-SY5Y cells (den Hollander et al., 2014; Rosas-Hernandez et al., 2016; Valente et al., 2017). Samples were done in triplicate and assessed in reference to a 1% Triton-X treated control.

Immunohistochemistry

Deparaffinized sections were incubated with rabbit polyclonal anti-TH (Pel-Freeze Biologicals; 1:250) overnight for identification of TH positive dopamine neurons. TH positive cells were visualized using the avidin biotin complex (ABC) reaction and

diaminobenzidine (DAB) substrate solution kits (Vector laboratories). All sections were counterstained with the nissl stain Neutral Red (Sadasivan et al., 2012).

Quantification of TH-positive neurons. A model-based stereological method was used to estimate the total number of DA neurons within the SNpc sections (Baquet et al., 2009). Briefly, TH-positive and TH-negative cells of the DA phenotype were counted from both the right and left sides of one SNpc section per slide using a 40x objective. The same section was counted on each slide, and the Abercrombie correction factor (Abercrombie, 1946) was used to correct for split nuclei.

Data Analysis

All statistical analyses were performed using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA, USA). LDH data were analyzed using a two-way ANOVA followed by Bonferroni's *post-hoc* test. The effect of each drug treatment on tissue DA, DOPAC, HVA, levels and relative expression levels of DAT, TH, MAO-B, COMT, VMAT2, and GFAP were tested for significance using one-way ANOVA, followed by Tukey's *post-hoc* test. DA turnover values were calculated as $[DOPAC+HVA]/[DA]$, and the effect of treatments on DA turnover were evaluated by one-way ANOVA followed by Tukey's *post-hoc* test. The effect of combined cathinone treatment on DA neuron number was compared to saline by an unpaired t-test. The minimum criterion for statistical significance was $P < 0.05$.

Results

Effects of Chronic Individual and Combined Synthetic Cathinone Exposure on Mesolimbic and Nigrostriatal DA Concentration and Turnover

Dopamine concentration and turnover rates within discrete brain regions of mice chronically treated with the synthetic cathinones, alone and in combination, are presented in Figure 4.1 and 4.2, respectively. The main effect of treatment on DA and DA turnover values was highly significant ($p < 0.001$ - < 0.0001). Statistical analyses revealed significant increases in DA levels above control within all brain areas 2 days after repeated individual exposure to MDPV and mephedrone, with methylone, significantly increasing NAc and VTA DA levels. Moreover, MDPV produced the greatest elevation of DA levels in the nerve terminal regions when compared to mephedrone ($p < 0.001$) and methylone ($p < 0.001$) (Fig 4.1A). Conversely, DA levels were significantly reduced in the NAc, STR, SNpc, and VTA brain regions of mice 2 days after chronic treatment with cathinone cocktail, as compared to saline control animals (Fig 4.1A). The cathinone cocktail significantly increased DA turnover in all brain areas. The individual cathinones induced similar significant increases in accumbal and striatal DA turnover, with MDPV additionally increasing DA turnover in the VTA (Fig 4.1B).

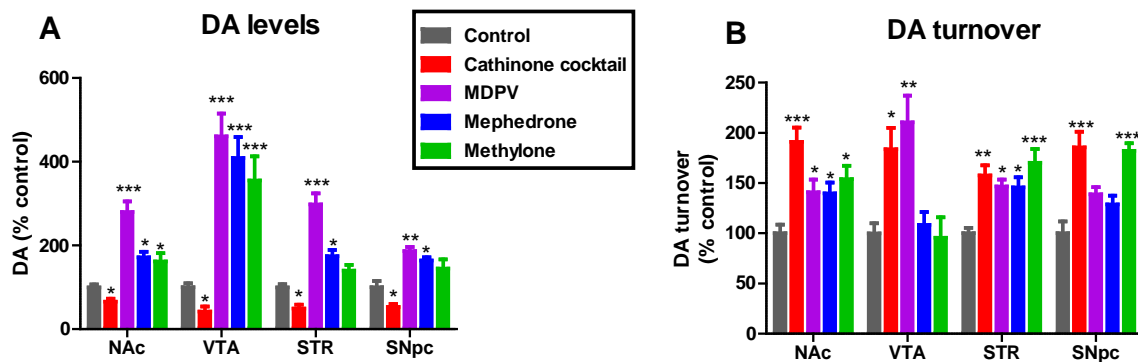


Figure 4.1 DA concentration (A) and turnover (B) in the NAc, VTA, STR, and SNpc following chronic dosing of MDPV, mephedrone, and methylone individually and in combination. Monoamine and metabolite levels were determined using HPLC analysis and are reported as % of control. Data are expressed as means for $n = 4 - 6$ mice per group \pm SEM ($< 5\%$ of means). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs control (saline).

Effects of Chronic Synthetic Cathinone Exposure on DA Degradation and Biochemical Markers of Neurotoxicity

MAO-B and COMT. Given the ability of amphetamine-like compounds to inhibit DA degradative enzymes, synthetic cathinones were evaluated for their individual and combined ability to dysregulate MAO-B and COMT expression levels (Fig 4.2). MDPV alone did not change the levels of MAO-B and COMT in any of the studied brain regions. Mephedrone treatment had no significant effects on MAO-B, but produced significant decreases in COMT in all regions examined (Fig. 4.2B). In contrast, significant reductions in both MAO-B and COMT levels were observed in all brain regions of mice treated with the cathinone cocktail (Fig 4.2A,B).

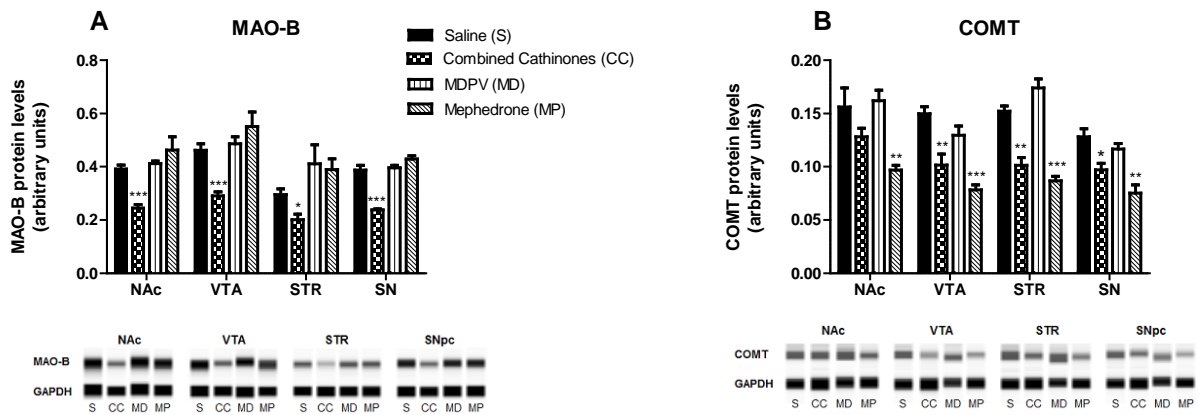


Figure 4.2 Relative expression levels of MAO-B and COMT in the mesolimbic and nigrostriatal brain pathways following repeated dosing of MDPV, mephedrone, and the cathinone cocktail (MDPV + mephedrone + methylone). Relative pixel densities were quantified using ProteinSimple compass software and were normalized to GAPDH. Data are means for $n = 4 - 6$ mice per group, \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. saline.

VMAT2. To assess the effects of repeated synthetic cathinone exposure on intracellular DA storage mechanisms, a target of METH and other amphetamines, protein expression levels of *VMAT2* were determined in mice chronically treated with the synthetic cathinones. As shown in Figure 4.3, enhanced striatal *VMAT2* expression was observed following exposure to the cathinone cocktail and MDPV, while mephedrone and MDPV treatment reduced expression in the VTA.

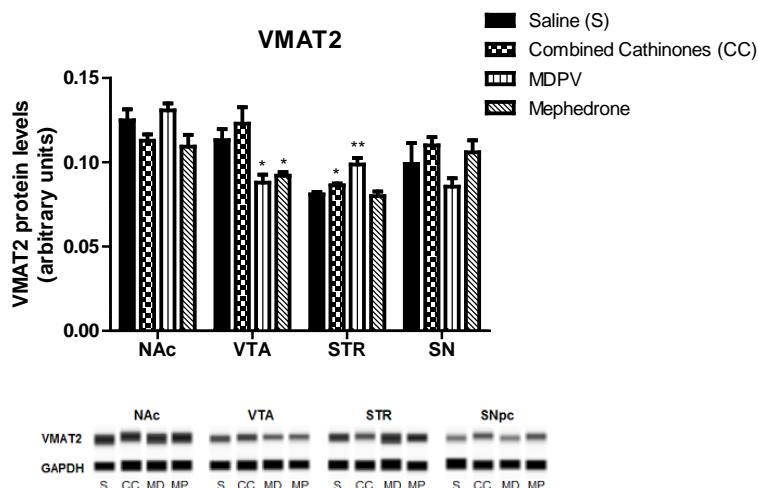


Figure 4.3 Relative expression level of VMAT2 in the NAc, VTA, STR, and SNpc of mice repeatedly administered MDPV, mephedrone, or the cathinone cocktail. Data are expressed as group means \pm SEM, $n = 4 - 6$ per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. saline.

DAT-1 and TH. To further investigate if chronic bath salt exposure affects the integrity of dopaminergic nerve terminals, expression of DAT-1 and TH, two major dopaminergic neuronal markers, were analyzed by ProteinSimple western blot analysis following repeated synthetic cathinone exposure (Figure 4.4). Statistical analysis showed significant reductions in mesolimbic and nigrostriatal DAT-1 levels following treatment with the cathinone cocktail, while only striatal reductions of DAT -1 were observed following individual exposure to MDPV and mephedrone (Fig 4.4A). Moreover, TH levels were significantly decreased in all brain regions of mice treated with the cathinone cocktail, as well as mephedrone alone (Fig 4.4B). Conversely, individual MDPV treatment significantly increased TH in all brain regions (Fig 4.4B).

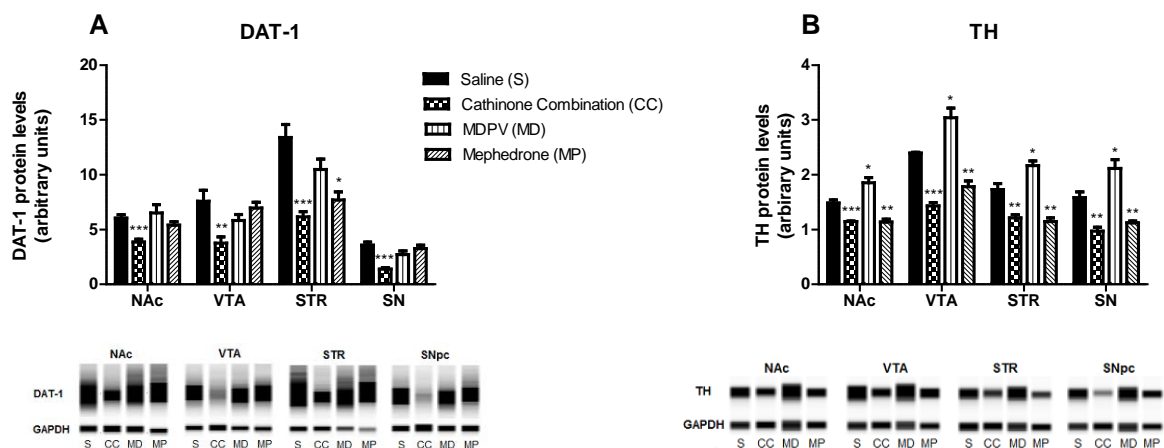


Figure 4.4 Effects of chronic exposure to MDPV, mephedrone, and the cathinone cocktail (10 mg kg⁻¹) on the relative expression levels of DAT-1 (A) and TH (B) in mice 48 h after dosing. Protein levels were normalized to GAPDH and are expressed at group means (n = 4 – 6) ± SEM. * p < 0.05, ** p < 0.01, *** p < 0.001 vs. saline.

Cytotoxic Effects of Synthetic Cathinones

The effects of the synthetic cathinones alone and in combination on LDH release over a 48 h time course are shown in Figure 4.5. The main effects of synthetic cathinone treatment, time after application, and their interaction were all highly significant (p < 0.0001). Post-hoc analysis revealed that all treatment groups increased LDH release over time (Fig 4.5A). Mephedrone (1 mM and 3 mM) and the cathinone cocktail significantly increased LDH release at all studied time points (6, 12, 24, and 48 h), while MDPV and methylone treatment significantly increased LDH release at 12, 24, and 48 h after exposure. Moreover, treatments including mephedrone trended significantly higher than either MDPV or methylone treatments, while LDH release due to 1 mM MDPV + 1 mM mephedrone + 1 mM methylone treatment was significantly greater than those observed with any of the individual drug treatments at 24 h and 48 h (Fig 4.5B).

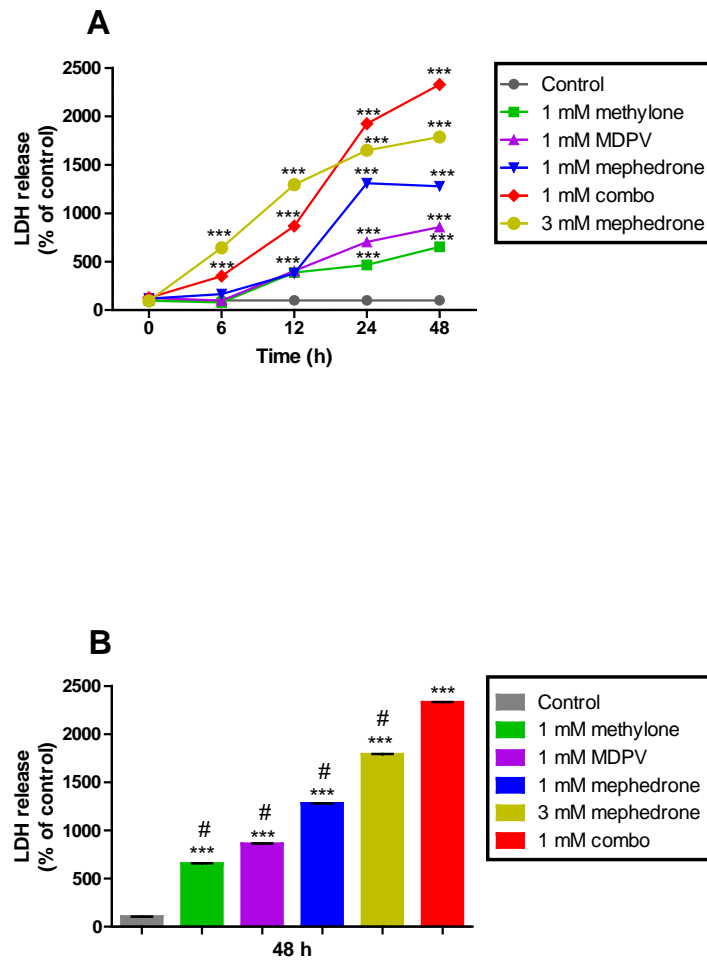


Figure 4.5 LDH leakage induced by MDPV, mephedrone, and methylone individually and in combination in dopaminergic SH-SY5Y cells 6, 12, 24, and 48 h after drug exposure (A). Experiments were performed in triplicate and the mean effects were normalized to Triton-X positive controls and untreated negative controls at each time point. (B) Represents all drug treatments at 48 h. *** $p < 0.001$ vs. control values, # $p < 0.05$ vs. combo (B).

Effects of Chronic Combined Administration of MDPV, Mephedrone, and Methylone on Neuroinflammation and Nigral DA Neuron Number

Glial reactivity. GFAP levels were measured in the striatum of mice chronically administered MDPV, mephedrone, methylone, or the cathinone cocktail to determine if

the synthetic cathinones induce neuroinflammatory responses at DA nerve terminals similar to other neurotoxic amphetamine derivatives. Data are presented in Fig 5 A. Treatment with MDPV, methylone, or the cathinone cocktail did not alter GFAP expression in regards to control, while striatal GFAP was significantly increased in the mephedrone treatment group.

DA neuron counts. To definitively assess the toxicity of drug mixtures containing MDPV, mephedrone, and methylone on DA neurons, systematic stereological analysis of TH-positive cells in the SNpc of mice treated with either saline or the cathinone cocktail was performed. As shown in Fig 5B, chronic exposure to the cathinone cocktail did not affect the number of TH-positive DA neurons in the SNpc.

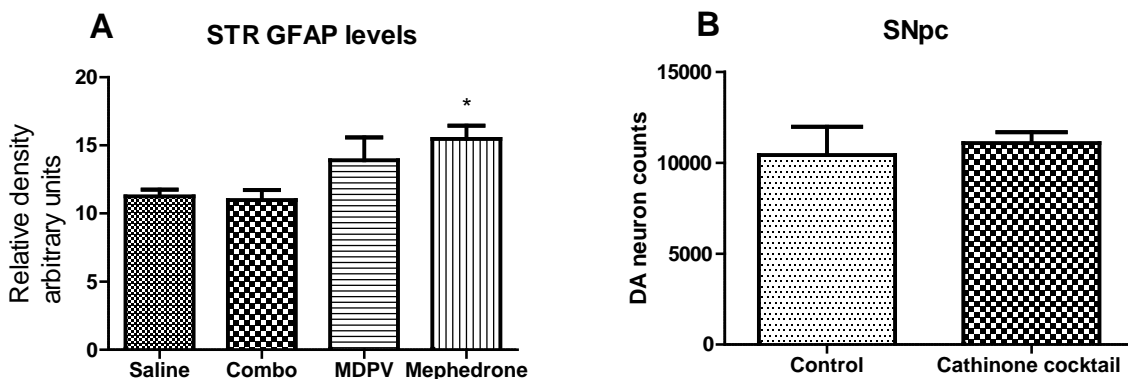


Figure 4.6 The effects of combined synthetic cathinone exposure on nigrostriatal toxicity. Mice were chronically treated with MDPV, mephedrone, or the cathinone cocktail and striatal levels of GFAP were determined 2 days later (A). GFAP levels were normalized to GAPDH and data are means for $n = 4 - 6$ mice per group (\pm SEM). Additionally, TH+ neurons were stained and counted in serial sections of the SNpc of mice repeatedly dosed with the MDPV + mephedrone + methylone cocktail (B). A nissl counter stain was used for landmark identification and data are expressed at group means \pm SEM (control $n = 2$, combo $n = 6$).

Discussion

The primary aim of the present study was to investigate the chronic effects of ternary mixtures of MDPV, mephedrone, and methylone on dopaminergic tone. While DEA statistics and numerous case reports indicate that many of the most commonly abused bath salt 'brands' contain various mixtures of these compounds (Araujo et al., 2015; Spiller et al., 2011), the majority of pre-clinical investigations on bath salts have focused on characterizing the effects of the individual constituents. Therefore, the rationale for analyzing the combined action of MDPV, mephedrone, and methylone on monoaminergic systems is compelling. Here, we have shown that chronic dosing of MDPV, mephedrone, or methylone alone induces prolonged DA elevations throughout the reward and motor control brain pathways. These data are supported by previous studies demonstrating that binge dosing regimens of cathinone, mephedrone, and methylone produce long-lasting elevations in extracellular DA levels (Angoa-Perez et al., 2012; Banjaw et al., 2003; López-Arnau et al., 2012), as well as studies reporting that MDPV administration precipitates persistent rises in extracellular DA and stimulates locomotor responses that persist for extended periods of time (Baumann et al., 2013; Schindler et al., 2016; Simmler et al., 2013).

Additionally, these persistent DA elevations appear to be associated with dysregulated expression of major DA regulatory proteins. Notably, repeated dosing of MDPV was accompanied by increased expression levels of the DA synthesis enzyme, tyrosine hydroxylase (TH), in all four brain regions. Given the potent blockade of DA reuptake by MDPV this is not surprising; cytosolic DA levels are depleted and increased TH densities may represent a compensatory mechanism for this effect (Vrana et al.,

1993). In contrast, decreased TH expression was observed in the DA nerve terminal regions of mice treated with mephedrone; however, the DA degradative enzyme COMT was also downregulated. These data are in support of previous findings indicating that mephedrone downregulates striatal TH levels (Lopez-Arnau et al., 2015) and causes long-term depletions in the COMT metabolite, homovanillic acid (HVA) (den Hollander et al., 2013). While COMT primarily functions to degrade DA and DOPAC, it can also degrade the DA precursor molecule, L-DOPA (Jones et al., 1998); thus, suppressed expression of COMT may contribute to the observed elevations in DA after repeated exposure to mephedrone by inhibiting degradation of both DA and the DA biosynthetic precursor molecule L-DOPA. Additionally, while mephedrone-induced reductions in TH have previously been suggested as evidence of its toxicity (Lopez-Arnau et al., 2015), we have shown here that this substrate cathinone can decrease TH without concurrently reducing DA and DAT. Thus, reduced TH expression may not be indicative of mephedrone toxicity.

Notably, repeated co-exposure to MDPV, mephedrone, and methyldone significantly reduced mesolimbic and nigrostriatal tissue levels of DA, TH, and DAT following a 48 h drug wash-out period. In addition, expression levels of MAO-B and COMT were also significantly reduced. These results are in contrast with the individual effects of MDPV and mephedrone and indicate a major dysregulation of the dopaminergic system when these compounds are co-abused in bath salt mixtures. As persistent depletions in DA and decreased expression of TH and DAT are well accepted gauges of neurotoxicity associated with chronic METH abuse (Cadet et al., 2007; Halpin et al., 2014), these data suggest that repeated co-abuse of bath salt constituents

MDPV, mephedrone, and methyldone, effectuates damage to dopaminergic nerve terminals. Therefore, it may be the case that while these compounds do not induce blatant DA toxicity individually, their co-abuse in bath salt mixtures produces potentially damaging interactions at dopaminergic terminals. This is supported by recent investigations that have described enhanced neurotoxicity when the synthetic cathinones were combined with other DAT-targeting drugs (Angoa-Pérez et al., 2013; Anneken et al., 2015). Additionally, recent studies have reported that self-administration of binary mixtures of MDPV and methyldone resulted in a high incidence of mortality (50 %), which was not observed with self-administration of either drug alone (Gannon et al., 2018).

To further assess if combined exposure to synthetic cathinone compounds enhances the toxic properties of individual drugs, LDH release assays were performed using dopaminergic SH-SY5Y cells treated with MDPV, mephedrone, and methyldone alone and in combination. All three compounds significantly increased LDH release over a 48 h time period. Notably, co-administration of MDPV, mephedrone, and methyldone exacerbated the cytotoxic effects of the individual drugs at both 24 and 48 h, suggesting that the toxic properties of these drugs at DA nerve terminals are enhanced when they are co-abused. To determine if this result was due to increased total synthetic cathinone exposure in the cocktail, which contained 1 mM doses of each individual drug (3 mM in total), a 3 mM dose of mephedrone was included in this study. Mephedrone dose-dependently increases LDH release (den Hollander et al., 2014) and has been shown by this study and others (den Hollander et al., 2014; Pantano et al., 2017; Valente et al., 2017) to be more cytotoxic than both MDPV and methyldone. Thus, the present finding

that the cathinone cocktail (1 mM) produced a cytotoxic response that was significantly greater than observed with 3 mM mephedrone at 24 and 48 h, supports the notion that the synthetic cathinones are more toxic at DAT-expressing terminals when co-administered in a cocktail.

However, in contrast to neurochemical and cytotoxicity findings, we found that chronic administration of the cathinone cocktail did not induce the loss of DA neurons in the SNpc, an area susceptible to METH and MPTP-induced toxicity (Ares-Santos et al., 2014; Thomas et al., 2004). While combined treatment may indeed promote degeneration of DA nerve terminals, DA nuclei counts were not reduced. Additionally, striatal levels of GFAP were not changed from control values in mice treated with the cathinone cocktail. This lack of blatant neurotoxicity may be due to the weak effects of mephedrone and methylone to increase cytosolic DA from vesicles in a VMAT2-dependent manner (Eshleman et al., 2013; Green et al., 2014), or of full drug wash out between doses, as METH toxicity is most notably observed following repeated, binge dosing (Halpin et al., 2014). However, in light of these findings and the enhanced DA responses with acute cocktail administration (Allen et al., in preparation), we suggest that initial exposures to the cathinone cocktail produced large, prolonged increases in DA that triggered a functional lesion in dopaminergic brain regions following later exposures and drug-wash out. More specifically, the excessive accumulation of DA in the synaptic cleft and enhanced DA receptor signaling triggers the downregulation of DAT, TH, MAO, and COMT, and results in decreased DA levels. However, a more detailed investigation regarding the downstream consequences of combined synthetic cathinone exposure on DA signaling cascades would be required to confirm this notion.

In summary, this study highlights the chronic neurochemical and biochemical consequences of individual versus combined synthetic cathinone abuse. We have shown that chronic individual exposure of MDPV, mephedrone, or methylone produced long-term elevations in DA that were accompanied by either increased TH levels or decreased COMT levels. In contrast, co-exposure of MDPV, mephedrone, and methylone resulted in depleted brain tissue DA levels 48 h following repeated dosing. We have also shown that these depletions are accompanied by decreased expression of TH, DAT, MAO-B, and COMT. These data together with findings of enhanced cytotoxicity with the cathinone cocktail, suggested a potential combined ability of MDPV, mephedrone, and methylone to induce DA toxicity. However, because combined treatment did not result in the loss of DA neurons in the SNpc or alter striatal GFAP expression, this does not appear to be the case. Thus, it appears that the cathinone cocktail may produce a “functional lesion,” downregulating many of the key players at the dopaminergic synapse while not inducing blatant neurotoxicity. Most significantly, this study has shown that the composition of bath salt mixtures can significantly influence the dopaminergic and toxic effects of the individual constituents.

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CHAPTER 5

SUMMARY AND CONCLUSIONS

In recent years, the abuse of synthetic cathinone derivatives, popularized as 'bath salts' has become a public health threat. Despite the designation of the three most prevalent synthetic cathinones, MDPV, mephedrone and methyldone, as schedule I controlled substances in 2011, abuse continues, with the internet serving as a central marketplace for these compounds (DEA 2014; Karila et al. 2015). Forensic analysis of bath salt products has revealed that many of the most popular bath salt 'brands' contain binary and ternary mixtures of MDPV, mephedrone, and methyldone; however, the majority of preclinical investigations on these designer drug mixtures has focused on elucidating the neurochemical and behavioral effects of each individual constituents (Spiller et al. 2011). Thus, to better model bath salt abuse, the effect of synthetic cathinone mixtures on the CNS should be assessed.

MDPV, mephedrone, and methyldone possess similar chemical and pharmacological characteristics to cocaine, methamphetamine, and MDMA. Namely, MDPV inhibits DA reuptake through direct binding at DAT, similar to cocaine but with greater potency (Baumann et al. 2013). Mephedrone and methyldone behave as DAT substrates and induce non-vesicular release of DA by reversing transporter flux, resembling the actions of both METH and MDMA (Baumann et al. 2012). Given that significant pharmacological interactions often occur following co-administration of drug compounds that share a primary biological target, their individual mechanisms offer opportunities for such interactions at monoamine transporters when taken together (Tallarida 2011). Specifically, while both non-substrate (MDPV) and substrate

(mephedrone and methylone) cathinones increase extracellular DA levels, the dichotomy of their interaction at the DAT provides an avenue by which these drugs may alter one another's dopaminergic effect when co-abused.

While interactions between the synthetic cathinones themselves are not well documented, evidence of enhanced neurotoxicity following co-exposure of the synthetic cathinones with other amphetamine derivatives has recently been described (Angoa-Pérez et al. 2016). In this case, while neither mephedrone nor methylone damage DA nerve endings, they accentuate the neurotoxic effects of METH, AMPH, and MDMA when co-administered (Angoa-Pérez et al. 2013; Anneken et al. 2015). While it could be predicted that these non-toxic substrates would compete for DAT-mediated uptake and thus weaken the toxic response of METH, AMPH, or MDMA, the findings in these studies did not indicate that effect. Therefore, it may be the case that while these compounds do not induce blatant DA toxicity individually, their co-abuse in bath salt mixtures produces potentially damaging interactions at dopaminergic terminals. Additionally, while the potent DAT-blocking action of MDPV has been shown to be neuroprotective against various amphetamine toxicities (Anneken et al. 2015), *in vitro* analyses suggest a synergistic interaction between mephedrone and MDPV at the DAT. Moreover, mephedrone enhances the locomotor stimulant effects of the DAT blocker, cocaine (Gregg et al. 2013b), and locomotor responses sensitize to drug mixtures of MDPV and mephedrone (Berquist et al. 2016). Overall, these preclinical findings demonstrate that the synthetic cathinones can significantly alter the DA effects of other DAT targeting drugs when they are co-administered. Therefore, considering the frequency of synthetic cathinone co-abuse in bath salts products, investigations into the

combined action of these drugs on dopaminergic systems are a necessary step in determining the pharmacology, abuse-related, and toxic effects of bath salts. The current work has addressed the lack of experimental data regarding the combined effects of MDPV, mephedrone, and methyldone on dopaminergic tone. Moreover, the studies described in this dissertation provide evidence of a significant pharmacological interaction between major bath salt constituents, MDPV, mephedrone, and methyldone.

The studies presented in Chapters 3 and 4 utilized a novel HPLC-ECD assay that was optimized for fast, sensitive, and simultaneous quantitative analysis of DA and its major metabolites in discrete DA nuclei including the substantia nigra pars compacta (SNpc) and the ventral tegmental area (VTA) (Allen et al. 2017). This method was also used to determine monoamine tissue content in the nucleus accumbens (NAc) and striatum (STR). Due to very sensitive limits of quantification for all monoamines and respective metabolites, right or left halves of discrete regions could be used for HPLC analysis while the other half could be used for protein expression or other various assays. Development and validation of this method in six discrete brain regions is described in chapter 2.

The study presented in Chapter 3 of this dissertation was the first to demonstrate a synergistic pharmacological effect of MDPV, mephedrone, and methyldone on DA levels throughout major dopaminergic brain pathways. These findings were extended to DA-mediated behavioral responses, as illustrated by the acute onset of robust stereotypic behavior and motor deficits with the cathinone cocktail. Such effects are primarily reported with very high doses of potent DAT- targeting drugs and have not been observed with any of the individual cathinones at the doses used in this study.

These findings shed light on two major issues: 1) bath salt formulations can have variable abuse liabilities based on their drug composition, and 2) chronic exposure to combined synthetic cathinones may be neurotoxic, given that excessive DA elevations have been attributed to meth-induced toxicity. The study detailed in Chapter 4 of this dissertation addresses the latter.

Chapter 4 evaluated the neurochemical and biochemical consequences of chronic co-exposure to MDPV, mephedrone, and methylone. Chronic dosing consisted of repeated drug injections, given every other day for 14 days (7 total injections) and analyses were conducted 48 h after the final exposure. In this study, chronic dosing of the individual synthetic cathinones induced persistent increases in DA that were accompanied by increased expression levels of the DA synthesis enzyme, tyrosine hydroxylase (TH), and decreased levels of DA degradative enzyme catechol-O-methyltransferase (COMT). However, when MDPV, mephedrone, and methylone were combined and chronically administered as a drug cocktail, DA, TH, DAT, MAO-B, and COMT levels were significantly reduced. Given that many of these effects are frequently used as neurochemical gauges of METH toxicity (Moratalla et al. 2017), we initially hypothesized that this indicated a potentially toxic interaction between these drugs at DA terminals. Furthermore, LDH release assays demonstrating that the individual cytotoxic effects of MDPV, mephedrone, and methylone are exacerbated when they are applied in conjunction with one another supported this notion. As such, further toxicity analyses were performed in the nigrostriatal brain regions, as these are especially susceptible to METH neurotoxicity (Ares-Santos et al. 2014). Surprisingly, we found that chronic dosing with the cathinone cocktail did not increase striatal levels of the

neuroinflammatory marker, GFAP, nor did it promote a loss of DA neuronal nuclei in the SNpc. Thus, it appears that DA, TH, and DAT levels are decreased without nerve terminal or nuclei toxicity. From these data, we proposed combined synthetic cathinones induce a “functional lesion,” whereby excessive increases in DA levels immediately following each injection of the cathinone cocktail results in a downregulation of many key players within the DA synapse after repeated dosing. This lack of blatant neurotoxicity may be due to the weak effects of mephedrone and methylone to increase cytosolic DA from vesicles in a VMAT2-dependent manner (Eshleman et al. 2013; Green et al. 2014), or of full drug wash out between doses, as METH toxicity is most notably observed following repeated, binge dosing (Halpin et al. 2014). Taken together, these data demonstrate that chronic abuse of synthetic cathinone mixtures produces a major dysregulation of the DA system and suggest that the toxicity of bath salts is largely dependent on the composition drugs within the mixture.

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