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DISCOVERY OF NOVEL PHARMACOTHERAPEUTICS FOR SUBSTANCE USE DISORDERS

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DISCOVERY OF NOVEL PHARMACOTHERAPEUTICS FOR SUBSTANCE USE DISORDERS

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Pharmacy
at the University of Kentucky

By
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Lexington, Kentucky
Director: Dr. Linda P. Dwoskin, Professor of Pharmaceutical Sciences
Lexington, Kentucky
2019

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ABSTRACT OF DISSERTATION

DISCOVERY OF NOVEL PHARMACOTHERAPEUTICS FOR SUBSTANCE USE DISORDERS

Substance use disorders are serious health concerns in the United States. Furthermore, the National Survey on Drug Use and Health reports a continuous increase in substance use disorders in the United States during the last 10 years. However, there are not many effective pharmacotherapeutics available for substance use disorders. The current dissertation is focused on research aimed at discovering pharmacotherapeutics for substance use disorders. First part of dissertation focused on discovering methamphetamine (METH) use disorder therapeutics targeting specific mechanism of METH action on dopaminergic neurons. The second part of dissertation focused on opioids and cocaine use disorder therapeutics targeting rewarding pathway commonly activated by opioids and cocaine.

With respect to METH, it induces release of dopamine (DA) in neuronal terminals by interacting with the vesicular monoamine transporter-2 (VMAT2) and DA transporter (DAT). VMAT2 inhibitors have been found by our research group to decrease METH-evoked DA release, METH-induced hyperlocomotion, and METH self-administration in rats. However, these VMAT2 inhibitors lacked selectivity and tolerance developed to these pharmacologic effects after repeated administration, thereby limiting their potential as pharmacotherapeutics for METH use disorders. In the current study, analogs from a novel scaffold were found to selectively inhibit VMAT2 and were evaluated using neurochemical and behavioral pharmacological approaches. *R*- and *S*-3-(4-methoxyphenyl)-*N*-(1-phenylpropan-2-yl)propan-1-amine (GZ-11610 and GZ-11608, respectively) exhibited 94- to 3450-fold selectivity for VMAT2 over human-ether-a-go-go (hERG) channel, DAT, serotonin transporter, and nicotinic acetylcholine receptors. GZ-11608 competitively and concentration-dependently inhibited METH-evoked DA release via VMAT2. Also, GZ-11610 (56-300 mg/kg, oral) and GZ-11608 (300 mg/kg, oral; 10-30 mg/kg, s.c.) reduced METH-induced hyperlocomotor activity in METH-

sensitized rats. Furthermore, GZ-11608 (1-30 mg/kg, s.c.) inhibited METH self-administration, cue- and METH-induced reinstatement in a dose-dependent manner, and 30 mg/kg (s.c.), 10 mg/kg (s.c.), and 17 mg/kg (s.c.) produced significant effect, respectively. Importantly, the GZ-11608-induced decrease in METH self-administration was not surmounted by increasing the amount of METH available. GZ-11608 did not substitute for METH and did not serve as a reinforcer in rats self-administering METH and drug naïve rats, respectively. Thus, these VMAT2 inhibitors incorporating a new scaffold are novel leads for new pharmacotherapeutics to treat METH use disorders.

Substances with high abuse potential including opioids and cocaine elevate extracellular DA concentration in the nucleus accumbens, and this mechanism has long been considered to underly substance-induced reward. DA in the nucleus accumbens originates from DA neuron cell bodies located in the ventral tegmental area in the midbrain. Interestingly, M5 muscarinic acetylcholine receptors (mAChRs) are proteins that are highly expressed on ventral tegmental area DA neurons. Also, studies investigating M5 mAChRs knockout mice showed reduced responding for cocaine in cocaine self-administration and decreased time spent in cocaine-paired and morphine-paired place preference studies. Pharmacological inhibition of M5 mAChRs function via microinfusing mAChR antagonists exhibiting no selectivity among M1-M5 mAChRs subtypes into the ventral tegmental area where expression of M5 mAChRs are dominant, reduced morphine-induced hyperlocomotion and cocaine seeking behaviors in rats. These studies support therapeutic potential of M5 mAChRs selectivity antagonists in opioids and cocaine use disorders. Thus, in the current study, affinity of a series of pethidine and quinuclidinyl *N*-phenylcarbamate analogs for M5 mAChRs was evaluated using *in vitro* and *ex vivo* neuropharmacological assays. Among the pethidine analogs, compound **6a** showed the highest binding affinity at M5 (Ki = 0.38 μM), but also high affinity at M1 and M3 mAChRs (0.67 and 0.37 μM, respectively). Among the quinuclidinyl *N*-phenylcarbamate analogs, compound **13c** exhibited the highest affinity at M5 (Ki = 1.8 nM), but also high affinity at M1, M2, M3 and M4 mAChRs (Ki = 1.6, 13, 2.6, 2.2 nM, respectively). Also, **13c** acted as an agonist of mAChRs on oxotremorine-induced DA release from rat striatal slices. In addition, compound **13b** was found exhibiting the highest selectivity (17-fold) at M3 over M2 mAChRs, suggesting potential of **13b** as a chronic obstructive pulmonary disease therapeutics. Taken together, these novel analogs serve as leads for further discovery of subtype-selective M5 mAChR antagonists that may have potential as therapeutics for substance use disorders, as well as for chronic obstructive pulmonary disease.

KEYWORDS: Substance Use Disorders, Methamphetamine, Cocaine, Opioids, Vesicular Monoamine Transporter-2, Muscarinic Acetylcholine Receptors.

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DISCOVERY OF NOVEL PHARMACOTHERAPEUTICS FOR SUBSTANCE USE DISORDERS

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LIST OF ABBREVIATIONS

[^{3}H]NMS	[^{3}H]N-methylscopolamine
5-HT	serotonin
6-OHDA	6-hydoxydopamie
ADHD	attention-deficit/hyperactivity disorder
ATP	adenosine 5'-triphosphate magnesium salt
AUC	area under the curve
cAMP	cyclic adenosine 5'-monophosphate magnesium salt
CDC	Centers for Disease Control and Prevention
COPD	chronic obstructive pulmonary disease
CPP	conditioned place preference
CS	conditioned stimulus
CR	conditioned response
CVD	conditional visual discrimination
D1R	D1 dopamine receptors
D2R	D2 dopamine receptors
DA	dopamine
DAT	dopamine transporter
DEA	Drug Enforcement Administration
delta-9-THC	delta-9-tetrahydrocannabinol
DOR	delta-opioid receptor
DOPAC	3,4-dihydroxyphenylacetic acid
Dofetilide	N-[4-[2-[methyl[2-[4-(methylsulfonamido)phenoxy]ethyl]amino] ethyl]phenyl]me-thanesulfonamide
DMT	dimethyltryptamine

DSM	Diagnostic and Statistical Manual Mental Disorders
ED	emergency department
EDTA	ethylenediamine tetraacetate
EGTA	ethylene glycol tetraacetate
FDA	Food and Drug Administration
GABA	gamma-aminobutyric acid
GBR-12935	1-[2-(diphenylmethoxy)ethyl]-4-(3-phenylpropyl)piperazinedihydro-chloride
GZ-11608	S-3-(4-methoxyphenyl)-N-(1-phenylpropan-2-yl)propan-1-amine
GZ-11610	R-3-(4-methoxyphenyl)-N-(1-phenylpropan-2-yl)propan-1-amine
GZ-793A	R-N-(1,2-dihydroxypropyl)-2,6-cis-di-(4methoxyphenethyl)pipe-ridine hydrochloride
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
hERG	human-ether-a-go-go-related gene
HPLC-EC	high-performance liquid chromatography with electrochemical detection
ICSS	intracranial self-stimulation
i.p.	intraperitoneal
i.v.	intravenous
KOR	kappa-opioid receptor
KO	knockout
L-dopa	L-3,4,-dihydroxyphenylalanine
LDT	laterodorsal terminal nucleus
LSD	lysergic acid diethylamide
mAChRs	muscarinic acetylcholine receptors
MAO	monoamine oxidase
MDMA	3,4-methylenedioxy-methamphetamine

MEM	minimum essential medium
METH	methamphetamine
MLA	methyllycaconitine
MP	mobile phase
MOR	mu-opioid receptor
NA	nucleus accumbens
nAChRs	nicotinic acetylcholine receptors
NE	norepinephrine
NET	norepinephrine transporter
NIC	nicotine
NIDA	National Institute on Drug Abuse
OFC	orbitofrontal cortex
PCP	phencyclidine
PEI	polyethyleneimine
PET	positron emission tomography
PFC	prefrontal cortex
RO4-1284	(2R,3S,11bS)-2-ethyl-3-isobutyl-9,10-dimethoxy-2,2,4,6,7,11b-hexahydro-1H-pyrido[2,1-α]isoquinolin-2-ol
ROS	reactive oxygen species
SAMHSA	Substance Abuse and Mental Health Services Administration
SAR	structure activity relationship
s.c.	subcutaneous
SERT	serotonin transporter
TH	hydroxylase
UNODC	United Nations Office on Drugs and Crime
UR	unconditioned response

US	unconditioned stimulus
U.S.	United States
VMAT2	vesicular monoamine transporter-2
VTA	ventral tegmental area

1. CHAPTER ONE: INTRODUCTION

1.1 Substance Use Disorders

The term, substance use disorders, is the appropriate terminology to refer to what used to be known as drug addiction or drug abuse. The National Institute on Drug Abuse (NIDA) defined substance use disorder as a brain disease manifested by compulsive substance seeking and obtaining behaviors, despite negative consequences (Volkow, Baler, et al., 2011; Volkow et al., 2016). In general, the word, drug, is used as two distinct definitions. One definition is substances or medications that potentially treat or prevent disease through modifying biochemical or physiological processes. The second definition is substances specifically altering mental processes (e.g., cognition, mood) with potential for misuse. In this dissertation, drugs and substances are used particularly to refer to the second definition. Also, since this dissertation is focused on discovering new pharmacotherapeutics/medications for patients who are diagnosed with substance use disorders, the terminology, substance use disorders instead of drug addiction, is used in this dissertation.

The Diagnostic and Statistical Manual Mental Disorders (DSM) provides standard criteria for mental health disorders diagnosis. Diagnostic criteria for substance use disorders are provided also by DSM, which has been published by the American Psychiatric Association since 1952.

Up until the 1970's, substance use disorders were commonly considered as a harmful habit of individuals or as reduced responsiveness of individuals, instead of as a brain disease (Stedman, 1976). Correspondingly, substance use disorders were classified as a secondary mental disease under 'Sociopathic Personality Disturbance' in DSM-I and under 'Personality Disturbance' in DSM-II, published in 1952 and 1968, respectively

(American Psychiatric Association, 1968; Robinson and Adinoff, 2016). However, over time, neurobiological observations on the long-lasting structural and functional brain changes following repeated substance use were accumulated, and which led perceptional changes in the point of view that substance use disorders as 'a lack of personal responsibility' to 'a brain disease' (neurobiological findings are discussed in a section 1.3).

In agreement with this change in perspective, substance use disorders were categorized as a primary mental disease beginning with the third edition of the DSM (DSM-III) published in 1980. In the DSM-III, two diagnostic terms were used to distinctly diagnose patients with substance use disorders depending on the severity of the disease (American Psychiatric Association, 1980). "Substance abuse" was the diagnostic term used for relatively less severe individuals. Substance abuse was diagnosed for individuals exhibiting the following behaviors: 1) pathological substance use pattern (i.e., repeated failure to stopping or reducing use of substances, and required daily use of substance to perform adequate function), 2) experiencing impaired social or job-related function due to the pattern of pathological substance use, and 3) longer than a month of duration for pathological substance use. On the other hand, "substance dependence" was the term for more severe cases. Substance dependence generally referred to more severe substance use disorders, which requires physiological dependence exhibiting either tolerance or withdrawal. Tolerance is defined as a diminished effect of equal amount of the substance used or as the necessity to use increased amount of substance to provide an equivalent effect in the same individual. Withdrawal is a series of substance-specific symptoms occurred in the abstinence of or reduced use of substances (American Psychiatric Association, 1980).

The DSM-IV published in 1994 retained these two diagnostic terms with minor modifications. The first minor modification was that substance use disorder, which was a

primary category in DSM-III, became a subcategory of a primary category, 'substance-related disorders'. Also, a 'substance-induced disorder' subcategory was added under substance-related disorders. Thus, the 'substance-related disorders' consist of 'substance use disorders' and 'substance-induced disorders'. The second minor modification in DSM-IV compared to DSM-III was that tolerance and withdrawal were added as diagnostic terms under substance-induced disorders. There were accumulated individuals who are seeking treatment for chronic substance use with exhibiting negative consequences without developing tolerance or withdrawal. Conversely, tolerance and withdrawal without producing addiction were observed following use of medications acting on the central nervous system (e.g., sedatives: benzodiazepines (Valium and Xanax) and barbiturates (Nembutal); antidepressants: selective serotonin reuptake inhibitors (Prozac, Soloft, Paxil, and Lexapro)). Regarding such modifications, the substance dependence criteria in the DSM-IV were 1) pattern of compulsive substance uses (i.e., larger amounts were taken often more than was intended, important social activities are reduced or given up due to substance use) with and without exhibiting 2) tolerance and/or 3) withdrawal. Whereas substance abuse diagnosis criteria include only the negative consequences of repeated substance use (i.e., repeated substance use resulting in failure to perform major obligations at work, school or home, repeated arrests for substance-related legal problems) without pattern of compulsive substance use, tolerance, or withdrawal (American Psychiatric Association, 1994). Also, the DSM-IV specifically defined the word "substance" as drugs exhibiting abuse liability, which include legal and illegal substances that modify mood or behaviors.

In the most recent edition, DSM-V, the two diagnostic terms, substance abuse and substance dependence were combined within "substance use disorder" to reduce confusion between dependence and addiction. The severity of the disease is indicated as

mild, moderate or severe instead of using two separate diagnostic terms (substance abuse or dependence). Due to the criteria difference between DSM-III, -IV verses -V it is difficult to directly compare; generally, substance abuse (DSM-III, -IV) refers to mild (DSM-V), and substance dependence (DSM-III, -IV) refers to moderate to severe substance use disorders (DSM-V). In DSM-V, 'non-substance-related disorders' was added, which include gambling disorders. Thus, the name of the primary category was changed from 'substance-related disorder' to 'substance-related and addictive disorders'. Thus, substance-related and addictive disorders consist of substance-related and non-substance-related disorders. The 'substance-related disorders' is further subcategorized to 'substance use disorders', 'substance intoxication', and 'substance withdrawal' and DSM-V provides specific diagnostic criteria for each subcategory. The diagnostic criteria of the DMS-V for substance use disorders are discussed in the section 1.1.2.

Notably, drug addiction has not been used as a diagnostic term in the DSM, except the first edition. According to the DSM-V, the term 'addiction' was omitted from the DSM due to the underlying negative connotation or stigma, and its ambiguous definition (American Psychiatric Association, 2013).

This dissertation describes research aimed at discovering potential pharmacotherapeutics for individuals diagnosed with substance use disorders. Thus, the official diagnostic term, "substance use disorders" is used instead of drug addiction. Also, the word "substances" in this dissertation specifically refers to drugs exhibiting abuse liability. In following subsections, various classes of substances (section 1.1.1) and the detailed diagnostic criteria for substance use disorders in DSM-V (section 1.1.2) are provided.

1.1.1 Substance Classifications

Self-administration of substances that have abuse potential could result in serious health problems including the development of substance use disorders. Thus, the United States (U.S.) Drug Enforcement Administration (DEA) classifies substances into five groups depending on their medical use and abuse potential in order to provide appropriate information to the public and to control the legal and illegal uses of substances. Also, the DSM-V provides substance classifications based on the similarity of effects induced by substances, which can aid in efficient diagnoses of substance abuse. There are several substances that result in considerably similar physical and psychological effects, although their chemical structures are not the same (e.g., amphetamine and methamphetamine (METH)). To provide a brief introduction of various substances and related substance use disorders, this section introduces the two substance classification systems provided by the DEA and DSM-V in section 1.1.1.1 and 1.1.1.2, respectively.

1.1.1.1 Substance Classification for Controlling and Trafficking of Substances

The Federal Comprehensive Drug Abuse Prevention and Control Act of 1970, commonly known as the "Controlled Substances Act," was activated on May 1, 1971 (Gabay, 2013; DEA, 2018). As part of the Act substances are categorized based on their medical use and relative abuse liability. This categorization provides appropriate substance-related information with the aim to reduce the spread of substance use disorders, while ensuring medical availability of substances (DEA, 2018).

According to the law, substances are classified into one of five classes (Schedules I-V). Substances that exhibit high abuse liability and have no accepted medical use are

classified as Schedule I. Substances that exhibit high abuse liability and also have legitimate medical uses, are classified as Schedule II. If substances have a legitimate medical use and relatively lower abuse liabilities compared to Schedule II substances, then they are classified as Schedule III, IV, or V depending on their relative abuse liability in descending order. For example, two substances exhibiting high abuse liability, heroin and lysergic acid diethylamide (LSD) have no accepted medical use, and thus are classified as Schedule I (DEA, 2017).

The abuse liability of substances is determined based on their ability to induce stimulant, depressant or hallucinogenic effects (DEA, 2018). Schedule II substances include morphine, codeine, fentanyl, methadone, hydrocodone, phencyclidine (PCP), oxycodone, cocaine, and METH, all of which have accepted medical uses, but have high abuse liability. For example, METH exhibits distinct stimulant effects in individuals at >10 mg (oral) or at 15 ng/mL plasma (Huestis and Cone, 2007; Sevak et al., 2009). However, METH is currently available under the product name, Desoxyn (5 mg/tablet, oral) as a legal medication for attention deficit disorder, severe obesity, and narcolepsy as a result of the accumulated scientific evidence supporting its pharmacological effects (Huestis and Cone, 2007; Sevak et al., 2009; Rau et al., 2016).

Schedule III consists of substances such as anabolic steroids, ketamine, and some barbiturates, which have moderate to low abuse potentials (Morgan, 1990; Lukas, 1996; Liu et al., 2016). Substances classified as Schedule IV have legitimate medical uses with relatively lower abuse liabilities compared to Schedule III substances and includes pentazocine, alprazolam, clonazepam, and pemoline (Chambers et al., 1971; Polchert and Morse, 1985; Mumford et al., 1995; Frauger et al., 2011). Schedule V includes substances used medicinally and exhibit the lowest abuse liabilities among controlled

substances. For example, cough medicines with codeine (<200 mg/100 mL) are examples of Schedule V substances (Gabay, 2013; DEA, 2017).

Laws surrounding the importation and exportation, manufacturing, distribution of substances are based on schedule and are controlled by law (DEA, 2018a; b). The synthesis, selling and use of Schedule I substances are illegal, with the exception of research purposes, which in turn requires DEA approval. However, the synthesis, selling and use of substances in Schedules II-V categories are legal when approved by the DEA. Thus, research in the current dissertation requiring the use of a Schedule II substance, METH, was conducted appropriately as allowed by law.

1.1.1.2 Classification for Substance Use Disorder Diagnosis

The DSM-V classifies problematic substances into 10 classes: alcohol, caffeine, cannabis, hallucinogens, inhalants, opioids, sedatives/hypnotics/anxiolytics, stimulants, tobacco and other or unknown substances (American Psychiatric Association, 2013). The alcohol class includes all beverages containing a compound called ethyl alcohol or ethanol (e.g., beer, wine, whiskey). Individuals who exhibit pathological use (see criteria in section 1.1.2) of any kind of ethyl alcohol containing beverage are diagnosed as having alcohol use disorder.

Caffeine (1,3,7-trimethylpurine-2,6-dione) is often consumed via coffee, tea, soda, and energy drinks. Due to the lack of clinical significance and the prevalence of caffeine use disorder, there is no diagnostic criteria for caffeine use disorder. However, there are diagnostic criteria for caffeine intoxication and withdrawal due to the sufficient prevalence and clinical significance of each (American Psychiatric Association, 2013).

Cannabis refers to all substances containing parts of cannabis plants (e.g., Cannabis sativa and Cannabis indica) as well as their corresponding synthetic derivatives. Cannabis plant products such as leaves, stems, flowers and seeds contain over 480 component (DEA, 2017). Delta-9-tetrahydrocannabinol (delta-9-THC) is a well-known psychoactive component of cannabis. The Food and Drug Administration (FDA) has approved pharmacotherapeutics containing synthetic delta-9-THC (a psychoactive component of cannabis) or its derivative; however, the pharmacotherapeutical use of the whole plant is not approved. FDA-approved pharmacotherapeutics containing delta-9-THC are controlled as either Schedule II or Schedule III depending on their relative abuse potential (Whiting et al., 2015). DSM-V provides diagnostic criteria for cannabis use disorder, intoxication and withdrawal.

The DSM-V also includes hallucinogens, which include many distinct chemical structures and different underlying neurochemical mechanisms of action. The definition of hallucinogen is based on their common effects, which are the ability to cause alterations in perception of the surrounding environment and of inner psychological processes (e.g., consciousness, cognition, and mood) (Martinotti et al., 2018). Interestingly, many research articles classify cannabis and its psychoactive component, delta-9-THC, as hallucinogens. The DSM-V classifies cannabis and hallucinogens separately due to their distinct psychological and behavioral effects (American Psychiatric Association, 2013; Garcia-Romeu et al., 2016). The DSM-V further provides separate diagnostic criteria for two different subclasses of hallucinogens (phencyclidines and other hallucinogens) based on their distinct physiological and behavioral consequences. The 'phencyclidines' subclass include PCP and other aryl-cyclohexyl amines such as ketamine, which commonly result in nystagmus, analgesia and remarkable hypertension (American Psychiatric Association, 2013). Other hallucinogens contain several chemical subclasses including

phenylalkylamines (e.g., mescaline, 3,4-methylenedioxy-methamphetamine (MDMA)), indoleamines (e.g., psilocybin, dimethyltryptamine (DMT)), and ergolines (e.g., LSD, morning glory seeds), all of which exhibit common hallucinations without specific physiological consequences observed in phencyclidines use disorders.

The inhalants class includes invisible and volatile hydrocarbon-based inhalant substances such as toluene, xylene, propane, butane, which are often contained in glue, fuels, spray paints, paint thinner, air conditioning refrigerant, and plastic cement (American Psychiatric Association, 2013). Although substances in different classes can be administered via inhalation, the inhalants class specifically includes substances taken only by inhalation.

Opioids refer to products of opioid-containing plants or pharmacologically similar synthetic chemicals including components extracted from the plant (also called as opiates; morphine, codeine), semi-synthetic opioids (derived from opiates; heroin, oxycodone, hydrocodone), and synthetic opioids (fully synthesized compounds exhibiting considerably similar effects with opiates; propoxyphene, fentanyl, methadone). This class of substances are often found in prescribed pain medications (see also section 1.6).

The seventh substance class in the DSM-V is the “sedatives, hypnotics, or anxiolytics” including all prescription sleeping and most antianxiety medications. Since there is no significant misuse reported, the DSM-V notes that nonbenzodiazepine antianxiety medications are not included in current substance class. However, benzodiazepine or benzodiazepine-like substances (e.g., zolpidem, zaleplon), carbamates (e.g., meprobamate, glutethimide), barbiturates (e.g., secobarbital), and barbiturate-like substances (e.g., glutethimide, methaqualone) are in the sedative, hypnotics, or anxiolytics class.

The eighth class is stimulants. The stimulant class consists of legal and illegal substances including cocaine, amphetamine, amphetamine-type substance (e.g., METH, dextroamphetamine, and other synthetic compounds containing a substituted-phenylethylamine structure). Stimulants class also include methylphenidate, which is not structurally similar but has stimulant like effects. Also, there are unspecified stimulants, which refer substances exhibiting stimulant-like effect, but have no structural similarity with cocaine, amphetamine, or methylphenidate. The plant-derived substance, khât is the example of unspecified stimulant. Additional information on cocaine and METH is provided in section 1.4 and section 1.5, respectively.

The ninth class in the DSM-V is tobacco. Tobacco is a common name of the plant Nicotiana, which includes more than 70 species worldwide. Among them, Nicotiana tabacum and Nicotiana rustica are the major species grown for commercial tobacco products around the world (Charlton, 2004). Tobacco contains nicotine (3-(1-methyl-2-pyrrolidinyl)pyridine), which is considered to be the main psychoactive substance leading to tobacco use disorders. Nicotine in tobacco is often consumed as cigarettes or cigars.

The last class is designated as ”other or unknown substances” and encompasses substances that are not able to be classified within the other classes above, but that produce clinical impairment or distress in individuals. This class includes anabolic steroids, cortisol, nitrous oxide, nitrates, nonsteroidal anti-inflammatory medications, and kava (from a South Pacific pepper plant).

The classification introduced by the DSM-V is based on similarity of physical and psychological effects induced by substances, which allows appropriate diagnosis for substance use disorders. The following section includes diagnostic criteria for substance use disorder introduced by the DSM-V. Depending on the class of substance consumed

by the individual, a specific substance use disorder is diagnosed (e.g., METH use disorder, cocaine use disorder). This dissertation is focused on METH and cocaine use disorders and on opioids use disorders. The DSM-V provides substance-specific diagnostic criteria. However, the essential feature of all of the substance use disorders is defined as continuous use of the substance despite negative consequences. Thus, DSM-V provides both general and substance-specific diagnostic criteria for substance use disorders for all substance classes. The general diagnostic criteria for substance use disorder are commonly applicable to all substances, with the exception of the caffeine class. The general diagnostic criteria for substance use disorder is introduced in the section

1.1.2 Diagnostic Criteria for Substance Use Disorders

Since this dissertation is focused discovering pharmacotherapeutics for substance use disorders including METH, cocaine, and opioids use disorders, the general diagnostic criteria for substance use disorder is introduced in this section.

In the DSM-V, there are a total of 11 diagnostic criteria, consisting of four different groups of pathological patterns of behaviors related to substance use. The four groups include impaired control (Criteria 1-4), social impairment (Criteria 5-7), risky use (Criteria 8-9), and pharmacological criteria (Criteria 10-11). The first group consists of behaviors that are evidence of impaired control over substance use and include four diagnostic criteria. Criteria 1 is that the individual obtains the substance over a longer period or in larger amounts than was initially intended, Criteria 2 is that the individual experiences multiple failures to discontinue or decrease use of the substance. Criteria 3 is that the individual spends excessive amounts of time on obtaining the substance, consuming the substance, or recovering from effects of the substance. Criteria 4 is that the individual

experiences cravings, which is manifest as experiencing a robust desire or urge for the substance and experiencing difficulty to think about anything else other than the substance.

The second group of behaviors demonstrate social impairment, which includes three diagnostic criteria 5-7. Criteria 5 is that the individual continues using the substance despite having difficulty fulfilling major obligations at school, work, or home, Criteria 6 is that the individual obtains the substance even when experiencing problems in social or interpersonal activities caused or exacerbated by the substance effects. Criteria 7 is that the individual shows reduced time or gives up the opportunity for important social and recreational activities due to the substance use.

The third group of behaviors reveal the risky use of the substance and is associated with Diagnostic Criteria 8-9. Criteria 8 is that the individual continues substance use despite resulting in physically hazardous situations. For example, the individual drinks alcohol while driving a car or operating machinery, Criteria 9 is that the individual exhibits recurrent substance use in spite of knowledge that obtaining the substance has caused or exacerbated their physical or psychological problems.

The last group of behaviors are the pharmacological indicators (tolerance and withdrawal). Criteria 10 is that the individual experiences tolerance, indicated by either obtaining increased amount of the substance to accomplish the desired effects or experiences decreased effect when the same amount of substance is consumed, Criteria 11 is withdrawal that results from reduced substance concentration in blood or tissue in the individual previously maintaining heavy use due to reduced or stopped substance use. Withdrawal manifests when the substance is used to attenuate or avoid withdrawal, or when reduced substance use results in specific withdrawal symptoms elicited. Withdrawal is differentially characterized depending on the substance consumed. For example, opioid

withdrawal is characterized by dysphoric mood, nausea or vomiting, muscle aches, lacrimation or rhinorrhea, pupillary dilation, piloerection or sweating, diarrhea, yawning, fever, or insomnia. If the individual exhibits three or more of these withdrawal symptoms after several weeks of cessation, opioid withdrawal is the diagnosis. Another example is cocaine, METH, or other stimulant withdrawal, which is characterized by dysphoric mood and two or more of symptoms including fatigue, vivid and unpleasant dreams, insomnia or hypersomnia, increased appetite, psychomotor retardation or agitation within a few hours to several days after the prolonged cessation of stimulant use. Substance specific withdrawal symptoms are included in the DSM-V.

In summary, an individual is diagnosed as having a mild, moderate, or severe substance use disorder if they exhibit 2-3, 4-5, or more than 6 of the diagnostic criteria, respectively, within a year. Based on these diagnostic criteria obtained in human clinic, there are multiple animal behavioral procedures designed with the aim of modeling the above criteria to support research including drug discovery (Lynch et al., 2010; Blanco-Gandia et al., 2015; Kenny et al., 2018). Thus, this dissertation also employed several of these animal behavioral procedures such as self-administration and reinstatement to evaluate the potential for the compounds to be evaluated as a pharmacotherapeutic for substance use disorder.

1.2 Epidemiology of Substance Use Disorders

According to the Substance Abuse and Mental Health Services Administration (SAMHSA) in 2016 in the U.S., the number of individuals using illegal substances and misusing prescription substances was 28.6 million, which was 10.6% of the population aged 12 or older (SAMHSA, 2017a). As such, in 2016, one American out of ten (aged 12

or older) used illicit substances or misused prescription substances, which was higher than in 2002 (8.3% of the population aged 12 or older) or in 2013 (9.4% of the population aged 12 or older). These reports indicate continued escalation of misuse substances (SAMHSA, 2014a).

Furthermore, multiple studies also report increased numbers of substance-related emergency department (ED) visits and substance overdose deaths. According to the Centers for Disease Control and Prevention, the number of nationwide ED visits involving illegal and prescription substance misuse increased from 3.3 million to 5.1 million between 2006 and 2011 (Crane, 2013). Rates of substance-related ED visits showed a pattern of increases (1,838 in 2006 to 2,519 in 2013 per 100,000 population aged 15-year and older) (Weiss et al., 2016). ED visits specifically associated with substance related disorder diagnoses increased by 73.7% in 2014 compared to 2006 (Moore et al., 2017).

According to the Centers for Disease Control and Prevention (CDC), the number of substance overdose-induced deaths increased by 86.1% from 2006 to 2016 (CDC, 2017). Specifically, the top two classes of substances, opioids and stimulants, contributed to the death of Americans of more than 40,000 per year since 2011 (Office of National Drug Control Policy (ONDCP), 2016). Also, between 2000 and 2014, every 15 min one American died due to use of illicit opioids or misuse of prescribed opioids (Worley, 2017). Since 2009, a greater number of individuals died due to the misuse of substances than motor cycle accidents (DEA, 2017).

Among the 24.6 million Americans (aged 12 or older) reporting past-month illicit substance use in 2013, 22.6% were 18 to 20 years of age. With respect to the 21 to 25 years of age individuals, they represent 20.9% of the total population of illicit substance users (NIDA, 2015). Taken together, in 2013, individuals between 18 and 25 years of age,

represented 43.5% of population of illicit substance users. When comparing to the total population, one out of four (23.2%) individuals 18 to 25 years of age in the U.S. in 2013 reported past-month illicit substance use, which was increased compared to 2006 in which 19.8% of American 18 to 25 years of age reported past-month illicit substance use (SAMHSA, 2017a; b). Additionally, 7% of young adults aged 18 to 25 were diagnosed for illicit substance use disorders in 2016 (SAMHSA, 2017b). The 26-year or older age group showed lesser increases (7.3% of total population aged 26 years or older in the U.S.) in 2016 compared to 2013 (8.9% of total population aged 26 years or older in the U.S.). Furthermore, according to the NIDA, there were 2.8 million new illicit substance users in 2013, and 7,800 individuals initiated illicit substance uses per day (NIDA, 2015). More than half (54.1%) of new substance users were teenagers (10 to 18 years old) (NIDA, 2015).

Moreover, substance use disorders are responsible for high social costs. Estimated annual costs of overall substance use disorders have been as much as $740 billion including costs related to health care, lost work productivity, and crime (ONDCP, 2016). Among that , the estimated social costs of “illicit substance use” was total $193 billion (ONDCP, 2016). Notably, social costs ($78.5 billion) associated with prescription opioid misuse has increased by more than $20 billion every year between 1997 and 2013. Along with recent dramatic increases in opioid use disorder, social costs associated specifically with heroin use disorder increased also from $21.9 billion in 1996 to $51.2 billion in 2015 (Jiang et al., 2017; Knipper et al., 2017).

There are 162 to 324 million people reporting substance use including illicit substance and misuse of prescribed medications worldwide in 2013. Among them, 10% (16 to 39 million) are diagnosed as having substance use disorders (United Nations Office on Drugs and Crime (UNODC), 2014). In addition, relapse rate of substance use disorders

is 40-60% (McLellan et al., 2000). These support that substance use disorder is a long-term disorder in a considerable number of individuals. For example, METH use disorder is characterized by high relapse rates, but there are no FDA-approved medications available to treat them. For METH users, 87% experience relapse within 5 years (Wang et al., 2012; Brecht and Herbeck, 2014).

Taken together, the number of substance users is continuing to grow in the U.S., especially among the young adults. Furthermore, a 40-60% rate of relapse has been reported in individuals with substance use disorders (McLellan et al., 2000). Thus, this dissertation focuses on pharmacotherapeutic discovery for opioids use disorder, which is the current second most common substances in the United States after cannabis, and cocaine and METH use disorders, which have no FDA accepted pharmacological therapeutics to treat cocaine and METH use disorders currently (SAMHSA, 2017a).

1.3 Neurochemical and Behavioral Pharmacology Underlying Substance Use Disorders

As described in section 1.1.1, there are various classes of substances where following repeated administration has the potential to result in substance use disorders. In the process of developing a substance use disorder, there are shared underlying mechanisms for many classes of substances. In this section, the progress of development of substance use disorder is introduced as three separated stages, including: (1) voluntary phase of substance intake, (2) transition phase from voluntary to compulsive substance seeking and taking, and (3) the craving and relapse phase that occurs after abstinence. The relevant neurochemical and behavioral pharmacology are discussed.

1.3.1 Voluntary Phase: Substance Intake

1.3.1.1 Substance-Induced Reward

Initially, substance intake results in rewarding effects, like other natural rewards such as food, via activating specific neuronal circuits in brain. This is known as the brain reward circuitry (Koob and Volkow, 2016; Volkow et al., 2016). From the behavioral neuropharmacology perspective, the term reward is defined as a stimuli that elicits approach responses (White, 1989). Multiple neuropharmacological studies reveal that the midbrain dopamine (DA) system is highly involved in substance-induced rewarding effects (Wise and Rompre, 1989; Leone et al., 1991; Di Chiara, 2002; Wise, 2008). The mesocorticolimbic DA system includes DA projections from the ventral tegmental area (VTA) to the nucleus accumbens (NA), frontal cortex, and amygdala. Specifically, substances increase extracellular DA in the NA, which is one of the major mechanisms mediating substance-induced rewarding effects (Koob, 1992; Di Chiara, 2002; Wise, 2008; Volkow et al., 2016).

Human brain imaging studies using positron emission tomography (PET) scans found that substances induce DA level increase in the NA. The amount of [^{11}C]raclopride (an antagonist of DA receptors) bound to DA receptors in the NA was detected by PET prior to and after amphetamine or alcohol administration. [^{11}C]Raclopride binding in the NA was decreased after amphetamine (0.3 mg/kg, i.v.; a stimulant) or alcohol (1 mL/kg in orange juice, oral) administration relative to baseline (prior to substance administration), indicating substance induced increase of DA extracellular concentrations in the NA (Drevets et al., 2001; Boileau et al., 2003). Importantly, this increase in DA transmission in the NA as a response to amphetamine (0.3 mg/kg, i.v.) is highly correlated with

responses for “hedonic feeling” and “substance wanting” in humans (Drevets et al., 2001; Leyton et al., 2002).

Most of substances, that engender substance use disorders in human, increase extracellular DA in studies using experimental animals (Balster et al., 1976; Ettenberg et al., 1982; Kenny et al., 2018). Thus, various animal behavioral models have been used to understand underlying the mechanisms and pathophysiology of substance use disorders. One of the most popular animal behavioral models to study substance use disorders is self-administration (Panlilio and Goldberg, 2007; Lynch et al., 2010). The self-administration procedure is conducted in an enclosed apparatus called an operant conditioning chamber or operant chamber. To conduct self-administration, experimental animals are placed in the chamber containing a lever(s) or a nose-poke hole(s). The voluntary behavior of the animal in the chamber, such as pressing the lever, results in delivery of reinforcers, typically food pellets, sucrose solution, or intravenous (i.v.) infusion of substances. The underlying principal of the self-administration method is that if a voluntary behavior results in delivery of reward (a positive reinforcer), then this behavior producing reinforcement is repeated with high probability (Panlilio and Goldberg, 2007). Self-administration is considered as the gold standard method in research on substance use disorders (Mews and Calipari, 2017). Often, food pellets or infusion of substance are used as a positive reinforcer during the initial training for substance self-administration. When food pellets are used for initial training, then the food pellets are replaced with i.v. infusion of substances in the later phase of training. Experimental animals including rodents and nonhuman primates acquire and maintain operant behavior to self-administer most substances including METH, cocaine, and opioids (Flagel et al., 2010; Kenny et al., 2018). As such, this dissertation includes self-administration studies.

Nonhuman primates, male Macaca Fasciularis monkeys, acquire cocaine (0.5 mg/kg/infusion) self-administration behavior and exhibit increased extracellular DA in the NA during the self-administration as determined using microdialysis (Bradberry et al., 2000). Likewise, rodents also readily acquire self-administration behavior, which have shown by numerous studies, with substances such as METH, cocaine, and opioids (Flagel et al., 2010; Kenny et al., 2018). Also, a series of in vivo microdialysis studies in rats revealed that DA in the NA was increased not only during self-administration, but also following acute injections of many classes of substances including stimulants (e.g., cocaine at 5.0 mg/kg, subcutaneous (s.c.) and amphetamine at 0.25-3.0 mg/kg, s.c.), opioids (e.g., morphine at 0.5-10.0 mg/kg, s.c. and methadone at 1.0-5.0 mg/kg, intraperitoneal (i.p.)), nicotine (0.6 mg/kg, s.c.), and alcohol (0.5-2.5 mg/kg, i.p.) (Imperato and Di Chiara, 1986; Imperato et al., 1986; Di Chiara and Imperato, 1988b; a; Di Chiara, 1991; Solinas et al., 2002).

Conversely, when selective lesion of the DA neurons in the NA occurred in drug naïve rats by infusing 6-hydoxydopamie (6-OHDA) bilaterally into the NA prior to self-administration operant training, the acquisition of self-administration of amphetamine (0.125 mg/kg/infusion, i.v.) was impaired, indicating that DA in the NA serves a crucial role in acquisition of substance self-administration behavior (Lyness et al., 1979). When the same lesion occurred in rats trained for amphetamine (0.125 mg/kg/infusion, i.v.) or cocaine (0.75 mg/kg/infusion, i.v.) self-administration, responses of rats for amphetamine or cocaine infusions were reduced to 5% or 30% of their previous baseline, respectively; thus, indicating a considerable role of DA increase in the NA for maintaining substance self-administration behavior (Lyness et al., 1979; Pettit et al., 1984). Additionally, DA transporter (DAT) knockout (KO) mice showed a decrease in cocaine self-administration, but not in mice with serotonin transporter (SERT) KO, supporting the important role of DA

not only in acquisition, but also in maintaining self-administration, relative to serotonergic transmission (Thomsen et al., 2009).

Conditioned place preference (CPP) is a behavioral procedure often used to study the underlying mechanisms of substance use disorder, which has been used to measure rewarding effects of substances (Bardo and Bevins, 2000; Lynch et al., 2010). CPP is conducted in a chamber consisting of two outer compartments, having distinct visual and tactile cues, and a center compartment. Prior to starting the CPP conditioning, experimental animals are placed in the center compartment and allowed entree to both compartments freely and inherent preference of the animals for each compartment are determined. Based on the inherent preference, to perform the unbiased design study, the environmental cues are manipulated to eliminate inherent preference of animals, but sustaining distinguishable differences between two outer compartments (Cunningham et al., 1999; Kõks, 2015). Although pre-existing preferences for one compartment over another would have a significant role in determining conditioned place preference, a bias may arise during the repeated access to environment instead of emerging on the initial test (Bardo and Bevins, 2000; Cunningham et al., 2003). Also, genetic differences among inbred mouse strains may affect to innate preferences to one environment (Bardo and Bevins, 2000). The biased design also is used often in research, which does not modify environmental cues. In the biased design, when the study assumes that a substance induces positive reinforcement, then the preferred environment by each animal is paired with a vehicle, and the nonpreferred environment is paired with substances. When the substance is assumed to produce aversive effects, then the preferred environment is paired with the substance to minimize the effect of intrinsic motivational values of environment on the study (Napier et al., 2013; Kõks, 2015). Following the preconditioning procedures, on the first conditioning day of CPP, substance is administered to

experimental animals; then, and then immediately, the animal is placed in one of the two outer compartments to form a paring between the substance administration and the respective compartment. On the following conditioning day, the animal receives a vehicle administration, and then immediately placed in the other outer compartment. By repeating these conditioning sessions over 2-3 days, the paring of substance and vehicle with each specific compartment is generated, which is called the conditioning process. Once, each outer compartment has been paired with either a substance or vehicle, then a choice test is conducted. On the choice test, the animal is placed in the center compartment and allowed to freely access both outer compartments, and the time spent in each compartment is recorded. The time spent in each compartment is compared to determine the preference for one compartment over the other. The observed preference on the choice test for one compartment is considered as evidence for a rewarding effect induced by the substance or vehicle that was paired with the preferred compartment (Bardo and Bevins, 2000; Lynch et al., 2010).

Lesioned DA neurons 6-OHDA in the NA bilaterally pretreatment of haloperidol (0.2 mg/kg, i.p.; an antagonist of DA receptor) attenuated heroin (2.0 mg.kg, i.p.)-induced place preference in CPP by 40% and 30%, respectively, relative to control rats (Spyraki et al., 1983). Also, bilateral infusion of 6-OHDA into the NA prior to conditioning decreased amphetamine (0.75 mg/kg, i.p.)-induced CPP by 70% compared to the control group of rats (Sellings and Clarke, 2003). In contrast, systemic administration of 6-OHDA (100 mg/kg, i.p.) in neonatal rats depleted central and peripheral norepinephrine (NE) without altering DA content in striatum, which did not alter heroin-induced CPP. This indicates the crucial role of DA transmission relative to NE for heroin-induced reward (Spyraki et al., 1983). These observations indicate the important role of substance-induced extracellular DA increase in the NA of substance-induced rewarding effects.

Altogether, multiple experimental observations demonstrated that substance administration increased extracellular DA in the NA brain reward circuitry. When the DA system is disturbed by neuronal lesion in the NA or DAT was KO, acquisition and maintenance of self-administration, and substance induced CPP were decreased. Thus, the substance induced extracellular DA increase in the NA provides reinforcing and rewarding effects of substances, and which serves a critical role in acquisition and maintenance of substance use disorders. On the other hand, although DA systems in the midbrain area serve critical roles in substance-induced rewarding effects, multiple neuronal systems and various brain areas also are involved to substance-induced rewarding effects. Involvements of acetylcholine, gamma-aminobutyric acid (GABA), glutamate, ghrelin, orexin, norepinephrine, and serotonin (5-HT) on substance-induced rewarding effects have been reported in addition to the DA system (Bardo, 1998; Marquez et al., 2017; Zallar et al., 2017; Farzinpour et al., 2019; Foster and Weinshenker, 2019). Thus, DA is not the only neuronal system involved in substance-induced rewards and development of substance use disorders. However, importantly, legion of DA neuron in the NA significantly reduced substance self-administration behaviors and preference for substance-induced CPP, further suggesting the crucial role of DA on substance-induced reinforcing and rewarding effects, respectively. Hence, this dissertation focus on the DA system in substance use disorders (see section 1.3).

1.3.1.2 The Law of Effects and Learning Underlying Substance Use Disorders

From the perspective of behavioral psychology, behaviors resulting in a pleasant consequence (i.e., reward) are more likely be repeated, whereas behaviors resulting in an unpleasant consequence are less likely be repeated, which is known as the law of effect

(Thorndike, 1898). Learning refers to an association forming between stimuli and responses, which leads to a relatively long-lasting change in individuals' behaviors. Additionally, B.F. Skinner, a behavioral researcher, employed several terms and specified types of stimuli depending on their effects on responses. B.F. Skinner defined reinforcer and reinforcement as stimuli that increases the tendency of a specific behavior and as a process resulting in a pleasant consequence following a voluntary behavior, respectively. When reinforcement occurred by adding/presenting a desired stimulus (i.e. food, substances) and removing undesired stimulus (i.e. anxiety, withdrawal), they are defined as a positive and a negative reinforcement, respectively (Holland and Skinner, 1961). A punishment is defined by B.F. Skinner as a process resulting in an unpleasant consequence following a voluntary behavior, which decrease tendency of behavior (Holland and Skinner, 1961). Also, the addition of unpleasant consequences and removing desired consequences are defined as positive and negative punishment, respectively (Holland and Skinner, 1961).

Ultimately, during the process learning, the reinforcement strengthens and punishment weakens the association between a stimulus (i.e., consequence, experience, reward, reinforcer) and a response (i.e., a specific behavior resulting in the pleasant or unpleasant consequence) (Landauer, 1969; White, 1989).

During the substance use disorder development, as well as relapse, both positive and negative reinforcement are involved. Initially, as a result of substance intake (a behavior), the individual experiences substance-induced rewarding effects (a pleasant consequence), which is how the positive reinforcement initiates the acquisition of substance use behaviors. After repeated substance intake, substance abstinence results in negative states, such as anxiety or withdrawal, which promotes maintenance and relapse of the substance use behaviors via negative reinforcement process. Together,

substance-induced reinforcement augments continued substance intake (Meyer et al., 2016).

Substance-induced rewarding effects repeatedly occurred during repeated substance use, which strengthens the association between stimuli (substance or substance-related cues) and responses (approaching and taking the substance) through learning process. There are two forms of learning involved: Pavlovian (classical) conditioning and instrumental (operant) conditioning (Rescorla and Solomon, 1967; Smith and Aston-Jones, 2014). Pavlovian conditioning refers to a learning process that associates an unconditioned stimulus (US) and a conditioned stimulus (CS) by repeatedly presenting them together. Thereby, the response for US (unconditioned response; UR) becomes a response for the CS, then called a conditioned response (CR). For example, a bell (CS) rang when food (US) was served to a dog. Salivation (UR) of the dog is observed. After repeated presentation of the bell ringing (CS) with food (US), the bell ringing (CS) results in salivation (UR) of the dog. Then, the salivation following the bell ringing (CS) is called a CR. In the other word, after the Pavlovian conditioning the CS became a predictor of the US, thus the CS elicited CR.

In contrast to the Pavlovian conditioning that results in an association between two stimuli (CS-US), instrumental conditioning (also called as operant conditioning) associates a voluntary behavior and the consequence of the behavior. Thus, instrumental conditioning requires a voluntary behavior to be conditioned, and the probability of the occurrence of the behavior is affected by the consequences of the behavior. Pavlovian conditioning measures reaction such as salivation, whereas operant conditioning measures voluntary behaviors of subjects. As an example, a rat presses a lever, if the behavior resulted in food delivery, then the rat tends to press the lever more often, which is an example of a positive reinforcement. Whereas, when lever pressing resulted in

elimination of existing electric foot-shock producing pain, then the rat tends to press the lever often, which is an example of a negative reinforcement.

Regarding substance use disorders, Pavlovian conditioning initially strengthens associations between substances-induced rewarding effects (US) and substance-related cues (CS), and CS became a predictor of US. In parallel, through the instrumental conditioning (substance-induced rewarding effects following a volunteer behavior) then the substance-induced rewarding effects stimulate the occurrence of the reward-approaching behaviors (i.e., substance seeking and intaking), which is a positive reinforcement. Later, the reward-approaching behaviors are stimulated also through punishment processes, such as anxiety and withdrawal, as instrumental conditioning. Additionally, Pavlovian conditioning is able to strengthen instrumental conditioning, which suggests that learned Pavlovian conditioning is transferred to the instrumental responses (Rescorla and Solomon, 1967; Lovibond, 1983; Cartoni et al., 2013). For instance, a rat experiences cue light illumination (CS) when food (US) is delivered. Thus, the rat learned cue light and food association through the Pavlovian condition. Then, the rat undergoes instrumental conditioning by lever pressing, resulting in food delivery without cue light illumination. Later, when the rat is exposed to a lever presented operant chamber with or without cue light illumination, then the rat showed higher response for the lever when the cue light is illuminated (Cartoni et al., 2013). The rat underwent no training to associate cue light and lever pressing, however, because the cue light illumination was paired previously with the food delivery in the initial Pavlovian conditioning. The cue light illumination was able to enhance the lever-pressing behavior through Pavlovian-to-instrumental transfer (Rescorla and Solomon, 1967; Lovibond, 1983). This Pavlovian-instrumental transfer contributes to the cue-induced rise on substance-seeking and taking behaviors, indicating the important role of Pavlovian and instrumental conditioning on

initiation and maintenance of substance use behaviors (Belin et al., 2009; Hogarth et al., 2014).

In experimental animals, an impaired Pavlovian conditioning has been observed when the DA system is disrupted by the genetic modification of tyrosine hydroxylase (TH) gene in KO mice.TH is the rate-limiting enzyme of DA synthesis. When TH KO mice showed decreased (5% of basal DA levels) DA levels in the NA, TH-KO mice were not able to learn the association between visual and sound cues and a 20-mg food pellet delivery (Darvas et al., 2014). The TH-KO mice exhibited no alteration of normal feeding or locomotor behaviors but exhibited specific impairment of Pavlovian conditioning between cues and reward delivery. However, interestingly, when L-3,4,-dihydroxyphenylalanine (L-dopa; the product of TH enzyme activity in the DA synthesis pathway) was injected daily into the NA of TH-KO mice prior to the Pavlovian training session, the impaired Pavlovian conditioning in the TH-KO mice was improved, which restored DA content in the NA to ~30% of the levels in wild-type mice. The level was maintained for up to 9 h following L-dopa administration (Darvas et al., 2014). When L-dopa injections were stopped, the conditioned response (CR; learned reward-approaching behavior) expression rate in the TH-KO mice after CS presentation was decreased gradually over days (Darvas et al., 2014). On the third day without L-dopa daily administration, the CR expression rate was lower compared to control mice, indicating that DA in the NA is required not only for acquisition, but also for maintenance of learned CR as a response to the CS through Pavlovian conditioning. In agreement with findings of Daravs et al (2014), heroin-induced CPP was decreased after bilateral microinfusion of 6-OHDA into the NA compared to the baseline (prior to the lesion) (Spyraki et al., 1983). In addition, when the lesion occurred after CPP conditioning in the NA through microinfusion of 6-OHDA, the expression of CPP for amphetamine was decreased

(Sellings and Clarke, 2003). These findings suggest an important role of DA in the NA for acquisition and expression of association between CS and CR in Pavlovian conditioning. Thus, inhibition of substance-induced DA increase in the NA would prevent acquisition and expression of cue-induced substance-seeking and taking behaviors. As such, this dissertation is focused on the DA system to discover pharmacotherapeutics for substance use disorders.

The instrumental incentive learning was impaired also by the lesion of DA neurons in the NA (Lyness et al., 1979). Bilateral microinfusion of 6-OHDA into the NA resulted in impaired acquisition and maintenance of amphetamine self-administration when the lesion occurred either before or after amphetamine self-administration training (Lyness et al., 1979). In further investigation, desmethylimipramine (25 mg/kg, i.p.), an inhibitor of NE transporter (NET), was administered to rats prior to bilateral 6-OHDA infusion into NA to avoid NE content alteration following 6-OHDA (Roberts et al., 1975, 1980; Kelly and Iversen, 1976). The desmethylimipramine administered rats resulted in an average of 18% of NE decrease compared to the control rats, and 3 out of 8 total rats receiving desmethylimipramine showed no difference on NE content relative to control. However, 80% of reduction in DA content compared to control groups (Roberts et al., 1980). Another group of rats receiving 6-OHDA without desmethylimipramine administration also exhibited 80% of reduction in DA content (Roberts et al., 1980). Bilateral infusion of 6-OHDA into NA with or without desmethylimipramine reduced the response of cocaine by 20% baseline when 6-OHDA occurred after cocaine self-administration (Roberts et al., 1980). These findings indicate the important role of DA in the NA over NE on maintaining cocaine self-administration behavior. Altogether, substance-induced increase in DA transmission in the NA is critical for the acquisition and maintenance of substance use behaviors learned via instrumental conditioning process.

During the learning process, when rewards (US) are food or intracranial VTA stimulation, and rewarding events occur repeatedly as predicted (quantity, quality, and timing) with cue (CS) presentation, then the DA release in the NA results from reward receipt (US), and is shifted gradually to the cue presentation (CS) that predicts the reward receipt (Schultz et al., 1997; Day et al., 2007; Schultz, 2007; Flagel et al., 2011). In microdialysis studies in rats, unpredicted consumption of palatable snack foods (Fonzies) resulted in a 150% increase in the DA levels in the NA compared to baseline (Bassareo and Di Chiara, 1999). Also, early in conditioning, results from fast-scan cyclic voltammetry studies showed that extracellular DA concentration in the NA were increased to 25-50 nM and 150-200 nM from baseline in response to food reward and intracranial electrical stimulation of the VTA, respectively (Day et al., 2007; Owesson-White et al., 2008). However, after repeated CS-US paired presentations, the US-induced increase in extracellular DA in the NA was reduced over time in both rats trained to press a lever to deliver food pellets and intracranial VTA stimulation (Day et al., 2007; Owesson-White et al., 2008). The In contrast, the cue that was simultaneously paired with food reward or intracranial stimulation increased DA release over time in both rats trained to press a lever to deliver the food and intracranial VTA stimulation following at least 10 trials (Day et al., 2007; Owesson-White et al., 2008). Together, the data indicate that DA release in the NA was shifted from reward receipt to cue presentation following repeated presentation of the cue and expected reward over time.

When the reward is administration of substance, the presentation of CS alone is able to result in an increase in the DA in the NA following repeated presentations, similar to electrical stimulation or a natural reward. For instance, after cocaine self-administration training using 0.25 mg/infusion (i.v.) in rats for 6-9 days, the CS presentation alone that previously was associated with cocaine i.v. infusion resulted in an increased extracellular

DA in the NA during cocaine extinction (Weiss et al., 2000). In contrast, due to the pharmacological properties of substances, the increase of DA in the NA as a response of the substance is greater than the natural reward. Cocaine (5 mg/kg, s.c.), morphine (2.5 mg/kg, s.c.), ethanol (1 g/kg, i.p.), nicotine (0.6 mg/kg, s.c.), and amphetamine (1 mg/kg, s.c.) administration results in increases of 250%, 80%, 100%, 100%, and 900%, respectively, of basal DA levels in the NA (Di Chiara and Imperato, 1988a). Substances, especially stimulants, maintain high levels of DA for a longer period than does natural reinforcers. This outcome results in higher motivation to use substances relative to natural reinforcers. On the other word, substance-induced DA levels increased in the NA results in strong association between cue (CS) and reward (US) in substance intake behavior, compared to the natural reward approaching behavior with Pavlovian conditioning (Di Chiara and Imperato, 1988a; Bassareo and Di Chiara, 1999). For example, the pharmacological properties of cocaine include inhibition of DA transporter (DAT) function. Thus, cocaine administration results in increased extracellular levels of DA in the NA. Also, cocaine (0.25-2.0 mg/kg, i.v.) inhibits GABA neurons by interacting with voltage-sensitive sodium channels in the VTA, which normally inhibits activity of VTA DA neurons. Thus, cocaine via inhibiting GABA neurons, also acts to disinhibit DA neurons in VTA (Steffensen et al., 2008).

Amphetamine and METH inhibit DAT function and promote DA release from the cytosol into extracellular space by reversing the activity of DAT. Furthermore, METH (2 and 5 mg/kg, i.p.) dose-dependently increases extracellular acetylcholine concentration in the VTA (250% and 400% of baseline VTA acetylcholine, respectively). Cholinergic neurons in the VTA innervate the VTA DA neurons which express muscarinic acetylcholine receptors (Dobbs and Mark, 2008). Thus, METH increases DA in the NA, not only via acting directly on DA neuron terminals in the NA, but also by acting indirectly

at VTA DA neurons. These actions of substances in the NA and VTA synergistically increase DA extracellular concentration in the NA (Steffensen et al., 2008).

Similarly, opioids indirectly activate VTA DA neurons projecting to the NA by inhibiting the GABA input onto the DA neurons (Johnson and North, 1992; Melis et al., 2000). Morphine administration (1-4 mg/kg, i.v.) dose-dependently increased NA DA neuron activity via this disinhibition pathway (Melis et al., 2000). Detailed mechanisms of action of cocaine, opioids, and METH are discussed further in sections 1.4, 1.5, and 1.6, respectively.

Taken together, based on the law of effect, behaviors resulting in pleasant (i.e., reward, substance, food) or unpleasant (i.e., anxiety, pain) consequences are more likely or less likely repeated, respectively (Thorndike, 1898). Substances increase DA in the NA, resulting in rewarding effects. Also, substances, like opioids, reduce pain. Thereby, intake of substances resulting in rewarding effects and/or attenuating anxiety and pain is more likely repeated, which is named as positive and negative reinforcement. Additionally, withdrawal occurred during substance abstinence following chronic use (an unpleasant consequence) motivates continuous substance use. This is called relapse and that is an example of negative reinforcement.

Learning refers to the association forming between a behavior and a consequence through repetition, which leads long-lasting changes on behaviors. The learning process includes Pavlovian conditioning and instrumental conditioning. Through Pavlovian conditioning, associations between CS-US is formed, which results in CR following US presentation. Through instrumental conditioning, association between a voluntary behavior and the consequence is formed. Also, association formed through Pavlovian conditioning is able to be transferred to instrumental conditioning. Importantly, inhibition

of substance-induced DA increase in the NA decreases acquisition and maintenance of Pavlovian conditioning and instrumental conditioning: thus, indicating the crucial role of DA in the NA during the learning process. Substance use, substances increases DA in the NA, which serves an important role in acquisition and maintenance of substance seeking and intake behaviors. Furthermore, during the learning process, substances interacts with neuronal systems directly and indirectly to produce greater increase in extracellular DA in the NA relative to natural rewards (i.e., food), which may explain why substances are able to form stronger association between cues and rewards and engender consumption in a compulsive manner (Volkow and Morales, 2015). Thus, this dissertation focuses on discovering the pharmacotherapeutic inhibiting effects of substances on the DA system to treat substance use disorders. Moreover, the greater increase in extracellular DA in the NA following substance intake relative to natural rewards results in long-lasting neuronal adaptation after chronic substance use, which is discussed in sections 1.3.2 and 1.3.3.

Of note, a theory proposing obesity (>30 Body Mass Index) as a “food addiction” received increasing attention recently (Meule and Gearhardt, 2014; Lerma-Cabrera et al., 2016; Volkow et al., 2017). Although food is considered as a natural reward rather than substances having abuse potential, obesity shares multiple pathological behaviors with substance use disorders such as loss of control over food intake, continued food consumption despite negative consequences, and inability to discontinue eating behavior despite desire to do so (Meule and Gearhardt, 2014; Lerma-Cabrera et al., 2016). Also, consumption of particular food types including fatty, sugary and salty food showed potential of resulting pathological behaviors in experimental animals and humans (Gearhardt et al., 2011; Narayanaswami et al., 2013; Lerma-Cabrera et al., 2016). However, there are a range variation regarding environmental, behavioral, physiological, and genetic factors that contribute to the obesity development in individuals. For instance,

sugar-sweetened drinks consumption is correlated with rising rates of obesity in humans and in experimental animals, but social reasons for the drink consumption may underlying the observation, especially in humans (Malik et al., 2006; Brownell et al., 2009; Wilson, 2010). As such, feeding and eating disorders are a primary mental disorder in DSM-V, but obesity is not considered as a mental disorder. Obesity is highly associated with several mental disorders including binge-eating disorder and depressive disorders, thereby obesity is considered as a risk factor for development of mental disorders (i.e., depressive disorders) and side effects in DSM-V (Meule and Gearhardt, 2014).

1.3.2 Transition Phase from Voluntary to Compulsive Substance Seeking and Taking

Initial intake of substance results in rewarding effects, which increases tendency of the substance seeking and taking behaviors through positive and negative reinforcement, which is the voluntary substance use. During the repeated occurrence of substance-induced rewarding effects and substance seeking and taking behaviors, the association between reward and behaviors are strengthen. However, chronic substance use results in neuronal adaptations. The transition from voluntary substance use to compulsive substance seeking and taking behaviors are mediated by numerous neuronal adaptations including activation of DA in dorsal striatum causing habit formation (section 1.3.2.1), depressed DA neuron activity in the NA (section 1.3.2.2), and decreased DA neuron activity in prefrontal cortex causing decreased executive function with increased impulsivity (section 1.3.2.3).

1.3.2.1 Habit Formation for Substance Seeking and Taking via Activating DA Neuron in the Dorsal Striatum

Repeated substance seeking and taking behavior as a response to CS presentation also results in increased DA release in the dorsolateral striatum, a brain region mediating habit formation (Everitt and Robbins, 2005, 2013; Yager et al., 2015).

After establishment and learning from the CS-US association by training, CS predicted cocaine (0.75 mg/kg/infusion, i.v.) infusion (Ito et al., 2000, 2002). The cumulated amount of lever pressing for cocaine infusion (reward, US) was increased linearly during the cocaine self-administration session (total 90 min). Escalation in response included the CS-only-presentation period and occurred during the initial 20 min of self-administration session, which measured cocaine seeking behavior in the absence of access to cocaine. Cocaine infusion increased extracellular DA concentrations in the NA and dorsal striatum as determined with in vivo microdialysis. However, the cue (CS) presentation in the absence of cocaine during the initial 20 min did not alter extracellular DA levels in the NA (Ito et al., 2000). In contrast, extracellular DA level increased in dorsal striatum during the CS presentation in the absence of cocaine, which resulted in cocaine seeking behavior as a response to the CS presentation during the initial 20 min of the session (Ito et al., 2002). In the dorsal striatum, extracellular DA increased to a maximum of 270% above baseline DA levels during cocaine seeking, and 310% above baseline DA levels during cocaine self-administration (Ito et al., 2002). These findings indicate that, after establishment of a learned relationship between CS presentation and substance seeking behavior, CS presentation elicits seeking behavior not through a reward induced by a DA increase in the NA, but through increased DA in the dorsal striatum associated with habit formation and automated response (Schiltz, 2006; Smith and Laiks, 2017).

Repeated stimulation of the NA reward circuitry via training of intracranial self-stimulation (ICSS) results in a gradually decreased DA neuron activation in the NA (Ljungberg et al., 1992; Redgrave et al., 1999). ICSS is an animal model that is often used in substance use disorder studies to mimic substance-induced reinforcing effects by providing short electric pulses in the target brain area related to the reinforcement as a result of an operant response of the experimental animal (Garcia Pardo et al., 2017; Kenny et al., 2018). ICSS uses similar operant training procedures as with self-administration, but the infusion of the substance as a result of operant response is replaced with electric pulses directly in the target brain region (Garcia Pardo et al., 2017; Kenny et al., 2018). In contrast to substance self-administration, which leads a numerous of neuronal modifications including substance-induced reinforcing effects, the ICSS allows the measurement of reinforcing effects without additional modifications on numerous neuronal cells.

Impairment of dorsal striatal DA transmission by injection of 6-OHDA into dorsal striatum in rats resulted in impaired CS-response association in a conditional visual discrimination (CVD) task (Robbins et al., 1990). The CVD task required 'procedural memory', also called 'stimulus-response habit', to form an association between the visual stimulus (fast or slow flashing lights) and an arbitrary lever response (left or right) (Dudchenko and Sarter, 1991). In rats where the dorsal striatum was lesioned, the stimulus-response habit formation was impaired (Robbins et al., 1990). In another study, restoration of DA levels in the dorsal striatum of TH-KO mice resulted in improvement of instrumental incentive learning, which requires stimulus-response habit formation (Darvas et al., 2014). D1 receptor agonist, SKF-38393, or D2 receptor agonist, LY 171555 infusion into dorsal striatum enhanced learning of habitual behaviors in a two-8-arm radial maze tasks (Packard and White, 1991). These observations indicate that after forming CS-US

association via repeated substance intake (US) in the presence of cues (CS), the CS presentation is able to increase extracellular DA in the dorsal striatum, which mediates stimulus-response (CS-CR) habit formation. Thus, after repeated substance intake in the presence of cues, cue presentations result in DA increase in the dorsal striatum, which elicit substance taking or seeking behavior (CR) as an automated response.

Further support of the involvement of striatal DA in habit formation comes from studies in which monkeys self-administering cocaine (0.03 mg/kg/infusion) for 5 days (initial), 3.3 months (chronic), or 1.5 years (long-term) showed different DA neuronal adaptations depending on duration of substance use (Letchworth et al., 2001). Density of DAT expression was decreased mainly in the NA at the initial stage. In contrast, at chronic and long-term stages, DAT density was increased in the NA and the dorsal striatum, respectively. Depending on the duration of cocaine exposure, the progressive change in DAT density from the NA to the dorsal striatum was observed, suggesting a differential DA increase and activation of DA neurons at each stage over time (Letchworth et al., 2001). This different neuronal adaptation, depending on the duration of stimulant use, supports the role of DA in dorsal striatum as important in habit formation of substance seeking and taking behaviors in chronic to long-term stages of substance self-administration. Together, chronic substance intake behavior results in DA release in the dorsal striatum, in contrast to the consequence of acute substance intake behavior. Striatal DA release mediates stimulus-response habit formation, and consequently, contributes to the transition from voluntary to compulsive substance seeking and taking.

1.3.2.2 Depression of DA Neuron Activity in the NA during Withdrawal Following Chronic Substance Use

When the CS-US association is established, presentation of the CS becomes a predictor of the receipt of a reward (US). However, when the predicted reward is missed or omitted after the CS presentation, DA neuron activity in the NA is depressed below the basal firing rate at exactly the timing of the expected reward delivery (Hollerman and Schultz, 1998). In the latter study using male Macaca fascicularis monkeys, lever pressing for 0.15 mL apple juice served as the natural reward. The CS was a paired visual cue. When the expected reward was omitted, DA neuron firing was depressed. In another study, rats were trained to self-administer cocaine (0.25 mg/kg/infusion, i.v.), and 10-15 days were required to reach stable responding (Weiss et al., 1992). After unlimited-access to cocaine for 12 h, there was a 12 h-long cocaine withdrawal session. During cocaine withdrawal, DA release in the striatum and NA were reduced and a maximal decrease in the NA DA release was observed at 4-6 h of withdrawal (Weiss et al., 1992). In the rat model of cocaine self-administration, cocaine withdrawal also increased ICSS reward thresholds, indicating an 'anhedonia' status during withdrawal of cocaine (Kornetsky and Esposito, 1981). Rats previously exposed to either 5 or 10 mg/kg/day of amphetamine via implanted osmotic minipump for 6 days exhibited increased ICSS thresholds for reward during amphetamine withdrawal, indicating anhedonia during withdrawal of amphetamine (Paterson et al., 2000). The decrease in basal DA neuron activity in the NA and DA release in striatum reported using animal models may be related to the depressive symptoms and anxiety observed during the several days of cocaine withdrawal in humans with cocaine use disorders (Gawin and Kleber, 1986). Depression of DA neuronal activity in the NA has been observed in patients with major depression (Drevets et al., 1992; Mayberg et al., 2000; Russo and Nestler, 2013). Thus, omission of the predicted reward after CS

presentation decreases DA neuron activity in the NA, which consequently results in anhedonia, and which is a consequence of withdrawal following repeated substance exposure and can lead to compulsive substance seeking and taking behavior through negative reinforcement process associated with reduced DA neuronal activity in the NA (Der-Avakian and Markou, 2012; Volkow and Morales, 2015).

1.3.2.3 Increased Impulsivity and Decreased Executive Function Result from Altered DA Receptor Expression Pattern Following Chronic Substance Use

Altered DA release induced by repeated substance intake also alters DA receptor expression patterns in brain, which contributes to the transition from voluntary to compulsive substance use via increasing impulsivity and decreasing executive function. Increases in extracellular DA concentrations, either directly induced by substance administration or by stimulus-response association-related processes, interacts with DA receptors to produce downstream signaling ultimately ending in the experience of reward. DA receptors are G-protein coupled receptors (GPCRs). There are two types of receptors depending on their Gα-protein binding preference, including D1-like receptors (D1, and D5 receptors) and D2-like receptors (D2, D3, and D4 receptors) (Missale et al., 1998). D1-like receptors couple with Gαs-protein activating adenylate cyclase and increase intracellular cyclic AMP (cAMP). D1 receptors (D1R) expressed in the NA are critical for rewarding effects and incentive learning processes, which are activated by substances and natural rewards (Di Chiara et al., 2004). D2-like receptors couple with Gαi-protein inhibiting adenylate cyclase and resulting in reduced cAMP intracellular levels (Missale et al., 1998; Neves et al., 2002). Activation of D1R in the NA was sufficient to induce

substance-induced rewarding effects by itself, but activation of only D2Rs was not (Volkow and Morales, 2015).

The peak of substance-induced rewarding effects have been associated with both D1R and D2R activation (Welter et al., 2007; Steinberg et al., 2014). Since D2R exhibits higher affinity for DA than D1R, higher DA levels are required to activate both receptor subtypes. Cocaine (8 mg/kg, i.p.) rapidly increased DA levels in NA and activated both D1R- and D2Rs in the NA in mice (Luo et al., 2011). D1R activation by DA resulted in increased calcium ion entry into the D1R-expressing neurons. In contrast, D2R activation decreased calcium ion entry into the D2R-expressing neurons. The cocaine-induced increase in DA reached a peak within 10 min, and then decreased gradually for following 30 min. During the first 10 min, the increase in calcium ion concentration in D1R-expressing neurons was rapid, followed by a lower slope in the change in DA concentration. In contrast, D2R-expressing neuron activity was consistent during the 30 min after cocaine administration (Luo et al., 2011). These findings indicate that both D1R and D2R were stimulated during the peak DA concentration after cocaine, and then later D2R predominantly was activated when the DA concentration was decreasing after the peak response; thus, indicating the important role of D2R in decreasing the cocaine-induced extracellular DA level increase.

Additionally, in human subjects, brain imaging studies show a fast increase of substance-induced DA concentration in the striatum (NA and dorsal striatum) within the first 10 min, which correlated with verbal responses of subjects (e.g., I feel “high”; Volkow et al., 2008). Although the duration of the substance-induced increase in extracellular DA levels was over 60 min, only the increase during the early time period was correlated with responses of “high” (Fowler et al., 2008; Bello et al., 2011; Volkow and Morales, 2015). In agreement with studies in humans, D1R antagonists (SCH23390 and SCH39166), but

not D2R antagonists (raclopride, eticlopride, and spiperone), selectively decreased responding for cocaine (0.25 mg/kg/infusion, i.v.) in self-administering rats, without altering responding for food pellets (Caine and Koob, 1994). Together, these findings using experimental animals and human subjects indicate that D1Rs, activated by the substance-induced rapid initial increase in extracellular DA in the NA, mediates the reinforcing effects of substances including cocaine. In contrast, high affinity D2Rs mediate inhibition of DA release, and D2Rs are activated longer than D1R, leading to a reduction over time in substance-induced DA release in the NA.

Furthermore, evidence that D2Rs are involved in the acute response to substance comes from studies showing that D2R gene KO in mice exhibited increased sensitivity to cocaine-induced hyperlocomotion after acute cocaine (5 and 15 mg/kg, i.p.) compared to control mice (Bello et al., 2011). Also, increased dorsal striatal DA release evoked by an electronic stimulus pulse on the brain slice and increased DA synthesis were observed in the D2R KO mice compared to control mice (Bello et al., 2011).

After chronic substance use, downregulation of D2Rs in the striatum (NA and dorsal striatum) and prefrontal cortex (PFC) results in reduced inhibition of the substance-induced increase of DA release in humans (Volkow et al., 2008; Volkow and Morales, 2015) and monkeys (Nader et al., 2006). In rhesus monkeys (Macaca mulatta), D2Rs expression levels in the NA was negatively correlated with response for cocaine during self-administration (Nader et al., 2006). Human brain imaging studies using PET scans revealed that multiple substances including cocaine, METH, and opioids downregulate D2Rs in striatum and PFC (Volkow et al., 1990, 1996; Wang et al., 1997; Volkow, Chang, Wang, Fowler, Ding, et al., 2001; Thanos et al., 2017). In healthy human subjects, who had never used substances before, low striatal D2R expression levels were correlated with a high “drug-liking” response after methylphenidate (a psychostimulant, 0.5 mg/kg,

i.v, infusion); (Volkow et al., 1999, 2002). Furthermore, in rats previously trained for alcohol self-administration, D2R overexpression in the NA through adenoviral vector delivery of the D2R gene resulted in a 52% increase of D2R expression and in reduced alcohol intake by 64% of previous baseline, indicating critical involvement of D2R expression level on repeated substance use (Thanos et al., 2001). Also, in rodents, low D2R expression level in the NA was correlated with high impulsivity measured by a five choice serial reaction time test (5-CSRT) (Everitt et al., 2008). Rats with low D2R expression did not show fast acquisition of cocaine self-administration, but exhibited increased intake of cocaine compared to control rats (Everitt et al., 2008). Increased cocaine intake presumably would result in an escalation of substance-induced neuronal adaption and eventually compulsive substance intake.

In the absence of any exposure to substance, intrinsically high impulsive rats expressed lower D2 expression level in NA compared to non-impulsive control rats based on 5-CSRT task prior to cocaine self-administration training. Although both high- and non-impulsive rats determined by previous 5-CSRT task underwent cocaine self-administration training, high impulsive rats exhibited greater cocaine intake during cocaine self-administration relative to non-impulsive controls (Dalley et al., 2007). Thus, these findings suggest that decreased D2R levels in the striatum mediate the high impulsivity, the subjective “substance-liking” response, and greater substance intake.

Furthermore, in humans with substance use disorders, downregulated D2Rs support the transition from voluntary to impulsive substance seeking and intake. The connection between impulsivity and substance use is supported by observations on high impulsive rats. Substances are self-administered by high impulsive rats during the withdrawal period to avoid the effect of a negative emotional state and mood dysregulation, which induced by high impulsivity instead of staying substance abstinent for longer. These

findings are consistent with observations in humans that self-medication of substances is observed often in patients diagnosed with obsessive-compulsive disorders (Everitt et al., 2008). Overall, these findings indicate that repeated substance intake decreases D2R expression levels in the striatum and PFC, and contributes to longer lasting substance-induced extracellular DA increases.

In an important study, the downregulated striatal D2R levels in humans with METH use disorders were correlated with decreased neuronal activity of orbitofrontal cortex (OFC) DA neurons, a subdivision of PFC (Volkow, Chang, Wang, Fowler, Ding, et al., 2001; Black et al., 2010). Downregulated D2Rs in the striatum have been associated with decreased baseline activity of a DA contained neuron in PFC, and PFC is involved in executive function including decision making and inhibitory control (Black et al., 2010; Volkow, Wang, et al., 2011). Thus, impairment of PFC function increases the propensity for exhibiting impulsive and compulsive behaviors (Volkow, Chang, Wang, Fowler, Ding, et al., 2001). In animal models, decreased baseline activity of PFC has been correlated with impairment of inhibitory control over substance seeking (Jentsch and Taylor, 1999; Goldstein and Volkow, 2011). Rats continuously seeking and taking cocaine despite electronic foot-shock resulted from cocaine seeking, and taking behaviors exhibited lower PFC activity compared to foot-shock sensitive rats (Chen et al., 2013). The rescuing activity of the PFC using optogenetic method reduced the compulsive cocaine seeking behavior in foot-shock resistant rats (Chen et al., 2013). Furthermore, based on a computerized gambling task study, stimulant use disorders showed decreased decision-making cognition (Rogers et al., 1999). The most favored option in the gambling task was chosen less frequently by individuals diagnosed with a stimulant use disorder compared to healthy control subjects, indicating that individuals with a stimulant use disorder have difficulties in approximating outcome probability or lack of concern for the consequences

of their actions (Rogers et al., 1999). Similarly, reduced concern for consequences of actions was found in patients with OFC damage (Rogers et al., 1999). Also, in Huntington's disease patients, a brain imaging study and a behavioral task study revealed a positive correlation between low D2R expression levels in the striatum and impaired planning accuracy, an executive cognition function regulated by PFC (Lawrence et al., 1998; Pavese et al., 2003). Thus, the transition from voluntary to compulsive substance seeking and taking that often is observed in substance use disorders appears to involve decreased D2R expression in stratum because of repeated substance use. Downregulated D2R in the stratum increases impulsive substance seeking and taking, which results in an increase in amount of substances consumed, ultimately resulting in escalation of compensatory neuronal adaptations. Also, decreased D2R levels in the stratum results in consequent hypoactivity in PFC; which, ultimately results in impaired cognitive and executive function, and contributes to the transition to compulsive substance seeking and taking.

1.3.3 Craving and Relapse Phase after Abstinence: Consequent Long-lasting Neuronal Adaptations Underlying Substance Craving

In addition to positive and negative reinforcements for acquisition and maintaining substance use behaviors (section 1.3.1 and 1.3.2), the formed habit between stimulus-response behaviors during repeated substance self-administration triggers craving during substance abstinence when the substance is no longer self-administered.

Numerous human brain imaging studies support the idea that changes in brain blood oxygenation levels, rate of glucose metabolism, and DA receptor selective antagonists binding to receptors occur after substance-related cue presentation (Breiter et al., 1997;

Volkow et al., 1999, 2006; Risinger et al., 2005; Sinha et al., 2005). Importantly, substance-related cue presentation evokes changes on DA neuron activity in the dorsal striatum, which was correlated with self-reported craving scores in individuals with cocaine use disorders (Grant et al., 1996). However, in healthy volunteers not using substances, such changes were not observed following presentation of either substance-related or neutral cue presentation (Grant et al., 1996). Additionally, individuals with low craving scores during the cocaine-related cue presentation did not exhibit a change in the rate of glucose metabolite production in the dorsal striatum. In contrast, individuals with high craving scores showed remarkably changed glucose metabolism in the dorsal striatum (Grant et al., 1996). These observations are consistent with findings leading to the suggestion that DA in the dorsal striatum mediates stimulus-response habit formation. Thus, the presentation of substance-related cues triggers activation of dorsal striatum through an automatized habit response, which results in craving (Tiffany, 1990; Volkow et al., 2006).

In animal models, chronic substance use results in impaired reversal learning (Schoenbaum et al., 2004; Stalnaker et al., 2006; Calu et al., 2007; Everitt and Robbins, 2013). Rats repeatedly exposed to cocaine (30 mg/kg, i.p.) for 14 days learned an initial association between the cue and reward during the acquisition period. However, these rats failed to adjust their responses based on the reversed outcome during the reversal period (Stalnaker et al., 2006). Importantly, in contrast to control rats, OFC neurons in rats administered cocaine failed to signal the undesired outcomes during their decision making, which resulted in the cocaine group of rats repeating behaviors that resulted in adverse outcomes. Similarly, in studies evaluating humans with a cocaine use disorders, inability to reverse responses during a probabilistic reversal-learning task also was observed (Ersche et al., 2008). Furthermore, rats were trained to self-administer cocaine (1.0 mg/kg,

i.v.) for 10 days and then they underwent extinction for 30 days, during which time they showed robust cocaine seeking behavior and markedly impaired reversal learning, indicating the long-lasting effect of cocaine self-administration and a high level of craving response (Calu et al., 2007).

Altogether, chronic substance use results in long-lasting modification on neuronal activity, which triggers craving in chronic substance users. Furthermore, the long-lasting alterations in the brain contributes to the high relapsing rate of substance use disorders (40-60%) (McLellan et al., 2000). Also, substance use disorders result in significant social costs, and harmful effects on public health in the U.S. However, there are no pharmacological treatments for cocaine or METH use disorders. Opioid use disorders have several pharmacological treatments, but the number of opioid use disorders have increased rapidly in the past 10 years. Therefore, this dissertation is focused on discovering pharmacotherapeutics for opioids, cocaine, and METH use disorder.

Although substances activate the rewarding circuitry of the brain by using learning processes, each substance has a specific mechanism of action depending on its pharmacological properties. Thus, the underlying mechanisms for opioids, cocaine, and METH are discussed in following sections.

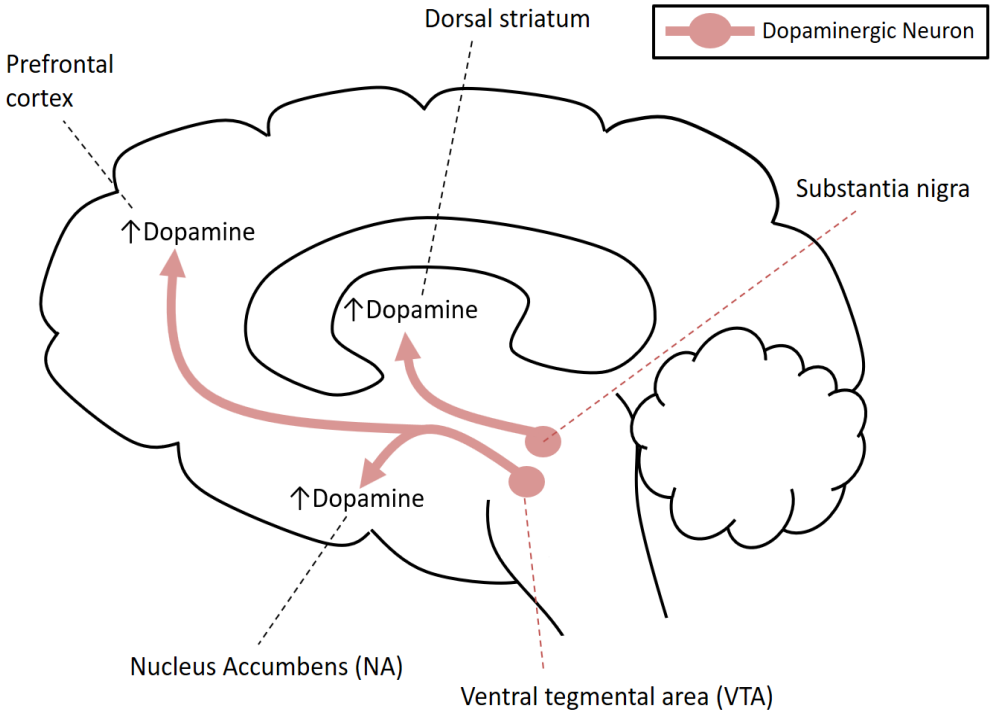


Figure 1.1. Dopaminergic neurons and brain region. Substance-induced DA increase in the NA is important in substance-induced rewarding effect and voluntary substance intake. Repeated substance use leads DA increase in the dorsal striatum and mediates habit formation: which consequently, results in compulsive substance seeking and taking behaviors. Repeated substance use decreases DA neuronal activity in prefrontal cortex, which is the underlying mechanism of decreased executive function (i.e., self-control) and increased impulsive substance seeking and taking behavior.

1.4 METH Use Disorder

Since 2008, numerous studies have reported a rapid increase in METH use, and the number of people who reported nonmedical use of METH in U.S. has also risen to 1.9-fold in 2016 relative to in 2008 (Maxwell and Brecht, 2011; ONDCP, 2016). Presently, global METH use also has escalated (UNODC, 2015, 2017). However, there is no pharmacotherapeutics for the treatment of METH use disorders. As a psychostimulant exhibiting high abuse liability, METH has a simple and easily synthesized chemical structure, which has contributed to the rapid increase of METH use disorder (Gonzales and Rawson, 2005). Furthermore, protocols for METH synthesis are available via the Internet. Thus, many state governments have restricted over-the-counter purchases of the precursors since 2004. Then, the Combat Methamphetamine Epidemic Act was passed by the Federal government in 2005 to regulate the sale of precursors. This resulted in stable indicators including ED visits between 2005 and 2008 (Cunningham and Liu, 2008; Gonzales et al., 2010). However, a more recent rapid growth in METH use disorder shows that there are significant needs for pharmacotherapeutics for treatment of METH use disorder (DEA, 2018). Thus, the following Chapters (2 and 3) focus on discovering therapeutics for METH use disorder using novel compound scaffold. This section reviews the brief history and epidemic of METH use disorders, its mechanism of action, and research to discover treatments for METH use disorders.

1.4.1 History of METH

METH is derived from amphetamine, and amphetamine was synthesized first in 1887 by Romanian chemist Lazar Edeleanu (Rassool, 2009). During World War II,

amphetamine was used by soldiers to reduce fatigue and increase alertness. Additionally, amphetamine was sold as an over-the-counter medication for use to treat nasal congestion, schizophrenia, mild depression, obesity, migraine, alcoholism in the 1930s (Rassool, 2011; Vearrier et al., 2012).

In 1919, a derivative of amphetamine named METH (N-methyl-amphetamine) was synthesized by a Japanese pharmacologist, Akira Ogata (Anglin et al., 2000; Vearrier et al., 2012). Initially, the METH was synthesized with the goal of improving the therapeutic property of amphetamine as a central nervous system stimulant, bronchodilator, and nasal vasoconstrictor. Due to the chemical structural and pharmacological similarity, amphetamine, METH, and its stereoisomers such as dextroamphetamine collectively are called as amphetamines or amphetamine-type stimulants. Amphetamines commonly contain the ephedrine skeleton. Ephedrine is a natural product and alkaloid extracted from Ephedra sinica, which often is used as a precursor of METH synthesis (Vearrier et al., 2012). During World War II, METH also was used by soldiers and war-related workers to improve shift work ability by increasing alertness, decreasing fatigue, and suppressing appetite (Anglin et al., 2000). Amphetamines also were legally manufactured, which led to a variety of individuals using METH including factory workers, truck drivers, housewives, students, and other professionals for the purpose of increasing wakefulness, mood, attention, and even weight loss. The number of METH users increased during the 1940s and 1950s. During the late 1960s, many METH users reported tolerance to the effects of METH. As a result, tolerance-related problematic METH use occurred, which led to serious physical and psychological health issues including tachycardia, chest pain, arrhythmias, seizures, stroke, skin ulcerations, pulmonary edema, dental caries, hallucinations, anxiety, and suicidality (Gonzales et al., 2010). METH-related violent crimes increased (Vearrier et al., 2012):thus, the Comprehensive Drug Abuse Prevention

and Control Act of 1970 limited the use of amphetamines, which markedly reduced amphetamines-related health problems in the US (Gonzales et al., 2010). Then illegal METH was manufactured in a few limited areas, such as California and Oregon in the late 1970s. Illegal use of METH resurfaced in the 1980s, often used by motorcycle gangs, truck drivers, and construction workers (Gonzales et al., 2010; DEA, 2017). During the 1990s, METH was synthesized easily in small mom-and-pop home laboratories using precursors such as pseudoephedrine (a component of common cold medicine), leading to increases in the METH epidemic. Thus, the Combat Methamphetamine Epidemic Act was passed by the federal government in 2005 to regulate METH precursors containing products (Gonzales et al., 2010; DEA, 2017). Although a slight decrease in the number of METH-related ED visits occurred in the mid-2000s, increases in ED visits associated with METH were reported in 2016 (Richards et al., 2017). Furthermore, rapid increases in METH use since the late 2000s were reported by multiple studies in the US, including increases in the number of ED visits, treatment facility admissions for METH, and METH-related seizures (Maxwell and Brecht, 2011; SAMHSA, 2014; ONDCP, 2016). Also, there were worldwide increases in the number of METH-related poisoning deaths and METH-related seizures since 2008 (UNODC, 2015; DEA, 2017). Currently, use of METH is under the Controlled Substance Act in the United States. METH is categorized as a Schedule II compound (DEA, 2018b), and is prescribed for a limited number of medical uses, including for attention deficit hyperactivity disorder (ADHD) and narcolepsy (sleep disorder) (Richards et al., 2017).

Altogether, multiple reports have pointed out that there are many people affected by acute overdose or chronic METH use, which would imply needs for pharmacotherapeutics to treat METH users, along with laws regulating its use. Especially, METH shows high abuse liability, which suggests acute METH users may have high

potential to become overdosed or chronic METH user (Huskinson et al., 2014). Thus, pharmacotherapeutics for METH use disorders would contribute to combat METH use. However, there are no FDA-approved pharmacotherapeutics for the treatment of METH use disorders.

1.4.2 Epidemiology

Illegal METH use has increased since the 1980s. Widespread use of amphetamines including METH was reduced due to the designation of amphetamines as Schedule II substances by the Comprehensive Drug Abuse Prevention and Control Act in 1970. However, the use of METH reappeared in Hawaii and the west coast of the United States in the late 1980s (Derlet and Heischober, 1990). METH use spread across the country towards the northwest and southwest during the 1990s, and then the midwest, south, and northeast of the United States during the 2000s (Maxwell et al., 2008). Also, based on the most recent report available, there are 1.7 million Americans who have used METH during the year in 2015, which was increased from 1.1 million in 2014 (CDC, 2017a). Moreover, during 2015, there were 225,000 Americans who initiated METH use, which was 0.1% of total population ages12 or older (CDC, 2017a).

During 2015, there were 872,000 persons in the United States ages 12-years or older who were diagnosed as having a METH use disorder, which was 0.3% of Americans in that age group (CDC, 2017a). In 2015, 135,000 people were admitted to a publicly-licensed treatment facility seeking treatment for METH use disorders (DEA, 2018). However, there are no FDA-approved pharmacotherapeutics for METH use disorders.

Importantly, METH use disorders showed higher risks for a psychiatric diagnosis compared to cocaine use disorders (Copeland and Sorensen, 2001). Hence, chronic METH use contributes to the increase in mental illnesses including anxiety, depression, paranoia, delusions, and hallucinations due to the effect of METH and its withdrawal syndrome (Glasner-Edwards et al., 2010; Su et al., 2017). Moreover, 79% of substance-related crimes were related to METH use disorders in 2013 (SAMHSA, 2014b). Among Americans who used METH in the past month, there were 13,000 adolescents (ages 12-17 years), 128,000 young adults (ages 18-25 years), and 757,000 adults (ages 26 and older) in 2015 (SAMHSA, 2017a). The demographics of this group include 631,000 males and 266,000 females, which were 0.5% and 0.2% of each gender population in 2015. The estimated rates of METH users were 1.2%, 0.6%, 0.4%, and 0.2% of populations living in the west, south, midwest, and northeast, respectively (CDC, 2017a).

Additionally, between 2009 and 2014, the number of METH-related ED visits with chest pain, psychosis, and trauma increased from 64,000 to 110,000 (SAMHSA, 2014; CDC, 2017; Richards et al., 2017). Furthermore, METH users showed higher rate of heart failure and dental diseases compared to the general ED populations and general patients in dental clinics (Clague et al., 2017; Richards et al., 2018). Of the 5,700 people who died due to stimulants overdose, 85-90% were related to METH in 2015 (DEA, 2017). There was a 225% increase in stimulant-related deaths between 2000 and 2015 (DEA, 2017). Furthermore, global METH use and METH-related problems continued to increase. Between 2010 and 2015, there was a 3.1-fold increase in the number of METH-related poisoning deaths, and a 158% increase in global METH seizures (UNODC, 2015; DEA, 2017). Also, worldwide users of amphetamine-type substances reached 37 million, METH being the dominant substance.

1.4.3 Mechanism of Action

METH has two enantiomers, (S)- and (R)-METH. (S)-METH exhibits psychostimulant effects, but (R)-METH does not. In this dissertation, METH refers to (S)-METH specifically when there is no additional designation. METH exhibits high lipophilicity (logP = 2.10), which is higher than the parent compound amphetamine (logP = 1.76) (Gulaboski et al., 2007). Due to its high lipophilicity, METH penetrates the blood-brain barrier, allowing a high concentration to reach the brain (Gulaboski et al., 2007). In rats, the concentration of METH in the brain was 7-, 13-, and 8-fold higher compared to serum at 2 min, 20 min (peak of METH concentration in brain), and 2 h, respectively after 1 mg/kg post i.v. injection (Riviere et al., 2000). Furthermore, area under the curve (AUC) ratio of serum to brain was 1:9.7, which was based on data collected from 1 min to 3 h after METH administration (Riviere et al., 2000). These data show that METH is able to accumulate in the brain 9.7-fold higher compared to serum (Riviere et al., 2000). Smoked METH exhibits higher bioavailability (90.3 ± 10.4%) than oral administration (bioavailability, 67.2 ± 3.1%; Cook et al., 1993). The half-life of METH (i.v.) in rats and humans is 70 min and 12 h, respectively (Cho et al., 2001). When METH reaches the brain, it accesses the neuronal cytosol by penetrating the plasmalemmal membrane or by being taken up into the neuron via monoamine transporters, such as DAT, SERT, and NET (Sulzer et al., 2005). METH acts as a substrate at monoamine transporters, resulting in inhibition of monoamine uptake from the extracellular space into the neuronal cytosol. METH exhibits comparable affinity for DAT (Ki = 0.46 ± 0.06 μM) and NET (0.11 ± 0.01 μM), but lower affinity for SERT (31.7 ± 2.40 μM) (Han and Gu, 2006). This profile for METH at neurotransmitter transporters is comparable to that for amphetamine, whereas cocaine shows higher affinity for SERT than METH or amphetamine. Ki values for amphetamine and cocaine DAT were 0.64 ± 0.14 and 0.23 ± 0.03 μM, for NET were 0.07 ± 0.06 and 0.48 ± 0.05 μM,

and for SERT 38.5 ± 3.84 and 0.74 ± 0.03 μM, respectively (Han and Gu, 2006). These findings support pharmacodynamic differences between METH and cocaine, but comparable mechanisms of action between METH and amphetamine at these transporters.

METH-induced inhibition of DAT function results in increased extracellular DA concentrations in the NA, which is known to play a key role in METH reward, contributing to its high abuse liability (Di Chiara et al., 2004). Similarly, amphetamine-induced increases in extracellular DA concentrations in the NA results in acquisition of self-administration behavior (Lyness et al., 1979). Also, the highest concentration of METH in striatum including the NA was found at 5 min post i.v. injection, which was correlated with an increased DA concentration in the striatum at 10 min post-METH injection (Melega et al., 1995).

Once METH enters the cytosol of neuron, it penetrates the synaptic vesicle membrane and inhibits function of the vesicular monoamine transporter-2 (VMAT2), which is expressed on the vesicle membrane (Eshleman et al., 1994; Sulzer et al., 2005; Fleckenstein et al., 2007) METH penetration into the synaptic vesicle increases the pH within the vesicles, which changes the pH gradient between cell cytosol and vesicle. Since the pH gradient is the driving force for DA uptake, METH penetration contributes to DA release from the vesicle into the neuronal cytosol (Nickell et al., 2014). Concurrently, METH inhibits DA uptake by VMAT2. Also, METH inhibits the metabolism of cytosolic DA by inhibiting the mitochondrial enzyme, monoamine oxidase (MAO) (Mantle et al., 1976). METH increases the activity of tyrosine hydroxylase (TH), which is the tightly regulated rate-limiting enzyme in the DA synthesis pathway (Larsen et al., 2002). Overall, the action of METH is to increase cytosolic DA concentrations. This increase in cytosolic DA concentration is available for reverse transport by DAT, which then increases extracellular

DA concentrations (Sulzer et al., 2005; Fleckenstein et al., 2007). Consequently, the METH that was released from the vesicle ultimately is transported to the extracellular compartment and activates D1 and D2 DA receptors expressed on the postsynaptic and presynaptic neuronal membrane. Activation of DA receptors expressed on the postsynaptic membrane in the NA then leads to downstream signaling in GABAergic medium spiny neurons and cholinergic interneurons, ultimately producing reward (Imperato and Di Chiara, 1986; Di Chiara and Imperato, 1988a; Di Chiara, 1991). METH-induced inhibition of VMAT2 allows DA concentrations to increase to a level higher than does cocaine, which does not interact with VMAT2 (Pifl et al., 1995). Cocaine does not increase cytosolic DA levels nor interact with VMAT2 at the vesicle membrane. When VMAT2 is present in the cell, METH produces a prolonged DA release compared to cocaine, especially compared to the absence of VMAT2 expression (Pifl et al., 1995). The multiple mechanisms of METH use contribute to its high abuse liability. The METH-induced increase in cytosolic DA concentrations via interaction with VMAT2 is an additional mechanism where METH ultimately increases extracellular DA concentrations, culminating in a reward response.

Chronic METH administration results in the degeneration of DA neurons. The cytosolic oxygen species related neurotoxicity has been observed only in METH users, but not in cocaine users (Giovanni et al., 1995; Larsen et al., 2002; Miyazaki et al., 2006). Accumulated cytosolic DA forms reactive oxygen species (ROS), which are known to induce apoptosis (Giovanni et al., 1995; Larsen et al., 2002; Miyazaki et al., 2006). Also, repeated METH use induces DA neuron degeneration by promoting autophagy in DA neurons (Larsen et al., 2002). DA neurotoxicity has been observed in chronic METH users, as demonstrated by brain imaging studies (Volkow, Chang, Wang, Fowler, Franceschi, et al., 2001; Chang et al., 2007). Decreased DAT, VMAT2 and SERT density in the striatum

and NA has been observed in chronic METH users (Chang et al., 2007). Also, post-mortem analysis of the brain from chronic METH users shows elevated antioxidants such as copper-zinc superoxide dismutase and glutathione, indicating that METH increases oxidative stress (Mirecki et al., 2004).

1.4.4 Clinical and Preclinical Pharmacotherapeutics

There are no pharmacotherapeutics approved by the FDA for METH use disorder. With great need for pharmacotherapeutics, there are multiple research-based approaches being investigated with the goal of discovering the first medication for METH use disorder. There are several different strategies including an anti-METH antibody and VMAT2 inhibitors.

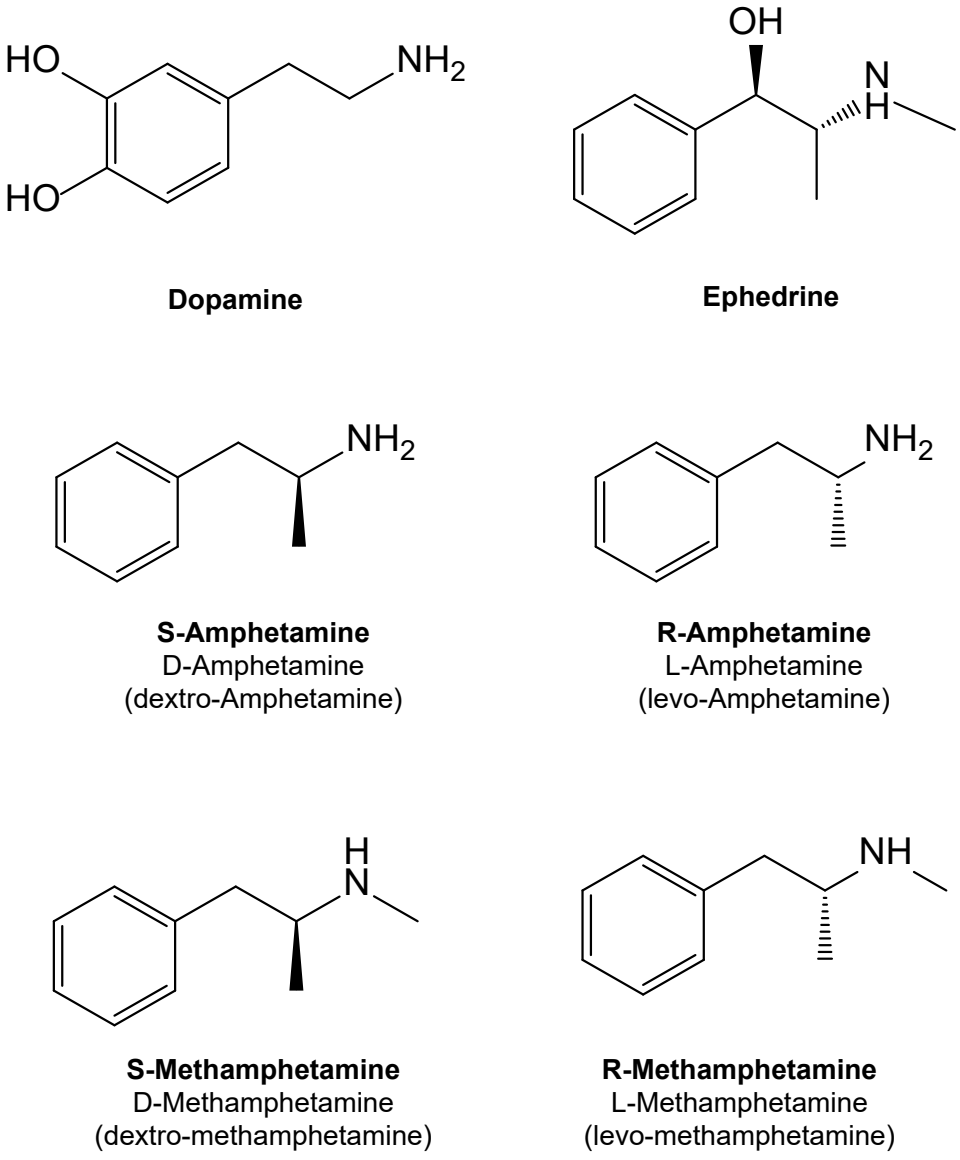
The anti-METH antibody, a pharmacokinetic approach, is aimed at reducing METH concentration in brain, which is expected to reduce the METH-induced rewarding effects in individuals using METH (Kosten and Owens, 2005; Owens et al., 2011; Y-H Chen et al., 2013). Without METH action in the brain, there would be no METH-induced reward, which is critical for both acquisition and maintenance of METH self-administration (Lyness et al., 1979). Also, this approach would eliminate the METH-induced rewarding effect if there was a lapse in use during abstinence preventing a relapse of METH seeking and taking behaviors (Gentry et al., 2009). Thus, decreasing METH concentration in the brain is an important factor as a pharmacotherapeutic for METH use disorders. Based on a study using rats, anti-METH antibody administration decreased METH self-administration when METH was accessible at low unit doses (0.01-0.03 mg/kg/infusion), but not high unit doses (0.06 mg/kg/infusion) (McMillan et al., 2004). The first human study investigating anti-METH antibodies (17-19 day half-life) showed that there were no concerns regarding

safety or tolerability (Stevens et al., 2014). The long half-life of the antibody would theoretically provide benefit to patients with respect to adherence to the treatment and reduction of the potential for a relapse. In the most recent report, the METH specific anti-METH antibody reduced METH (0.56 mg/kg, i.p.) concentration in the brain by 40-73% of sham mice at 21 days post-injection of antibody (Hay et al., 2018).

Another approach to the discovery of therapeutics to treat METH use disorder is using a small molecule that targets VMAT2 to inhibit METH action. A natural alkaloid extracted from Lobelia plant, lobeline was the first compound found to inhibit responses for METH in rats by inhibiting VMAT2 (Harrod et al., 2001; Wilhelm et al., 2008; David B. Horton et al., 2011). Lobeline completed a Phase 1B Clinical Trial (Jones, 2007), and showed no major concerns with regards to safety for individuals with METH use disorder. However, relatively minor undesired side effects were observed including nausea, which may have been due to the high affinity of lobeline for nicotinic acetylcholine receptors (nAChRs) (Travagli et al., 2006; Babic and Browning, 2014). Thus, further structure-activity relationship studies were performed and ultimately VMAT2 selective analogs of lobeline were discovered that did not develop tolerance or cardiotoxicity (Dwoskin and Crooks, 2002; Nickell et al., 2014).

GZ-793A is an analog of lobeline exhibiting selective VMAT2 inhibition, inhibiting METH-evoked DA release from rat brain slices, reducing response for METH in METH self-administration and inhibiting METH reinstatement in rats following both oral and s.c. administration routes. No tolerance developed and no reinforcing effects were produced (D. B. Horton et al., 2011; Alvers et al., 2012; Beckmann et al., 2012; Horton et al., 2013; Meyer et al., 2013; Wilmouth et al., 2013; Nickell et al., 2014). Unfortunately, GZ-793A exhibited affinity for the hERG channel expressed by HEK cells, suggesting the potential for cardiotoxicity (Nickell et al., 2017). Thus, this dissertation focuses on the further

investigation of lobeline analogs as a VMAT2 inhibitor to discover pharmacotherapeutics for METH use disorder (Chapters 2 and 3).



Dopamine

Ephedrine

S-Amphetamine
D-Amphetamine
(dextro-Amphetamine)

R-Amphetamine
L-Amphetamine
(levo-Amphetamine)

S-Methamphetamine
D-Methamphetamine
(dextro-methamphetamine)

R-Methamphetamine
L-Methamphetamine
(levo-methamphetamine)

Figure 1.2. Chemical structures of DA, ephedrine, S- and R-amphetamine, and S- and R-METH.

1.5 Cocaine Use Disorder

Since 2013, cocaine use indicators have rebounded (DEA, 2018). Both the number of cocaine poisoning deaths and new cocaine initiates were 2-fold increased within 3 years, between 2013 and 2016 (DEA, 2018). Furthermore, within a year, cocaine overdose poisoning deaths were 53%, increased between 2015 and 2016. Based on DSM-IV criteria, one in six persons who self-administered cocaine develop cocaine dependence, which is equivalent to a moderate to severe cocaine use disorder in DSM-V (Anthony et al., 1994). Due to the high abuse liability of cocaine, increased cocaine use indicators suggest increased numbers of patients having cocaine use disorder. Also, there was 3.6-fold increase in 2017 compared to 2013 in the cultivation of a coca plant, the primary resource needed for cocaine production in Colombia, from where 93% of seized domestic cocaine originates. This suggests increased domestic cocaine availability (DEA, 2018). Cultivation of the coca plant in Colombia has increased 20% between 2016 and 2017, which has been the highest level of cocaine production in the last 10 years (DEA, 2018). Despite decreasing trends (3.7-fold) in cocaine-related admission to publicly-funded facilities from 2005 to 2015 (DEA, 2018), recent increases in cocaine use indicators, its high abuse liability, and the highest cocaine availability in the US since 2007 reveals the increased need for treatments for cocaine use disorder. However, there are no pharmacotherapeutics approved by FDA to treat cocaine use disorder. Thus, this dissertation conducts a series of studies to discover pharmacotherapeutics for cocaine use disorder by targeting M5 muscarinic acetylcholine receptors (mAChRs) (Chapters 4 and 5). Since inhibition of M5 mAChRs decreases cocaine- and opioid-induced rewarding effects, both cocaine and opioid use disorders are reviewed in the current and next sections. The current section reviews a brief history of cocaine, the epidemic of cocaine

use disorder, the mechanism of action of cocaine, and research to discover treatments for cocaine use disorder.

1.5.1 History of Cocaine

Cocaine is a natural product and tropane ester alkaloid obtained from the leaves of Erythroxylum coca plant, traditionally cultivated in Andean region where Columbia and Peru are located (Redman, 2011). The people of ancient civilizations chewed the leaves of the plant for recreational or religious purpose and achieved stimulant effects (Redman, 2011). In the mid-1800s in Germany, cocaine was isolated from the plant, and was used as an anesthetic since 1884 (Just and Hoyer, 1977; Calatayud and Gonzalez, 2003; Goldstein et al., 2009). Until the late 1800s, cocaine was used as an analgesic for nerve block and spinal anesthesia. On the other hand, due to the rewarding effects of cocaine, it was sold also as a powder, in a cigarette form and as chocolate cocaine tablets for recreational use in the late-1800s. Interestingly, a wine named Vin Mariani was sold (circa 1863) in France, which included cocaine as an ingredient. Later, in 1886, a beverage containing cocaine with sugar syrup was marked with as name of Coca-Cola® in the United States (Goldstein et al., 2009). A glass of Coca-Cola® contained 9 mg of cocaine. However, due to accumulated reports of adverse effects of cocaine reported in the 20th century, cocaine was removed from Coca-Cola® around 1903 (Lundberg et al., 1977; Shuster et al., 1977; Redman, 2011). Also, the Harrison Narcotic Tax Act in 1914 was passed containing regulations regarding cocaine among other substances. Cocaine also has other medical uses rather than anesthetic. In 1879, cocaine was used therapeutically to treat morphine use disorders. Until 1916, cocaine was available as an over the counter medication in the United States to treat toothache and nausea.

Accumulated evidence supports the high abuse liability of cocaine as well as its aversive physical effects including stroke, cardiac arrest, and sudden death (McClenny, 1991). Also, effects of cocaine on society have been recognized, including serious crimes associated with cocaine use and trafficking (Grinspoon and Bakalar, 1981). The Harrison Narcotic Tax Act led to a decline in cocaine use rates in the 1920s, and since the 1930s, amphetamine became popular.

Currently, the use of cocaine is regulated by the Controlled Substance Act in the United States as a Schedule II drug due to its high abuse liability (Redman, 2011; DEA, 2017). Cocaine is prescribed rarely as a local anesthetic and blood vessel constrictor in the US. Since the epidemic of cocaine use disorders continues to be a growing health issue in the United States, numerous research studies have been conducted on cocaine, focusing on the effects of cocaine as a substance having abuse potential. Cocaine was used widely as an anesthetic in the clinic until the end of the 19th century. Currently, most of its anesthetic role has been replaced generally by other synthetic compounds (Redman, 2011).

1.5.2 Epidemiology

Between 2007 and 2009, cocaine initiates were decreased from 906,000 to 617,000, and then it was stable until 2012 at around 630,000. However, a noticeable trend of an increase was reported between 2013 and 2016 (DEA, 2018). The number of past year cocaine initiates were 601,000 in 2013, and 906,000 in 2016 (DEA, 2018). The number of current cocaine users, as indicated by cocaine use within the past month, has increased from 1.53 million in 2014 to 1.9 million in 2015 (DEA, 2017). Due to the cardiac toxicity of cocaine, with the increase in the cocaine use indicators, cocaine-involved

overdose deaths increased also from 4,400 to 13,900 between 2012 and 2017 (CDC, 2018; DEA, 2018). Since cocaine exhibits high abuse liability, the increase in number of cocaine initiates was considered as an important factor in the number of cocaine use disorder patients (Anthony et al., 1994). In accordance with these studies, in the US, 833,000 Americans ages 18 or older met cocaine use disorder diagnostic criteria in 2013, which has increased to 947,000 in 2017 (Lipari and Van Horn, 2017; CBHSQ, Center for Behavioral Health Statistics and Quality, 2018). Moreover, the quantity of cocaine imported into the US increased between 2013 and 2015, which was parallel with the increase of cocaine initiates during the same period of time (DEA, 2017). Also, cultivation of the coca plant in Columbia, was the highest level in 2017 since 2007, which suggests the potential of further increase in the number of cocaine use disorder patients (DEA, 2018). More recently, there has been an emergence of a cocaine and fentanyl mixture in the US, which resulted in an increase in the rate of opioid involvement among the cocaine overdose deaths in 2017 (70%) compared to 2012 (55%) (CDC, 2018; DEA, 2018). These findings suggest that the recent rapid increasing in opioid epidemic since 2013 (section 1.6.2.) also accelerated cocaine overdose deaths (Dowell et al., 2017; Schiller and Mechanic, 2018).

Taken together, cocaine use has increased rapidly since 2013, which was parallel with increase in cocaine use disorder patients and cocaine overdose deaths. The recent opioid epidemic has accelerated the number of cocaine overdose deaths, revealing the need for pharmacotherapeutics to treat cocaine use disorder, as well as, opioid use disorders. Furthermore, cocaine use is a worldwide health issue, and has been used by 18.2 million of global population aged between 15 and 64 (Milano et al., 2017). Thus, discovery of pharmacotherapeutics for cocaine use disorder would contribute to improving not only health in the US, but also worldwide cocaine use disorder-related health concerns.

1.5.3 Mechanism of Action

Cocaine is administered via multiple routes. Insufflation (snorting), smoking, and i.v. injection results in rapid absorption, whereas oral administration and topical application delay absorption (Jeffcoat et al., 1989). Bioavailability of smoked and orally administered cocaine are 70% and 30%, respectively (Leikin and Paloucek, 2008). Onset of action for cocaine is rapid with inhalation and intravenous injection, requiring 3-5 sec and 10-60 sec, respectively. Intranasal administration of cocaine requires 5 min for onset (Leikin and Paloucek, 2008; Goldstein et al., 2009). The half-life for cocaine is 0.7-1.5 h (Jeffcoat et al., 1989).

Administered cocaine crosses the blood brain barrier and dispositions into the brain (Ikegami and Duvauchelle, 2004). According to a rat study, ratio of the brain to plasma distribution of absorbed cocaine was 2.0 ± 0.59, indicating accumulation of cocaine in brain tissues (Pan and Hedaya, 1998; Kulkarni et al., 2016). Cocaine in the brain acts as a psychostimulant by inhibiting the reuptake into cytosol of catecholamines including DA, NE, and serotonin (5-HT), which results in increased concentration of these neurotransmitters in the synaptic cleft and extracellularly (Goldstein et al., 2009). Catecholamines are transferred from the synaptic cleft into cytosol via Na^+/Cl^- dependent monoamine transporters expressed on the plasmalemmal membranes (i.e., DAT, SERT, and NET). To reuptake a DA from synaptic cleft into cytosol, two sodium ions and a chloride ion have to bind to DAT. To reuptake a 5-HT and NE, a sodium and a chloride ion are needed to bind to SERT and NET, respectively (De Felice, 2016). Cocaine exhibited high affinity for DAT and SERT (IC_{50} = 256 ± 43 and 299 ± 10 nM, respectively), and relatively lower affinity for NET (IC_{50} = 4700 ± 721 nM) (GM Miller et al., 2001). Monoamines including DA, 5-HT, and NE are synthesized in neuron cell bodies and terminals, in neuron terminals, and in neuron terminals, respectively. Subsequently,

monoamines are stored in vesicles in neuron terminals, which are released into the synaptic cleft as tonic and phasic transmission. Monoamine released from neuronal terminals to synaptic cleft binds to specific receptors (i.e., DA binds to DA receptors) to activate downstream signaling. Monoamine transporters reuptake monoamines from the synaptic cleft into cytosol, which is essential to terminate the signaling mediated by the released monoamines. Thus, inhibition of reuptake by cocaine results in increased extracellular concentrations (Ikegami and Duvauchelle, 2004). The increase in extracellular concentration of DA in the NA contributes to cocaine-induced rewarding effects and abuse liability of cocaine (Di Chiara and Imperato, 1988a; Wise and Rompre, 1989; Warner, 1993; Eshleman et al., 1994). The percent occupancy of DAT by cocaine was highly correlated with human subjects' reports of "high" (Volkow, Wang, Fischman, et al., 1997). At least 47% of the DAT occupancy by cocaine resulted in human subjects' response "high" (Volkow, Wang, Fischman, et al., 1997). The i.v. cocaine doses (0.3-0.6 mg/kg) that showed abuse liability in human, resulted in 60-80% of DAT occupancy by cocaine (Verebey and Gold, 1988; Volkow, Wang, Fischman, et al., 1997). Cocaine also Increases extracellular 5-HT concentrations in multiple brain areas including the ventral pallidum, NA, substantia nigra, and orbitofrontal cortex, which would contribute to the cocaine reward (Matsui and Alvarez, 2018). Pretreatment of fluoxetine (5 and 10 mg/kg, i.v.), a SERT inhibitor, to rats trained for cocaine self-administration decreased cocaine-self-administration by 50% of baseline, suggesting increased extracellular 5-HT contributes to cocaine reinforcing effects (Carroll et al., 1990). DAT KO mice were able to acquire cocaine self-administration, however, DAT and SERT double KO mice were not able to acquire cocaine self-administration, suggesting 5-HT involvement to cocaine-induced reinforcement (Rocha et al., 1998; Sora et al., 2001). Increased extracellular NE concentrations in the VTA and prefrontal cortex are critical for cocaine sensitization (Robinson and Berridge, 2001; Drouin et al., 2002; Jimenez-Rivera et al., 2006). Although

NET inhibitors (desipramine and nisoxetine; 1 and 3 mg/kg, i.v.) did not alter cocaine self-administration, disruption of NE signaling by administering an antagonist of alpha-1 adrenergic receptor (prazosin, 0.5 mg/kg, i.p.) decreased development of cocaine sensitization on locomotor activity (Tella, 1995; Jimenez-Rivera et al., 2006). Also, the alpha-1 adrenergic receptor KO mice were given daily cocaine (5 mg/kg, s.c.) administration for 5 days failed to develop cocaine-induced sensitization on locomotor activity, but wild-type mice developed (Drouin et al., 2002). The alpha-1 adrenergic receptor KO mice and wild-type mice showed no difference on DA receptor expression levels and DAT sites (Drouin et al., 2002). These observations indicate critical role of NE signaling on development of cocaine-induced sensitization.

Cocaine also inhibits voltage-gated ion channels in membranes of cells that conduct action potentials (Mittleman and Wetli, 1984; Luft and Mendes, 2007). The cocaine inhibits the voltage-gated sodium channels in neurons, which results in inhibition of depolarization of the neuron and blocks nerve impulses conveying pain to the brain, and cocaine was able to be used as an anesthetic (Luft and Mendes, 2007). Also, cocaine modulates voltage-gated cardiac ion channels including sodium, potassium and calcium channels expressed in cardiac tissues, which contributes to development of cardiac arrhythmias by disrupting maintenance of electrical excitability of cardiac tissues and sudden deaths (O'Leary and Hancox, 2010).

Administered cocaine is metabolized through three main pathways. Benzoylecgonine (~40% of absorbed cocaine) and ecgonine methyl ester (~50% of absorbed cocaine) are two major metabolites. Norcocaine (~10% of absorbed cocaine) is a minor metabolite, relative to benzoylecgonine and ecgonine methyl ester (Goldstein et al., 2009). Cocaine is metabolized to benzoylecgonine by hepatic carboxylesterase. Benzoylecgonine produces vasoconstriction, but does not penetrate the blood-brain

barrier readily (Rohrig and Hicks, 2014). The half-life of benzoylecgonine is 5-6 h. Ecgonine methyl ester is formed by butyrylcholinesterase and is an inactive metabolite, exhibiting no pharmacological properties. Cocaine is metabolized to norcocaine by cytochrome P450, specifically by CYP3A4, which produces hepatotoxicity (Riezzo et al., 2012). Norcocaine is able to penetrate the blood-brain barrier, and self-administered by monkeys (0.05, 0.2, and 0.8 mg/kg/infusion, i.v.) (Bedford et al., 1980; Ikegami and Duvauchelle, 2004). However, norcocaine (20 and 40 mg/kg, i.p.) showed lack of stimulatory effect on locomotor activity in rats (Bedford et al., 1980). In contrast, cocaine (0.2 mg/kg, i.v.) was self-administered by monkeys, and increased locomotor activity at 20 and 40 mg/kg (i.p.) in rats. Also, the amount of norcocaine produced following cocaine administration is ~5% of absorbed cocaine. Thus, cocaine serves reinforcing effects predominantly, instead of metabolites of cocaine.

Repeated cocaine administration results in decreased function of DA in brain, which contributes to craving for cocaine with anhedonia, loss of control, severe psychomotor retardation, and psychiatric disorders (Volkow, Wang, Fowler, et al., 1997; Goldstein et al., 2009). Based on PET images, the level for an antagonist of D2R ([^{11}C]raclopride) in cocaine dependent human subjects' brain was lower than control subjects (Volkow, Wang, Fowler, et al., 1997). The cocaine dependent in DSM-IV is equivalent to moderate to severe cocaine use disorder in DSM-5 (American Psychiatric Association, 2013). Also, the methylphenidate-induced changes in the [^{11}C]raclopride binding were higher in cocaine dependents than control subjects. Since methylphenidate increases extracellular DA by inhibiting DAT, the decreased methylphenidate-induced changes in the cocaine dependents suggests decreased DA availability in the baseline state and decreased responsiveness to psychostimulants (Volkow, Wang, Fowler, et al., 1997). Also, cocaine dependent human subjects showed about 50% decreased DA

neuronal activity in striatum and thalamus, which were correlated with restlessness and craving, respectively (Volkow, Wang, Fowler, et al., 1997; Volkow et al., 2007). About 80% of cocaine users also reported psychotic symptoms including paranoia, delusions, and hallucinations (Smith et al., 2009; Roncero et al., 2012).

When cocaine is administered with alcohol, the activity of carboxylesterase enzyme is reduced, resulting in reduced relative amounts of benzoylecgonine, but increased relative amounts of cocaethylene. The cocaethylene inhibits DAT, which contributes to cocaine-induced rewarding effects. The half-life of cocaethylene was 3.5-5.5 h, which was longer than cocaine. Also, cocaethylene exhibited hepatotoxicity (Ponsoda et al., 1999). Thus, cocaine administration with alcohol further increases hepatotoxicity and the rewarding effects of cocaine.

1.5.4 Clinical and Preclinical Pharmacotherapeutics

There are no FDA-approved pharmacotherapeutics for cocaine use disorders. In the preclinical research arena, there are several therapeutic candidates being considered as treatments for cocaine use disorders, including agonist replacements (amphetamine and METH), anti-cocaine vaccines, and engineered enzyme approaches, which are suggested reduced cocaine-rewarding effects.

As an agonist replacement approach, d-amphetamine and d-METH (acting as a substrate of monoamine transporters) have been considered as therapeutic candidates. Due to one chiral center in amphetamine, there are two forms of enantiomers named as d- and l-amphetamine, which are also called as S- and R-amphetamine, respectively. Racemic mixture of d- and l-amphetamine is a Schedule II substance, which acts as a

substrate of monoamine transporter and increases extracellular monoamines (Courtney and Ray, 2016). The d-amphetamine exhibited higher reinforcing effects relative to l-amphetamine in rats, monkeys and humans (Balster and Schuster, 1973; Kirkpatrick et al., 2012). The d-amphetamine and a mixture of d- and l-amphetamine (3:1) are approved by FDA to treat attention-deficit/hyperactivity disorder (ADHD) and narcolepsy in the early 20th century (Sharbaf Shoar and Molla, 2019). Accumulated preclinical data indicated that d-amphetamine is also effective to reduce cocaine self-administration in rodents, nonhuman primates, and humans (Howell and Negus, 2014). In human subjects, oral d-amphetamine (0, 30 and 60 mg/day; each dose was administered for a week before the test) decreased choice for cocaine and response to "want drug again" after intranasal cocaine (100 mg), indicating potential for d-amphetamine to decrease cocaine self-administration (Greenwald et al., 2010). For a study using nonhuman primates, d-amphetamine was infused through the implanted i.v. catheters, once every 20 min for 23 h per day for 3 consecutive days, and the data from the third day were reported. The lowest dose of amphetamine test (0.01 mg/kg/h, i.v.) didn't decrease per cent choice of cocaine (0.032 mg/kg/infusion), but the middle and highest doses (0.032 and 0.1 mg/kg/h, i.v.) decreased per cent choice of cocaine (Negus, 2003). In another study examined longer periods of time (28 consecutive amphetamine treatment days), although during the first 7 days of d-amphetamine (0.1 mg/kg/h) administration by s.c. minipump in monkeys, it reduced both food-maintained responding and cocaine (0.01 mg/kg/infusion, i.v.) self-administration (Negus and Mello, 2003). However, response for food pellets was tolerated a week after; whereas decreased response for cocaine infusions were maintained for 28 days, indicating a specific reduction for cocaine intake (Negus and Mello, 2003). In rats, d-amphetamine (5 mg/kg/day) administered by the implanted osmotic mini-pumps decreased cocaine (0.75 mg/kg/infusion, i.v.) self-administration (Chiodo et al., 2008). Although d-amphetamine has abuse liability, the dose range decreasing cocaine self-

administration was 30-60 mg/day, which was overlaps with d-amphetamine doses used to treat attention deficit hyperactivity disorder (5-80 mg/day in 2-4 divided doses) (Greydanus et al., 2009; Greenwald et al., 2010). Also, METH, a Schedule II substrate, is a potential replacement therapeutic for cocaine use disorder. METH has one chiral center like amphetamine, and the d-METH, which is also called as S-METH, exhibits high abuse liability; however, l-METH (also called as R-METH) does not show abuse potential (Mendelson et al., 2006). Without discrimination of specific enantiomer, METH is also Schedule II substrate. In nonhuman primates, d-METH (0.056 mg/kg/h, i.v., one injection every 20 min for 23 h per day) specifically reduced cocaine (0.032 mg/kg/infusion, i.v.) self-administration, but did not alter food-maintained responding (Negus et al., 2007). Daily METH infusion did not alter food-maintained responding for 7 consecutive days (Negus et al., 2007). These experimental animal studies indicate potential of d-amphetamine and d-METH as therapeutics for cocaine use disorder. Also, amphetamine and METH are approved by FDA to treat ADHD and narcolepsy, indicating relatively lower potential for safety issues. However, amphetamine and METH exhibit high abuse liability. Thus, there is a potential of misuse of amphetamine and METH, which would ultimately cause development of amphetamine or METH use disorder or overdose deaths. Thus, sustained-release formulations of amphetamine (i.e., Adderall XR capsules) are used to prevent rapid increase of amphetamine blood concentration, which would contribute to the reinforcing effect of amphetamine (Berman et al., 2009; Sharbaf Shoar and Molla, 2019). Amphetamine and METH exhibited efficacy to reduce cocaine use, but their abuse potential is the limitation of these replacement therapeutics. Indeed, although amphetamine improved narcolepsy patients by 65-85%, modafinil are preferred by physicians due to relatively low abuse potential of modafinil (Mitler et al., 1994; Jasinski, 2000; Berman et al., 2009). Thus, discovering therapeutics have no abuse potential would provide advantage to patients compared to these replacement therapeutics in treating

cocaine use disorder. On the other hand, a small molecule, modafinil inhibiting DAT (IC_{50} = 4.0 ± 0.39 μM) with low abuse potential should be proposed as a therapeutic for cocaine use disorder (Zolkowska et al., 2009; Stoops and Rush, 2013; Shalabi et al., 2017). In contrast, cocaine inhibiting DAT (IC_{50} = 0.49 ± 0.03 μM) showed high abuse liability (Zolkowska et al., 2009). According to a Phase I clinical trial, modafinil was administered orally 400 mg every 12 h for 7 days, and then 800 mg modafinil was administered orally every 12 h for 7 days. On the sixth and seventh day of each modafinil pretreatment, effects for the 20 mg and 40 mg of cocaine (i.v.) were obtained, respectively. There was no modafinil dose-dependent effect, but both of modafinil doses abolished and decreased one of the visual analog scales, "worth of cocaine in dollars", for i.v. cocaine 20 and 40 mg in cocaine dependents, respectively (equivalent to moderate-to-severe cocaine use disorder) (Malcolm et al., 2006). Also, once daily oral modafinil (200 and 400 mg) for 12 weeks increased non-use days for cocaine to 13.5 and 15.2 days in patients diagnosed to have cocaine dependent, respectively. The non-use days for cocaine in the placebo group was 6.6 days (Anderson et al., 2009). These studies in cocaine use disorders suggest high potential of modafinil as a pharmacotherapeutic for cocaine use disorders. Moreover, in contrast to amphetamine and METH, modafinil exhibited low abuse liability, which encourage the use of modafinil as therapeutics for cocaine use disorders (Jasinski, 2000; Myrick et al., 2004; Reith et al., 2015). Although modafinil failed to show efficacy to increase the non-cocaine use days in the subgroup of patients who are co-diagnosed with cocaine and alcohol dependent, modafinil exhibited efficacy to increase non-cocaine-day in cocaine dependents who are not an alcohol dependent (Anderson et al., 2009). In more recent studies, nociception opioid receptor agonists showed potential as a therapeutic for cocaine use disorders. AT-312 (3 mg/kg, s.c.), a nociception opioid receptor agonist, blocked acquisition of cocaine (15 mg/kg, i.p.)-induced CPP in mice (Zaveri et al., 2018). Also, AT-202 (3, 10, and 30 mg/kg, s.c.), a nociception opioid receptor agonist, decreased

cocaine- and stress-induced using forced swim and yohimbine (2 mg/kg) reinstatement of CPP for cocaine in mice (Zaveri, 2016). An alpha-2 adrenergic agonist, guanfacine (3 mg), also showed efficacy to decrease cue- and stress-induced cocaine craving in cocaine dependents (Fox and Sinha, 2014). Interestingly, guanfacine (3 mg) decreased cue-induced cocaine and alcohol craving in females, but not among male cocaine dependents, indicating potential as a therapeutic for multi-substance use disorders in females (Fox and Sinha, 2014). Multiple studies are ongoing to discover small molecules as a therapeutic for cocaine use disorders, but currently no therapeutics are available in clinic. Especially since a recent rise in cocaine and opioid co-users (McCall Jones et al., 2017), a therapeutic molecule which inhibits more than one specific substance (i.e., cocaine and opioids) would be additionally beneficial to treat recent burden in cocaine use disorders.

Anti-cocaine vaccines are aimed to prevent cocaine-induced rewarding effects by blocking cocaine entering the brain(referred to as a pharmacokinetic antagonism) (Kosten and Owens, 2005; Carfora et al., 2018). In the study using mice , the anti-cocaine vaccine administered mice exhibited decreased cocaine-induced locomotor activity compared to the control mice (Kosten et al., 2014). However, the anti-cocaine vaccine was able to only reduce responding for cocaine (0.1 mg/kg/infusion, i.v.) infusion in one monkey out of 4 monkeys self-administering cocaine (Evans et al., 2016). While the anti-cocaine vaccine increased duration required for reacquisition of cocaine self-administration following extinction phase (21-29 sessions) in all four of vaccinated monkeys (19-94 sessions) compared to control vehicle monkeys (6 and 41 sessions, n=2), which would suggest potential of anti-cocaine vaccines as a treatment for relapse of cocaine use disorder (Evans et al., 2016). Higher sample size and a range of cocaine dose for self-administration would provide stronger evidence to support the potential for anti-cocaine vaccines as therapeutics. Especially, the blood level of anti-cocaine vaccines after

vaccinations would be critical to evaluate efficacy of the anti-cocaine vaccination in reducing cocaine self-administration. According to a human study, the blood level of anti-cocaine vaccine was related to the effect of cocaine (Haney et al., 2010). In the study, cocaine (25 and 50 mg) was smoked by participants who are diagnosed to have cocaine dependence one day per week for 13 weeks. The anti-cocaine vaccine was administered four times to these participants in Weeks 1, 3, 5, and 9 after the cocaine administration session of each week. A group of cocaine dependents exhibiting higher plasma levels of the anti-cocaine vaccine showed significantly lower ratings of "good drug effect" in Week 13 compared to Week 3. However, the other group of cocaine dependents exhibiting lower plasma levels of the anti-cocaine vaccine did not show difference on ratings of "good drug effect" between weeks 13 and 3. This study indicates that the blood level of the vaccine is an important factor to determine efficacy of the anti-cocaine vaccine in order to decrease effects of cocaine. The anti-cocaine vaccination may have higher potential as a therapeutic for cocaine use disorder when the individual maintains high levels of anti-cocaine vaccine. However, the cocaine self-administration study in monkeys did not include blood levels of the anti-cocaine vaccine. Thus, though anti-cocaine vaccine showed potential as a therapeutic for cocaine use disorder, efficacy of anti-cocaine vaccine to decrease cocaine self-administration was observed in one out of four total monkeys. Also, the shorter duration of onset of the antibody (i.e., two vaccinations instead of four vaccinations for 13 weeks) with longer duration of maintenance of the peak blood level of anti-cocaine vaccine after vaccination would improve clinical feasibility.

Another pharmacokinetic approach to antagonism of the rewarding effect of cocaine uses engineered enzymes that specifically metabolize plasma cocaine, since the enzyme is not able to penetrate the blood-brain barrier (Zheng and Zhan, 2012; Zhang et al., 2017). An endogenous enzyme, butyrylcholinesterase is a principal enzyme

metabolizing cocaine into ecgonine methyl ester, which exhibits no pharmacological properties in human (Goldstein et al., 2009). The engineered butyrylcholinesterase based on computational design exhibited 2000-fold improved turnover number (K_{cat}), indicating an enzyme hydrolyzes a 2000-fold higher number of cocaine molecules per min (catalytic efficiency) (Zheng et al., 2008). A high dose of cocaine (180 mg/kg, i.p.) resulted in 100% lethality and 100% convulsions in all teste control mice (n=6), whereas mice receiving the engineered butyrylcholinesterase pretreatment (0.01 and 0.03 mg, i.v.) 1 min prior to cocaine 180 mg/kg (i.p.) administration showed no lethality and 30% of occurrence to no convulsions (Zheng et al., 2008). These observations suggest potential for the engineered butyrylcholinesterase as a therapeutic for cocaine overdose and cocaine use disorders with improved catalytic efficiency. Also, the engineered butyrylcholinesterase was fused with the Fc region of the antibody that rescued the enzyme from degradation following endocytosis, which contributes to the improved half-life of the enzyme (Chen et al., 2018). A more recent study reported a single dose of the engineered butyrylcholinesterase (0.2 mg/kg, i.v.) exhibiting a half-life of 136 h in rats was able to decrease cocaine (15 mg/kg, s.c.)-induced hyperlocomotor activity for 11 days (268 h). Contrast to antibody, enzymes are able to metabolite multiple substrates while the enzymes are maintained in the blood (Zheng et al., 2017). Thus, these studies suggested high possibility for the enzymes as therapeutics for cocaine use disorders.

There have been years of accumulated studies aimed at discovering new therapeutics for cocaine use disorders. However, still there is no FDA-approved effective therapeutics to treat cocaine use disorders. The lack of a therapeutics indicates the need for a novel approach to discover new therapeutics for cocaine use disorders. Also, the recent epidemic of global cocaine overdose deaths reveals increased cocaine and opioid co-administration and a further increase on overdose deaths. Thus, current this

dissertation describes the research on uncovering novel therapeutics for cocaine and opioid use disorders by inhibiting DA projection underlying the rewarding effects of multiple substances, including cocaine and opioids (Chapters 4 and 5).

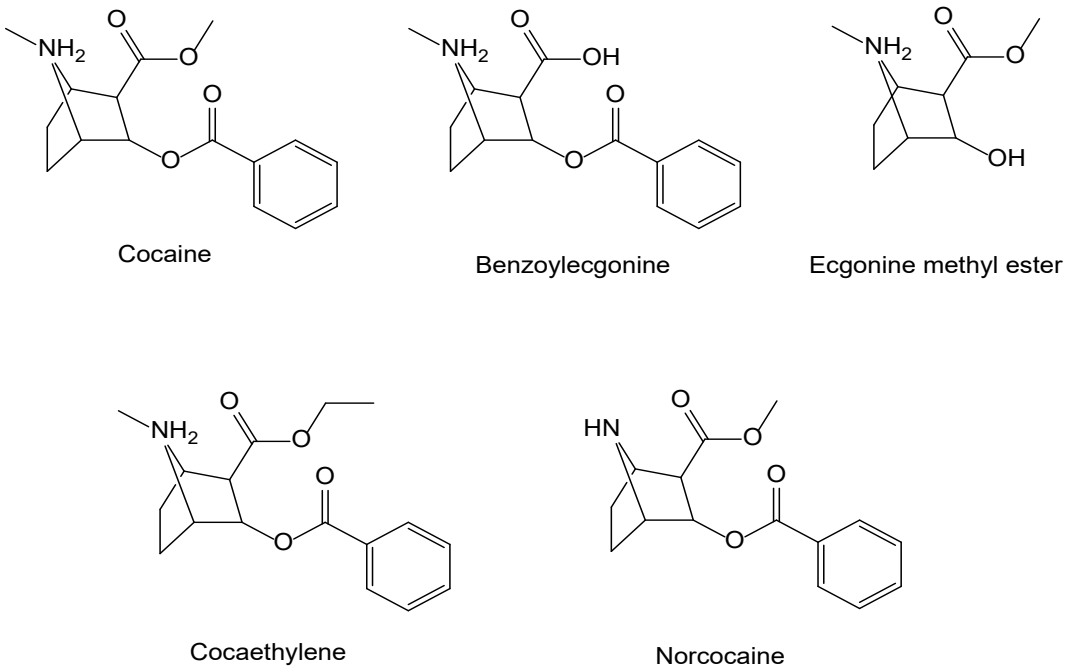


Figure 1.3. Chemical structures of cocaine and its metabolites.

1.6 Opioid Use Disorders

Pain relief medications containing opioids are the second top ranked substances being used by those initiating illicit substance use (NIDA, 2015). The first top substance being cannabinoids. Pharmacotherapies are available to treat opioid use disorders. Goals of treatment are a longer period in the opioid-free condition through reduction of the withdrawal syndrome and rescue from opioid overdose. However, opioid use disorder has continued to dramatically escalate since 1999, leading to the current epidemic. Thus, to improve the outcome of opioid use disorder treatments, this dissertation discusses a novel approach to pharmacotherapeutics for opioid use disorder. Below, a review of opioid compound classes, mechanisms of action, the current epidemic of opioid use disorders, and current treatments for opioid use disorders is presented.

1.6.1 History of Opioids

Opioids are a class of substances including four groups of chemicals that interact with opioids receptors: endogenous opioids, opiates (opioids extracted from opium), semi-synthetic opioids, and synthetic opioids (Martin, 1967; Feng et al., 2012; NIDA, 2018a).

1.6.1.1 Endogenous Opioids

Endogenous opioids are a family of peptides including endorphins, enkephalins, and dynorphins, which act as analgesics in animals and humans by preferentially interacting with mu- (MOR), delta- (DOR), and kappa- KOR) opioid receptors (Feng et al., 2012). Endogenous opioids are derived from large protein precursors, such as

proopiomelanocortin (POMC), proenkephalin (PENK), prodynorphin (PDYN), which are cleaved and from endorphins, enkephalin, and dynorphin, respectively (McDonald and Lambert, 2005; Le Merrer et al., 2009). Synthesized peptide precursors are cleaved, and modified further (i.e., glycosylation, phosphorylation, methylation, acetylation), and then stored in vesicles in opioid-containing neurons. Posttranslational modifications allow endogenous peptides to exhibit various pharmacological profiles including different selectivity at opioid receptor subtypes and different affinities at the subtypes (Akil et al., 1984). POMC, the precursor of endorphins (endogenous MOR preferring ligands), is synthesized only in neurons located in hypothalamus and dorsal medulla. These neurons project to the NA, amygdala, hypothalamus, periaqueductal gray, VTA, brainstem and spinal cord.

PENK and PDYN are synthesized and expressed in neurons in multiple different brain regions. PENK, the precursor of enkephalins (endogenous DOR preferring ligands), is expressed highly in the thalamus. PDYN, the precursor of dynorphin (endogenous KOR preferring ligands), is expressed highly in hippocampus, hypothalamus, and NA (Benarroch, 2012). These endogenous opioids are released by the central and peripheral nerve systems when painful, stressful, or traumatic stimuli are presented. Endogenous opioid-induced modulation of multiple neurotransmitters in CNS and PNS, including various glands, results in reduced sensitivity to those stimuli by producing analgesic and rewarding effects (euphoria) (Froehlich, 1997).

1.6.1.2 Opiates

Opiates refer to two classes of chemicals interacting with opioid receptors, which are identified from exudate obtained by unripe capsules of opium poppy, Papaver

somniferum. Opium has been cultivated by humans since 3400 B.C., and has been used to reduce pain and anxiety, induce sleep, relieve bowels and reduce coughing, as well as to produce pleasurable effects (Bodnar, 2011). Opium consists of opiate alkaloids (also called opiates, ~20% of total weight), water (20%), various sugars (20%), and several simple organic acids (oxaloacetic acid, lactic acid, fumaric acid, and meconic acid) (Najafipour and Beik, 2016). Opiates consist of around 50 different chemicals that are obtained from opium and are divided into two chemical groups: phenanthrenes (i.e., morphine, 12% of total weight; codeine, 2%; and thebaine, 0.5%) and benzylisoquinolines (i.e., noscapine, 5% and papaverine, 1%) (European Food Safety Authority, 2011; Hodgson, 2012; Najafipour and Beik, 2016). The pharmacological effects of opium are attributed to its predominant ingredient, morphine. In 1806, morphine was isolated as an active ingredient with analgesic and rewarding effects (Pathan and Williams, 2012). Importantly, morphine acting as an agonist of MOR is the main component of currently available prescribed pain medications. Hence, morphine is under government control as a Schedule II substance due to its analgesic effects, rewarding effects and high abuse potential (DEA, 2018a). Morphine administration results in analgesia and rewarding effects at the same or higher dose of analgesia (European Food Safety Authority, 2011).

In 1832, codeine was isolated from opium extract. Codeine is 3-methylmorphine and exhibits 200-fold lower affinity for MOR compared to morphine. Codeine is metabolized to form codeine-t-glucuronide (50-70%) and morphine (15%) in the liver by UGT2B7 and CYP2D6 enzymes, respectively (Mignat et al., 1995; Thorn et al., 2009). These two codeine metabolites, codeine-t-glucuronide and morphine are the compounds that mediate codeine-induced analgesic effects, which exhibited 440-fold lower and 200-fold higher affinity for MOR compared to codeine, respectively (Meyer, 2000; Vree et al., 2000). Overall, codeine is a less potent analgesic and rewarding substance compared to

morphine. However, morphine and codeine are contained in prescribed pain medications. Tolerance develops easily to the analgesic and rewarding effects of morphine and codeine, which contributes to the misuse of these prescribed pain medications. Furthermore, opioids produce respiratory depression, the direct effect resulting in opioid related overdose deaths.

Other ingredients of opium include noscapine and papaverine, which have cough suppressant effects and vasodilator properties, respectively (Hao et al., 2015). Also, thebaine is used also as a substrate for semi-synthetic compounds such as oxycodone and hydrocodone (Najafipour and Beik, 2016).

1.6.1.3 Semi-Synthetic Opioids

Semi-synthetic opioids refer to derivatives of opiate alkaloids including one of the widely used illicit opioid substances, heroin, and prescribed opioids (oxycodone and hydrocodone). Heroin was synthesized initially to discover an improved morphine and was marketed by the Bayer Company in 1889. Heroin was considered a valuable treatment for respiratory diseases (coughs, asthma, bronchitis, dyspnea, and tuberculosis) and to treat morphine use disorders (de Ridder, 1994). Heroin exhibited higher potency compared to morphine (logP = 1.07), due to heroin's greater lipophilicity (logP = 1.88), resulting in a higher absorption rate into brain (Peckham and Traynor, 2006; Najafipour and Beik, 2016). Intravenously administered heroin accumulated (68% of observed heroin) into brain, in contrast with 5% of observed morphine (i.v.) located into brain (Oldendorf et al., 1972). Also, heroin passes through the blood-brain barrier rapidly, which allows heroin to achieve peak concentration within 3-5 min after intravenous injection and 5-10 min after subcutaneous injections in mice (Way et al., 1960). After either subcutaneous or

intravenous administration, heroin is metabolized to morphine over a 20-30 min time course (Way et al., 1960). Rapid disposition of heroin and its metabolites to brain contributes to its analgesia, rewarding effects and toxicity, including respiratory depression (Sporer, 1999). In the early 1900s, numerous studies reported the habit-forming properties and abuse liability of heroin. In 1906, Sollier published on the toxicity of heroin (UNODC, 1953). Thus, though heroin produces greater analgesic effects per equivalent amount relative to morphine, heroin is not used clinically due to its high liability for misuse, and is controlled as a Schedule I compound by the U.S. DEA (UNODC, 1953; DEA2018a).

Oxycodone was introduced in the clinic first in Germany in 1917 and in the US in 1981 as an "over-the-counter non-opioid analgesics" (Poyhia et al., 1993; Kalso, 2005). Oxycodone was synthesized from thebaine, an opiate, and exhibits comparable lipophilicity to morphine (logP = 0.91) (Peckham and Traynor, 2006). Oxycodone has high bioavailability (60%) (Poyhia et al., 1993).Oxycodone is an agonist primarily at MOR (Ki = 18 ± 4 nM) and possibly KOR (Ki = 677 ± 326 nM) (Monory et al., 1999). Oxycodone is prescribed as an orally available sedative analgesic to treat moderate to severe pain (Ross and Smith, 1997; Najafipour and Beik, 2016). Oxycodone exhibits a 5.5-fold higher ED50 for antinociception compared to morphine (Kuo et al., 2015). Research revealed that oxycodone serves as a reinforcer and has been found to be misused (Comer et al., 2010; Knipper et al., 2017). Thus, oxycodone is controlled as Schedule II compound.

Hydrocodone was synthesized in the early 1920s by Knoll, a Germany pharmaceutical company, with the goal of discovering an improved codeine with less side effects. Hydrocodone is oxygenated codeine. Comparable with heroin, hydrocodone exhibited higher lipophilicity (logP = 1.75) compared to morphine (Peckham and Traynor, 2006). In 2013, the most commonly prescribed opioids for pain management are hydrocodone (128 million prescriptions) and oxycodone (32 million) (Grant Welker, 2018).

Hydrocodone is a Schedule II substance and is used to treat moderate pain; however, it is prescribed only as a combination formulation with acetaminophen (Lortab® and Vicodin®), which are Schedule III compounds (Schiller and Mechanic, 2018).

1.6.1.4 Synthetic Opioids

Synthetic opioids are compounds exhibiting analgesic effects by interacting as agonists at opioid receptors but are structurally unrelated to opiates. Fentanyl and methadone are well known synthetic opioids, synthesized in 1939 and 1960, respectively (Chen et al., 1993; Raynor et al., 1994). Both opioids exhibit higher affinity for MOR compared to morphine. Based on the competitive binding inhibition assays using radiolabel receptor specific ligands, the Ki of methadone, fentanyl and morphine for MOR was 0.72, 0.39, and 14 nM, respectively, showing 9 to 36-fold higher affinity for MOR compared to morphine (Chen et al., 1993). Also, compared to morphine lipophilicity (logP = 1.07), methadone (logP = 4.77) and fentanyl (logP = 4.28) exhibited higher lipophilicity, indicating that higher amounts of methadone and fentanyl are able to pass through the blood-brain barrier relative to morphine when equivalent amounts are administered (Peckham and Traynor, 2006). Fentanyl exhibits faster onset of analgesic effects compared to morphine. Peak analgesia was observed at 0.6 h and 1 h after subcutaneous administration of fentanyl and morphine, respectively, in rats (Haazen et al., 1999). Furthermore, the analgesic peak dose for fentanyl and morphine was 0.032 mg/kg (s.c.) and 8 mg/kg (s.c.), respectively, indicating 250-fold higher potency of fentanyl relative to morphine in rats (Haazen et al., 1999). Also, human studies report 70- to 100-fold greater potency of fentanyl relative to morphine (Jeal and Benfield, 1997). Importantly, the elimination half-life of fentanyl was longer (8-10 h) compared to morphine (2-3 h),

contributing to the higher risk of fentanyl for overdose relative to morphine (Kharasch, 2011). Due to the high lipophilicity of fentanyl, transdermal administration resulted in an even shorter onset of action (10-15 min) compared to subcutaneous injection (Stanley, 2014). Fentanyl is prescribed only to treat severe pain including cancer pain, pain after surgery, and chronic pain. The first incidence of prescribed fentanyl misuse was reported in the mid-1970s by clinicians; and more recently, escalation of fentanyl misuse and overdose deaths have been reported (Silsby et al., 1984; SAMHSA, 2017b). The illegal manufacturing of fentanyl has paralleled the recent increase in overdose deaths (CDC, Centers for Disease Control and Prevention, 2015). Currently fentanyl is controlled as a Schedule II compound, but was classified temporally (February 2018) as a Schedule I compound due to the epidemic of its illegal use and overdose rate (DEA, 2018).

This synthetic opioid, methadone exhibits unique pharmacological properties. Methadone was synthesized first at a pharmaceutical company, IG Farben, in Germany in 1939. Methadone was approved by the FDA in 1947 for use as an analgesic and to treat cough (antitussive agent) (Rettig and Yarmolinsky, 1995). In contrast to opioids such as morphine or fentanyl, methadone-induced analgesic effects are mediated by MOR agonism and N-methyl-D-aspartate (NMDA) receptor antagonism (Gorman et al., 1997). MOR mediates the majority of analgesic effects of methadone, but inhibition of NMDA receptors expressed in the pain signal transmission pathway contribute to methadone-induced analgesia (Gorman et al., 1997; Carpenter et al., 2000). Thus, NMDA receptor antagonism by methadone is one of its notable differences in mechanism of action compared to other opioids (Gorman et al., 1997). Importantly, NMDA receptor antagonism on top of MOR agonism has allowed methadone to become an effective pain medication.

In terms of pharmacology, methadone exhibits 75% bioavailability and is absorbed mostly by the stomach; peak plasma concentration occurs 2.5-4 h after oral administration,

which allows methadone to be suitable for oral administration (Eap et al., 2002). In comparison, bioavailability of morphine is 26% after oral administration (Gourlay et al., 1986). Morphine is metabolically converted to methadone at a ratio of 10 to 1. Thus, a patient who chronically used a total of 440 mg/day (oral) of morphine would need 44mg/day of methadone (15 mg/8 h, oral) for moderate to severe pain (Toombs and Kral, 2005). Moreover, methadone-induced analgesic effects have an 8-12 h duration, which is longer than for morphine (3 h duration) for moderate to severe pain in humans (Toombs and Kral, 2005). As a pain medication, methadone exhibited comparable efficacy with morphine, but 1.5-fold lower than fentanyl in mice tail-flick studies (Madia et al., 2009). Methadone exhibited significantly improved pain reduction in opioid naïve patients compared to fentanyl (Haumann et al., 2016).

Methadone (maximum dose of 30 mg/kg/day) prevented and reversed the acquired morphine tolerance on chronic pain (Chatham et al., 2013; Wallace et al., 2013). Since the 1980s, methadone has been used for opioid-tolerance chronic pain patients, who use opioids chronically (Portenoy and Foley, 1986). Development of methadone tolerance for analgesia through its chronic use has been shown to be slower compared to the rate of tolerance development with other opioids (Inturrisi et al., 1990).

Since 1962, methadone was studied for its use as a treatment for narcotic use disorders, and specifically, it was promoted to treat heroin use disorders. In March 1973, the FDA approved methadone as a treatment for opioid use disorder (Rettig and Yarmolinsky, 1995). Methadone showed reductions in heroin craving, symptoms of heroin withdrawal, and mortality (Magura et al., 1998; Williamson et al., 2006). Methadone exhibits a long duration of action (24-36 h in human) in reducing symptoms of opioid use disorders (Kharasch, 2011). Methadone is a racemic mixture, containing one chiral center, when it was approved by FDA in 1947 as an analgesic and in 1973 as a therapeutic for

opioid use disorder. However, the racemic mixture of methadone exhibited 89-fold higher affinity for hERG channel compared to heroin (Zunkler and Wos-Maganga, 2010). Importantly, R-Methadone exhibits 10-fold higher affinity for MOR and 50-fold greater analgesic efficacy compared to the S-enantiomer (Davis and Walsh, 2001; Eap et al., 2002). R- and S-enantiomers inhibit NMDA receptors with comparable affinity, indicating that both enantiomers prevent and reverse acquisition of morphine tolerance with comparable efficacy and potency (Chatham et al., 2013; Wallace et al., 2013). Numerous studies have shown that the S-methadone inhibits hERG channels with greater potency than the R-enantiomer, and as such, is anticipated to have greater risk of cardiac toxicity relative to the R-enantiomer (Kornick et al., 2003; Zunkler and Wos-Maganga, 2010; Mccance-Katz, 2011). In 1992, the FDA recommended that the biologically active isomer as a medication instead of the racemic mixture (Food and Drug Administration, 1992; Rettig and Yarmolinsky, 1995). The R-enantiomer of methadone was responsible for its analgesic effects, whereas the S-enantiomer exhibited cardiotoxicity (Davis and Walsh, 2001; Eap et al., 2002). Thus, in 2006, the FDA warned against the potential lethal cardiotoxicity of racemic methadone to reduce associated deaths from overdose (Food and Drug Administration Public Health Service Department of Health and Human Services, 2007).

Although multiple studies including studies performed by Eli Lilly in the late 1940s proved that the R-enantiomer exhibited superior analgesic effects compared to S-enantiomer, the R-enantiomer has not been commercially available in the US due to the substantial costs (Chem, 1948; Van Dyke, 1949). To be approved by FDA, the R-enantiomer must pass through safety testing and clinical trials to prove that the R-enantiomer is an improved medication compared to racemic mixture methadone. The R-enantiomer of methadone is commercially available in the European Union including

Germany, Austria and Switzerland, and has a 5-times higher cost compared to racemic methadone (Lisberg and Scheinmann, 2013).

With chronic use, racemic methadone produces rewarding effects like other opioids (Jasinski and Preston, 1986; Holuj et al., 2013). Interestingly, unlike morphine which produces conditioned place preference that becomes stronger with repeated treatment, methadone does not show accumulating reward over repeated treatments (Holuj et al., 2013). Based on a self-administration study using progressive ratio in monkeys, breakpoints of methadone (0.03 and 0.1 mg/kg/infusion) was 3-fold lower than for heroin (0.05 and 0.10 mg/kg/infusion) (Mello et al., 1988; Panlilio and Goldberg, 2007). These findings indicate that methadone had abuse liability like other opioids, but the labiality is lower relative to heroin. Thus, racemic methadone is categorized currently as a Schedule II compound (DEA, Drug Enforcement Administration, 2018b). Racemic methadone is prescribed to treat moderate to severe pain and to treat opioid use disorders.

1.6.2 Epidemiology

Since 1999, the opioid epidemic has had a positive trajectory. On October 16, 2016, the U.S. government has responded by officially recognizing the opioid epidemic as a national public health emergency (Jones et al., 2018). According to the data collected in 2015, 92 million people aged 18 or older used prescription opioids during the past year, which is 38% of American adults, (Han et al., 2017). Among them, 11.5 million reported misuse of opioids, and 1.9 million were diagnosed as having an opioid use disorder during the last year (Han et al., 2017). One year later, an increased number (2.0 million) of American adults were diagnosed with an opioid use disorder (SAMHSA, 2017b). The number of adolescents between 12 to 17 years old who were diagnosed with an opioid

use disorder during the past year also increased from 127,000 to 153,000 between 2015 and 2016 (SAMHSA, 2017b). Ninety-two million adults in U.S. used opioids, and among them at least 1 out of 10 (12.5%) reported misuse of opioids.

Along with the increase in the diagnosis of opioid use disorders in the U.S., the number of ED visits for opioid overdose have increased. Between 2016 and 2017, opioid-involved ED visits for individuals ages 11 or older were 142,557 in the United States, revealing a 29.7% increase compared to the previous year. An increase in opioid-involved ED visits was noted in both males (30.2%) and females (24.0%), and across all age groups (15-24 years of age (7.3%), 25-34 years (30.7%), 35-54 years (36.3%), and 55 or older (31.9%)) between July 2016 and September 2017 (CDC, 2018b).

There has been three waves or opioid overdose related deaths since 1999. The first wave was in 1999, which was accordance with increased opioid prescriptions for pain. At that time, opioid overdoses were due to opioids extracted mostly from opium and to semi-synthetic opioids. The second wave was in 2010, which mainly involved heroin. Reports indicated that 5.1 million people aged 12 or older reported heroin use during their lifetime, which account for 1.9% of Americans in 2015. Among them, 882,000 and 329,000 reported heroin use in the last year and month, respectively; thus, revealing the heroin epidemic (SAMHSA, 2017b). Compared to 2010 and 2014, in 2015, there was a 328% and 22.8% increase, respectively in heroin overdose deaths (NIDA, 2017). The third wave was in 2013 and is related to the increase in illegal manufacturing of fentanyl (Dowell et al., 2017; Schiller and Mechanic, 2018). With this rapid and dramatic increase in fentanyl deaths, the DEA in February 2018 temporally categorized fentanyl as a Schedule I compound (DEA, 2018). Fentanyl and fentanyl-related synthetic opioids were responsible for a 72.2% increase in fatal overdoses in one year (2014-2015) (Schiller and Mechanic, 2018).

Interestingly, cocaine overdose deaths rates have stabilized since 2008, but increased by 1.8-fold between 2012 and 2015, paralleling the use of opioid and cocaine combinations (Schiller and Mechanic, 2018). Specifically, overdose rates resulting from the combination of prescription opioids and cocaine combinations has decreased by 3.9%; however, combinations of cocaine with synthetic opioids including fentanyl and fentanyl related compounds were associated with increased (22.5%) overdose deaths between 2014 and 2015 (Kandel et al., 2017). According to the CDC, overdose deaths from the use of fentanyl and cocaine combinations has increased (17.1-fold) between 2013 and 2016, paralleling the rapid increase in cocaine-induced overdose deaths (Lopez, 2018). In contrast, methadone-related death has decreased by 9.1% between 2014 and 2015 (CDC, 2016).

Overall, opioid overdose deaths, including prescription and illicit opioids, has increased more than 425% in 2016 compared to 1999 (NIDA, 2017). Opioid overdose deaths in 2016 was more than 42,200, which was increased compared to 28,647 in 2014 and 33,091 in 2015 (NIDA, 2017; Jones et al., 2018). In the substance overdose related deaths in 2016, the 65% of overdose deaths were opioid-related (Schiller and Mechanic, 2018). Taken together, rapid increase in opioids use in U.S. is leading the fast growth of opioids overdose deaths. Also, the increased opioids use contributed to the growth of the national substance overdose deaths.

1.6.3 Mechanism of Action Underlying Opioid Use Disorder

Opioid-induced rewarding effects have resulted in an epidemic of opioid use disorder and overdose deaths. Three opioids are problematic, including morphine (an extracted opioid from opium), heroin (a semi-synthesized opioid), and fentanyl (a

synthesized opioid) (CDC, 2017). In this section, the mechanism of action of these opioids is reviewed, as well as the mechanism of action of endogenous opioids.

Greater expression of DOR and KOR, but relatively lesser expression of MOR has been found in NA (Svingos et al., 2001; Le Merrer et al., 2009). DOR and MOR expression on cholinergic neurons cell bodies have been found, but KOR found on the DA neuron terminals (Dourmap et al., 1997; Svingos et al., 2001; Britt and McGehee, 2008). Indeed, cholinergic neuron lesion in the NA, intra-NA infused a MOR agonist (DAMGO) were not able to increase DA levels in the NA (Dourmap et al., 1997). Thus, the activation of DOR and MOR increased DA release in the NA by increasing cholinergic input into the DA neuronal terminal in the NA (Longoni et al., 1991; Pentney and Gratton, 1991; Spanagel et al., 1992). Whereas KOR activation decreased DA release in the NA by decreasing responses to the intended action potential from the VTA (Spanagel et al., 1990; Svingos et al., 2001; Britt and McGehee, 2008).

Historically, the primary use of opioid receptor agonists from ancient to current times is inhibition of pain signaling, which is associated with a considerable respiratory depression side-effect (Jones et al., 2018). Based on research with KO mice, the analgesic effects induced by opioid receptor activation are mainly mediated via MOR (Kieffer and Gaveriaux-Ruff, 2002). The analgesic effects of opioid receptor agonists are accompanied by rewarding effects at the same doses that produce analgesia. Also, MOR activation results in increased extracellular DA concentration in the NA.

Morphine, an opiate, exhibits high affinity for MOR (Ki = 1.8 ± 0.6 nM) compared to DOR (160 ± 90 nM) or KOR (Ki = 47 ± 3 nM), evidenced by competitive binding assays (Mignat et al., 1995). Systemically administered morphine passes through the blood-brain barrier due to its lipophilicity (logP = 1.07) and activates brain opioid receptors (Peckham

and Traynor, 2006). Also, morphine-induced analgesic and rewarding effects were abolished in MOR KO mice, indicating a critical role of MOR in the mechanism of action of morphine (Matthes et al., 1996; Schuller et al., 1999). Morphine binding to MOR results in coupling to Gi protein. Thus, morphine reduced cytosolic calcium and cAMP, but increased potassium efflux. Consequently, morphine reduces neurotransmitter release and hyperpolarizes neurons, which mediates its analgesic effects, when activated MOR are located on the pain signaling pathways, such as the pain circuitry (spinal cord, periaqueductal gray (PAG), brain stem, medulla, hypothalamus, amygdala (Basbaum and Fields, 1978). When morphine activates brain MOR, specifically midbrain MOR, it produces rewarding effects. Activation of MOR expressed on terminals of GABA neurons in the VTA results in a reduction of GABA release onto DA neurons in the VTA. GABA is an inhibitory neurotransmitter, so that GABA release into the VTA decreases firing rate of VTA DA neurons. Inhibition of GABA neuron in the VTA by microinfusion of a MOR agonist, [D-Ala2,N-methyl-Phe4,Gly5-ol]enkephaline (DAMGO; 0.1 and 1.0 nmol), resulted in a dose-dependent increase in DA concentration in the NA (Spanagel et al., 1992). Also, an agonist of MOR, morphine activates VTA DA neurons in rats (Gysling and Wang, 1983). Thus, MOR activation disinhibits DA neuron in the VTA, which elevate DA extracellular concentration in the NA (Johnson and North, 1992). Intravenous morphine (1 and 5 mg/kg) increased DA levels in the NA to 60-100% of baseline, which was completely inhibited by pretreatment with naloxone (1-3 mg/kg, i.p.; a nonselective opioid receptor antagonist) in rats (Kosterlitz, 1985). In contrast, DOR selective antagonist (naltrindole, 1 mg/kg, i.p.) partially inhibited morphine-induced DA increase in the NA (Borg and Taylor, 1997). Also, morphine injection (0.5-1.5 mg/kg, i.v.) significantly reduced activity of VTA neurons, except DA contained neurons in the VTA, which was reversed by naloxone (Gysling and Wang, 1983). Additionally, activation of GABA neurons in the VTA by GABA receptor agonist (baclofen, 0.1nmol/side) into the VTA inhibited acquisition and expression of

morphine (10 mg/kg, s.c.)-induced hyperlocomotion, indicating that morphine-induced DA release in NA is mediated via inhibition of VTA GABA neurons (Leite-Morris et al., 2004). In addition, intra-NA microinfusion of morphine dose-dependently (125, 250 and 500 ng) increased DA levels in the NA, which was significantly inhibited by pretreatment with naltrindol (i.p., a DOR antagonist) but not naloxone (Borg and Taylor, 1997). Taken together, these findings indicate that morphine activation of MOR in VTA and DOR in NA resulted in DA release in NA via both DA dependent and independent mechanisms. Also, acute morphine did not cause MOR internalization, while endogenous opioids rapidly promoted receptor endocytosis after its activation, indicating higher abuse liability of morphine by producing longer lasting DA level increased (Keith et al., 1996, 1998; Sternini et al., 1996). Further, systemic morphine injections induced CPP in multiple animal models. Also, numerous studies show that animals acquire intravenous morphine self-administration behavior, like human opioid use disorders. Thus, MOR activation by morphine resulted in rewarding effects by disinhibiting DA neurons in the VTA.

Heroin is a semi-synthetic morphine analog also known as diacetylmorphine ((5α,6α)-7,8-didehydro-4,5-epoxy-17-methylmorphinan-3,6-diol diacetate (ester)). Acetylation of morphine at 3- and 6-positions provides heroin with greater lipophilicity (logP = 1.88) compared to morphine (logP = 1.07) (Peckham and Traynor, 2006). Thereby, 68% of intravenously administered heroin was absorbed into the brain, which is higher than morphine (less than 5%) (Oldendorf et al., 1972). Also, according to studies in chronic pain patients or healthy volunteers, peak concentrations of heroin in blood were within 1 min, 3-5 min, and 5-10 min after intravenous, intranasal and intramuscular, and subcutaneous administration, respectively; thusindicating rapid absorption of heroin (Way et al., 1960; Inturrisi et al., 1984; Skopp et al., 1997). When heroin enters the brain, it is hydrolyzed into 6-monoacetylmorphine within 5-10 min by carboxylesterase, and then it is

metabolized into morphine within 20-30 min by carboxylesterase (Umans and Inturrisi, 1981, 1982). The first metabolite of heroin, 6-monoacetylmorphine, produces analgesic effects by interacting with MOR and DOR (Rady et al., 1994, 1997). Both metabolites of heroin are active and considered responsible for analgesic and rewarding effects of heroin (Inturrisi et al., 1983, 1984). Thus, heroin has a 30 min half-life and 4-5 h duration of action (Reisine and Pasternak, 1996). The heroin metabolites, 6-acetylmorphine (IC_{50} = 73 nM) and morphine (IC_{50} = 53 nM) exhibited higher affinity for opioid receptors than heroin (IC_{50} = 483 nM) demonstrated using binding assays with radiolabeled naltrexone, a non-specific opioid receptor antagonist (Inturrisi et al., 1983). These findings suggested that heroin produces its effects via its metabolites, 6-acetylmorphine and morphine. In agreement with this, the half-life of heroin, 6-acetylmorphine, and morphine after inhalation of heroin were 3.2 ± 0.3 min, 26 ± 0.9 min, and 184 ± 7.2 min, respectively (Rook et al., 2006). Also, heroin (10 μM) produced 10% of MOR internalization, comparable with morphine (10 μM, >5%), but lower than beta-endorphin (1 μM, 28%) (Keith et al., 1998). Overall, the effects and mechanism of action of heroin overlap with morphine but has a greater efficacy due to the rapid absorption and high penetration rate through the blood-brain barrier.

Fentanyl is a synthetic opioid involved in the most recent opioid epidemic, and is a opioid (Stanley, 2014). Fentanyl is not structurally related to morphine or heroin, but produces analgesic and rewarding effects by acting as an agonist at opioid receptors (Yoshida et al., 1999). Fentanyl exhibited 2- to 4-fold higher lipophilicity (logP = 4.28) compared to heroin and morphine, respectively, which allows fentanyl to readily penetrate the blood-brain barrier compared to morphine and heroin (Peckham and Traynor, 2006). Also, fentanyl exhibits 16-fold higher affinity for MOR compared to morphine (Bot et al., 1998). Although, fentanyl (10 μM) produced 18% of MOR internalization, more clinically relevant analgesic doses of fentanyl (1-50 nM) did not produce detectable MOR

internalization (Keith et al., 1998; Martini and Whistler, 2007). Clinical studies with postsurgical patients show that intramuscularly (i.m.) administered fentanyl (0.2 mg) exhibits equivalent analgesic effects with 10 mg (i.m.) of morphine, indicating 50-fold higher potency of fentanyl compare to morphine in humans (Finch and DeKornfeld, 1967). Although, affinity of fentanyl (Ki for MOR = 1.2 ± 0.2 nM) for MOR was comparable to morphine (Ki for MOR = 1.8 ± 0.6 nM), the high lipophilicity of fentanyl and low rate of fentanyl-induced MOR internalization results in analgesia with 50-fold higher potency relative morphine (Maguire et al., 1992; Mignat et al., 1995).

Respiratory brainstem neurons express MOR and opioids activate MOR depressing ventilation (van der Schier et al., 2014). Dose-dependent analgesia and respiratory depression produced by morphine was not observed in MOR KO mice (Romberg et al., 2003). Fentanyl exhibits high affinity at MOR and induced respiratory depression at the same or higher dose as that producing analgesic effects, similar to morphine (Downes et al., 1967). In healthy volunteers, fentanyl (1, 2, and 4 μg/kg, i.v.)-induced analgesic effects lasted for 90 min after administration. With the same dose range of fentanyl, respiratory depression had a duration of 120 and 240 min, respectively (Bailey et al., 1990).

Microdialysis showed fentanyl (5 and 10 ug/kg iv, or intra-NA infusion 0.5-5 nmol) -induced dose-dependent increases in extracellular DA in the NA in rats, indicating that fentanyl activated MOR and DOR (Yoshida et al., 1999). In contrast to morphine exhibiting 89- and 26-fold selectivity for MOR over DOR or KOR, respectively, fentanyl showed higher selectivity for MOR over KOR (Maguire et al., 1992; Mignat et al., 1995). Fentanyl exhibited 150- and 242-fold selectivity for MOR over DOR and KOR, respectively (Maguire et al., 1992). Due to low affinity of fentanyl for KOR, fentanyl increased DA in NA more effectively than morphine (Yoshida et al., 1999). Local NA injection of fentanyl (5 nmol)

increased DA release in NA up to 682% of baseline, which was higher than intra-NA infusion of morphine (Yoshida et al., 1999). Intra-NA infusion of morphine (250 and 500 ng) increased DA in the NA by 70 to 80% of baseline (Borg and Taylor, 1997). Moreover, fentanyl (0.01 mg/kg, s.c.) or morphine (1 mg/kg) increased DA in NA by 90% or 70% of baseline, respectively, suggesting higher abuse liability of fentanyl compared to morphine (Di Chiara and Imperato, 1988b).

Endogenous opioids consisted of endorphins, enkephalins, and dynorphins act by activating opioid receptors. MOR, DOR, and KOR, respectively, which are G-protein coupled receptors, coupling to Gi proteins. Activation of opioid receptors results in inhibition of adenylyl cyclase activity, which decreases cAMP concentration in the neuronal cytosol. Activation of opioid receptors decreases calcium ion influx and increases cytosolic potassium ion concentration by closing calcium channel and opening potassium ion channels, respectively. Consequently, activation of opioid receptors expressed on presynaptic neuronal membranes reduces neurotransmitter release by inhibiting a response of inward action potential. Activation of opioid receptors located on postsynaptic neuronal membranes decreases downstream signaling of the response to excitatory neurotransmitters (Kapitzke et al., 2005; McDonald and Lambert, 2005). Depending on where the opioid receptors are expressed, multiple effects are observed after opioid receptor activation (Froehlich, 1997; Le Merrer et al., 2009).

To summarize, MOR agonists, including morphine, heroin, and fentanyl, exhibit analgesic and rewarding effects. MOR are expressed in numerous regions of the central and peripheral nervous systems including within the pain circuitry (spinal cord, PAG, brain stem, medulla, hypothalamus, and amygdala) and reward circuitry (midbrain, VTA and NA). Other commonalities of MOR agonist administration are increased extracellular DA levels in NA, indicating an important role of the mesocorticolimbic DA system in opioid

reward and indicating that affinity for MOR underlies the abuse liability of these opioids. Activation of MOR by endogenous opioids results in rapid internalization, which decreases signaling transduction mediated by MOR (Sternini et al., 1996; Keith et al., 1998). In contrast to endogenous opioids, exogenous opioids, including morphine, heroin, and fentanyl, show a low rate of internalization of MOR after acute administration, which results in enhanced analgesic and rewarding effects induced by exogenous opioids compared to endogenous opioids (Hashimoto et al., 2006). Chronic morphine administration (day 1-2, 10mg/kg; day 3-4, 20 mg/kg; day 5-6, 40 mg/kg; and day 7, 80 mg/kg, s.c. to Porcelluus guinea pigs) results in increased internalization, which contributes to tolerance development to morphine effects including analgesia and reward (Patierno et al., 2011; Allouche et al., 2014). Heroin and fentanyl exhibit 4- to 2-fold greater lipophilicity compared to morphine and heroin, respectively, indicating a faster rate of these opioids penetrating the blood-brain barrier when an equivalent dose is administered. Although affinity for MOR of fentanyl and morphine are comparable, fentanyl exhibits 4-fold higher lipophilicity and higher selectivity for MOR over KOR (242-fold) than morphine (26-fold), which results in 50-fold higher potency in analgesia. The affinity of heroin and it's a metabolite (6-acetylmorphine) mediating rewarding effects are also lower than fentanyl by 10- and 1.5-fold, respectively. Thus, the MOR activation by exogenous opioids including fentanyl, morphine, and heroin results in rewarding effects and tolerance development following chronic administration of exogenous opioids, which contribute to the development of opioid use disorders. Importantly, respiratory depression, which is also mediated by MOR, is of the greatest concern as a side effects due to its contribution to the epidemic of opioid overdose deaths.

1.6.4 Clinical and Preclinical Therapeutics

The government of United States has made two political efforts including Prescription Drug Monitoring Programs (PDMPs) and the National All Schedules Prescription Electronic Reporting Act (NASPER) to reduce opioid prescriptions and to prevent prescription opioid-induced overdose deaths. With these systems in place, opioid prescriptions and opioid overdose deaths have decreased by 8% and 12%, respectively (Dowell et al., 2016). In addition, four pharmacotherapeutics have been approved by the FDA to treat opioid use disorders and opioid overdoses. There are two types of pharmacotherapeutics approved: agonist substitution therapy to reduce withdrawal, and antagonists to reverse overdose effects of opioids.

Historically, the first pharmacotherapy for heroin use disorders was methadone, a synthetic opioid agonist at MOR. Methadone use was approved by FDA in 1947 and was approved for treatment of heroin use disorders in the 1960s. Methadone is a long-lasting (half-life of 22 h) opioid agonist with high bioavailability (75%) following oral administration (Eap et al., 2002). Also, oral administration of levomethadyl acetate (also known as levo-alpha-acetylmethadol or LAAM), an analog of methadone, exhibited 92-h of half-life and was approved to treat heroin use disorder as an alterative to methadone by FDA in 1993. However, it was withdrawn in 2007 due to cardiac adverse effects including increases in the cardiac QT interval (Tetrault and Fiellin, 2012).

Buprenorphine is a semi-synthetic opioid derivative of thebaine and a partial agonist at MORs with about 20-fold higher affinity for MOR compared to morphine (Vardanyan and Hruby, 2016). Buprenorphine displaces the binding of opioids (such as morphine, methadone) at MOR (Bickel et al., 1988; Rosen et al., 1994; Strain et al., 2002). Buprenorphine also exhibits a low dissociation rate, which indicates that it can produce a

prolonged inhibition of opioid withdrawal and that it requires a less frequent dosing schedule than once per day (Amass et al., 1994). When administered via sublingual and buccal routes, buprenorphine showed high bioavailability, 51% and 28%, respectively (Kuhlman et al., 1996). In addition, buprenorphine is a partial agonist of MOR, which indicates that buprenorphine-induced MOR activation efficacy is lower compared to full agonists such as morphine, heroin, or methadone. Thus, the side effects including respiratory depression of buprenorphine show a plateau, indicating that a higher dose is able to induce less severe side effects compared to full agonists (Center for Substance Abuse Treatment, 2004). Buprenorphine was approved to treat pain and opioids dependence (equivalent to moderate to severe opioids use disorders in DSM-5) as a monotherapy by FDA in 2002 (Center for Substance Abuse Treatment, 2004). Thus, the MOR agonist buprenorphine prevents relapse by reducing opioid withdrawal symptoms caused by decreased endogenous opioid signaling, including VTA DA neuronal activity during withdrawal. Sublingual buprenorphine (16 mg per day) for 12 months was administered to opioids dependents. Retention in treatment for 12 months in buprenorphine and placebo groups were 75% and 0%, respectively. Also, the urine screens revealed 75% negative for opioids, stimulants, and cannabinoids among the patients who maintained buprenorphine for 12 months (Kakko et al., 2003). However, buprenorphine is a Schedule III substance, indicating potential to develop moderate or low substance use disorders. Heroin dependents who maintained heroin abstinence for about a week self-administered 2 and 8 mg of buprenorphine instead of making a choice for $20, indicating potential of buprenorphine to substitute heroin in patients who detoxified recently (Comer and Collins, 2002). Also, buprenorphine produced mild elevation of the liver enzymes AST and ALT, which would suggest benefits for liver function tests prior to buprenorphine treatment (Welsh and Valadez-Meltzer, 2005). Thus, buprenorphine exhibited efficacy in clinic to increased opioids abstinence rate, but strict control is

necessary to prevent misuse of buprenorphine. Also, liver testing and drug-drug interaction should be considered prior to buprenorphine treatment to prevent hepatic toxicity and unexpected medical condition, respectively.

Naltrexone is an antagonist at opioid receptors (Ki for MOR, DOR, and KOR are 1, 149 and 3.9 nM, respectively), and was approved by FDA in the 1970s as a treatment for heroin use disorder (Raynor et al., 1994; Newman and Rothman, 2007). Based on analyses of 13 studies using oral naltrexone, only 28% of initial participants were retaining with the oral naltrexone treatment, which concluded no difference between placebo and oral naltrexone (Minozzi et al., 2011). While in a study using intramuscular injectable extended-release naltrexone (required once a month injection, maintaining plasma naltrexone level to 1-2 ng/mL), naltrexone and placebo groups achieved 51% and 31% opioids abstinence for 24 weeks, respectively (Nunes et al., 2015). Another study also showed that 62% of patients retained intramuscular extended-release naltrexone for 12 months, and 51% of remaining participants achieved opioid abstinence (Krupitsky et al., 2013). The extended-release naltrexone achieved higher retention rate to naltrexone treatment relative to oral naltrexone by altering the dosing schedule to once monthly injection (380 mg, i.m.) from once daily oral administration (40-200 mg). The intramuscular administration of naltrexone exhibited higher bioavailability and increased elimination half-life (100% and 4.9 days, respectively) compared to oral administration of naltrexone (5-40% and 9 h, respectively) (Dunbar et al., 2006). However, administration of opioids with naltrexone has potential to lead a fatal overdose (O'Connor and Kosten, 1998; Boyce et al., 2003). Since naltrexone is an antagonist of opioid receptors, patients may administer large quantity of opioids to obtain reinforcing effects of opioids, indicating importance of maintaining opioid abstinence condition during the naltrexone treatment to avoid life-threatening risk.

Naloxone is an antagonist at opioid receptors (Ki for MOR, DOR, and KOR are 1.1, 16 and 12 nM, respectively) (Tam, 1985). FDA approved i.v. and i.m. naloxone in 1971 and nasal spray naloxone in 2017 to treat opioid overdoses. Naloxone exhibits a shorter onset of action (1-2 or 2-5 min after i.v or s.c. administration, respectively) compared to naltrexone (1-2 h, oral), indicating advantage of naloxone to treat opioid overdoses by rapidly reversing opioid binding on opioid receptors (Licko, 1981; Koyyalagunta, 2007). Rapid association-dissociation kinetics of naloxone and high affinity for MOR (Ki = 1.1 nM) of naloxone allow effective reversal of opioid-induced respiratory depression (Rzasa Lynn and Galinkin, 2018). Naloxone also exhibits 100-fold higher affinity for MOR compared to heroin, indicating efficient replacement of heroin by naloxone on MOR to reverse heroin-induced respiratory depression (Kleber et al., 1985). However, naloxone affinity for MOR is comparable to morphine and fentanyl (Ki = 1.8 and 1.2, respectively), and 20-fold lower than buprenorphine (Vardanyan and Hruby, 2016). Along the affinity and association-dissociation kinetics of opioids, multiple factors such as the duration of effect of opioids administered, quantity of opioids presented in blood resulting respiratory depression, and patients' ability to clear opioids should be considered to determine the adequate dose of naloxone (Rzasa Lynn and Galinkin, 2018). Importantly, the elimination half-life of naloxone is 30-80 min. Thus, when opioids exhibiting longer elimination half-life (i.e. fentanyl 8-10 h) was administered, or unknown opioids were administered, then observing patients at least for 80 min would reduce risk of reoccurrence of respiratory depression (Kharasch, 2011). As a therapeutic for opioids overdose, naloxone exhibits beneficial properties including rapid onset of action, high affinity for MOR, and rapid elimination, but as a mono-therapeutic for opioid use disorders naloxone has limitations with the rapid elimination.

The buprenorphine and naltrexone as a combination product was approved by the FDA in 2002 (Center for Substance Abuse Treatment, 2004). Since a partial agonist of MOR, buprenorphine also exhibits abuse liability, like other full MOR agonists, naltrexone was included in the combination product to reduce misuse of buprenorphine among those with opioid use disorders (Alho et al., 2007). Bioavailability following sublingual administration of naloxone is lower than bioavailability of buprenorphine, resulting in predominant effects of buprenorphine in patients when it is administered through a sublingual route (Preston et al., 1990). However, when injected, naloxone provides the predominant effects over buprenorphine due to the high parenteral bioavailability of naloxone (Stoller et al., 2001). Thus, patients would experience opioid withdrawal symptoms with dysphoria when the combination sublingual tablet was misused via i.v. administration. The combination therapy shows no additional safety or side effect issues compared to buprenorphine monotherapy (Harris et al., 2000). Thus, the buprenorphine and naloxone combination treatment provides advantages compared to monotherapy of buprenorphine, naloxone, and naltrexone by reducing abuse potential, improving duration of action, and reducing opioids antagonist-induced dysphoria (Center for Substance Abuse Treatment, 2004).

Patients who received pharmacotherapeutics with behavioral therapy remained opioid abstinent longer and exhibited a reduction in their symptoms of opioid withdrawal compared to the control group who received behavioral treatment only, which emphasize importance of pharmacotherapeutics in treating substance use disorders (Mattick et al., 2014). Although there has been some success combating the opioid use disorder epidemic with available pharmacotherapeutic treatments, the opioid use disorder epidemic is currently growing overall in the United States. Continuous growth of the opioid use disorder epidemic is due to the limitation of existing pharmacotherapeutics such as

low adherence rates and failure on blocking cue-, stress-, or drug-induced relapse (Chartoff and Connery, 2014). Thus, this current dissertation focuses on discovering pharmacotherapeutics for opioid use disorders by inhibiting cholinergic systems in brain, and is further discussed in Chapters 2 and 3 as a potential treatment for the opioid use disorder epidemic.

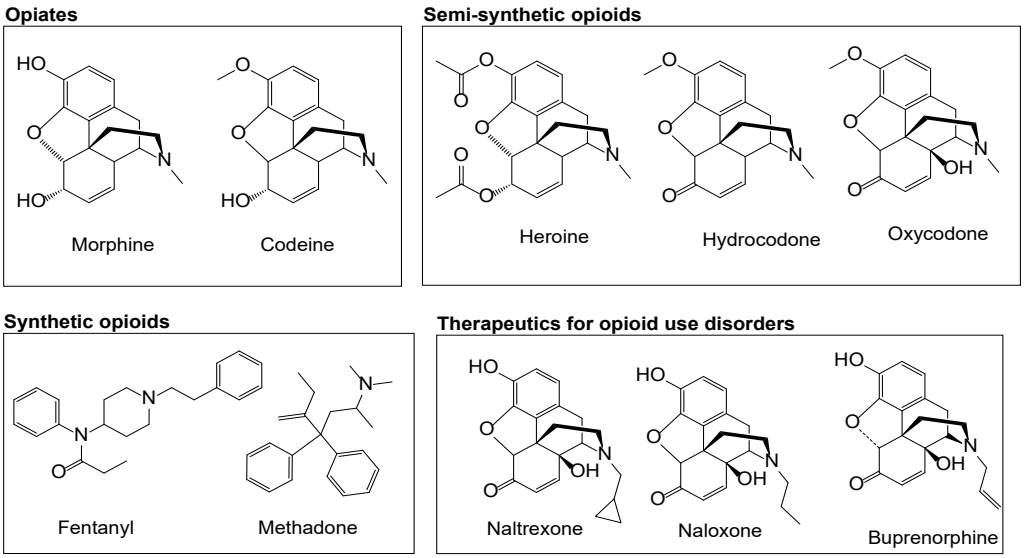


Figure 1.4. Structures of opioids.

1.7 Hypothesis and Specific Aims

Substance use disorders are a growing health concern in the United States, which lead high social costs in our society (Florence et al., 2016; CDC, 2017). Although much research has shown the high abuse liability of METH and cocaine, there are no FDA-approved pharmacological medication for METH or cocaine use disorders. Thus, millions of Americans are currently using METH or cocaine, and suffering currently or in near future with METH or cocaine use disorders: there is no pharmacotherapeutic help for them. Furthermore, opioid use disorders are the most recent growing issue in the U.S. There have been several pharmacotherapeutics for opioid use disorders, but it is still one of the highly ranked substances resulting in substance use disorders in the U.S.

1.7.1 Project 1: Discovering Pharmacotherapeutics for METH Use Disorder

1.7.1.1 Background Information

In contrast to cocaine or opioids, METH is able to release DA into the synaptic cleft in the NA without altering DA projection from the VTA. Thus, a different pharmacological target based on the specific pharmacodynamic profile of METH and a different hypothesis are investigated in this dissertation (Chapters 2 and 3) to discover pharmacotherapeutics for METH use disorders. Since there was no FDA-approved medication for METH use disorder, there is a great need for this current research. As reviewed in section 1.6, METH increased DA in the cytosol in the neuronal terminal, which was released into the synaptic cleft. Especially, METH-induced DA in the NA served a critical role for METH-induced rewarding effects. When the substance-induced DA release in the NA was absent, acquisition of substance self-administration behavior was not formed (Lyness et al., 1979).

Also, maintaining self-administration behavior was reduced when the DA release was absent after self-administration training, suggesting that inhibition of substance-induced rewording effects reduced maintenance of self-administration behavior (Lyness et al., 1979). Since the inhibition of VMAT2 DA uptake by VMAT2 inhibitor, lobeline reduced METH-evoked DA release, reduced METH self-administration, and VMAT2 was evaluated as a novel target for discovering medication for METH use disorders (Harrod et al., 2001; Wilhelm et al., 2008). There were continued years of effort to discover VMAT2 selective lobeline analogs as a pharmacotherapeutic for METH use disorders. In this current dissertation, two analogs of lobeline were investigated based on the hypothesis below and specific aims to discover new medication for METH use disorders.

VMAT2 is one subtype in a family of transmembrane proteins consisting of 12 transmembrane regions, which are highly expressed in the central nervous system (Schütz et al., 1998). VMAT1 is often found in the peripheral nerve system exhibiting high affinity for DA, 5-HT, and NE (Schütz et al., 1998); whereas VMAT2 and VMAT1 exhibit 60% of amino acid sequence homologue, which contributed different uptaking affinity (Anne and Gasnier, 2014). VMAT2 uptakes monoamines including DA, 5-HT, and NE from cytosol into synaptic vesicles via proton-dependent process (Erickson et al., 1992). The amino acids sequence homology of VMAT2 is conserved well across mammalian. The sequence of VMAT2 in mice are 96% and 92% identical with rats and humans, respectively (Takahashi and Uhl, 1997). The proton-dependent process is mediated via the H^+-ATPase enzyme, which is expressed on the synaptic vesicle membrane (Anne and Gasnier, 2014) . The enzyme uptakes a proton using the energy obtained by hydrolyzing one ATP molecule to ADP from the cytosol, which allows the inside of the vesicle to stay in an acidic pH of 5.5, compared to cytosol (pH 7.4). This pH gradient between inside and outside of vesicles is the driving force of DA uptake from cytosol into vesicle via VMAT2

(Chaudhry et al., 2008; Wimalasena, 2011; Chaudhury et al., 2013). A proton in vesicle binds to the VMAT2, which leads conformational change of VMAT2. This allows DA presented in cytosol binding on the DA binding site at VMAT2. Subsequently, when the VMAT2 converts its conformation, the proton bound to VMAT2 is released into the cytosol and DA is released into the vesicles. Monoamines stored in vesicles are released via exostosis into the synaptic cleft following action potential. After the action potential, the released DA is uptaken via DAT into cytosol, and then, it is either metabolized into 3,4-dihydroxyphenylacetic acid (DOPAC) via monoamine oxidase (MAO) or uptaken via VMAT2 into vesicles for storage (Wimalasena, 2011; Anne and Gasnier, 2014).

1.7.1.2 Hypothesis 1 and Specific Aims

Hypothesis 1 (Chapters 2 and 3). Selective and potent inhibition of the VMAT2 function reduces the neurochemical and behavioral response to METH.

Specific Aim 1. Identify selective inhibitors of VMAT2 using neurochemical approaches.

Specific Aim 1.1. Determine if analogs exhibit high affinity for VMAT2 and high potency at inhibiting VMAT2 DA uptake function.

Specific Aim 1.2. Determine selectivity of analogs for VMAT2 over human ether-a-go-go-related gene (hERG) channel, DAT, SERT and nAChRs.

Specific Aim 1.3. Determine if VMAT2 selective inhibitors inhibit METH-evoked vesicular DA release.

Specific Aim 2. Determine if selective VMAT2 inhibition exacerbates METH-induced striatal DA depletion.

Specific Aim 2.1. Determine if selective VMAT2 inhibitors alter striatal DA content.

Specific Aim 2.2. Determine if selective VMAT2 inhibitors exacerbate METH-induced striatal DA depletion.

Specific Aim 3. Determine if selective VMAT2 inhibition decreases response to METH in animal models using behavioral pharmacological approaches. Determine if VMAT2 selective inhibitors,

- block METH-induced hyperactivity in METH-sensitized rats (Specific Aim 3.1).
- decrease METH self-administration, but food-maintained response (Specific Aim 3.2).
- substitute METH in METH self-administered rats (Specific Aim 3.3).
- serve as a reinforcer (Specific Aim 3.4).
- develop tolerance by repeated injections to decrease METH self-administration (Specific Aim 3.5).
- decrease cue-induced or METH-induced METH reinstatement (Specific Aim 3.6).
- are surmountable by increased METH unit doses (Specific Aim 3.7).

In **Chapter 2**, potency and selectivity of R-3-(4-methoxyphenyl)-N-(1-phenylpropan-2-yl)propan-1-amine (GZ-11610, R-enantiomer of GZ-888) are studied and the effect of subcutaneous and oral administration of GZ-11610 on METH-induced hyperlocomotor activity in METH-sensitized rats is examined. **Chapter 3** describes and studies potency and selectivity of S-3-(4-methoxyphenyl)-N-(1-phenylpropan-2-yl)propan-1-amine (GZ-11608, S-enantiomer of GZ-888) for VMAT2 over DAT, SERT, and hERG. The ability of GZ-11608 to inhibit METH-evoked vesicular DA release is measured. Toxicity of GZ-11608 on METH-induced striatal DA depletion is evaluated. The effect of subcutaneously

administered GZ-11608 on METH-induced hyperlocomotor activity in METH-sensitized rats, METH SA, food-maintained response are evaluated. Also, abuse liability of GZ-11608 is examined using a METH substitute study and a GZ-11608 self-administration study. An evaluation to see if GZ 11608 administration is able to develop tolerance is presented. Finally, surmountability of GZ-11608 effects on METH self-administration is studied using a range of METH unit doses.

The purpose of this dissertation is to discover pharmacotherapeutics for opioid, cocaine and METH use disorders by investigating analogs inhibiting substance-induced rewarding effects. For that, two different novel therapeutic targets, M5 mAChRs and VMAT2, are investigated. Due to differences of the underlying rewarding mechanism among substances, two different hypotheses are proposed; One for discovering opioid and cocaine use disorder therapeutics, and the other for METH use disorder treatments. The overall hypothesis of this dissertation is that inhibition of substance-induced rewarding effects by inhibiting substance-induced DA release in the NA would reduce substance seeking and taking behaviors.

1.7.2 Project 2: Discovery Pharmacotherapeutics for Cocaine and Opioid Use Disorders

1.7.2.1 Background Information

As reviewed by previous sections, especially, substance-induced DA increased in the NA served a crucial role in compulsive substance seeking and using (Wise and Rompre, 1989; Di Chiara and Bassareo, 2007). Thereby, inhibition of substance-induced DA release in the NA by DA neuron specific lesion or pharmacological approaches

resulted in inability of acquirement of substance self-administration or maintenance of substance self-administration behaviors. Importantly, DA release in the NA was projected from the VTA DA neurons. The DA neuron activation in the VTA was depending on multiple factors including acetylcholine inputs. Interestingly, among five subtypes of mAChRs (M1-M5), M5 subtypes were highly expressed on the DA neurons in the VTA (Vilaro et al., 1990; Weiner et al., 1990; Yasuda et al., 1993; Lein et al., 2007; Yeomans, 2012). In contrast, M1-M4 subtypes were found on cholinergic neurons instead of dopaminergic neurons (Vilaro et al., 1990; Yeomans, 2012). Although, in comparison of mAChR subtype expression levels in the brain, the M5 mAChRs expression level was 1% of total M1-M5 mAChRs in the brain: M5 mAChRs were highly concentrated in the VTA (Yasuda et al., 1993; Lein et al., 2007). The cholinergic axons placed in the VTA frequently innervated DA synapses in the NA, but rarely prefrontal DA neurons (Omelchenko and Sesack, 2006). Indeed, electrical stimulation of the laterodorsal terminal nucleus (LDT) from where the cholinergic input in the VTA projected increased extracellular DA in the NA (Oakman et al., 1995; Forster and Blaha, 2000). Certainly, LDT-evoked DA release in the NA was significantly reduced in the M5 mAChRs KO mice (Forster and Blaha, 2000). Specifically, LDT stimulation induced three phages of DA release in the NA: a rapid initial increase of DA, a subsequent decrease, and final prolonged increase of DA. In the M5 mAChR KO mice, cholinergic input following LDT stimulation was not able to induce the final prolonged DA release (Forster et al., 2002). Reversely, activation of M5 mAChRs using oxotremorine (an agonist of mAChRs without subtype preferences) resulted in DA release from striatal slices, which was significantly (>50%) reduced in the M5 mAChRs KO mice (Yamada et al., 2003). These findings suggest the possibility of the critical role of M5 mAChRs on rewarding effects. Additionally, the LDT innervation that resulted in DA release in the NA via activating DA neurons in the VTA was known to mediate natural reinforcement, such as food-induced rewarding effects (Sakurai, 2007). Interestingly, the

M5 mAChR KO mice exhibited significantly reduced morphine-induced rewarding effects, which was measured by CPP without altering morphine-induced analgesia (Basile et al., 2002). Furthermore, the M5 mAChRs KO mice showed significantly reduced responses for cocaine (i.v.) in self-administration study in mice (Fink-Jensen et al., 2003). Importantly, the M5 mAChRs KO mice presented no significant abnormal behaviors including food-maintained responses (Basile et al., 2002; Fink-Jensen et al., 2003; Thomsen et al., 2005; Raffa, 2009). Taken together, the M5 mAChRs would be a novel target for substance use disorders including cocaine and opioids. These observations drive the first hypothesis (1) of this dissertation, which is discussed in Chapters 4 and 5. This hypothesis was investigated through the specific aims below.

1.7.2.2 Hypothesis 2 and Specific Aims

Hypothesis 2 (Chapters 4 and 5). Selective and potent antagonists of M5 mAChRs expressed in the VTA provide novel treatments for opioid and cocaine use disorders.

Specific Aim 1. Determine the affinity and selectivity of analogs for M5 over M1-M4 mAChRs.

Specific Aim 1.1. Optimize competitive [^{3}H]N-methylscopolamine ([^{3}H]NMS) binding assay using each human mAChR subtype (M1-M5) expressed on Chinese hamsters ovary (CHO) cell membranes.

Specific Aim 1.2. Evaluate binding affinity and selectivity of analogs for M5 mAChRs over M1-M4 mAChRs.

Specific Aim 2. Determine if the M5 mAChR selective antagonists inhibit mAChR agonist-induced DA release.

Specific Aim 2.1. Determine if M5 mAChRs selective antagonists inhibit oxotremorine, a mAChR agonist without subtype preference, induced DA release from rat striatal brain slices.

Chapter 4 describes the binding affinity of analogs of pethidine structure scaffold for M1-M5 human mAChR subtypes. The structure activity relationship (SAR) and selectivity for M5 versus M1 or M3 is discussed. **Chapter 5** describes binding affinity and selectivity of novel methyl phenylcarbamate structure scaffold for M5 over M1-M4 mAChRs subtypes, and functional potency of the most potent analogs for striatal DA release in the rat brain slices were studied.

2. CHAPTER TWO: NOVEL SCAFFOLD FOR LEAD COMPOUNDS TO TREAT METH USE DISORDERS

2.1 Introduction

The United Nations reported that METH seizures worldwide increased by 158% between 2010 and 2015 (UNODC, 2015), revealing recent dramatic increases in METH use. However, there are no pharmacotherapies approved by the U.S. FDA to treat METH use disorder. The abuse liability of METH is the result of its rewarding effects, which are mediated by increases in DA release in the mesocorticolimbic system (Di Chiara and Imperato, 1988a; Wise and Rompre, 1989). METH penetrates the blood-brain barrier and dopaminergic neuronal cell membranes due to its high lipophilicity (LogP = 2.10) (Gulaboski et al., 2007) and its ability to act as a substrate for the DAT (Johnson et al., 1998). Once inside dopaminergic neurons, METH inhibits MAO activity, leading to a reduction in DA metabolism and increased cytosolic DA concentrations (Mantle et al., 1976). Moreover, increased cytosolic DA concentrations result from METH-induced inhibition of DA transport from the cytosol into presynaptic storage vesicles via the VMAT2 (Peter et al., 1994; Sulzer et al., 2005; Horton et al., 2013). Also, METH stimulates DA release from the vesicles into the cytosol via reverse transport at VMAT2 and via its weak base properties, which reduce the vesicular pH gradient and driving force for VMAT2 function and retention of DA in the storage vesicles (Sulzer and Rayport, 1990; Schuldiner et al., 1993; Sulzer et al., 2005). Increased cytosolic DA concentrations are released from the presynaptic terminals into the extracellular space via reversal of DAT, the functional outcome being METH-induced reward (Sulzer et al., 1995; Fleckenstein et al., 2007; Volkow, Wang, et al., 2011). Support for the concept that METH-induced reward requires an increase in extracellular DA is derived from studies in which bilateral injection of the dopaminergic neurotoxin, 6-OHDA into reward-relevant brain regions (e.g., NA) results in

a decrease in amphetamine self-administration (Lyness et al., 1979). Thus, a reduction in METH-evoked DA release is a desired property of a pharmacotherapy for METH use disorder.

Based on the complex mechanism of action of METH at dopaminergic presynaptic terminals, we identified VMAT2 as a novel target for the discovery of therapeutics for METH use disorder (Dwoskin and Crooks, 2002). Moreover, we identified lobeline (chemical structure shown in Fig. 6), the major alkaloidal natural product from Lobelia inflata, as a potent inhibitor of VMAT2 function using an isolated synaptic vesicle preparation from rat brain; as such, lobeline was identified from in vitro studies as having potential therapeutic efficacy in the treatment of METH use disorder (Teng et al., 1997; Dwoskin and Crooks, 2002). Further evidence supporting the concept that VMAT2 is a viable pharmacological target comes from studies showing that METH-evoked DA efflux from dissociated cells co-expressing VMAT2 and DAT was reduced by 60% in the presence of either lobeline or dihydrotetrabenazene, another VMAT2 inhibitor (Wilhelm et al., 2004). Furthermore, lobeline decreased METH-evoked DA release from the intact rat brain slice preparation and importantly attenuated METH-induced hyperactivity and METH self-administration in preclinical animal models (Miller et al., 2001; Harrod et al., 2003; Wilhelm et al., 2004; Nickell et al., 2010).

Although lobeline decreases the neurochemical and behavioral effects of METH by inhibiting VMAT2 function, lobeline has limitations as a therapeutic candidate, in that it acts nonselectively, inhibiting nicotinic acetylcholine receptors (nAChRs) and opioid receptors (Teng et al., 1997, 1998; Zheng et al., 2005; Miller et al., 2007; Nickell et al., 2014). Also, lobeline has aversive side-effects, including conditioned taste avoidance in rats (Harrod et al., 2004) and nausea in humans, resulting from its bitter taste (Glover et al., 2010). Furthermore, lobeline has a relatively short plasma half-life necessitating

multiple daily dosing, which would likely decrease medication compliance (Miller et al., 2003).

To address these limitations, our research group began a drug discovery program and embarked on structure-activity relationship studies aimed to discover compounds with improved selectivity for VMAT2 and enhanced drug likeness. Lobelane (Fig. 2.1), a chemically defunctionalized lobeline analog, exhibited increased affinity for VMAT2 and low affinity for nAChRs, i.e., improved selectivity for VMAT2 relative to lobeline (Miller et al., 2004; Zheng et al., 2005). Lobelane also inhibited VMAT2 function and METH-evoked DA release from rat brain slices, and importantly, decreased METH-induced hyperactivity and METH self-administration in rats (Miller et al., 2004; Neugebauer et al., 2007; Beckmann et al., 2010). However, tolerance developed to lobelane's behavioral efficacy following repeated administration (Neugebauer et al., 2007). Upon further iterative investigation of the chemical scaffold, GZ-793A (Fig. 2.1) was identified as a lead compound, having high affinity and selectivity (>1030-fold) for VMAT2 over nAChRs (Nickell et al., 2017). GZ-793A inhibited METH-evoked DA release from striatal vesicle preparations and from nucleus accumbens using in vivo microdialysis (Horton et al., 2013; Meyer et al., 2013). Importantly, GZ-793A decreased METH-induced hyperactivity, METH reward in conditioned place preference studies, and METH self-administration without the development of tolerance (Beckmann et al., 2012). Further preclinical research showed that GZ-793A decreased METH-induced and cue-induced reinstatement of METH seeking, indicating its potential efficacy in the treatment of relapse of METH seeking (Alvers et al., 2012; Beckmann et al., 2012; Wilmouth et al., 2013). However, GZ-793A also exhibited affinity for the human-ether-a-go-go related gene (hERG) channel, indicating the potential for cardiotoxicity (Nickell et al., 2017), prohibiting its further development as a medication.

In our continued pursuit of a pharmacotherapy to treat METH use disorder, the current study evaluated in vitro inhibition of VMAT2 function produced by a novel, but related chemical scaffold represented by the lead compound, R-3-(4-methoxyphenyl)-N-(1-phenylpropan-2-yl)propan-1-amine (GZ-11610; Fig. 2.1). Selectivity of GZ-11610 for VMAT2 over DAT, the serotonin transporter (SERT), nAChRs, and the hERG channel was determined. In addition, the ability of GZ-11610 to reduce METH-induced hyperactivity in METH-sensitized rats was assessed as an initial in vivo preclinical evaluation of its therapeutic potential as a treatment for METH use disorder.

2.2 Material and Methods

2.2.1 Animals

Male Sprague-Dawley rats (200-250 g upon arrival) were purchased from Harlan Inc. (Indianapolis, IN, USA) and individually housed with ad libitum access to food and water. Following arrival, rats acclimated to the environment for 1 week prior to the start of experiments. Experimental protocols involving the animals were in accordance with the 2011 National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the University of Kentucky.

2.2.2 Chemicals

Radiolabeled DA ([^{3}H]DA, dihydroxyphenylethylamine, 3,4-[7-3H], specific activity 34.8 Ci/mmol), serotonin ([^{3}H]5-HT, 5-hydroxytryptamine creatinine sulfate, 5-[1,2-3H[N]], specific activity 29.5 Ci/mmol), nicotine ([^{3}H]NIC, L-(-)-[N-methyl-3H]; specific activity, 80.4 Ci/mmol) and MicroScint 20 cocktail were purchased from PerkinElmer (Waltham, MA,

USA). [^{3}H]Dofetilide ([N-methyl-3H], specific activity, 80 Ci/mmol) and methyllycaconitine ([^{3}H]MLA, [1α,S,6β,14α,16β]-20-ethyl-1,6,14,16-tetramethoxy-4-[[[2-([3-3H]-[3-3H]-methyl-2,5-dioxo-1-pyrrolidinyl)benzoyl] oxy]methyl]-aconitane-7,8-diol; specific activity, 60 Ci/mmol) were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). (+)-METH hydrochloride (METH), sucrose, N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), tris[hydroxymethyl]-aminomethane hydrochloride (Trizma HCl), tris[hydroxymethyl]aminomethane base (Trizma), sodium chloride, magnesium sulfate, α-D-glucose, disodium ethylenediamine tetraacetate (EDTA), ethylene glycol tetraacetate (EGTA), magnesium sulfate, potassium hydroxide, potassium tartrate, adenosine triphosphate (ATP-Mg^{2+}), geneticin, polyethyleneimine (PEI), DA hydrochloride, pargyline hydrochloride, catechol, 5-hydroxytryptamine creatinine sulfate (5-HT), amitriptyline, nomifensine maleate (nomifensine), 1-(2-[bis(4-fluorophenyl)methoxy]ethyl)-4-(3-phenylpropyl)piperazine dihydrochloride (GBR-12909), 1-[2-(diphenylmethoxy)ethyl]-4-(3-phenylpropyl)piperazine dihydrochloride (GBR-12935), cytisine, and (-)-nicotine hydrogen tartrate salt were purchased from Sigma-Aldrich (St. Louis, MO). Sodium bicarbonate, potassium chloride, calcium chloride, monopotassium phosphate, sodium hydroxide, and hydrogen chloride were purchased from Fisher Scientific Co. (Pittsburgh, PA). Ascorbic acid was purchased from AnalaR-BHD Ltd. (Poole, UK). Scintillation cocktail 3a70B was purchased from Research Products International Corp. (Mount Prospect, IL). Minimum essential medium, 10% fetal bovine serum and Hanks' Balanced Salt solution were purchased from Gibco (Grand Island, NY). Also, 1% non-essential amino acids was purchased from Thermo Fisher Scientific (Waltham, MA). A VMAT2 inhibitor, 2-Ethyl-9,10-dimethoxy-3-(2-methylpropyl)-1,3,4,6,7,11b-hexahydrobenzo[a]quinolizine-2-ol (RO4-1284) was a kind gift from Hoffman-La Roche, Ltd. (Basel, Switzerland),

2.2.3 Vesicular [^{3}H]DA Uptake

Rat striatal synaptic vesicles were prepared as previously described (Teng et al., 1997) and used to determine GZ-11610-induced inhibition of [^{3}H]DA uptake into the isolated presynaptic vesicles. Briefly, striata from individual rats were homogenized in 14 mL of 0.32 M sucrose solution containing 5 mM sodium bicarbonate (pH 7.4) with 10 up-and-down strokes of a Teflon pestle homogenizer (clearance ~0.009 inch) using a Maxima Digital Overhead Stirrer (400 rpm; Fisher Scientific Co., Pittsburgh, PA). Homogenates were centrifuged (2,000 *g* for 10 min at 4°C), and the resulting supernatants were centrifuged (10,000 *g* for 30 min at 4°C). Pellets were resuspended in 2 mL of 0.32 M sucrose solution and were subjected to osmotic shock by transferring samples to tubes containing 7 mL of ice-cold MilliQ water. Then, samples were homogenized with 5 up-and-down strokes of the Teflon pestle homogenizer. After 5 min, osmolarity was restored by transferring the samples to tubes containing 900 µL of 0.25 M HEPES and 900 µL of 1.0 M potassium tartrate solution. Samples were centrifuged (20,000 *g* for 20 min at 4°C) and resulting supernatants centrifuged (55,000 *g* for 1 h at 4°C), followed by addition of 100 µL of 10 mM magnesium sulfate, 100 µL of 0.25 M HEPES, and 100 µL of 1.0 M potassium tartrate solution, followed by a final centrifugation (100,000 *g* for 45 min at 4°C). Final pellets were resuspended in 2.4 mL of assay buffer (25 mM HEPES, 100 mM potassium tartrate, 50 µM EGTA, 100 µM EDTA, 1.7 mM ascorbic acid, and 2 mM ATP-Mg2+, pH 7.4). Vesicular suspension (100 µL) was added to tubes containing assay buffer (300 µL), various concentrations (0.1 nM - 0.1 mM; 50 µL) of GZ-11610 and 0.1 µM [^{3}H]DA (50 µL) to obtain a final assay volume of 500 µL. Nonspecific uptake was determined using RO4-1284 (10 µM). After incubation for 8 min at 37°C, uptake was terminated by rapid filtration. Scintillation cocktail was added to filters (presoaked GF/B filters in 0.5% PEI for 1 h). Radioactivity retained by the filters was determined by liquid scintillation spectrometry

(TRI-CARB 2100 TR Packard scintillation counter; Packard BioScience Company, Meriden, CT).

2.2.4 Synaptosomal [^{3}H]DA and [^{3}H]5-HT Uptake

Inhibition of [^{3}H]DA and [^{3}H]5-HT uptake via DAT and SERT, respectively, was determined using previously published methods (Teng et al., 1997; Norrholm et al., 2007). Briefly, striata from individual rats were homogenized in 20 mL of 0.32 M sucrose containing 5 mM sodium bicarbonate (pH 7.4) with 16 up-and-down strokes of a Teflon pestle homogenizer (clearance ~0.003 inch) using the Maxima Digital Overhead Stirrer (400 rpm). Homogenates were centrifuged (2,000 *g* for 10 min at 4°C). Resulting supernatants were centrifuged (20,000 *g* for 17 min at 4°C). Pellets were resuspended in 2.4 mL (DA uptake assay) or 1.4 mL (5-HT uptake assay) of assay buffer (125 mM sodium chloride, 5 mM potassium chloride, 1.5 mM magnesium sulfate, 1.25 mM calcium chloride, 1.5 mM monopotassium phosphate, 10 mM α-D-glucose, 25 mM HEPES, 0.1 mM EDTA, 0.1 mM pargyline hydrochloride, and 0.1 mM ascorbic acid, and saturated with 95% O2/5% CO2, pH 7.4). To determine [^{3}H]DA uptake, synaptosomal suspension (25 µL) was added to tubes containing assay buffer (375 µL) and various concentrations (0.1 nM – 0.1 mM; 50 µL) of GZ-11610. To determine [^{3}H]5-HT uptake, synaptosomal suspension (50 µL) was added to tubes containing assay buffer (125 µL) and GBR-12935 (25 µL, 100 nM; a DAT inhibitor). After incubation, tubes were placed on ice for 2 min. [^{3}H]DA (50 µL, 100 nM) or [^{3}H]5-HT (25 µL, 100 nM) was added to each tube, and then incubated at 34°C for 10 min. Assays were performed in duplicate in a total volume of 500 µL for the DA uptake assay, or 250 µL for the 5-HT uptake assay. Uptake was terminated by addition of 3 mL of ice-cold assay buffer and subsequent filtration. Nonspecific [^{3}H]DA and [^{3}H]5-HT uptake

were determined in the presence of nomifensine (100 μM) and fluoxetine (10 μM), respectively. Radioactivity retained by the filters (presoaked in assay buffer containing 1mM catechol for 1 h) was determined.

2.2.5 [^{3}H]NIC and [^{3}H]MLA Binding

GZ-11610-induced inhibition of [^{3}H]NIC and [^{3}H]MLA binding assesses the interaction with α4β2 and α7 nAChRs, respectively. Binding assays employed previously published methods (David B. Horton et al., 2011). In brief, rat whole brain excluding cortex and cerebellum was homogenized in 20 vol of ice-cold assay buffer (2 mM HEPES, 14.4 mM sodium chloride, 0.15 mM potassium chloride, 0.2 mM calcium chloride and 0.1 mM magnesium sulfate at pH 7.5) for 90 sec using a Tekmar polytron (Tekmar-Dohrmann, Mason, OH, USA). Homogenates were centrifuged (31,000 *g* for 17 min at 4°C). Pellets were resuspended in 20 vol of assay buffer by sonication (Vibra Cell, Sonics & Materials Inc., Danbury, CT). Samples were incubated at 37 °C for 10 min (Reciprocal Shaking Bath Model 50, Precision Scientific, Chicago IL, USA). Samples were centrifuged (31,000 *g* for 17 min at 4°C). Resulting pellets were resuspended in 20 vol assay buffer by sonication, and centrifuged (31,000 *g* for 17 min at 4°C). Final pellets were resuspended and stored in incubation buffer (40 mM HEPES, 288 mM sodium chloride, 3.0 mM potassium chloride, 4.0 mM calcium chloride and 2.0 mM magnesium sulfate (pH 7.5). Membrane suspensions (100-140 μg protein/100 μL) were added to tubes containing a single concentration of GZ-11610 (7-9 concentrations, 0.1 nM – 0.1 mM), 3 nM (50 μL) [^{3}H]NIC or [^{3}H]MLA, and incubation buffer for a final assay vol of 250 μL. Samples were incubated for 60 min at room temperature. NIC or MLA (10 pM – 100 μM) concentration-response curves were obtained as positive controls. Nonspecific [^{3}H]NIC or [^{3}H]MLA binding was

determined using 100 µM cytisine and 10 µM nicotine, respectively. Reactions were terminated by filtration on Unifilter-96 GF/B filter plates presoaked in 0.5% PEI using a Packard Filter Mate Harvester (Perkin Elmer, Inc., Waltham, MA). Plates were washed 3 times with 350 µL of ice-cold assay buffer, dried for 60 min at 45 °C, bottom sealed, and each well filled with 40 µL MicroScint 20 cocktail. Bound radioactivity on the filter was determined via liquid scintillation spectrometry (Top Count NXT scintillation counter; PerkinElmer, Inc.).

2.2.6 [^{3}H]Dofetilide Binding

GZ-11610-induced inhibition of [^{3}H]dofetilide binding to hERG channels assessed potential cardiotoxicity. HEK293 cells stably expressing hERG channels were cultured according to the Millipore protocol (Millipore, Billerica, MA, USA). The method for determining [^{3}H]dofetilide binding to hERG protein expressed by the cell membranes was described previously (Sviripa et al., 2014; Nickell et al., 2017). In brief, frozen cells were thawed at 37 °C and immediately transferred into T-75 cm2 flasks containing minimum essential medium supplemented with 10% fetal bovine serum, 1% non-essential amino acids, and 400 µg/mL geneticin. Cells were allowed to adhere for 4-8 h in a humidified atmosphere with 5% CO2. Cells were passaged every 6 days, and medium was replaced every 2 days. At least three passages were performed before membrane collection. On the last passage, cells were seeded into 150 x 25 mm dishes at 2.5 x 106 cells per dish and placed at 30 °C, 5% CO2, for 40-48 h prior to membrane preparation. Cells were rinsed twice with Hanks' Balanced Salt solution at 37 °C and collected by scraping the dishes in ~20 mL of ice-cold 0.32 M sucrose solution containing 5 mM sodium bicarbonate (pH 7.4). Cell membranes were homogenized on ice with a Teflon pestle (~0.003 inch)

using a Maximal Digital homogenizer at 280 rpm for 30 sec. Homogenates were centrifuged (300 *g* and 800 *g* for 4 min each at 4 °C). Pellets were resuspended in 9 mL of ice-cold MilliQ water, and osmolarity was restored by adding 1 mL of 500 mM Tris buffer (pH 7.4), followed by resuspension and centrifugation (20,000 *g* for 30 min at 4 °C). Pellets were resuspended in 2 mL assay buffer (50 mM Tris, 10 mM potassium chloride, and 1 mM magnesium chloride, pH 7.4, at 4 °C). Aliquots of cell membrane suspension were stored at -80 °C and thawed the day of the [^{3}H]dofetilide binding assay. Protein content was determined prior to the assay using a Bradford protein assay with bovine albumin as the standard. On the day of the binding assay, thawed cell membrane suspension (5 µg) were added to duplicate tubes containing assay buffer (150 µL), a single concentration (25 µL; 0.1 nM – 0.1 mM) of GZ-11610 or amitriptyline (0.1 nM – 0.1 mM, as the positive control; Teschemacher et al., 1999; Jo et al., 2000), and 25 µL of [^{3}H]dofetilide (5 nM) for a final assay vol of 250 µL, and incubated for 60 min at room temperature. Amitriptyline (1 mM) was used to determine nonspecific binding. Reactions were terminated by rapid filtration through Whatman GF/B filters presoaked in 0.5% PEI. Filters were washed 3 times with 1 mL ice-cold assay buffer. Radioactivity retained by the filters was determined as described.

2.2.7 METH-Induced Hyperactivity

The ability of GZ-11610 to decrease METH-sensitized locomotor activity was determined using a mixed factor design with METH treatment as a between-subjects factor and GZ-11610 as within-subjects factor (Alvers et al., 2012). Briefly, distance traveled was measured in locomotor activity chambers (42 x 42 x 30 cm) with clear acrylic walls and floor. Chambers contained a horizontal 16 x 16 grid of photo beam sensors

located 2.5 cm apart and 7 cm above the chamber floor. Photo beam breaks were recorded automatically and expressed as distance traveled using Versamax and Digipro System software (AccuScan Instruments Inc., Columbus, OH, USA). Rats were assigned randomly to METH treatment or saline control groups. On day 0 (habituation day, no injection), rats were placed in the locomotor activity chamber for 60 min and then returned to their home cages. On days 1-10, METH (1 mg/kg, s.c.) or saline (1 mL/kg, s.c.) injections were administered based on group assignment, and rats were placed immediately in the chamber for 60 min. On day 11 (first test day), GZ- 11610 (either 1, 10 or 30 mg/kg, s.c.) was administered in a randomized order 15 min prior to METH or saline injection, and then, rats were placed immediately into the activity chamber. Between test days, 2-3 washout days occurred in which METH (1 mg/kg, s.c.) or saline (1 mL/kg, s.c.) was administered in the absence of GZ-11610. To obtain full dose-response curves, additional doses (0, 3 and 5.6 mg/kg) of GZ-11610 were evaluated following s.c. administration using the same group of rats and the same procedures.

In a separate drug-naive group of rats, the ability of oral GZ-11610 to decrease METH-induced hyperactivity was evaluated using a mixed factor design, with METH treatment as a between-subjects factor and GZ-11610 dose a within-subjects factor. GZ-11610 was administered using an ascending dose order. Initially, rats were habituated to the gavage procedure on 5 consecutive days during the METH-sensitization period (Wilmouth et al., 2013). Food was removed from the home cage 2 h prior to each oral gavage. GZ-11610 (5.6 - 300 mg/kg or sterile water vehicle) was administered by oral gavage followed 15 min later by either METH (1 mg/kg, s.c.) or saline (1 mg/kg, s.c.) injection, depending on group assignment, and then rats were placed immediately into the activity chamber. On habituation days and on washout days between test days, 2 mL of vehicle was administered 15 min prior to the locomotor activity session.

2.2.8 Data Analysis

Specific [^{3}H]DA and [^{3}H]5-HT uptake as well as [^{3}H]dofetilide, [^{3}H]NIC, and [^{3}H]MLA binding were obtained by subtracting nonspecific uptake or binding from total uptake or binding, respectively. Concentration of GZ-11610 that produced 50% inhibition of uptake or binding (IC_{50} values) was obtained from the concentration-response curves via an iterative curve-fitting program (Prism 4.0; GraphPad Software Inc., San Diego, CA, USA). Inhibition constants (Ki values) were determined using the Cheng-Prusoff equation (Cheng and Prusoff, 1973). Distance traveled (in meters) during the last 45 min of the 60 min locomotor activity session was analyzed using two-way or one-way ANOVA followed by Tukey's or Dunnett's post hoc analysis, as noted. Data from the first 15 min of the session was considered to be a habituation period and thus, was not included in the analysis. Data analysis was performed using GraphPad Prism 7.03 (GraphPad Software, Inc., La Jolla, CA, USA).

2.3 Results

VMAT2 affinity and selectivity. GZ-11610 potently (Ki = 8.7 nM) inhibited [^{3}H]DA uptake at VMAT2, with a maximal inhibition (Imax) of >95% (Fig. 2.2). GZ-11610 also inhibited [^{3}H]DA uptake at DAT and [^{3}H]5-HT uptake at SERT (Ki = 2.51 and 5.55 μM, respectively), with an Imax at both transporters >95% (Fig. 2.2). GZ-11610 exhibited 288- and 637-fold greater affinity for VMAT2 relative to DAT and SERT, respectively, Thus, GZ-11610 is selective for VMAT2 over DAT and SERT. Also, GZ-11610 inhibited [^{3}H]dofetilide binding to hERG channels expressed on HEK-293 cell membranes, with a Ki of 9.50 μM

and an Imax of >80% (Fig. 2.2). Thus, GZ-11610 was 1090-fold selective for VMAT2 over hERG. Further, across a wide concentration range (0.1 nM – 0.1 mM), GZ-11610 exhibited an Imax of <20% inhibition of [^{3}H]NIC and [^{3}H]MLA binding to rat brain membranes (Fig. 2.2); as such, Ki values could not be obtained. Overall, GZ-11610 exhibited high affinity for VMAT2 and greater than two-orders of magnitude selectivity for VMAT2 over DAT, SERT, hERG, and α4β2 and α7 nAChRs.

METH sensitization. For Day 0, 1 and 10, data from the two experiments determining effects of s.c. and oral administration of GZ-11610 were combined to evaluate effects of acute and repeated administration of METH or saline, since these data were obtained prior to GZ-11610 administration and groups were handled identically in the two experiments. Two-way ANOVA revealed a significant treatment x day interaction [$F_{2,14}$ = 36.44, $p < 0.0001$]. On Day 0 (habituation, prior to METH or saline administration), no differences in distance traveled between the METH-treated and saline-injected groups were found (Fig. 2.3). On Day 1, acute METH (1 mg/kg, s.c.) increased ($p < 0.05$) distance traveled compared to saline control (Fig. 2.3). On Day 10, repeated administration of METH (1 mg/kg, s.c., once daily for 10 days) increased distance traveled ($p < 0.05$) compared to 10 consecutive daily saline injections (Fig. 2.3). On Day 10, distance traveled by the METH group was greater than distance traveled by this same group on Day 1 ($p < 0.05$; Fig. 2.3), indicative of sensitization.

Effect of GZ-11610 (s.c.) on METH-sensitized locomotor activity. After 10 consecutive daily administrations of METH or saline, GZ-11610 (1 - 30 mg/kg) or vehicle (sterile water) was administered s.c. to both groups of rats 15 min prior to injection (s.c.) of METH or saline, respectively, followed by placement in the activity chamber. Two-way ANOVA revealed a GZ-11610 pretreatment x METH treatment interaction [$F_{5,48}$ = 68.81, $p < 0.0001$] on distance traveled. Compared to vehicle injection, GZ-11610 dose-dependently

reduced the distance traveled following an injection of METH in the METH-sensitized group (Fig. 2.4). Tukey's test revealed that GZ-11610 at doses from 3 to 30 mg/kg were different from vehicle in the METH group. Although GZ-11610 appeared to decrease activity in the saline group, this decrease did not reach statistical significance as determined by Tukey's test, likely due to the high variably following the vehicle injection (Fig. 2.4, inset). Additionally, pair-wise comparison of the distance traveled between the METH group and the saline group after each dose of GZ-11610 revealed a greater distance traveled in the METH group ($p < 0.05$, Tukey's test), with the exception of the highest dose (30 mg/kg) of GZ-11610. However, using one-way ANOVA to assess the effect of GZ-11610 in the saline group revealed a significant dose effect [$F = 5.55$, $p < 0.005$], and Dunnett's test indicated a significant decrease in locomotor activity following 3-30 mg/kg (Fig. 2.4, inset). Thus, GZ-11610 (s.c.) decreased METH-induced hyperactivity in METH-sensitized rats and in the saline control group, such that the effect of GZ-11610 was not specific. The decrease in activity induced by GZ-11610 in saline group did not appear to be due to lethargy or pain, as noted by the experimenter.

Effect of GZ-11610 (oral) on METH-sensitized locomotor activity. After 10 consecutive daily administrations of METH or saline, GZ-11610 (5.6 – 300 mg/kg) or vehicle (sterile water) was administered orally to rats in both groups 15 min prior to injection (s.c.) of METH or saline, respectively, and placement in the activity monitor. Two-way ANOVA revealed a GZ-11610 pretreatment x METH treatment interaction [$F_{7,63} = 7.403$, $p < 0.0001$] on distance traveled. Compared to vehicle injection, GZ-11610 dose dependently reduced the distance traveled following an injection of METH in the METH-sensitized group (Fig. 2.5). Tukey's test revealed that GZ-11610 at doses from 56 – 300 mg/kg were different from vehicle in the METH group; whereas, GZ-11610 did not decrease significantly the distance traveled in the saline group compared to vehicle injection (Fig. 2.5, inset).

Furthermore, pair-wise comparison of the distance traveled between the METH group and the saline group after each dose of GZ-11610 revealed a greater distance traveled in the METH group. Using one-way ANOVA to assess the effect of GZ-11610 in the saline group also revealed that there were no significant GZ-11610 effects on locomotor activity in saline group [$F_{7,32} = 0.572$, $p > 0.05$]. Thus, oral GZ-11610 specifically decreased METH-induced hyperactivity in METH-sensitized rats.

Of note, there was a 2.3-fold greater amount of locomotor activity in the saline group following oral administration of vehicle (Fig. 2.5, insert) relative to that after s.c. administration of vehicle (Fig. 2.4, insert). Despite the habituation to the oral gavage, this procedure may have produced some stress, resulting in a greater mean amount of locomotor activity. However, the standard errors of the mean in the oral and s.c. vehicle conditions overlap, suggesting that there were not significant differences in locomotor activity between these control groups.

2.4 Discussion

Using an iterative drug discovery approach targeting VMAT2, the current study identified a new N-butyl(1-methyl-2-phenylethyl)amine, amphetamine-like scaffold, containing an important chiral center, and moreover, evaluated the neurochemical and behavioral effects of GZ-11610, the pure R-enantiomer. GZ-11610 was found to selectively inhibit VMAT2 function and to specifically attenuate METH-sensitized locomotor activity when given by the oral route, suggesting that this lead compound has potential as a candidate pharmacotherapy for METH use disorder.

The current in vitro neurochemical results demonstrate that GZ-11610 exhibited high affinity (Ki = 8.7 nM) for VMAT2 and high selectivity (290 to 3500-fold) for VMAT2 over DAT, SERT, hERG, α4β2 nAChRs and α7 nAChRs. The high selectivity for VMAT2 over DAT suggests that GZ-11610 may lack abuse liability. Inhibition of DAT function results in increased extracellular DA concentrations and stimulation of postsynaptic DA receptors leading to reward. Inhibition of DAT function is highly correlated with psychostimulant-induced behaviors, reward and abuse liability (Stathis et al., 1995; Swanson and Volkow, 2003; Volkow and Swanson, 2003; German et al., 2015).

The 4-orders of magnitude selectivity for VMAT2 over hERG suggests reduced risk for untoward cardiac arrhythmias at concentrations of GZ-11610 interacting with VMAT2 and providing therapeutic efficacy. Interaction at hERG channels has been associated with cardiac arrhythmias due to the role of these inward rectifying potassium channels during depolarization of the heart muscle and propagation of cardiac rhythm (Trudeau et al., 1995; Abbott et al., 1999; Doggrell and Hancox, 2014). Selectivity regarding hERG in the current study is particularly compelling, because previous lead compounds acting as VMAT2 inhibitors, including lobeline, lobelane and GZ-793A, provided only 3 to 26-fold selectivity for VMAT2 over hERG (Nickell et al., 2010, 2017).

Importantly, the greater than 4-orders of magnitude selectivity for VMAT2 over the most abundant nicotinic receptor subtypes (α4β2 and α7) supports the interpretation that these nAChR protein targets are not responsible for the decrease in METH's behavioral effects nor their potential therapeutic efficacy. Furthermore, the robust selectivity of GZ-11606 will allow validation of VMAT2 as an important pharmacological target in the discovery for METH use disorder therapeutics.

In the current study, GZ-11610 decreased the psychomotor response to METH in rats that previously had been sensitized to repeated METH administration. Based on the current findings, GZ-11610 appears to have improved potency and efficacy reducing METH-induced locomotor activity relative to our previous lead compounds, lobeline, lobelane and GZ-793A (DK Miller et al., 2001; Neugebauer et al., 2007; Alvers et al., 2012). However, the behavioral effects of the previous leads were evaluated in these earlier studies with respect to their ability to reduce the acute, dose-related hyperactivity induced by METH. Behavioral sensitization induced by repeated exposure to psychostimulants has been established as an animal model of human addiction due to the associated enduring alterations in nucleus accumbens DA neurochemistry leading to reward and persistent drug seeking (Kalivas and Stewart, 1991; Vezina et al., 2002; Volkow, Wang, et al., 2011; Wang et al., 2017). Thus, the GZ-11610-induced decrease in METH-stimulated locomotor activity in METH-sensitized animals provides greater face validity with respect to the clinical situation than does a reduction in the acute behavioral response to METH.

The assertion that the GZ-11610-induced decrease in METH-stimulated locomotor activity in METH-sensitized rats was specific is based on the observation that GZ-11610, following oral administration, did not significantly alter locomotor activity in the control group repeatedly administered saline. However, following s.c. administration, GZ-11610 was found to decrease locomotor activity in the saline control group. Thus, the GZ-11610-induced decrease in METH sensitized activity was specific only following oral administration of GZ-11610. This is important because the oral route is the preferred clinical route of administration.

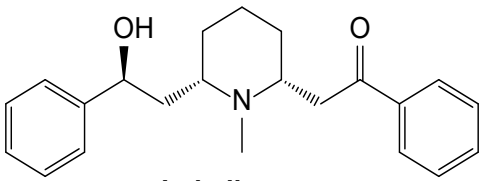
Consistent with previous findings regarding our earlier lead compounds (DK Miller et al., 2001; Neugebauer et al., 2007), GZ-11610 significantly decreased METH-sensitized locomotor activity following either s.c. or oral administration. The GZ-11610-induced

decrease in METH's behavioral response supports the interpretation that GZ-11610 penetrates the blood-brain barrier and accesses the brain and that the lead compound is sufficiently orally bioavailable to demonstrate efficacy. However, an approximately 10-fold higher dose was required following oral administration relative to s.c. administration. Further, the maximal effect after oral administration of GZ-11610 was about 50% of that following s.c. administration. Likely, the physicochemical characteristics (e.g., solubility, logP) and/or pharmacokinetic properties (e.g., half-life, clearance, plasma protein binding, metabolism) of GZ-11610 may be responsible for the observed reduced potency and efficacy following oral versus s.c. administration. Interestingly, it is possible that GZ-11610 (R-enantiomer) may be metabolized via oxidative N-CH_2 bond cleavage to R-amphetamine, which has relatively lower psychostimulant effects compared with S-amphetamine (Heal et al., 2013). Future pharmacokinetic and drug metabolism studies will determine the ADME profile of GZ-11610 and also identify its metabolites after oral administration, in order to assess the limitations and potential abuse liability of this promising lead compound.

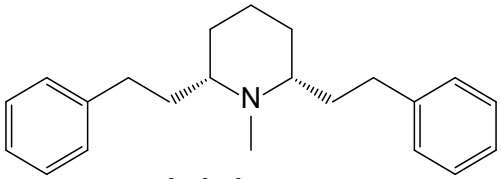
2.5 Conclusion

GZ-11610, a representative compound from a new VMAT2-selective inhibitor scaffold (N-butyl(1-methyl-2-phenylethyl)amine), was identified and its effects on METH-stimulated locomotion in METH-sensitized rats were evaluated following s.c. and oral administration. GZ-11610 exhibited high affinity (Ki = 8.7 nM) and high selectivity (290- to 3500-fold) for VMAT2 over hERG, DAT, SERT, and nAChRs. Of note, GZ-11610 exhibited robustly improved selectivity for VMAT2 over the hERG channel (>1000-fold) compared to previously reported VMAT2 inhibitors (3- to 26-fold). Oral administration of GZ-11610

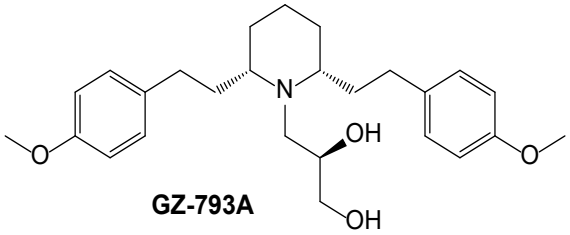
specifically reduced METH-stimulated locomotor activity in METH-sensitized rats. Further studies aimed at improving the bioavailability of GZ-11610 would contribute to the development of this lead compound as a treatment for METH use disorders.



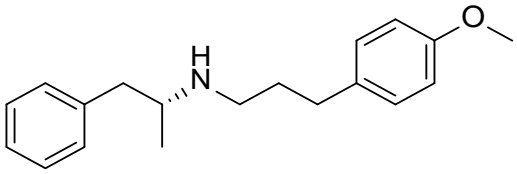
Lobeline



Lobelane



GZ-793A



GZ-11610

Figure 2.1. Chemical structures of lobeline, lobelane, GZ-793A and GZ-11610.

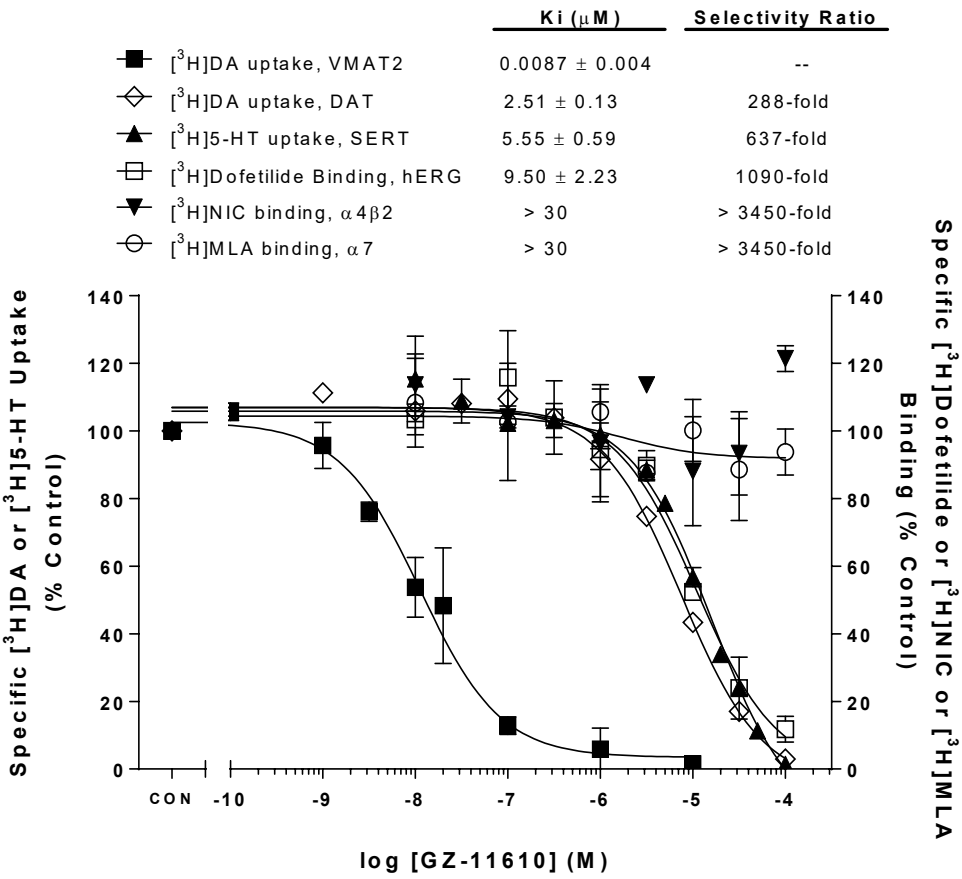


Figure 2.2. GZ-11610 potently inhibits [^{3}H]DA uptake at VMAT2 and is selective for VMAT2 over DAT, SERT, hERG, α4β2 nAChRs and α7 nAChRs. Ki values and selectivity ratios are provided in the symbol legend. Data are mean ± SEM specific uptake or binding expressed as % of the respective control (CON) uptake or binding in the absence of GZ-11610. Control values (mean ± SEM) are the following: for specific [^{3}H]DA uptake at VMAT2, 49.6 ± 8.35 pmol/mg of protein/min; for specific [^{3}H]DA uptake at DAT, 12.2 ± 0.54 pmol/mg of protein/min; for specific [^{3}H]5-HT uptake at SERT, 7.89 ± 0.51 pmol/mg of protein/min; for specific hERG binding, 1540 ± 178 fmol/mg; for specific [^{3}H]NIC binding at α4β2 nAChRs, 22.6 ± 3.38 fmol/mg; and for specific [^{3}H]MLA binding at α7 nAChRs, 36.4 ± 2.11 fmol/mg. (n=4 rats for neurotransmitter uptake assays, n=3 cell batches for hERG assays, and n=3 for nAChR binding assays).

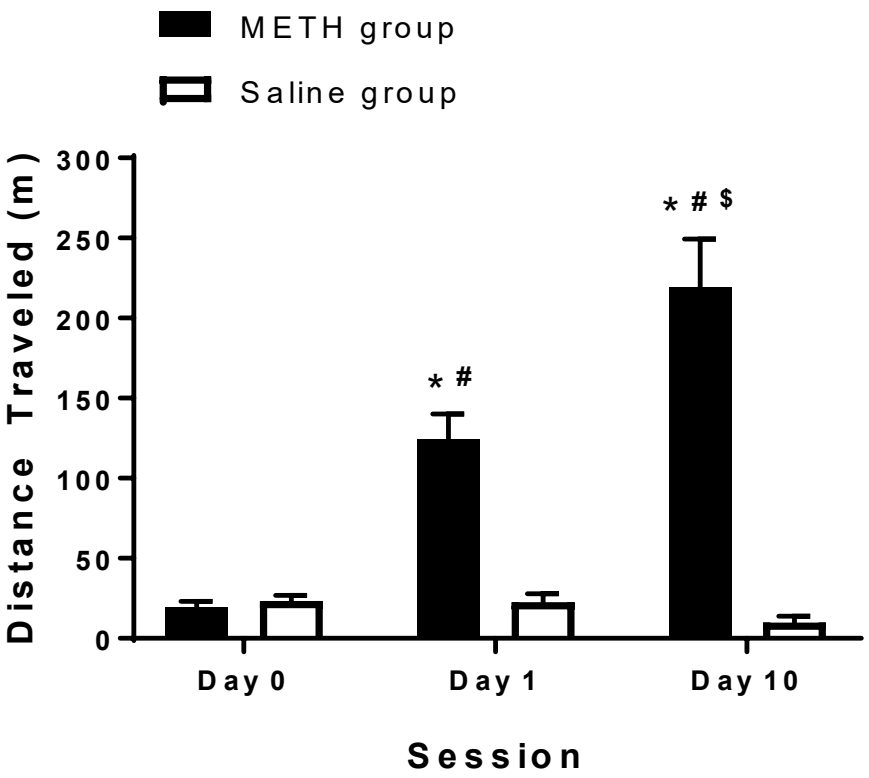


Figure 2.3. Locomotor sensitization to repeated METH administration. Day 0 (habituation day) represents distance traveled before METH or saline injection. Day 1 represents distance traveled following acute METH (1 mg/kg, s.c.) or saline (1 mL/kg, s.c.) injection. Day 10 represents distance traveled following 10 daily injections of METH or saline. Data are mean ± SEM distance traveled in meters. Data were analyzed using two-way mixed factor ANOVA followed by Tukey's post hoc tests. (*$p < 0.05$ compared to respective saline group;

# $p < 0.05$ compared to METH group on Day 0; $ $p < 0.05$ compared to METH group on Day 1; n=10/group; however, due to a computer problem, data for n=2 from each of the METH and saline groups are not included in the analysis).

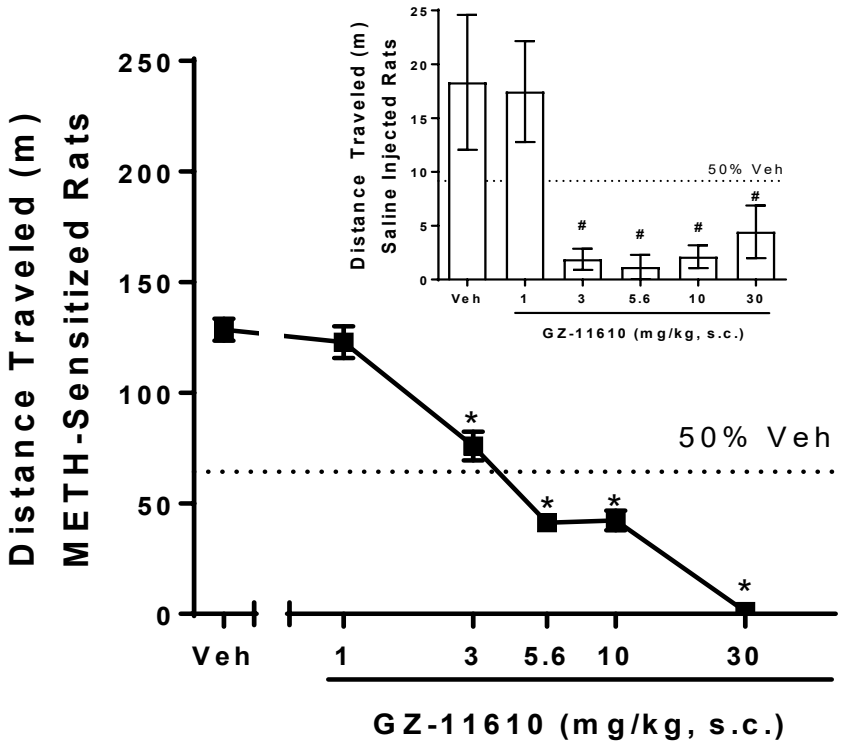


Figure 2.4. GZ-11610 (s.c.) decreased METH-induced hyperactivity in METH-sensitized and control rats. GZ-11610 (1 – 30 mg/kg) or vehicle (Veh, sterile water) was administered s.c. to the METH-sensitized group and the saline group (inset) 15 min before METH (1 mg/kg, s.c.) or saline (1 mL/kg, s.c.), respectively, followed by placement in the activity chamber. Data are mean ± SEM distance traveled in meters. Dotted line represents 50% of the distance traveled after vehicle injection. Data were analyzed by two-way mixed factor ANOVA followed by Tukey's post hoc tests (*$p < 0.05$ compared to vehicle within each group; n = 5/group) or by one-way ANOVA followed by Dunnett's test (#$p < 0.05$ compared to vehicle).

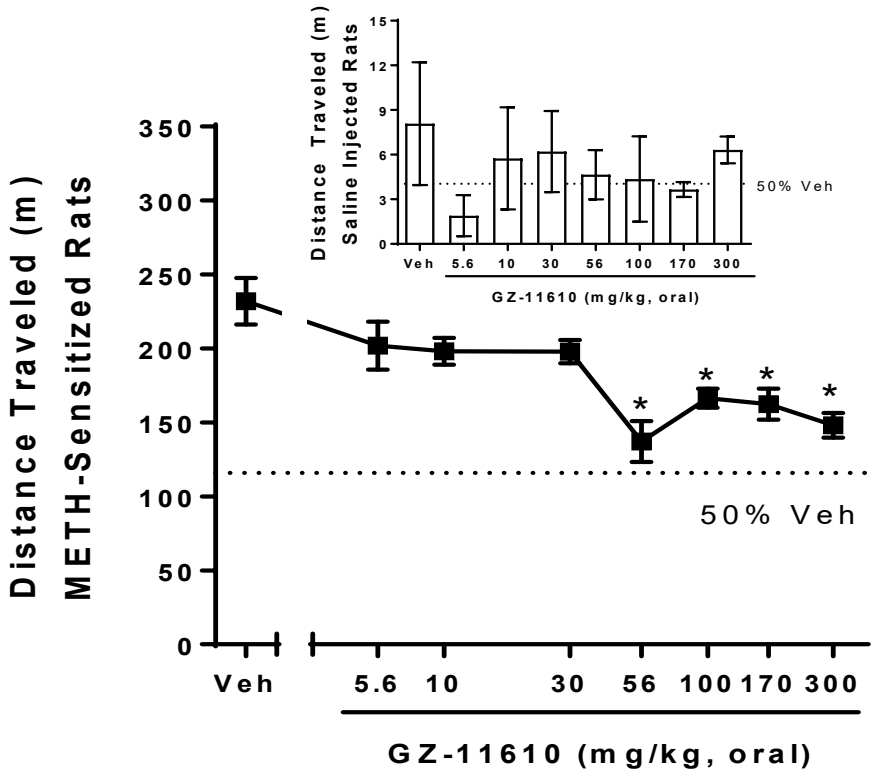


Figure 2.5. GZ-11610 (oral) specifically decreased METH-induced hyperactivity in METH-sensitized rats. GZ-11610 (5.6 – 300 mg/kg) or vehicle (Veh, sterile water) was administered orally to the METH-sensitized group and the saline group (inset) 15 min before METH (1 mg/kg, s.c.) or saline (1 mL/kg, s.c.), respectively, followed by placement in the activity chamber. Data are mean ± SEM distance traveled in meters. Dotted line represents 50% of the distance traveled after vehicle injection. Data were analyzed by two-way mixed factor ANOVA followed by Tukey's post hoc tests. (*$p < 0.05$ compared to vehicle within each group; n = 5/group), or by one-way ANOVA for the data in the saline control group.

3. CHAPTER THREE: GZ-11608, A VESICULAR MONOAMINE TRANSPORTER-2 INHBITOR, DECREASES THE NEUROCHEMICAL AND BEHAVIORAL EFFECTS OF METH

3.1 Introduction

METH use disorder is characterized by a constellation of symptoms including relapse, continued use despite adverse consequences, and social impairment (American Psychiatric Association, 2013). From 2010 to 2016, seizures of METH by United States law enforcement agencies increased 3.2-fold and METH overdose death rates increased 4.1-fold, indicative of escalating use (UNODC, 2014, 2018; NIDA, 2018). Importantly, METH use has increased among opioid users as access to opioids has diminished; and associations between these epidemics are recently being recognized (Ellis et al., 2018). In 2015, 135,000 Americans aged 12 and older sought treatment for METH use disorder at publicly-licensed facilities (DEA, 2018). Unfortunately, even now, FDA-approved pharmacotherapeutics for METH use disorder are not available.

Substantial effort has been directed towards discovering a pharmacotherapeutic for METH use disorder (see reviews: Ballester et al., 2017; Dwoskin et al., 2017; Reynolds et al., 2017). METH redistributes DA from synaptic vesicles into the cytosol by interacting with the VMAT2 and disrupting the vesicular pH gradient (Sulzer and Rayport, 1990; Sulzer et al., 1995; Dwoskin and Crooks, 2002). Also, METH reverses DAT function, resulting in transport of DA from the cytosol into the extracellular compartment, ultimately mediating METH reward and abuse liability (Wise and Rompre, 1989; Volkow et al., 2017).

The current study extends our iterative drug discovery research targeting VMAT2 with the goal of obviating the neuropharmacological effects of METH. Initially, lobeline, the major alkaloid in Lobelia inflata, was found to inhibit VMAT2 function (Ki = 470 nM) and

reduce METH-evoked DA release from superfused striatal slices and from nucleus accumbens following in vivo microdialysis in rats (Miller et al., 2001; Nickell et al., 2010; Meyer et al., 2013). Lobeline also decreased i.v. METH self-administration in rats without the development of tolerance (Harrod et al., 2001). Furthermore, increasing the METH unit dose did not surmount the lobeline-induced decrease in responding for METH (Harrod et al., 2001). Based on these preclinical findings, lobeline was evaluated in Phase 1 clinical trials and found to be safe in individuals actively using METH (Jones, 2007). Due to its physicochemical properties and pharmacokinetic profile, enthusiasm for its further clinical development diminished.

Lobelane, a chemically defunctionalized lobeline analog, was identified from SAR studies, as exhibiting greater potency (10-fold) and reduced affinity for α4β2 and α7 nicotinic acetylcholine receptors, thereby, augmenting selectivity for VMAT2 (Miller et al., 2004; Zheng et al., 2005; Nickell et al., 2010). Lobelane decreased METH-induced hyperlocomotion and decreased METH self-administration, however, the development of tolerance limited its therapeutic utility (Neugebauer et al., 2007). Further SAR identified R-N-(1,2-dihydroxypropyl)-2,6-cis-di-(4-methoxyphenethyl)-piperidine hydrochloride (GZ-793A), containing an N-1,2,-dihydroxypropyl in place of the N-methyl group in lobelane (Horton et al., 2011). GZ-793A exhibited high affinity (Ki = 29 nM) for VMAT2, inhibited METH-evoked DA release from striatal slices and synaptic vesicular preparations, and from nucleus accumbens using in vivo microdialysis (Horton et al., 2011, 2013; Meyer et al., 2013; Nickell et al., 2017). GZ-793A decreased METH self-administration and reinstatement of METH seeking behavior (Alvers et al., 2012; Beckmann et al., 2012). Importantly, tolerance did not develop to the GZ-793A-induced decrease in METH self-administration (Beckmann et al., 2012). Unfortunately, GZ-793A interacted with the human-ether-a-go-go-related gene (hERG) channel, revealing potential for cardiotoxicity

and precluding its further development (Abbott et al., 1999; Sanguinetti and Tristani-Firouzi, 2006; Nickell et al., 2017).

Expansion of the SAR focused on minimizing the hERG interaction and resulted in a new structural scaffold with a phenylalkyl moiety replacing the piperidine ring in GZ-793A (Lee et al., 2018). Additionally, the new scaffold has only one chiral center rather than three in GZ-793A. The enantiomerically-pure lead analog, R-3-(4-methoxyphenyl)-N-(1-phenylpropan-2-yl)propan-1-amine (GZ-11610), was identified as having high affinity (Ki = 8.7 nM) and selectivity (1090-fold) for VMAT2 over hERG. GZ-11610 (p.o.) specifically decreased METH-sensitized locomotor activity. Although limitations in efficacy and potency for GZ-11610 were noted, the new scaffold showed good potential for identifying a high value lead compound.

The current study investigated the pharmacology of enantiomerically-pure S-3-(4-methoxyphenyl)-N-(1-phenylpropan-2-yl)propan-1-amine (GZ-11608). VMAT2 affinity, selectivity and mechanism of inhibition of METH-evoked vesicular DA release were determined. Ability of GZ-11608 to exacerbate the METH-induced decrease in striatal DA content was evaluated ex vivo. Effects of GZ-11608 on METH-sensitized locomotor activity, METH self-administration and reinstatement also were determined. Development of tolerance and the potential for METH to surmount the efficacy of GZ-11608 to decrease METH self-administration were determined. Furthermore, the abuse liability of GZ-11608 was evaluated by determining its ability to substitute for METH in the self-administration assay and by the acquisition of i.v. GZ-11608 self-administration in drug naïve rats.

3.2 Material and Methods

3.2.1 Animals

Adult male Sprague-Dawley rats (body weight of 300-400 g during conduct of experiments, Harlan, Indianapolis, IN) were individually housed for behavioral studies and housed under standard conditions for neurochemical and pharmacokinetic assays. Upon arrival, rats were given free access to food and water in their home cages, which were maintained in an environment maintained at 24 ºC, 45% humidity, and 14/10 h light/dark cycle. Rats acclimated to the environment for one week prior to initiation of experiments, and when used in behavioral experiments, rats were handled daily. During operant training, food in the home cage was limited to 5-10 g/day to maintain bodyweight at ~85%, and then, free feeding continued once rats reached criteria for stable responding. Experiments were conducted during the light phase. Experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Kentucky and were in accordance with the 2011 National Institutes of Health Guide for the Care and Use of Laboratory Animals.

3.2.2 Chemicals

[^{3}H]Dopamine ([^{3}H]DA; dihydroxyphenylethylamine, 3,4-[7-^{3}H]; specific activity, 24.8 Ci/mmol), [^{3}H]5-hydroxytryptamine ([^{3}H]5-HT; 5-hydroxytryptamine creatinine sulfate, 5-[1,2-^{3}H[N]]; specific activity, 29.5 Ci/mmol), [^{3}H]nicotine ([^{3}H]NIC; (L-(-)-[N-methyl-^{3}H]; specific activity, 80.4 Ci/mmol), and MicroScint 20 cocktail were obtained from PerkinElmer Inc. (Waltham, MA). [^{3}H]Dofetilide ([N-methyl-^{3}H]; specific activity, 80 Ci/mmol) and [^{3}H]methyllycaconitine ([^{3}H]MLA; [1α,4S,6β,14α,16β]-20-ethyl-1,6,14,16-

tetramethoxy-4-[[[2-([3-^{3}H]-[3-^{3}H]-methyl-2,5-dioxo-1-pyrrolidinyl)benzoyl]oxy]methyl]-aconitane-7,8-diol; specific activity, 60 Ci/mmol) were obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). (+)-METH hydrochloride, 1-[2-(diphenylmethoxy)ethyl]-4-(3-phenylpropyl)piperazine dihydrochloride (GBR-12935), amitriptyline, cytisine, fluoxetine, S(-)-nicotine hydrogen tartrate salt, nomifensine, 1-octanesulfonic acid sodium salt, 3-(4-methoxyphenyl)propanoic acid, 5-hydroxytryptamine creatinine sulfate, adenosine 5'-triphosphate magnesium salt (ATP-Mg^{2+}), α-D-glucose, ammonium chloride, anhydrous sodium sulfate, ascorbate oxidase, catechol, celite, dichloromethane, diethyl ether, dimethylformamide, dopamine hydrochloride, ethyl acetate, ethylenediamine tetraacetate (EDTA), ethylene glycol tetraacetate (EGTA), hexane, hydrochloric acid, Kolliphor EL®, magnesium sulfate, methanesulfonyl chloride, methanol, methylene chloride, N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride, pargyline hydrochloride, phenyllithium, polyethyleneimine (PEI), potassium hydroxide, potassium tartrate dibasic hemihydrate, R-propylene oxide, sodium azide, silica, sodium chloride, sodium hydroxide, sucrose, tetrahydrofuran, triethylamine, triphenylphosphine, tris[hydroxymethyl]aminomethane base, tetrahydrofuran, and tris[hydroxymethyl]-aminomethane hydrochloride were obtained from Sigma-Aldrich (St. Louis, MO). Calcium chloride, citric acid, formic acid, hydrogen chloride, methanol, monopotassium phosphate, potassium chloride, sodium bicarbonate, and sodium hydroxide were obtained from Fisher Scientific Co. (Pittsburgh, PA). Ascorbic acid, 1% non-essential amino acids, and scintillation cocktail 3A70B were purchased from AnalaR-BHD Ltd. (Poole, UK), Thermo Fisher Scientific (Waltham, MA), and Research Products International Corp. (Mount Prospect, IL), respectively. Minimum essential medium (MEM), Hanks' Balanced Salt solution, and 10% fetal bovine serum were obtained from Gibco (Grand Island, NY). (2R,3S,11bS)-2-Ethyl-3-isobutyl-9,10-dimethoxy-2,2,4,6,7,11b-hexahydro-1H-pyrido[2,1-

a]isoquinolin-2-ol (RO4-1284) was a generous gift from Hoffmann-LaRoche Inc. (Nutley, NJ).

3.2.3 Vesicular [^{3}H]DA Uptake

To obtain the affinity (Ki value) of GZ-11608 for VMAT2, the ability of GZ-11608 to concentration-dependently inhibit [^{3}H]DA uptake into isolated synaptic vesicles, as previously described (Teng et al., 1997). Nonspecific uptake of [^{3}H]DA was determined in the presence of RO4-1284 (10 µM). Briefly, striata from individual rats were homogenized in 14 mL of ice-cold 0.32 M sucrose solution containing 5 mM sodium bicarbonate (pH 7.4) with 10 up-and-down strokes of a Teflon pestle homogenizer (clearance ~0.009 inch) using a Maxima Digital Overhead Stirrer (400 rpm; Fisher Scientific Co., Pittsburgh, PA). Homogenates were centrifuged (2,000 *g* for 10 min at 4°C), and the resulting supernatants were centrifuged (10,000 *g* for 30 min at 4°C). Pellets were resuspended in 2 mL of 0.32 M sucrose solution containing 5 mM sodium bicarbonate (pH 7.4) and were subjected to osmotic shock by transferring samples to tubes containing 7 mL of ice-cold MilliQ water. Samples were homogenized on ice with 5 up-and-down strokes of a Teflon pestle homogenizer. After 5 min, osmolarity was restored by transferring the samples to tubes containing 900 µL of 0.25 M HEPES and 900 µL of 1.0 M potassium tartrate dibasic hemihydrate solution. Samples were centrifuged (20,000g for 20 min at 4°C) and resulting supernatants centrifuged (55,000 *g* for 1 h at 4°C). To the resulting supernatants, 100 µL of 10 mM magnesium sulfate, 100 µL of 0.25 M HEPES, and 100 µL of 1.0 M potassium tartrate dibasic hemihydrate solution were added, followed by a final centrifugation (100,000g for 45 min at 4°C). Final pellets were resuspended in 2.4 mL of assay buffer (25 mM HEPES, 100 mM potassium tartrate dibasic hemihydrate, 50 µM EGTA, 100 µM

EDTA, 1.7 mM ascorbic acid, and 2 mM ATP-Mg^{2+}, pH 7.4 adjusted dropwise with 10 M potassium hydroxide). Aliquots of the resulting suspension of isolated synaptic vesicles (100 µL) were added to tubes containing assay buffer (300 µL), one of a range of concentrations of GZ-11608 (final concentration 0.1 nM - 0.1 mM; 50 µL) and 0.1 µM [^{3}H]DA (final concentration 10 nM; 50 µL) to obtain a final assay vol of 500 µL. After incubation for 8 min in a 37°C water bath (Reciprocal Shaking Bath Model 50, Precision Scientific, Chicago IL, USA), [^{3}H]DA uptake was stopped by rapid filtration through presoaked (0.5% PEI for 1 h at 4 ºC) Whatman® GF/B Glass microfiber filters (1.0 µm pore size; Clifton, NJ) via a cell harvester (MP-43RS; Brandel Inc., Gaithersburg, MD). Subsequently, filters were washed 3 times with 4 mL of ice-cold wash buffer (25 mM HEPES, 100 mM potassium tartrate dibasic hemihydrate, 50 µM EGTA, 100 µM EDTA, 1.7 mM ascorbic acid, and 2 mM magnesium sulphate; pH 7.4 adjusted dropwise with 10 M potassium hydroxide). Scintillation cocktail (5 mL) was added to tubes containing the filters, followed by shaking for 30 min at room temperature. Radioactivity retained on the filters was determined by liquid scintillation spectrometry (TRI-CARB 2100 TR Packard scintillation counter; Packard BioScience Company, Meriden, CT).

3.2.4 Synaptosomal [^{3}H]DA and [^{3}H]5-HT Uptake

To evaluate the selectivity of GZ-11608 at VMAT2 relative to DAT and SERT, GZ-11608 inhibition of [^{3}H]DA and [^{3}H]5-HT uptake, respectively, into rat striatal synaptosomes was determined using previously published methods (Teng et al., 1997; Norrholm et al., 2007). Briefly, striata from individual rats were homogenized in 20 mL of 0.32 M sucrose containing 5 mM sodium bicarbonate (pH 7.4) with 16 up-and-down strokes of a Teflon pestle homogenizer (clearance ~0.003 inch) using the Maxima Digital

Overhead Stirrer (400 rpm). Homogenates were centrifuged (2,000 *g* for 10 min at 4°C). Supernatants were centrifuged (20,000g for 17 min at 4°C) and pellets were resuspended (2.4 mL for DAT assay; 1.4 mL for SERT assay) in Krebs' buffer (125 mM sodium chloride, 5 mM potassium chloride, 1.5 mM magnesium sulfate, 1.25 mM calcium chloride, 1.5 mM monopotassium phosphate, 10 mM α-D-glucose, 25 mM HEPES, 0.1 mM EDTA, 0.1 mM pargyline hydrochloride, and 0.1 mM ascorbic acid, and saturated with 95% O_2/5% CO_2; pH 7.4 adjusted dropwise with 1 M sodium hydroxide). For DAT and SERT assays, aliquots of synaptosomal suspension (25 µL and 50 µL, respectively) were added to tubes containing Krebs' buffer (375 µL and 125 µL, respectively) and one of a range of concentrations of GZ-11608 (0, 0.1 nM – 0.1 mM) in 50 µl and 25 µl buffer, respectively. Uptake in the absence of GZ-11608 represents control. For nonspecific uptake, assay tubes contained nomifensine (final concentration, 100 µM in 50 µl for DAT assays) and fluoxetine (final concentration, 10 µM in 25 µl for SERT assays) in the absence of GZ-11608. For SERT assays, GBR-12935 (final concentration, 100 nM in 25 µL), a DAT inhibitor, was added to all assay tubes to prevent [^{3}H]5-HT uptake into dopaminergic terminals (Norrholm et al., 2007). DAT and SERT assay tubes (450 µL and 225 µL, respectively) were incubated at 34 °C for 5 min. After incubation, tubes were placed on ice for 2 min. [^{3}H]DA (final concentration, 10 nM in 50 µl) or [^{3}H]5-HT (final concentration, 10 nM in 25 µL) was added to each tube. DAT and SERT assay tubes (final assay vol, 500 µL and 250 µL, respectively) were incubated at 34 °C for 10 min. Uptake was stopped by addition of 3 mL of ice-cold assay buffer and subsequent filtration. [^{3}H]DA or [^{3}H]5-HT retained on the filters (presoaked in assay buffer containing 1 mM catechol for 1 h at 4 ºC) was determined as previously described.

3.2.5 [^{3}H]Dofetilide Binding

GZ-11608 inhibition of [^{3}H]dofetilide binding to hERG assessed potential cardiotoxicity and selectivity of GZ-11608 for VMAT2 over hERG. HEK-293 cells stably expressing hERG channel protein were purchased from Millipore (Catalog number CYL3006, Billerica, MA). Binding assays were performed as previously described (Sviripa et al., 2014; Nickell et al., 2017). Briefly, frozen cells were thawed at 37 ºC and placed in T-75 cm^2 flasks (Becton Dickinson and Company, Franklin Lakes, NJ), containing 20 mL of complete media (MEM supplemented with 10% fetal bovine serum, 1% non-essential amino acids, and 400 µg/ml geneticin), according to the Millipore protocol. For 4-8 h, cells adhered to flasks in a humidified atmosphere (5% CO_2 at 37 ºC), after which the media was replaced with 20 mL fresh media. Subsequently, media was replaced every 48 h. For routine passages, media was removed, cells rinsed with 2 mL of phosphate-buffered saline (137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM disodium hydrogen phosphate, 2 mM potassium dihydrogen phosphate), followed by addition of Hank's Balanced Salt Solution containing trypsin (0.5 g/L, porcine trypsin) and EDTA (0.5 mM). To dissociate the cells, flasks were placed in a 37 ºC incubator for 2-5 min, and then, fresh complete media (5 mL) was added to the cell resuspensions, followed by seeding onto new flasks at 2-3 x 10^6 cells/flask. Passages were conducted every 6 days. At least 3 passages occurred before cell membrane collection. On the last passage prior to membrane preparation, cells were seeded onto 150 x 25 mm culture dishes at 2.5 x 10^6 cells/dish, and culture dishes were incubated (5% CO_2 at 37 ºC) for 40-48 h. Media was removed and then culture dishes rinsed twice with 30 °C Hanks' Balanced Salt solution (13 mL). Then, a solution of ice-cold 0.32 M sucrose with 5 mM sodium bicarbonate (20 mL, pH 7.4) was added to each culture dish on ice. Cells were scraped gently from the dishes and then homogenized (30 s) on ice with a Teflon pestle (~0.003 inch) using a

Maximal Digital homogenizer (280 rpm). Homogenates were centrifuged (300g and 800g for 4 min each at 4 °C). Pellets were resuspended in 9 mL ice-cold MilliQ water, and osmolarity restored by adding 1 mL of 500 mM Tris buffer (pH 7.4). Samples were centrifuged (20,000g for 30 min at 4 °C). Pellets were resuspended in 2 mL assay buffer (50 mM Tris, 10 mM potassium chloride, and 1 mM magnesium chloride, pH 7.4, at 4 °C). Aliquots of membrane suspension were stored at -80 °C until use. To perform the [^{3}H]dofetilide binding assay, membrane suspension was thawed and protein content determined using a Bradford protein assay (Bio-Rad Laboratories, Inc., Hercules, CA), with bovine albumin (Sigma-Aldrich Corporation, St. Louis, MO) as the standard. Duplicate tubes containing membrane suspension (5 µg/100 µL), one of a range of concentrations of GZ-11608 (final concentrations 0, 0.1 nM – 0.1 mM in 25 µL) or amitriptyline (0, 0.1 nM – 0.1 mM; positive control), assay buffer (150 µL), and [^{3}H]dofetilide (5 nM in 25 µL) for a final assay vol of 250 µL. Amitriptyline (1 mM) was used to determine nonspecific binding (Teschemacher et al., 1999; Jo et al., 2000). Samples were incubated for 1 h at room temperature. Reactions were stopped by rapid filtration through Whatman® GF/B Glass microfiber filters presoaked in 0.5% PEI for 1 h at 4 ºC. Filters were washed 3 times with 1 mL ice-cold assay buffer. Radioactivity retained by the filters was determined as previously described.

3.2.6 [^{3}H]NIC and [^{3}H]MLA Binding

To evaluate the selectivity of GZ-11608 for VMAT2 over α4β2 and α7 nicotinic acetylcholine receptors (nAChRs), GZ-11608 inhibition of [^{3}H]NIC and [^{3}H]MLA binding was determined, respectively, using previously published methods (Horton et al., 2011a). In brief, whole brains excluding cortex and cerebellum from individual rats were

homogenized for 90 s in 20 vol of ice-cold assay buffer (2 mM HEPES, 14.4 mM sodium chloride, 0.15 mM potassium chloride, 0.2 mM calcium chloride and 0.1 mM magnesium sulfate, pH 7.5 adjusted dropwise with 1 M sodium hydroxide) using a polytron. Homogenates were centrifuged (31,000g for 17 min at 4 °C). Pellets were resuspended in 20 vol of assay buffer by sonication (Vibra Cell, Sonics & Materials Inc., Danbury, CT). Subsequently, duplicate samples were incubated in a 37 °C water bath for 10 min, and then centrifuged (31,000g at 4 °C for 17 min). Resulting pellets were resuspended in 20 vol of assay buffer by sonication, and centrifuged (31,000g for 17 min at 4 °C). Final pellets were resuspended and stored in 10 mL of incubation buffer (20 mM HEPES, 144 mM sodium chloride, 1.5 mM potassium chloride, 2 mM calcium chloride and 1 mM magnesium sulfate, pH 7.5 adjusted dropwise with 1 M sodium hydroxide) at -20 ºC until use. Thawed membrane suspensions (100-140 µg protein/100 µL) were added to tubes containing one of 7-9 concentrations of GZ-11608 (final concentration, 0, 0.1 nM – 0.1 mM in 50 µL) or nicotine (final concentration, 10 pM – 100 µM; positive control) or methyllycaconitine (final concentration, 10 pM – 100 µM; positive control), and [^{3}H]NIC or [^{3}H]MLA (final concentration, 3 nM in 50 µL), and incubation buffer (50 µL) for a final assay vol of 250 µL. Nonspecific binding of [^{3}H]NIC and [^{3}H]MLA was determined in the presence of 10 µM of cytisine (50 µL) and 10 µM of nicotine (50 µL), respectively. Samples were incubated for 1 h at room temperature. Unifilter-96 GF/B filter plates (1.0 µm pore size; PerkinElmer, Inc., Waltham, MA) were presoaked in 0.5% PEI for 1 h at 4 ºC. Reactions were stopped by filtration using a Packard Filter Mate Harvester (Perkin Elmer, Inc., Waltham, MA). Plates were washed 3 times with 350 µL of ice-cold assay buffer, and dried for 1 h at 45 °C. Plates were bottom sealed, and each well filled with 40 µL Microscint 20 cocktail. Bound radioactivity on the filter was determined via liquid scintillation spectrometry (Top Count NXT scintillation counter; PerkinElmer, Inc.).

3.2.7 METH-Evoked [^{3}H]DA Release

To further evaluate GZ-11608 efficacy as an inhibitor of the pharmacological effects of METH, the concentration-dependent effect of GZ-11608 to inhibit METH-evoked DA release from isolated striatal synaptic vesicles was determined using previously described methods (Teng et al., 1997; Horton et al., 2013). Also, the underlying mechanism of GZ-11608 inhibition was determined. Initially, the effect of GZ-11608 to release DA from synaptic vesicles was determined. Vesicles were prepared as described above for the vesicular DA uptake assay, except that final pellets were resuspended in a smaller vol (2.7 mL) of assay buffer. To preload the vesicles, [^{3}H]DA (final concentration,0.3 μM in 300 μL) was added to the vesicle suspension, and incubation proceeded at 37 °C for 8 min. Samples were placed on ice for 2 min to stop [^{3}H]DA uptake, and then centrifuged at 100,000g for 1 h at 4 °C to remove free [^{3}H]DA not transported into the vesicles. Pellets were resuspended in a final vol of 4.2 mL of assay buffer. Aliquots of [^{3}H]DA-preloaded vesicular suspension (180 μL) were added to tubes containing one of 11 concentrations of GZ-11608 (final concentrations, 0, 0.1 nM - 0.1 mM in 20 μL). Samples were incubated at 37 °C for 8 min. Reactions were stopped by adding 2.5 mL of ice-cold buffer (25 mM HEPES, 100 mM potassium tartrate, 50 μM EGTA, 100 μM EDTA, 1.7 mM ascorbic acid, 2 mM magnesium sulphate, pH 7.4), followed by rapid filtration onto PEI-presoaked GF/B filters, and rinsing of the filters with ice-cold buffer (3 times, 4 mL each). Scintillation cocktail was added, and radioactivity retained on the filters determined by liquid scintillation spectrometry. GZ-11608-evoked vesicular DA release was determined as the amount of [^{3}H]DA retained by the vesicles subtracted from the amount retained in control vesicles not exposed to GZ-11608. To determine METH-evoked [^{3}H]DA release and GZ-11608-induced inhibition of METH-evoked [^{3}H]DA release, [^{3}H]DA preloaded vesicles (180 μL) were incubated for 8 min at 37 °C with one of 11 METH

concentrations (final concentrations, 0, 0.1 µM - 20 mM in 10 µL) in the absence (control) and presence of a single concentration of GZ-11608 (final concentrations, 0, 10, 500 nM and 10 µM in 10 µL) in a total vol of 200 µL. Reactions were stopped, radioactivity retained on the filter determined, and vesicular [^{3}H]DA release calculated as described above to determine GZ-11608-induced inhibition of METH-evoked [^{3}H]DA release.

3.2.8 METH Sensitization

As a rapid means of determining if GZ-11608 decreases the in vivo effects of METH, locomotor sensitization following repeated METH injection was the initial assay employed (Alvers et al., 2012; Lee et al., 2018). Repeated METH administration results in a robust and stable increase in locomotor activity from day-to-day, allowing for reliable evaluation of the ability of compound to reduce the effects of METH on behavior. Locomotor activity was measured in a locomotor chamber (24 x 24 x 30 cm) with clear acrylic walls and floor. A horizontal 16 x 16 grid of photo beams was located 7 cm above the chamber floor, with each beam 2.5 cm apart. Movement in the chamber resulted in beam breaks, which were recorded and transformed into distance traveled (cm) by Versamax and Digipro System software (AccuScan Instruments Inc., Columbus, OH). The effect of GZ-11608 on METH-sensitized activity was determined using a mixed factor design with METH as a between-subjects factor and GZ-11608 as within-subjects factor. Rats were assigned randomly to METH or saline groups. After a week of acclimation, rats were habituated to the apparatus by being placed in the chamber for 1 h with no injection (Day 0). On Days 1-10, rats were injected (subcutaneous, s.c.) daily with either METH (1 mg/kg) or saline (1 ml/kg), immediately placed in the chamber, and activity measured for 1 h. METH dose and number of daily injections were chosen to provide stable, sensitized

locomotor activity, based on previous findings (Lee et al., 2018). On Day 11, GZ-11608 (0, 1-30 mg/kg, s.c.) in a quasi-randomized dose order was injected 15 min prior to the daily METH or saline injection, and then, rats were placed immediately into the chamber for 1 h. A washout period (2-3 days) intervened between testing of GZ-11608 doses to avoid potential drug accumulation. On washout days, METH or saline was injected and locomotor activity determined.

In a separate experiment employing a mixed factor design, the effect of GZ-11608 administration by oral gavage (p.o.) on METH locomotor sensitization was determined. Following repeated METH or saline for 5 days, rats were habituated to the oral gavage procedure. On Day 5-10, rats received vehicle (15% (v/v) Kolliphore EL in saline, p.o., 3 mL) 15 min prior to METH or saline injection (s.c.) and were placed in the activity chamber for 1 h (Wilmouth et al., 2013). On Days 11-27, GZ-11608 (0, 17-300 mg/kg, p.o., ascending dose order) was administered, followed 15 min later by either METH or saline injection (1 mL/kg, s.c.), depending on group assignment, and immediate placement into the chamber for 1 h. Between GZ-11608 doses, 2-3 days of washout occurred. On washout days, vehicle was administered p.o. and METH or saline was injected s.c.

3.2.9 Striatal DA Content

To determine whether GZ-11608 alters striatal DA content and/or exacerbates METH-induced striatal DA depletion, GZ-11608 was administered s.c. to rats in the absence and presence of a METH dose known to deplete rat striatal DA content (Bowyer et al., 1992, 1994; pilot study). The dose of GZ-11608 was chosen based on its ability to reliably decrease METH-sensitized locomotor activity. Following acclimation to the colony and 3 days prior to drug injection, a thermal transponder (Bio Medic Data Systems, Inc.,

Seaford, Delaware) was implanted (s.c.) beneath the scapula to monitor body temperature. In the first series of experiments using a between-groups design, GZ-11608 (17 mg/kg, s.c.) or saline (1 mL/kg, s.c.) was administered 15 min prior to METH (30 mg/kg, i.p.) or saline (1 mL /kg, i.p.), according to random assignment to treatment group. In the second series of experiments also using a between-groups design, GZ-11608 or saline was administered 15 min after METH or saline. Body temperature was monitored every 30 min for 8 h following METH injection. If the body temperature increased to 41.3 ºC or higher, rats were transferred to a cage placed on ice until body temperature decreased to 40.0 ºC or below (Bowyer et al., 1992, 1994; Fukumura et al., 1998). Striata were obtained 72 h after METH injection and were processed via high performance liquid chromatography with electrochemical detection (HPLC-EC). Striata were weighed, placed in 1 mL of perchloric acid and sonicated. Tissue samples were centrifuged at 31,000g for 30 min at 4 °C. Supernatant (50 µL) was injected onto the octadecylsillica Ultrasphere C18 reverse-phase column (80 x 4.6 mm, 3 µm ESA Inc., Chelmsford, MA) via autosampler (508 Beckman Coulter, Inc., Fullerton, CA). DA was detected by a coulometric-II detector with guard cell (model 5020; ESA, Inc., Chelmsford, MA) maintained at +0.60 V and an analytical cell (model 5011) maintained at E1 = +0.05 V and E2 = +0.35 V. Mobile phase (MP) consisted of 0.07 M citrate, 0.1 M acetate buffer with 175 mg/L octylsulfonic acid-sodium salt, 650 mg/L of sodium chloride and 7% methanol (pH 4.2). Flow rate was 1.2 mL /min, and 4-5 min were required to analyze each sample. DA standards were used to identify and quantify DA peak and amount using 32 Karat software (Beckman Coulter, Inc., Fullerton, CA).

3.2.10 METH Self-Administration

The ability of GZ-11608 to dose-dependently decrease the reinforcing effect of METH was determined using a within-subject design. Due to the high doses of GZ-11608 required in the METH sensitization studies and the assumed low oral bioavailability, s.c. rather than oral administration was employed for self-administration studies. Two-lever operant chambers were used to train rats once daily for 3 days in 1 h sessions to press a lever (active lever) for food pellet reinforcement (45 mg pellet, BIO-SERV, #F0021, Frenchtown, NJ) on a fixed ratio 1 (FR1) schedule, while responding on the other lever (inactive lever) had no programmed consequence. Subsequently, the operant schedule was incremented to a FR3 schedule for 3 days, then a FR5 schedule for 14 days until rats met the criteria for stable responding, which included: 1) $\geq$10 pellets earned/session and 2) a minimum of a 2:1 ratio of active:inactive lever presses. After delivery of each food reinforcer, the lights above both levers were illuminated for a 20-s signaled timeout period. After reaching stable responding for food on the FR5 schedule, rats underwent catheter implantation surgery. Rats were anesthetized (75 mg/kg ketamine, 7.5 mg/kg xylazine, and 0.75 mg/kg acepromazine; i.p.) and a silastic catheter was implanted into the jugular vein. The free end of the catheter was affixed with dental acrylic to the skull by metal screws and exited through the scalp. Rats were allowed to recover for 1 wk. Prior to the start and at the end of each behavioral session. Catheters were flushed daily with 0.1 mL heparinized saline to maintain patency. Following recovery from surgery, rats were trained to press a lever for i.v. METH (0.05 mg/kg/infusion) during 1 h daily sessions using a standard 2-lever procedure as previously reported (Harrod et al., 2001; Beckmann et al., 2012). The FR schedule was incremented across training sessions (3 days, FR1; 3 days, FR3; 14 days, FR5). A 20-s signaled timeout occurred after each METH infusion. Upon reaching criteria for stable responding ($\geq$ 10 infusions/session and a 2:1 ratio of

active:inactive lever presses), a dose of GZ-11608 (0, 1, 3, 10, 17, and 30 mg/kg, in ascending dose order, s.c.) was administered 15 min prior to the session. GZ-11608 vehicle (0 mg/kg) was 15% kolliphore in saline (1 mL/kg, s.c.).

3.2.11 Food-Maintained Responding

To evaluate the specificity of the GZ-11608-induced decrease in responding for i.v. METH, the ability of GZ-11608 to decrease food-maintained responding was determined using a within-subject design. GZ-11608 doses and pre-treatment time were as described for METH self-administration experiments. Experiments were conducted as described above, with exceptions that no surgery was performed, and rats did not self-administer METH. Instead, rats were trained to a terminal FR5 to respond for food pellets (45 mg pellet, BIO-SERV, #F0021) in daily 1-hr sessions.

3.2.12 Repeated GZ-11608 Administration

A within-subject design was used to determine if tolerance developed to the GZ-11608-induced decrease in METH self-administration and/or food-maintained responding. One group of rats underwent operant training for food reinforcement, catheter implantation surgery, and operant training for i.v. METH (0.05 mg/kg/infusion) self-administration as described above. In these experiments, GZ-11608 (30 mg/kg, s.c.; a dose that reliably decreased METH self-administration) was administered 15 min prior to 7 consecutive, daily METH self-administration sessions. Then, for 5 consecutive daily sessions, responding for METH was determined without GZ-11608 treatment. Another group of rats was trained for food-maintained responding, and the effect of repeated GZ-11608 (30

mg/kg, s.c.) was determined using the same dose and pre-treatment time as described above for METH self-administration.

3.2.13 Surmountability

To determine whether increasing the unit dose of METH would surmount the effect of GZ-11608 to decrease i.v. METH self-administration, another group of rats underwent operant training for food reinforcement, catheter implantation surgery, and operant training for METH (0.05 mg/kg/infusion) self-administration as described above. A within-subject design was employed to establish the METH dose-response across a range of METH doses (0.01-0.25 mg/kg/infusion) in the absence of GZ-11608. Each dose of METH was tested for 3 consecutive sessions. Then, the METH dose-response was re-evaluated in the same group of rats following treatment with GZ-11608 (30 mg/kg, s.c.) 15 min prior to the session. To maintain stable responding, two intervening maintenance sessions occurred between each session in which GZ-11608 was administered. For these maintenance sessions, no GZ-11608 treatment was administered prior to self-administration of each METH unit dose.

3.2.14 Reinstatement

The ability of GZ-11608 to decrease cue- and METH-induced reinstatement of METH seeking behavior was determined using previously published methods (Harrod et al., 2003). In brief, 3 groups of rats were trained to self-administer METH (0.05 mg/kg/infusion) as described above, except that cue lights were illuminated for 5 s at the beginning of each session prior to presentation of the levers. For cue-induced

reinstatement experiments, upon reaching the criteria for stable responding, rats underwent extinction for 14 days. During extinction, cue lights were not illuminated at the beginning or during daily 1 h sessions, and active lever presses did not result in METH infusion. The day after the last extinction day (test for reinstatement), the cue light was illuminated at the beginning and during the session, and the dose effect for GZ-11608 to decrease cue-induced drug seeking behavior was determined. Because drug seeking behavior is diminished with repeated testing, two groups of rats were needed to generate the complete dose response. In one group, low doses (0, 3, 5.6, and 10 mg/kg, s.c.) were evaluated in a randomized order 15 min prior to the session, and higher doses (0, 10, 17 mg/kg, s.c.) were evaluated in a second group. To maintain responding at extinction levels, 5 intervening sessions occurred between each session in which GZ-11608 was administered. On intervening sessions, there was no cue light illumination, no GZ-11608 pre-treatment and no METH infusion.

The effect of GZ-11608 (0, 10, 17, and 30 mg/kg, s.c.; in randomized order) on METH-induced reinstatement of drug seeking behavior was determined in a third group of rats. GZ-11608 or saline was injected 15 min prior to the session and METH (0.5 mg/kg, i.p.) was injected immediately prior to the session to reinstate drug seeking behavior. Experiments were conducted using similar procedures as in the experiments evaluating cue-induced reinstatement of drug seeking, except that the 20-sec contingent cue light illumination continued during the 14 days of extinction, as well as on reinstatement tests.

3.2.15 Substitution of GZ-11608 for METH

To determine whether GZ-11608 substitutes for METH in rats trained to self-administer i.v. METH, another experiment was conducted using a mixed factor design with

GZ-11608 treatment as a between-subject factor, and dose and session as within-subject factors, similar to previously published methods (Harrod et al., 2003; Beckmann et al., 2012). Rats were trained to stable performance for METH (0.05 mg/kg/infusion) self-administration under an FR5 schedule of reinforcement as described above. Upon reaching the criteria for stable responding, rats were assigned randomly to either the GZ-11608 or saline groups. For the GZ-11608 group, responding on the active lever under the FR5 schedule resulted in i.v. infusions of GZ-11608 (0.01, 0.05, 0.1, and 0.5 mg/kg/infusion; in ascending order), each dose was administered across 4 consecutive sessions. Only saline was available (i.v.) to the saline group across the same number of self-administration sessions. Then, for both GZ-11608 and saline groups, METH (0.05 mg/kg/infusion) was available for 4 consecutive self-administration sessions.

3.2.16 GZ-11608 Self-Administration

To determine whether GZ-11608 was self-administered in drug naïve rats, a mixed factor design with GZ-11608 treatment as a between-subject factor, and dose and session as within-subject factors was conducted, similar to previously published methods (Harrod et al., 2003). Rats underwent operant training for food reinforcement, catheter implantation surgery, and random assignment to operant training of GZ-11608 or saline i.v. self-administration. For the saline group, only i.v. saline was available across the experiment. For the GZ-11608 group, each GZ-11608 dose (0.5, 0.1 and 0.05 mg/kg/infusion) was available in a descending order on a FR1 schedule of reinforcement for 5 consecutive days, followed by availability on an FR2 schedule for 3 days. To maintain stable responding, intervening maintenance sessions occurred after the evaluation of each GZ-11608 dose. During the maintenance sessions, rats responded for food reinforcement

under FR1 for 2 days, and then, under FR2 for 1 day; GZ-11608 was not available. As a positive control, ability to self-administer METH (0.05 mg/kg/infusion) under an FR1 for 5 days, FR2 for 3 days, and FR5 for 10 days was determined in the GZ-11608 group. To further evaluate GZ-11608 as a reinforcer, responding for i.v. GZ-11608 (0.1 mg/kg/infusion) or saline (depending on random group assignment) was determined in drug naive rats under an FR1 for 5 days, FR2 for 3 days, and FR5 schedule for 10 days.

3.2.17 Data Analysis

Specific [^{3}H]DA uptake, [^{3}H]5-HT uptake, [^{3}H]dofetilide binding, [^{3}H]NIC binding, and [^{3}H]MLA binding were calculated by subtracting nonspecific uptake or binding from total uptake or binding, respectively. The GZ-11608 concentration that produced 50% inhibition of specific uptake or binding (IC_{50} values) was obtained from individual concentration-response curves via an iterative curve-fitting program (Prism 7.03; GraphPad Software, Inc., La Jolla, CA). Inhibition constants (Ki values) were determined using the Cheng-Prusoff equation (Cheng and Prusoff, 1973). The selectivity ratio for GZ-11608 relative to the off-target sites was determined as the Ki value for inhibition of VMAT2 divided by the Ki value for DAT, SERT, hERG and nAChRs, respectively. EC_{50} values from individual concentration-response curves for METH or GZ-11608 to evoke [^{3}H]DA release from synaptic vesicles was determined using Prism 7.0. Mechanism of GZ-11608-induced inhibition of METH-evoked vesicular [^{3}H]DA release was determined using Schild analysis. Dose ratios (DR) were obtained by dividing the EC_{50} for METH-evoked [^{3}H]DA release in the presence of each concentration of GZ-11608 by that in the absence of GZ-11608. Log (DR-1), plotted as a function of log GZ-11608 concentration, provided the Schild regression; linearity of the slope was significantly different from unity

if the 95% confidence intervals (CI) did not include 1.0 (Prism 7.03; Kenakin et al., 2006).

For all behavioral experiments, distance travelled, and number of responses were subjected to analyses of variance (ANOVA), followed by Tukey's post hoc tests when appropriate, unless otherwise indicated.

3.3 Results

GZ-11608 potently and selectively inhibits VMAT2 function relative to interactions at off-target sites. GZ-11608 potently inhibited (Ki = 25 ± 4 nM) [^{3}H]DA uptake at VMAT2 with maximal inhibition (I_{max}) of >95% (Fig. 3.2). GZ-11608 selectively inhibited [^{3}H]DA uptake at VMAT2, having at least 92-fold higher affinity at VMAT2 relative to off-target sites (Fig. 2; [^{3}H]5-HT uptake at SERT: Ki = 2.36 ± 0.29 μM, I_{max} > 95%, 92-fold selective for VMAT2; [^{3}H]dofetilide binding to hERG: Ki = 4.16 ± 1.68 μM, I_{max} > 90%, 163-fold selective for VMAT2; and, [^{3}H]DA uptake at DAT: Ki = 6.15 ± 0.74 μM, I_{max} > 90%, 241-fold selective for VMAT2). GZ-11608 did not inhibit [^{3}H]NIC and [^{3}H]MLA binding to α4β2 and α7 nAChRs, respectively (Ki > 30 μM; >1180-fold selective for VMAT2). Taken together, GZ-11608 exhibited high affinity and selectivity for VMAT2 over SERT, hERG, DAT, α4β2 nAChRs and α7 nAChRs.

GZ-11608 evokes vesicular [^{3}H]DA release and inhibits METH-evoked vesicular [^{3}H]DA release. Effects of GZ-11608 to release vesicular [^{3}H]DA and to inhibit METH-evoked vesicular DA release are illustrated in Fig. 3.3. GZ-11608 (0.1 nM – 0.1 mM) stimulated [^{3}H]DA release from isolated striatal synaptic vesicles with an EC_{50} value of 0.62 ± 0.14 μM and an E_{max} value of 76.8 ± 5.0%. The GZ-11608 concentration-response data fit a one-site model (non-linear regression, $R^2 = 0.87$, $p < 0.05$; Fig. 3.3A). The effect

of GZ-11608 to inhibit METH-evoked [^{3}H]DA release in the absence and presence of a low, medium and high concentration of GZ-11608 is illustrated in Fig. 3.3B. METH evoked [^{3}H]DA release from isolated vesicles with an EC_{50} value of 14.5 ± 4.10 μM, an E_{max} value of 90.0 ± 2.04%, and in the absence of GZ-11608, the METH concentration-response data fit a one-site model (non-linear regression, R^2 = 0.97, $p < 0.0001$; Fig. 3.3B). Kinetic parameters for methamphetamine-evoked [^{3}H]DA release from striatal synaptic vesicles were consistent with previously published values (Horton et al., 2013). Concentration-response data sets for METH in the presence of varying GZ-11608 concentrations (10 nM, 500 nM, or 10 μM) each fit a one-site model (non-linear regression, R^2 = 0.94, 0.95, 0.90, respectively, ps < 0.0001; Fig. 3.3.B). METH concentration-response curves for vesicular [^{3}H]DA release were shifted rightward, and EC_{50} values in the presence of GZ-11608 (0, 10, 500 nM and 10 μM) were 14.5 ± 4.10, 17.3 ± 4.15, 122 ± 32.3, and 388 ± 23.7 μM, respectively; whereas, the E_{max} values were not altered as the concentration of GZ-11608 increased (90.0 ± 2.04, 94.1 ± 1.25, 88.1 ± 1.27, and 88.4 ± 3.70%, respectively). Two-way repeated measures ANOVA revealed a METH x GZ-11608 interaction [$F_{33,99}$=11.2, $p < 0.0001$]. The Schild regression had a slope of 0.90 (CI: 0.603 to 1.20; Fig. 3.3B insert), consistent with a competitive mechanism of VMAT2 inhibition.

GZ-11608 decreases METH sensitization. GZ-11608 decreased METH sensitization (1.0 mg/kg, s.c., once daily for 10 days; Fig. 3.4). Locomotor activity (distance traveled) in the METH-treated group increased following acute METH and plateaued after 5 daily METH injections at an activity level greater than that observed following acute injection on Day 1 (i.e., sensitization; Fig. 3.4A). Across the same time period, the locomotor activity in the saline-injected control group was not changed (Fig. 3.4A, inset). Two-way ANOVA revealed a treatment x session interaction [$F_{10,90}$ = 21.7, $p < 0.001$]. Post hoc analysis revealed no differences between the treatment groups on the initial habituation day (Day 0). However, as expected METH increased distance traveled on

Day 1 relative to the saline-injected group ($p < 0.001$) and distance traveled was greater on Days 5-10 than on Day1 in the METH treatment group ($ps < 0.05$), but not in the saline treatment group. There were no significant differences in activity between Days 5-10 in the METH treatment group, indicating that sensitization occurred and stabilized by Day 5.

GZ-11608 (s.c.) decreased METH sensitized locomotor activity in a dose-dependent manner without altering activity in the saline-injected control group (Fig. 3.4B). Two-way ANOVA revealed an interaction between METH treatment x GZ-11608 dose [$F_{5,28} = 23.5$, $p < 0.001$]. Post hoc analysis revealed that GZ-11608 (10, 17, and 30 mg/kg) decreased METH sensitization relative to vehicle (15% Kolliphore EL in saline) ($p < 0.005$), and activity reached the criteria of a 50% reduction relative to activity following vehicle (Fig. 3.4B). In the saline-injected control group, no effect of GZ-1108 at any dose relative to vehicle was found (Fig. 3.4B, inset), indicating a specific effect of GZ-11608 on the METH-sensitized response. Activity at the highest dose of GZ-11608 (30 mg/kg) in the METH group was not different from activity following vehicle injection in the saline group, demonstrating GZ-11608 blockade of the expression of METH sensitization.

To assess oral bioavailability, the effect of GZ-11608 following oral administration on METH sensitization was determined. GZ-11608 (17-300 mg/kg, p.o.) decreased METH sensitization in a dose-dependent manner relative to vehicle; however, activity tended to increase following oral administration of GZ-11608 in the saline-injected control group (Fig. 4C). An interaction was found between METH treatment x GZ-11608 dose [$F_{6,53} = 10.1$ $p < 0.001$] (Fig. 3.4C). Post hoc analysis revealed that GZ-11608 (300 mg/kg) decreased sensitized locomotor activity relative to vehicle in the METH treatment group ($p < 0.05$; Fig. 3. 4C). Activity following the highest dose (300 mg/kg) of GZ-11608 reached the criteria of a 50% reduction relative to vehicle. Activity following GZ-11608 (17-300 mg/kg) in the saline-injected group tended to increase, but did not reach statistical significance (Fig. 3.4C; inset). Activity at the highest dose of GZ-11608 (300 mg/kg) in the METH group

was not different from activity following vehicle in the saline group, demonstrating GZ-11608 blockade of the expression of METH sensitization. Taken together, GZ-11608 following s.c. and p.o. administration specifically decreased METH sensitization.

GZ-11608 does not alter striatal DA content and does not exacerbate the decrease in DA content produced by METH. To determine the effect of GZ-11608 on striatal DA content, a dose of GZ-11608 (17 mg/kg, s.c.) that both reliably and specifically decreases METH sensitization was employed (Fig. 3.5). One-way ANOVAs revealed significant differences in DA content after either GZ-11608 pretreatment or post-treatment and a relatively high dose (30 mg/kg, i.p.) of METH (pretreatment, $F_{3,34} = 6.26$, $p < 0.05$, Fig. 3.5A; post-treatment, $F_{3,21} = 16.5$, $p<0001$, Fig. 3.5B). Post hoc analysis revealed that DA content was decreased (40-50%) following METH alone compared with the respective saline control groups. GZ-11608 alone did not alter DA content compared with the respective saline control. DA content following GZ-11608 pretreatment or post-treatment and METH was not different from that following METH alone (Fig. 3.5A and 3.5B, respectively), indicating that GZ-11608 did not exacerbate the effect of METH to decrease striatal DA content.

GZ-11608 decreases responding for i.v. METH, but not for food. To determine if GZ-11608 specifically decreases METH self-administration, the effects of GZ-11608 (1-30 mg/kg, s.c.) on METH (0.05 mg/kg/infusion) self-administration and food-maintained responding were evaluated. One-way ANOVAs revealed that GZ-11608 dose-dependently decreased METH self-administration, but not food-maintained responding ([$F_{5,45} = 3.92$, $p < 0.005$], Fig. 3.6A; [$F_{5,60} = 3.23$, $p = 0.0119$], Fig. 3.6B, respectively). Post hoc analysis revealed that after the highest dose of GZ-11608 (30 mg/kg), responding for METH was lower than following vehicle ($p < 0.05$, Fig. 3.6A). Post hoc analysis also revealed that GZ-11608 did not alter responding for food ($p > 0.05$, Fig. 3.6B). Thus, GZ-11608 specifically decreased METH self-administration.

Tolerance does not develop to the effect of GZ-11608 to decrease responding for i.v. METH. One-way ANOVA revealed that following acute administration, GZ-11608 (30 mg/kg, s.c.) decreased responding for METH (0.05 mg/kg/infusion) and that responding continued to be decreased across 7 consecutive, once/daily GZ-11608 treatments [$F_{7,43}$ = 3.68, $p < 0.05$], indicating that tolerance did not develop to this effect of GZ-11608 (Fig. 3.7). Upon cessation of GZ-11608 treatment, responding for METH returned to baseline ($F_{5,26}$ = 3.67, $p < 0.05$; Fig. 3.7). Post hoc analysis revealed that responding during post-treatment sessions 1 and 2 was decreased compared to baseline ($p < 0.05$), and that responding during post-treatment sessions 3-5 was not different from baseline ($p > 0.05$). Of note, repeated administration of GZ-11608 (30 mg/kg, s.c., once daily for 7 days) initially decreased responding for food, but tolerance developed to this effect after 5 daily administrations (Fig. 3.12).

Increasing the unit dose of METH does not surmount the effect of GZ-11608 to decrease responding for i.v. METH. This experiment assessed if the GZ-11608-induced decrease in METH self-administration could be surmounted by increasing the unit dose of METH. Inverted U-shaped METH dose-response curves were obtained with no treatment and following GZ-11608 (30 mg/kg, s.c.) treatment. Following GZ-11608 treatment, the METH dose-response curve was downward and rightward shifted relative to the curve obtained with no treatment (Fig. 3.8). Two-way ANOVA on the data expressed as number of METH infusions revealed an interaction between GZ-11608 treatment x METH unit dose [$F_{5,68}$ = 2.55, $p < 0.05$]. Post hoc analysis revealed that GZ-11608 decreased the number of METH infusions when low unit doses (0.01-0.05 mg/kg/infusion) were available, whereas GZ-11608 had no effect on number of infusions when higher unit doses (0.1 and 0.25 mg/kg/infusion) of METH were available. Without GZ-11608 treatment, the peak number of METH infusions (20 infusions) occurred with a unit dose of 0.025 mg/kg/infusion. Following GZ-11608 treatment, the peak number (9 infusions) of METH occurred with 0.1

mg/kg/infusion, representing a ~50% decrease relative to no GZ-11608 treatment (Fig. 3.8). Thus, increasing the METH unit dose did not surmount the effect of GZ-11608.

GZ-11608 decreases cue- and METH-induced reinstatement of METH-seeking behavior. These experiments determined if GZ-11608 decreases cue- and METH-induced reinstatement (models of relapse). In a dose-related manner, GZ-11608 decreased cue- and METH-induced reinstatement of METH seeking (Fig. 3.9A and 3.9B, respectively). For both cue- and METH-induced reinstatement experiments, one-way ANOVAs revealed that METH seeking was decreased in a dose-related manner by GZ-11608 ([$F_{6,99}$ = 34.5, $p < 0.001$] and [$F_{5,54}$ = 17.1, $p < 0.001$], respectively). Post hoc analyses revealed that lever pressing was decreased following 14 extinction sessions compared to baseline and that both the cue and METH (0.5 mg/kg, i.p.) reinstated METH seeking following a vehicle pretreatment (ps < 0.05). GZ-11608 dose-dependently decreased both cue- and METH-induced reinstatement (ps < 0.001). Thus, GZ-11608 dose-dependently decreased cue- and METH-induced reinstatement of METH seeking.

GZ-11608 does not substitute for METH. One potential mechanism underlying the GZ-11608-induced decrease in METH self-administration may be due to an action as a substitute reinforcer. In rats trained to self-administer METH, METH was replaced by either i.v. GZ-11608 or saline. Fig. 3.10 illustrates that responding across a range of GZ-11608 doses was not different from responding for saline. Two-way ANOVA revealed no main effect of treatment [$F_{1,3}$ = 0.206, $p = 0.681$] and no treatment x session interaction [$F_{20,60}$ = 0.368, $p = 0.99$]; however, there was a main effect of session [$F_{20,60}$ = 7.46, $p < 0.001$]. Post hoc analysis revealed no differences between the groups at baseline ($p > 0.05$) and no differences between groups responding for the range of doses of GZ-11608 and saline across sessions 1-16. During sessions 17-20, in which METH was available and responding returned to baseline, there were also no differences in responding between groups. Thus, GZ-11608 did not substitute for METH as a reinforcer.

GZ-11608 does not serve as a reinforcer. To assess the abuse liability of GZ-11608, drug naïve rats were trained to respond for GZ-11608 using the same procedure as that used for METH self-administration. Fig. 3.11 shows that GZ-11608 was not self-administered, and may have had aversive properties at the highest dose evaluated. Two-way ANOVA on the number of responses at the highest GZ-11608 dose (0.5 mg/kg/infusion) revealed a main effect of session [$F_{7,70} = 4.50$, $p < 0.05$], a trend for a GZ-11608 x session interaction [$F_{7,70} = 2.01$, $p = 0.0658$], but no main effect of GZ-11608 [$F_{1,10} = 2.30$, $p = 0.160$]. Post hoc analysis revealed that responding during sessions 1 and 2 was decreased when GZ-11608 (0.5 mg/kg/infusion) was available compared to the saline group ($p < 0.05$), supporting that the high dose of GZ-11608 served as a punisher (Fig. 3.11A). Analysis of the intermediate dose of GZ-11608 (0.1 mg/kg/infusion) revealed no main effects of treatment or session ($F_{1,10} = 0.51$, $p = 0.492$ and $F_{7,70} = 2.09$, $p = 0.056$, respectively), and no treatment x session interaction ($F_{7,70} = 0.42$, $p = 0.884$; Fig. 3.11B). Analysis of the lowest dose of GZ-11608 (0.05 mg/kg/infusion) revealed no main effects of treatment or session ($F_{1,9} = 0.69$, $p = 0.426$; $F_{7,59} = 1.92$, $p = 0.082$, respectively) and no treatment x session interaction ($F_{7,59} = 0.20$, $p = 0.984$; Fig. 3.11C). Thus, responding for GZ-11608 (0.05-0.1 m/kg/infusion) was not different from the saline group. The lack of responding for GZ-11608 was not due to lack of i.v. catheter patency, because as expected, responding increased subsequently when METH was available (Fig. 3.11D). During the FR1/FR2 components of the session, a main effect of session [$F_{7,42} = 2.56$, $p < 0.05$] was found, with no main effect of treatment [$F_{1,6} = 2.87$, $p = 0.14$] and no treatment x session interaction [$F_{7,42} = 1.22$, $p = 0.31$]. During the FR5 component, a main effect of treatment [$F_{17,102} = 2.09$, $p < 0.05$] was found with no main effect of session [$F_{1,6} = 5.66$, $p = 0.055$] and no treatment x session interaction [$F_{17,102} = 1.11$, $p = 0.37$]; Fig. 3.11D). Thus, GZ-11608 does not have reinforcing properties.

3.4 Discussion

The current drug discovery program probed VMAT2 as a therapeutic target for identification of medications for METH use disorder. While a previous lead, GZ-793A, showed VMAT2 potency, selectivity and efficacy, without development of tolerance (Horton et al., 2011b; Alvers et al., 2012; Meyer et al., 2013; Nickell et al., 2017), it had potential cardiotoxicity (Nickell et al., 2017). Structural modification aimed at eliminating hERG affinity resulted in GZ-11610 and GZ-11608. GZ-11610 exhibited 9 nM Ki and 1090-fold selectivity for VMAT2 over hERG, and specifically decreased METH sensitization (Lee et al., 2018), revealing advantages of this structural scaffold. In the current study, GZ-11608 potently inhibited VMAT2 (Ki = 25 nM), with >100-fold selectivity at VMAT2 over nAChRs and hERG, and also decreased METH sensitization, METH self-administration and reinstatement, without exhibiting intrinsic reinforcing properties itself.

Interaction of METH with the DA system underlies its abuse liability (Koob and Volkow, 2016). METH increases cytosolic DA concentration by releasing vesicular DA (EC_{50} = 9-15 μM, E_{max} = 90%) and inhibiting vesicular uptake (Ki = 2.5 μM) (Nickell et al., 2010; Horton et al., 2013; current study). At VMAT2, METH inhibits uptake ~4-fold more potently than it evokes release. In contrast, GZ-11608 inhibits uptake 25-fold more potently than it evokes release (EC_{50} = 0.62 μM, E_{max} = 77%). GZ-11608 releases vesicular DA 23-fold more potently and inhibits uptake at VMAT2 100-fold more potently than METH. Moreover, the current study shows that GZ-11608 inhibits METH-evoked vesicular DA release and shifts rightward by 100-fold the METH concentration-response curve, with no change in E_{max}. The Schild slope not being different from unity suggests a competitive mechanism, such that GZ-11608 and METH appear to act at the same site on VMAT2.

Although most compounds known to interact with VMAT2, both release and inhibit its function, they are categorized as either releasers (substrates) or inhibitors based on

relative E_{max} (Partilla et al., 2006). Generally, as compared to inhibitors, releasers have greater vesicular DA release E_{max} values. Classified as a releaser, METH releases 65% of preloaded DA and 90% of tyramine. In contrast, tetrabenazine and reserpine, classified as inhibitors, release DA and tyramine by 45% and 50%, respectively (Partilla et al., 2006). Current work shows that METH releases 90% of DA; whereas tetrabenazine and reserpine release 49% and 28%, respectively (Horton et al., 2013). Since GZ-11608 and GZ-793A release 77% and 86%, respectively, they would be classified likely as releasers. The current study employed classical pharmacological methods to determine mechanism of action (Kenakin, 1997). Schild analysis revealed that GZ-11608 and tetrabenazine competitively inhibit METH at VMAT2, whereas GZ-793A exhibited allosteric inhibition (Horton et al., 2013; current study).

The in vitro neurochemical effects of GZ-11608 translated to in vivo behavioral efficacy. GZ-11608 decreased METH sensitization following repeated administration. METH sensitization reflects dynamic brain changes associated with METH use disorder (Robinson and Berridge, 2008; London et al., 2015). GZ-11608 specifically decreased (>50%) METH sensitization. Interestingly, the R-enantiomer GZ-11610, was not specific following s.c. administration, reducing activity in non-sensitized saline controls at doses that decreased METH sensitization (Lee et al., 2018).

METH self-administration is considered the gold standard animal model of MUD (Panlilio and Goldberg, 2007; Mews and Calipari, 2017). GZ-11608 dose-dependently decreased METH self-administration. Behavioral efficacy was found within the maximal concentration-exposure window, as indicated by the dose-dependent linear pharmacokinetics, including similar E_{max} values across doses. Tolerance did not develop to GZ-11608's efficacy in the METH self-administration assay. Further, GZ-11608 specifically decreased METH self-administration, especially when given across repeated injections. While acute high doses of GZ-11608 (10-30 mg/kg) decreased food-maintained

responding by ~20%, this effect was not significant. Moreover, repeated high dose (30 mg/kg) administration significantly decreased food-maintained responding, but tolerance developed after 5 sessions. Different results between acute and repeated studies on food-maintained responding using the 30 mg/kg dose of GZ-11608 may be explained by the escalating-dose design of the acute study, such that some tolerance may have developed across the incrementing 1-17 mg/kg doses given prior to the 30 mg/kg dose of GZ-11608. Interestingly, during the post-treatment sessions after repeated administration, an apparent increase (10-40%) above baseline in food-maintained responding was observed. Since MUD is often associated with decreased body weight (Sommers et al., 2006), increases in food intake may be a beneficial side effect.

METH use disorder is characterized by high relapse rates with ~90% relapsing within 5 years (Wang et al., 2012; Brecht and Herbeck, 2014). Preclinical models characterize relapse using both surmountability and reinstatement assays. Prevention of a lapse developing into a relapse was evaluated by determining if higher METH doses could surmount the inhibitory effect of GZ-11608 on METH self-administration. GZ-11608 produced a downward and rightward shift of the dose-response curve for METH self-administration. Thus, increasing the METH dose did not surmount the GZ-11608-induced decrease in responding for METH. In that experiment, it was notable that GZ-11608 tended to suppress responding for saline infusions compared to the no treatment condition (Fig. 3.8), suggesting a non-specific suppressant effect. However, the locomotor data do not support this interpretation, as GZ-11608 did not decrease activity. An alternative explanation is that GZ-11608 accelerated the within-session rate of extinction that occurred when saline was substituted for METH. Moreover, GZ-11608 dose-dependently blocked both cue- and METH-induced reinstatement of METH seeking, supporting its potential utility in preventing relapse to METH seeking.

Decreases in METH self-administration and reinstatement may have been due to reinforcing effects of GZ-11608, such that it acts as a substitute for METH. Consistent with this possibility, GZ-11608 tended, although not significantly, to increase locomotor activity in the control group repeatedly injected with saline. However, in additional experiments, GZ-11608 did not substitute for METH in the self-administration assay and did not engender self-administration in drug naïve animals. The lack of reinforcing effects of GZ-11608 was not due to faulty cannula, since availability of METH resulted in a return to maintenance levels of responding. Thus, GZ-11608 appears to decrease METH self-administration by inhibiting the reinforcing effect of METH, rather than by producing reinforcement. The lack of intrinsic reinforcing effects of GZ-11608 and the prediction of low abuse liability are consistent with its >200-fold selectivity for VMAT2 over DAT, with DAT inhibition being most closely associated with abuse liability (Seeman and Lee, 1975; Stathis et al., 1995).

METH is well known to deplete striatal DA content (Bowyer et al., 1992, 1994). The current study shows that GZ-11608 neither reduces DA content nor exacerbates the METH-induced decrease in content. Thus, during a relapse event when both GZ-11608 and METH may be onboard, no additional dopaminergic neurotoxicity would be predicted to occur. In contrast, tetrabenazine, a classical and reversible VMAT2 inhibitor with only 2-fold lower affinity for DAT, exacerbates METH-induced DA depletion (Kenney and Jankovic, 2006; Guay, 2010). Importantly, at low doses, tetrabenazine increases responding for METH, whereas at high doses responding for METH decrease; however, responding for food also decreases, indicating a lack of specificity (Meyer et al., 2011). Thus, relative to this classical VMAT2 inhibitor, GZ-11608 has considerable advantages as a potential METH use disorder therapeutic.

Several lead compounds, i.e., lobeline, lobelane, GZ-793A and GZ-11608, have been identified from our iterative drug discovery program (Harrod et al., 2001, 2003;

Neugebauer et al., 2007; Meyer et al., 2011; Alvers et al., 2012; Beckmann et al., 2012; Horton et al., 2013; current study). VMAT2 affinity has increased, reaching the low nM range with GZ-793A and GZ-11608. Schild regression on METH-evoked vesicular DA release revealed a surmountable allosteric mechanism for GZ-793A and an orthostatic mechanism for GZ-11608. Generally, the leads exhibit good selectivity for VMAT2 over DAT, do not exacerbate METH-induced striatal depletion, and have behavioral efficacy and specificity decreasing METH self-administration, without decreasing food-maintained responding. Interestingly, lobeline and GZ-793A produced downward shifts of the METH self-administration dose-response curve, whereas GZ-11608 produced a downward and rightward shift, suggesting potentially different underlying mechanisms. Nevertheless, the GZ-11608-induced decrease in METH self-administration was not surmounted by increasing the unit dose of METH. Although tolerance developed to lobelane's efficacy in decreasing METH self-administration, this was not the case for lobeline, GZ-793A and GZ-11608. Although lobeline was not efficacious in decreasing reinstatement of METH-seeking behavior, GZ-793A and GZ-11608 showed efficacy. However, since GZ-793A was eliminated as a potential therapeutic for METH use disorder due to its potential cardiotoxicity, these new results advance GZ-11608 as a potential therapeutic due its ability to specifically decrease METH self-administration and reinstatement. Moreover, GZ-11608 does not have intrinsic reinforcing properties and is expected to have low abuse liability.

3.5 Conclusion

GZ-11608, a potent and selective VMAT2 inhibitor, specifically decreases METH reinforcement and tolerance does not develop to its efficacy. METH does not surmount the GZ-11608-induced decrease in responding for METH, and GZ-11608 decreased both

cue- and METH-induced reinstatement of METH-seeking behavior. GZ-11608 also appears to have low abuse liability. These preclinical findings suggest that GZ-11608 has good efficacy and potential as a therapeutic for METH use disorder with the exception of its low oral bioavailability.

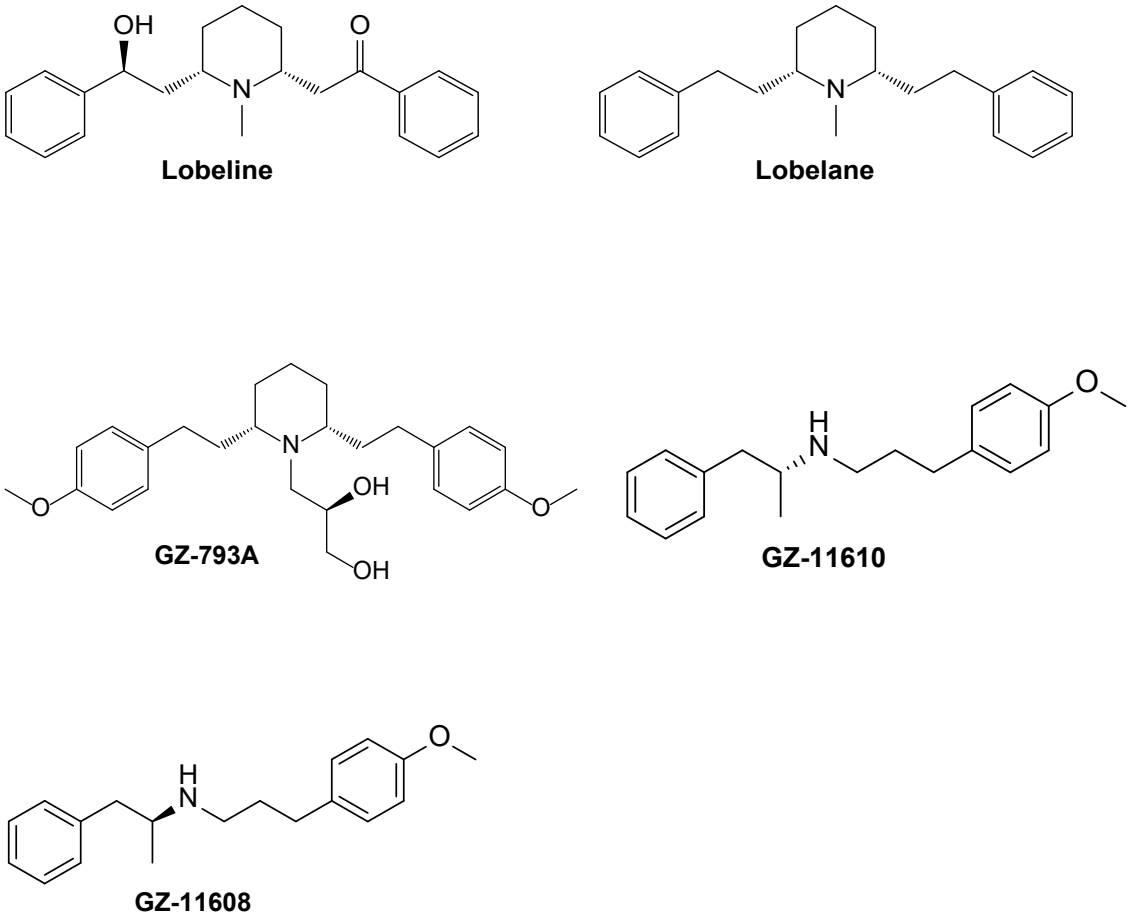


Figure. 3.1. Structures of lobeline, lobelane, GZ-793A, GZ-11610 and GZ-11608

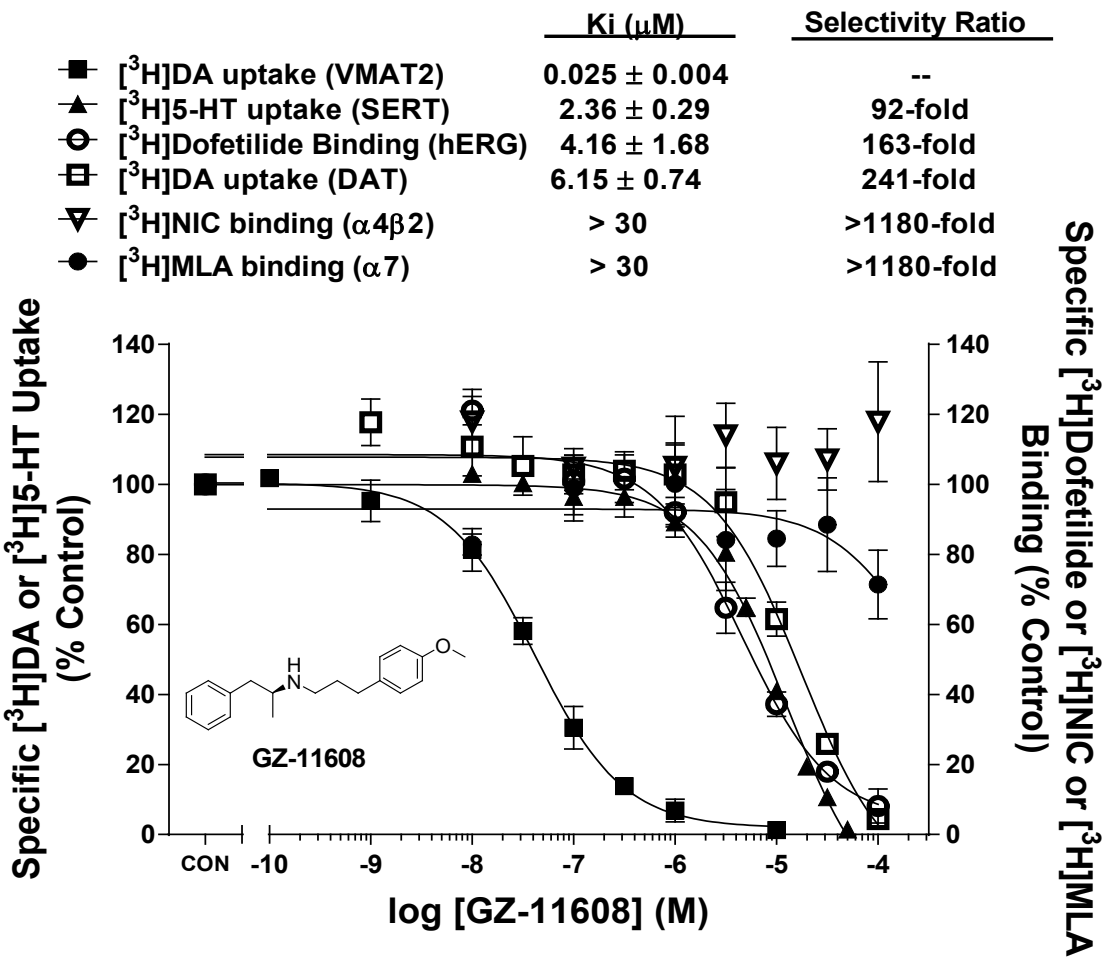


Figure. 3.2. GZ-11608 potently and selectively inhibits VMAT2 relative to SERT, hERG, DAT, α4β2 nAChRs, and α7 nAChRs. Chemical structure for GZ-11608 is shown in the insert. Ki values for GZ-11608 from the various neurochemical assays are provided in an ascending order in the legend. Data are mean ± SEM specific uptake or binding as a percentage of the respective control (Con), in the absence of GZ-11608. Control values for each assay are as follows: VMAT2, 56.3 ± 10.9 pmol/min/mg; SERT, 54.5 ± 7.16 fmol/min/mg; hERG, 888 ± 80.3 fmol/mg; DAT, 91.0 ± 10.1 fmol/min/mg; α4β2 nAChRs, 21.5 ± 3.28 fmol/mg; α7 nAChRs, 31.4 ± 2.72 fmol/mg. n = 4 rats for neurotransmitter uptake assays, n = 3 cell batches for hERG binding assays, and n = 3 rats for nAChR binding assays.

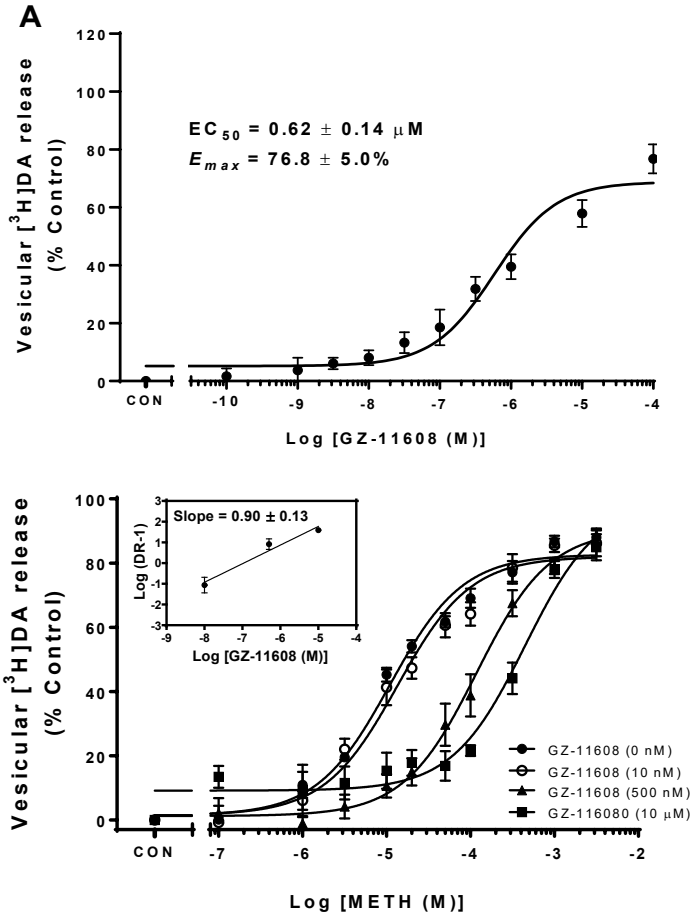


Figure. 3.3. GZ-11608 evokes vesicular DA release (panel A) and competitively inhibits METH-evoked vesicular DA release (panel B). Data are mean ± SEM [^{3}H]DA release from striatal vesicles as a percentage of control. Control (CON) values for [^{3}H]DA release in the absence of GZ-11608 or METH (METH) were 3290 ± 564 dpm (panel A) and 3380 ± 708 dpm (panel B), determined in duplicate in each experiment. Panel A: EC_{50} and E_{max} for GZ-11608 are provided in the insert. Panel B: GZ-11608 concentrations are provided in the legend, and Schild regression and slope are shown in the insert. n = 6 rats, panel A; n = 4 rats/assay, panel B.

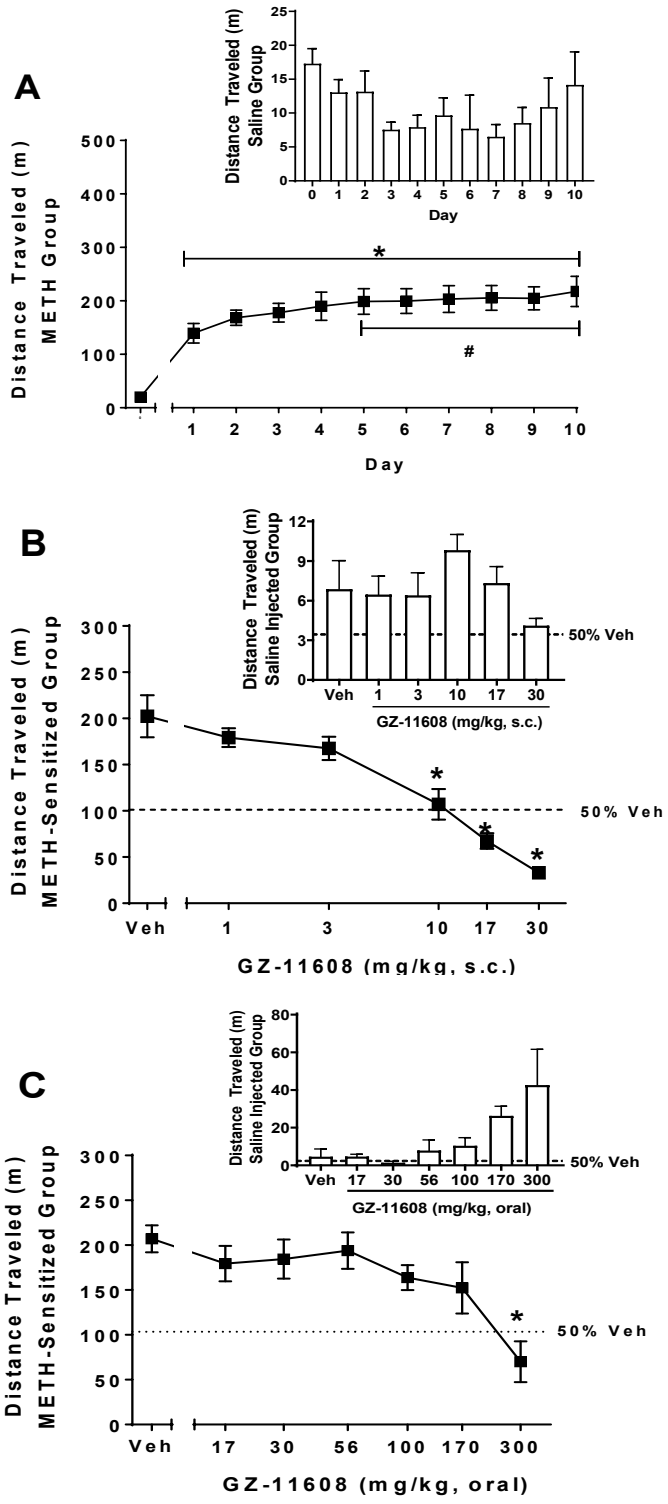


Figure. 3.4. Locomotor sensitization following repeated METH administration (panel A) is decreased in a dose-dependent manner by GZ-11608 (s.c., panel B; oral, panel C). Data are mean ± SEM distance traveled in meters (m) during the last 45

min of the 60-min sessions. Panel A: Day 0 shows activity on a habituation day prior to the first METH or saline injection. METH (1 mg/kg, s.c.) or saline (1 mL/kg, s.c.) was administered once daily for 10 days (Days 1-10). Locomotor activity for the saline group is shown in the insert. Panels B and C: GZ-11608 or vehicle (Veh, 15% (v/v) Kolliphor EL®:/saline; 1 mL/kg) was administered s.c. (panel B) or p.o. (panel C) 15 min prior to METH (METH; 1.0 m/kg, s.c.) or saline injection. Dashed line represents 50% of the distance traveled following vehicle. Locomotor activity for the saline group following GZ-11608 or vehicle is shown in panel B and C inserts. $^{+}p < 0.05$ compared to Day 0; #$p < 0.05$ compared to Day 1 within group (panel A). *$p < 0.05$ compared to vehicle within group (panel B and C); n = 10 rats/group in panel A, which were subdivided into n = 5 rats/group for panels B and C. Note: METH was administered inadvertently to two of the rats in the saline group and saline was administered inadvertently to one rat in the METH group on the day before the 300 mg/kg dose of GZ-11608 (panel C), and these data were not included in the analysis.

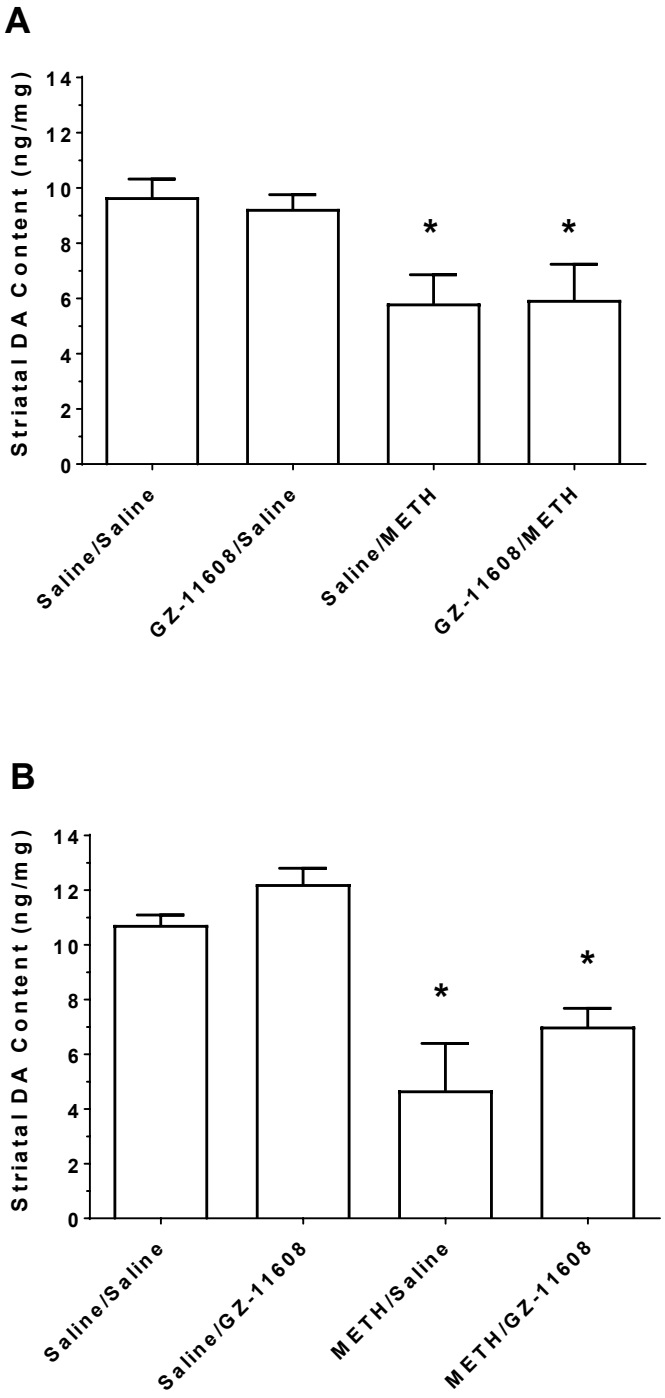


Figure. 3.5. GZ-11608 does not exacerbate METH-induced striatal DA (DA) depletion.

Data are mean ± SEM striatal DA content expressed as ng/mg tissue. GZ-11608 (17

mg/kg, s.c.) was administered 15 min prior to METH (METH, 30 mg/kg, i.p.) or saline (1 mL/kg, i.p.) (panel A, n=7-12 rats/group) or 15 min after METH (30 mg/kg, i.p.) or saline (1 mL/kg, i.p.) (panel B, n = 4-8 rats/group). As a result of the lethality associated with METH, data were not collected for 18 of the 80 rats in the experiment (5 in the METH/saline group; 5 in the METH/GZ-11608 group; 3 in the saline/METH group; and 4 in the GZ-11608/METH group). *$p < 0.05$ compared to the respective saline/saline control group.

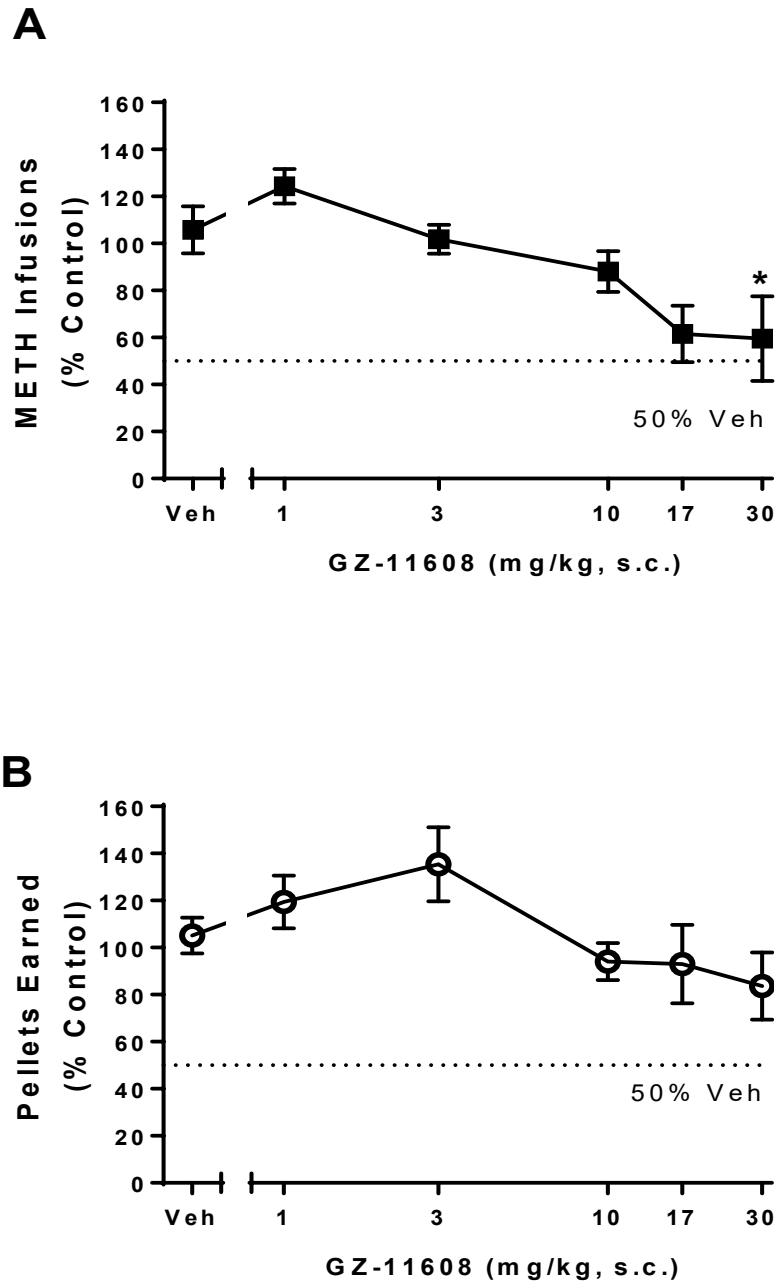


Figure. 3.6. GZ-11608 specifically decreases responding for i.v. METH, without altering responding for food. GZ-11608 or vehicle (Veh, 15% (v/v) Kolliphor EL®:in

saline, 1 mL/kg) was administered (s.c.) to rats trained to self-administer METH (METH, 0.05 mg/kg/infusion; panel A) or to respond for food pellet reinforcers (panel B) during 60 min FR5 operant sessions. Data are mean ± SEM number of reinforcers earned as a percentage of the respective vehicle control (Veh; 16.4 ± 2.6 METH infusions, panel A; 38.4 ± 5.0 food pellets, panel B). Dotted line represents 50% of the reinforcers earned following vehicle injection. The complete GZ-11608 dose-response curve was not collected for 1 rat in the METH self-administration experiment due to an insecure head-mount; data following head mount loss were not included in the analysis. *$p < 0.05$ compared to vehicle control; n = 8 - 9 rats, panel A; n = 11 rats, panel B).

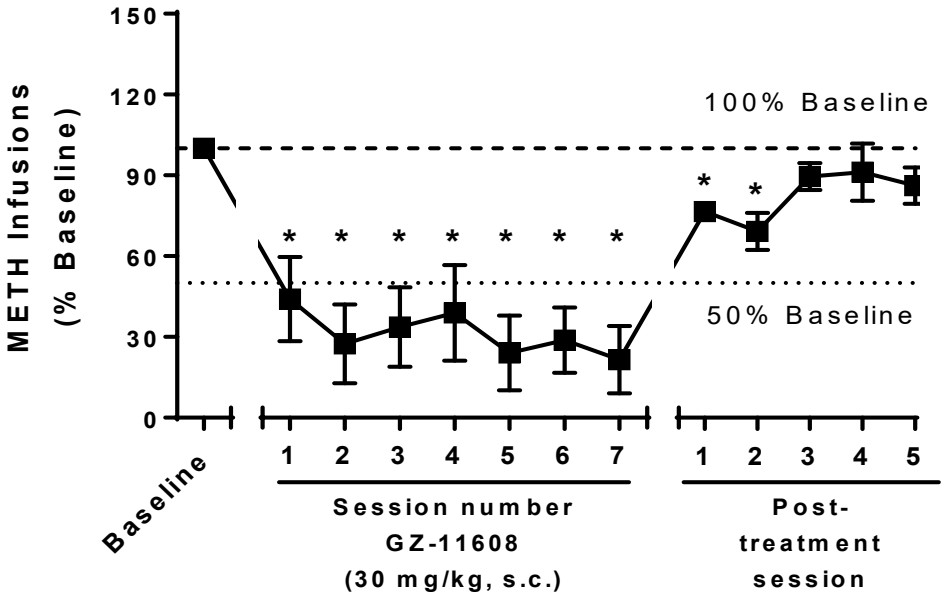


Figure. 3.7. Repeated GZ-11608 decreases i.v. METH self-administration, without the development of tolerance. Baseline represents the number of METH infusions (22.2 ± 4.0) after vehicle injection (15% (v/v) Kolliphor EL®:in saline, 1 mL/kg) 15 min prior to 60-min FR5 sessions. GZ-11608 (30 mg/kg, s.c., once daily for 7 days) was administered 15 min prior to METH self-administration sessions, followed by 5 METH self-administration sessions with no GZ-11608 treatment. Data are presented as mean ± SEM METH infusions earned as a percentage of baseline. Dotted line represents 50% of baseline responding for METH, and dashed line represents 100% of baseline. GZ-11608 treatment decreased (55-75%) responding for METH. Responding for METH returned to baseline levels after discontinuation of GZ-11608 treatment. Complete data were not collected for 2 rats due to insecure head mounts; data following head mount loss were not included in the analysis. *$p < 0.05$ compared to baseline. n = 5 - 7 rats.

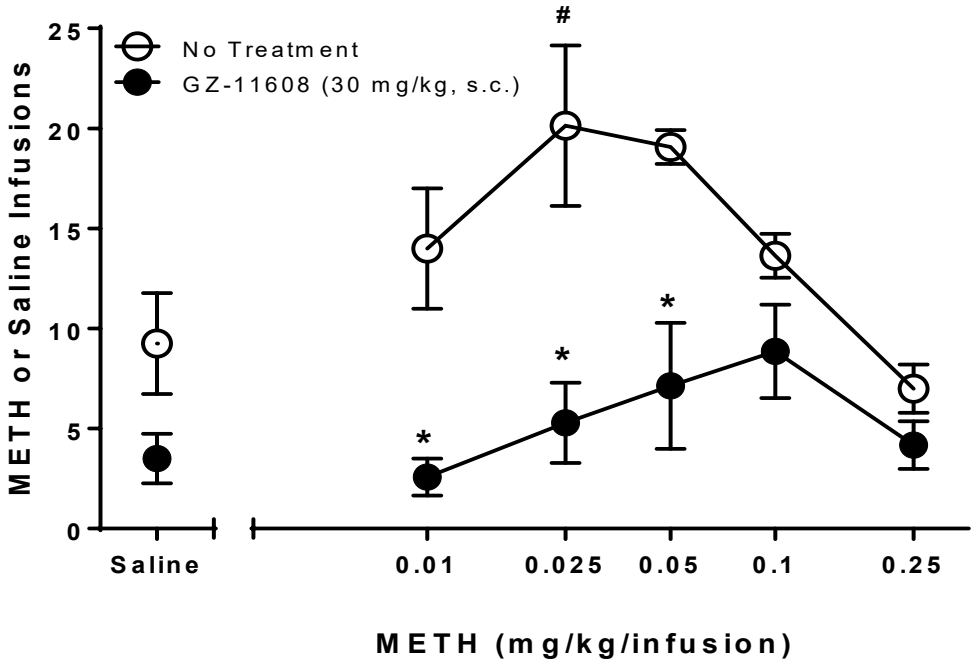


Figure. 3.8. Increasing the unit dose of self-administered i.v. METH does not surmount the GZ-11608-induced decrease in responding for METH. Data are presented as mean ± SEM METH or saline infusions earned during 60-min FR5 operant sessions. The initial training dose of METH was 0.05 mg/kg/infusion, followed by varying unit doses of METH or saline presented in a randomized order of presentation. Open circles represent no GZ-11608 treatment, and closed circles represent GZ-11608 (30 mg/kg, s.c.) treatment 15 min prior to the session. To maintain stable responding, two intervening maintenance sessions occurred between each GZ-11608 treatment session, in which METH was available and no GZ-11608 was administered. Complete data were not collected for 1 rat due to an insecure head-mount; data following head mount loss were not included in the analysis. #$p < 0.05$ compared to saline infusion for the respective group; *$p < 0.05$ compared to the no treatment condition for each unit dose of METH; n = 6 - 7 rats.

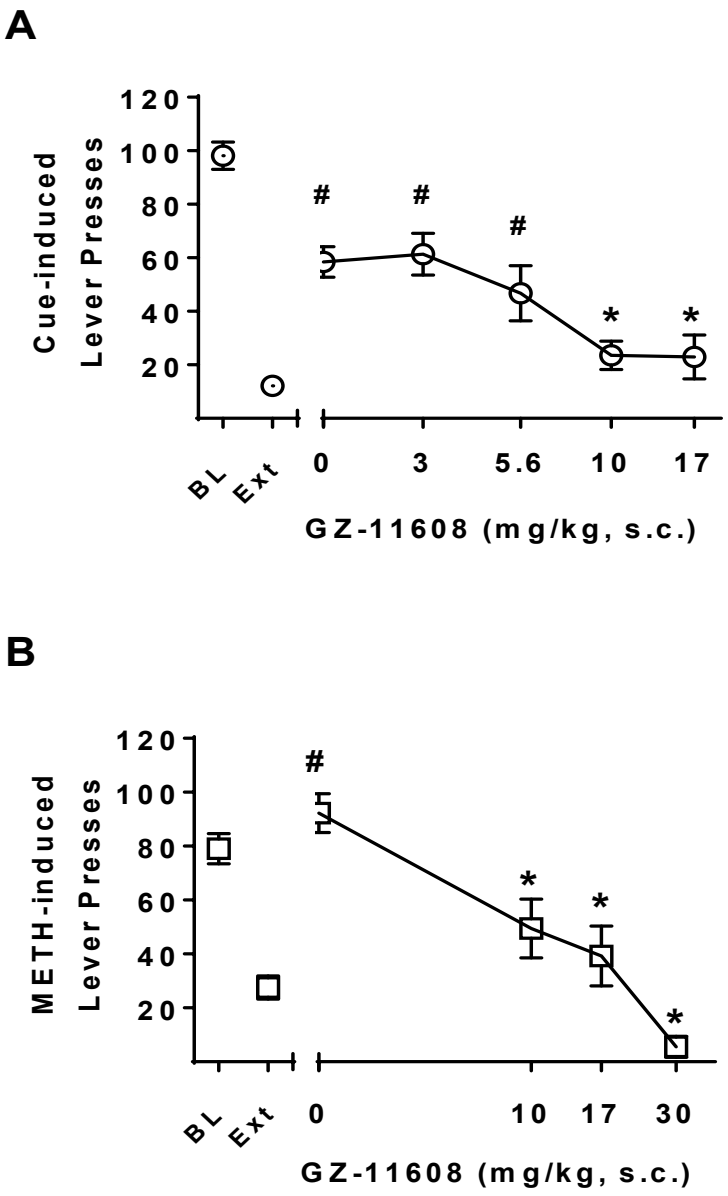


Figure. 3.9. GZ-11608 dose-dependently decreases cue-induced and METH-induced reinstatement of METH seeking. Data are presented as mean ± SEM number of lever presses. Baseline (BL) represents lever presses on the last day of maintenance during which METH (0.05 mg/kg/infusion) was available for self-administration. For cue-induced reinstatement (panel A), extinction (Ext) represents lever presses on the last of 14 days in which no cue light was presented and no METH was available. GZ-11608 treatment decreased cue-induced lever presses relative to vehicle injection (0 dose; Kolliphor EL).

Number of cue-induced lever presses following GZ-11608 (10 mg/kg) was not different between the two groups of rats evaluated ($p > 0.05$), and these data were combined for analysis. Between each session in which the effect of GZ-11608 was evaluated, 5 extinction sessions occurred. For METH-induced reinstatement (panel B), extinction (Ext) represents lever presses on the last day of 14 days in which the cue light was presented, but no METH was available. GZ-11608 decreased METH-induced lever presses relative to vehicle injection (0 dose). Between each session in which the effect of GZ-11608 was evaluated, 5 extinction sessions occurred. No reinstatement was exhibited by 3 rats (defined as <10 cue-induced responses following vehicle); data from these rats were not included in the analysis. The complete GZ-11608 dose-response curve was not collected for 2 rats in the cue-induced reinstatement experiment (panel A) due to an insecure head-mount; data following head mount loss were not included in the analysis. *$p < 0.05$ compared to the respective vehicle control, #$p < 0.05$ compared to respective last day of extinction prior to GZ-11608 treatment. n = 6 - 12 rats/experiment.

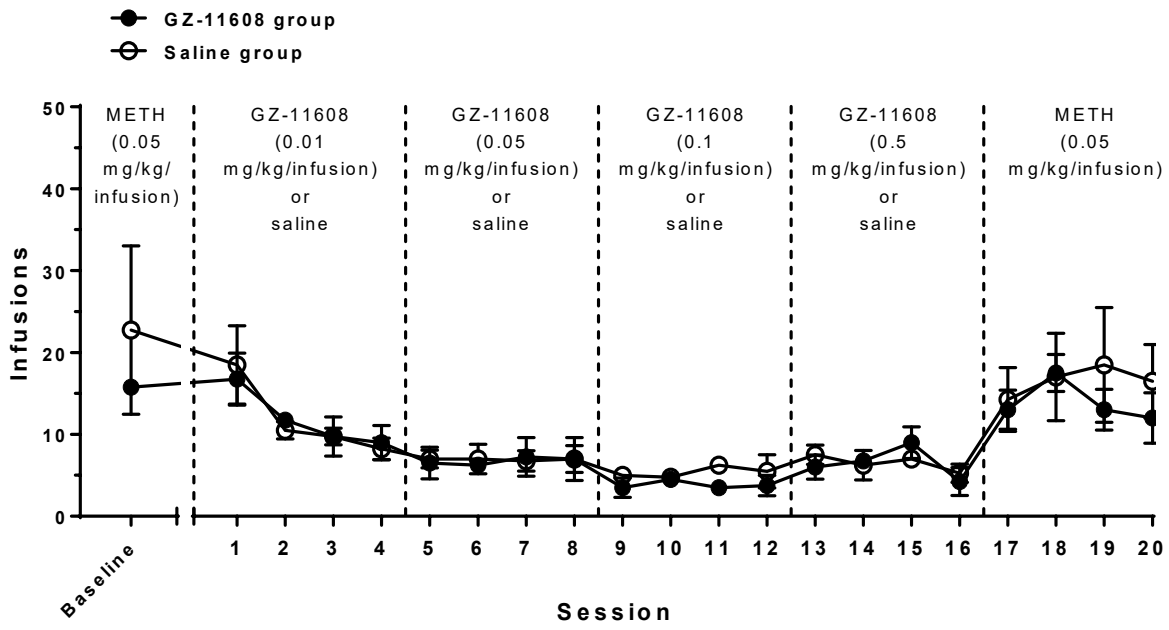


Figure. 3.10. GZ-11608 does not substitute for METH self-administration. Data are presented as mean ± SEM number of i.v. infusions (METH, GZ-11608, or saline) across 60-min, FR5 sessions. Baseline represents the number of METH infusions on the last day of maintenance for both the GZ-11608 and saline groups. Ascending doses of GZ-11608 for the GZ-11608 group or saline for the saline group were available as reinforcers for 4 sessions/unit dose. Then, METH was available during the final 4 sessions of the experiment. n = 4 rats/group.

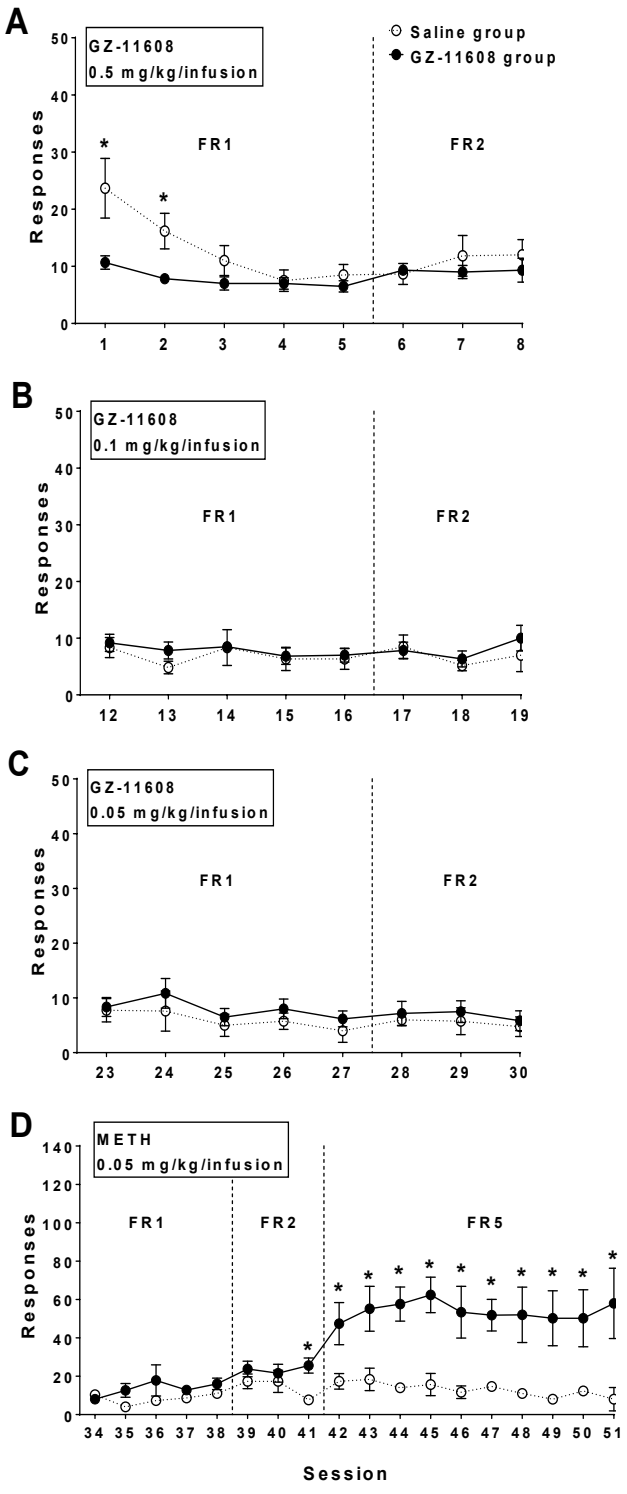


Figure. 3.11. GZ-11608 is not self-administered by drug naïve rats. Responding during successive operant sessions is illustrated across panels A-D, with the

exception of 3 intervening sessions between each GZ-11608 unit dose and prior to the METH self-administration sessions, in which responding for food pellet reinforcers occurred (data not shown). Data are presented as mean ± SEM number of i.v. infusions of GZ-11608 (descending dose order) for the GZ-11608 group or saline (1 mL/kg/infusion, i.v.) for the saline group across 60-min FR1 and FR2 sessions (panels A-C). METH self-administration was available to the GZ-11608 group, and saline was available to the saline group under the FR1, FR2 and FR5 schedules of reinforcement (panel D). Complete data were not collected for 1 rat in the GZ-11608 group due to a faulty catheter and for 3 rats in the saline group due to an insecure head mount; data following procedural interruptions were not included in the analysis. *$p < 0.05$ compared to the saline group on the respective session, n = 3 - 6 rats/group.

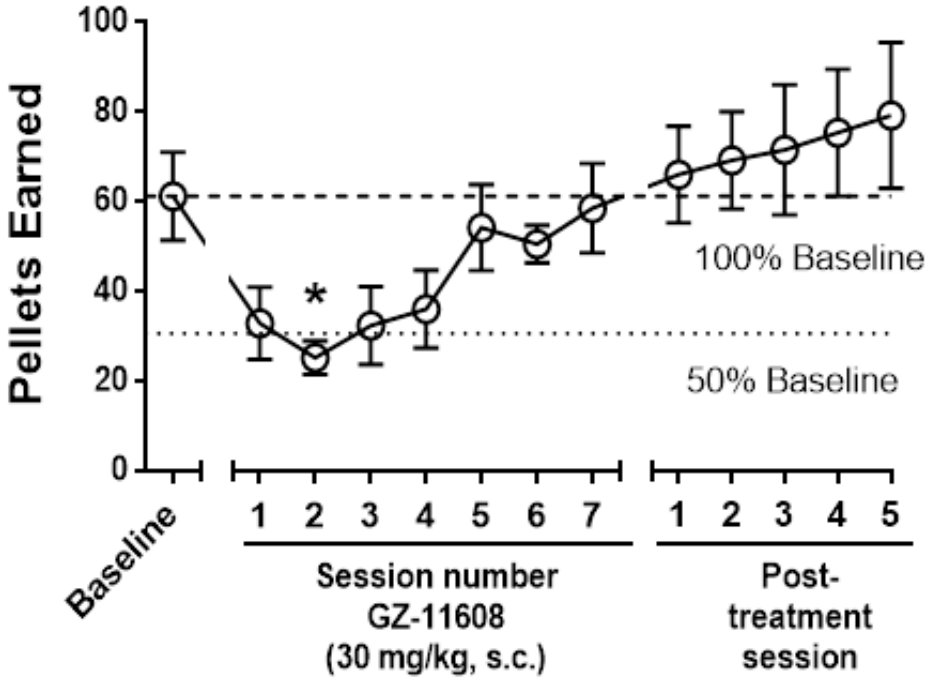


Figure. 3.12. GZ-11608 decreased food-maintained responding and tolerance developed to this effect over repeated administration. GZ-11608 (30 mg/kg, s.c., once daily for 7 days) was administered 15 min prior to 60-min sessions, in which rats responded for food pellets under an FR5 schedule. Data are presented as mean ± SEM food pellets earned. Baseline was the number of food pellets earned following vehicle (15% (v/v) Kolliphore EL in saline) injection. Dotted and dashed lines represent 50% and 100% of baseline responding, respectively. One-way ANOVA revealed a main effect of GZ-11608 relative to baseline [$F_{7,40} = 2.81$, $p < 0.05$]. One-way ANOVA also revealed no alterations in responding for food during post-treatment sessions relative to baseline [$F_{5,30} = 0.25$, $p > 0.05$]. (n = 6 rats).

4. CHAPTER FOUR: MUSCARINIC AGONIST, (±)-QUINUCLIDIN-3-YL-(4-FLUOROPHENETHYL)-(PHENYL)CARBAMATE: HIGH AFFINITY, BUT, LOW SUBTYPE SELECTIVITY FOR HUMAN M1 - M5 MUSCARINIC ACETYLCHOLINE RECEPTORS

4.1 Introduction

Many drugs of abuse including cocaine, amphetamine, METH, and morphine increase extracellular DA in the NA at doses that produce rewarding effects (Di Chiara and Imperato, 1988a). Indeed, bilateral microinjection of 6-OHDA which produces dopaminergic neuron damage in the NA inhibited initiation of amphetamine self-administration in rats when 6-OHDA was administered before self-administration training, and disrupted responding during maintenance of amphetamine self-administration (Lyness et al., 1979). DA containing VTA neurons project to NA, and are important for drug seeking behavior. Electrical stimulation of acetylcholine-containing laterodorsal tegmental nucleus (LDT) neurons, which innervate VTA dopaminergic neurons, results in an increase in extracellular DA concentrations in the NA (Oakman et al., 1995; Forster and Blaha, 2000). Important to the current study, M5 mAChRs, among five different subtypes of mAChRs (M1, M2, M3, M4, and M5), are highly expressed on the postsynaptic DA neurons in VTA (Vilaro et al., 1990; Weiner et al., 1990; Lein et al., 2007; Yeomans, 2012). In M5 mAChRs KO mice, LDT stimulation and morphine-induced DA release in the NA is reduced relative to wild-type mice (Basile et al., 2002; Forster et al., 2002). M5 KO mice also have decreased cocaine self-administration and cocaine- or morphine-induced CPP when compared to wild-type controls (Basile et al., 2002; Fink-Jensen et al., 2003). Microinfusion into VTA of scopolamine, a mAChR antagonist, robustly decreased cocaine-seeking behavior during withdrawal in rats (Solecki et al., 2013). Together, these findings

led us to hypothesize that selective antagonism of M5 mAChRs represents a novel target for the treatment of drug abuse.

Recently reported a class of M5-preferring orthostatic antagonists was based on the scaffold of 1,2,5,6-tetrahydropyridine-3-carboxylic acid (Zheng et al., 2013). Compound **1** (Figure 4.1., Table 1) was identified as the most selective M5 mAChRs antagonist in this series. Interestingly, removal of the meta-methoxy group in 1 (compound **2**) significantly increased binding affinities at both M1 and M5 receptors, but resulted in a complete loss in selectivity for M5 over M1. To further explore the structure-activity relationship (SAR), studies were planned to reposition the carboxylate group in **1** and **2** from C-3 to C-4 of the piperidine ring. New analogs resulted from such rearrangement resembling pethidine (**3**, Figure 4.1.), a once popular analgesic. Interestingly, pethidine has been identified as an antagonist at mAChRs in guinea-pig ileum assays (Hustveit and Setekleiv, 1993). Thus, analogs based on the pethidine scaffold may afford interesting SAR at mAChRs. In addition to ester containing analogs (**4** and **6**), we also planned to evaluate amides (**5** and **7**), carbamates (**8** and **9**), and carbamides (**10**). Herein, binding affinity and selectivity for M5 over M1 and M3 mAChRs were evaluated.

4.2 Method

Analog affinities for M1, M3 and M5 mAChRs were determined by measuring inhibition of [^{3}H]N-methylscopolamine (NMS) binding to Chinese hamster ovary (CHO) cell membranes expressing M1, M3, or M5 recombinant human mAChRs. CHO cells stably expressing each of the human mAChRs were obtained from Dr. Tom Bonner of National Institute of Mental Health (NIMH). Detailed materials and methods for cell culture and cell membrane preparation were described previously (Zheng et al., 2013). Briefly, [^{3}H]NMS

binding assays were performed using 96-well plates. Membrane aliquots containing 10 µg of protein per well for M1 or M5 subtypes and 3 µg of protein per well for M3 subtype were added to wells containing 1 nM to 100 µM of test analog, 0.3 nM [3H]NMS (specific activity 84.2 Ci/mmol; Perkin-Elmer/NEN, Boston, MA), and buffer (50 mM Tris-HCl, pH 7.4), and then incubated for 2 h at 25 ˚C. Nonspecific binding of [^{3}H]NMS was determined in the presence of 1 µM atropine. Reactions were terminated by rapid filtration onto GF/B filters using a Filtermate harvester (PerkinElmer Life and Analytical Sciences, Boston, MA) and washed three times with buffer (50 mM Tris-HCl, pH 7.4). Subsequently, 40 µL of MicroScint 20 (PerkinElmer Life and Analytical Sciences, Waltham, MA) was added to each well and radioactivity bound determined using liquid scintillation spectrometry. IC_{50} values were obtained and Ki values were calculated using the equation of Cheng and Prusoff (Cheng and Prusoff, 1973).

4.3 Results and Discussion

Results are summarized in Table 1. Similar to the SAR generated for the parent compounds **1** and **2**, mono-methoxy substituted analogs (**4b**, **5b**, **6b**, **7b**, and **10b**) consistently exhibited higher affinity (2 to 9-fold at M1; 3 to 19-, fold at M3; 2 to 8-fold at M5) when compared to their corresponding di-methoxy substituted analogs (**4a**, **5a**, **6a**, **7a**, and **10a**, respectively). The corresponding carboxylate moiety repositioned in molecule **4a** exhibited 2- and 19-fold higher affinity at M1 and M3 mAChRs, respectively, compared with compound **1**. However, the affinity of **4a** at M5 mAChRs was decreased by 30%. Thus, compound **4a** was not subtype selective.

In addition, replacement of the ester link in **4a/b** or **6a/b** with an amide link (**5a/b** and **7a/b**, respectively) resulted in a loss of affinity at all three mAChR subtypes (**4a** vs **5a**,

2 to 4-fold; **4b** vs **5b**, 5 to 8-fold; **6a** vs **7a**, 3 to 7-fold; **6b** vs **7b**, 4 to 5-fold). In general, analogs with carbamate and carbamide linkers exhibited up to an 8-fold lower affinity compared to the esters. Furthermore, the reverse ester of 4a (i.e., **6a**) exhibited a moderate 1 to 3-fold increase in affinity at all three mAChRs. A similar increase in affinity was observed for the other reverse ester/amide series, i.e., **4b** vs **6b** and **5b** vs **7b**. Analog **6b** was identified as the most potent compound at M5 in this series.

4.4 Conclusion

A series of pethidine analogs was synthesized and evaluated to determine binding affinity for the [^{3}H]NMS binding site on M1, M3, and M5 human mAChRs expressed by CHO cell membranes. Compound **6b** showed the highest binding affinity at M1, M3 and M5 mAChRs (Ki = 0.67, 0.37, and 0.38 μM, respectively). However, this series of new analogs did not exhibit selectivity for M5 mAChRs over M1 and M3 subtypes. Further SAR and pharmacological evaluations are needed to identify potent and selective M5 mAChR antagonists.

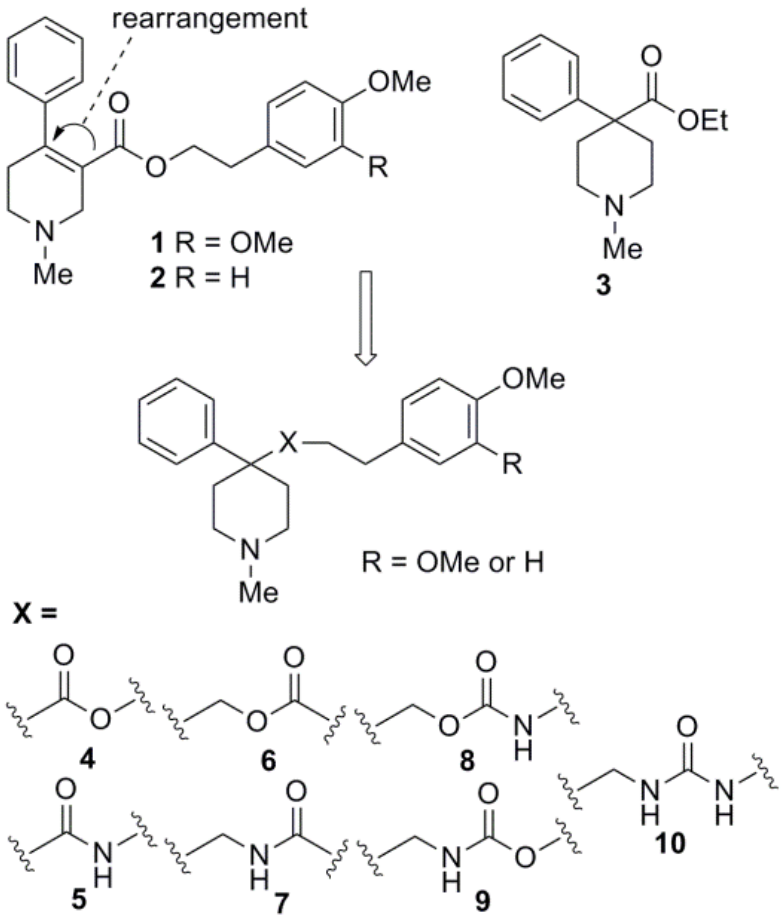
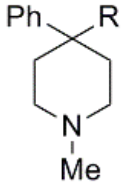
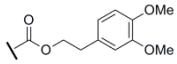
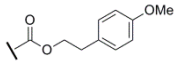
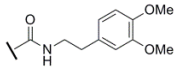
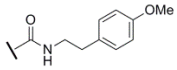
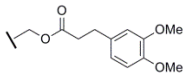
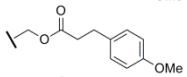
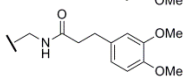
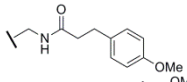
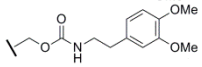
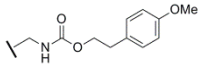
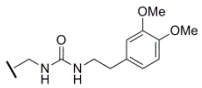
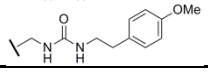


Figure 4.1. Structure of compounds 1 and 2, pethidine (3), and design of pethidine analogs 4-10 as novel mAChR ligands.

Table 1. Structures and binding affinity for analogs at M1, M3, and M5 mAChRs[a]



compd	R	[^{3}H]NMS binding Ki ±SEM (μM) M1	M3	M5
1[b]	-	25.3	>100	2.24
2[b]	-	0.02± 0.002	ND[c]	0.03± 0.005
4a		10.8 ± 0.67	5.26 ± 0.33	6.95 ± 0.47
4b		1.20 ± 0.11	0.64 ± 0.037	0.87 ± 0.053
5a		> 30	> 10	> 30
5b		> 10	3.29 ± 0.80	6.97 ± 0.77
6a		3.63 ± 0.22	4.60 ± 0.61	2.14 ± 0.22
6b		0.67 ± 0.078	0.37 ± 0.045	0.38 ± 0.011
7a		> 10	> 30	> 10
7b		3.44 ± 0.93	1.54 ± 0.19	1.81 ± 0.11
8		5.09 ± 0.29	4.03 ± 0.57	5.41 ± 0.59
9		2.91 ± 0.17	2.30 ± 0.22	2.05 ± 0.30
10a		> 10	> 10	> 10
10b		5.40 ± 1.22	3.55 ± 0.11	4.30 ± 0.29

[a] Three independent experiments, each experiment included duplicate samples, were performed to obtain Ki values (Mean ± SEM)
[b] Data from reference Zheng et al., 2013
[c] Not determined

5. CHAPTER FIVE: NOVEL METHYL PHENYLCARBAMATE ANALOGS BINDING AFFINITY AT HUMAN M1-M5 MUSCARINIC ACETYLCHOLINE RECEPTORS EXPRESSED ON CHO CELLS

5.1 Introduction

Muscarinic acetylcholine receptors (mAChRs) consist of five subtypes (M1, M2, M3, M4 and M5 mAChRs). Each mAChR subtype has a unique expression pattern and activates distinct cholinergic signaling mechanisms and cellular functions (Eglen, 2006). Each mAChR subtype can be considered as a therapeutic target for relevant diseases that are associated with specific cell functions modulated by the mAChR subtype. For example, M1 mAChRs expressed in cerebral cortex, striatum and hippocampus mediate learning and memory processes (Levey, 1996). M1 mAChRs allosteric agonists, VU0357017 and VU0364572, enhance spatial learning in rats in the Morris water maze (Digby et al., 2010; Lebois et al., 2010). Agonists at M1 mAChRs have been proposed as pharmacotherapeutics for Alzheimer's disease and schizophrenia (Fisher et al., 2003; Eglen, 2006; Jiang et al., 2014). Another example is the M3 mAChR, which mediates contraction of smooth muscles in the respiratory system (Barnes, 1989). M3 mAChR antagonists, such as tiotropium or umeclidinium have been approved by the FDA as therapeutics for chronic obstructive pulmonary disease (COPD) (Eglen, 2006; Food and Drug Administration, 2015; Chin et al., 2016). Also, a combination therapy including umeclidinium and vilanterol, a β2-adrenergic agonist, was approved by the FDA for COPD (Goldenberg, 2014). Thus, mAChRs are viable targets for drug discovery.

M5 mAChRs are specifically and highly expressed in the VTA, a brain region containing DA neuronal cell bodies, which project to the NA (Vilaro et al., 1990; Weiner et al., 1990; Yasuda et al., 1993; Omelchenko and Sesack, 2006; Lein et al., 2007; Yeomans,

2012). Importantly, NA DA release mediates the rewarding effects of many substances with high abuse liability, including opioids (e.g., morphine) and psychostimulants (e.g., cocaine and amphetamines) (Lyness et al., 1979; Di Chiara and Imperato, 1988a; Koob et al., 1998; Chevrette et al., 2002). With respect to opioids, morphine-induced DA release in the NA is absent in M5 mAChR KO mice, linking M5 mAChRs to opioid-induced reward (Steidl et al., 2011). Direct infusion into VTA of a virus containing M5 mAChR DNA increased both M5 mAChR expression and morphine-induced locomotor activity compared to control mice, suggesting that M5 mAChRs mediate DA-related behaviors (Wasserman et al., 2013). Relative to wild-type mice, M5 mAChR KO mice exhibit a decreased amount of time spent in the cocaine-paired compartment in the conditioned place preference assay (Fink-Jensen et al., 2003; Raffa, 2009) and decreased cocaine i.v. self-administration (Thomsen et al., 2005), suggesting M5 mAChRs mediate cocaine reward and reinforcement, respectively.

Since M5 mAChRs are highly expressed in VTA, micro infusion of nonselective mAChRs antagonists (e.g., scopolamine and atropine) into VTA allows for quasi-selective inhibition of M5 mAChR function. Scopolamine microinfused unilaterally into VTA decreased morphine-evoked DA release in the NA in mice (Steidl et al., 2011). Atropine microinfused bilaterally into VTA also decreased morphine-induced hyperlocomotion in mice, supporting a critical role for VTA M5 mAChRs in modulating opioid effects on behavior (Steidl and Yeomans, 2009). Thus, both pharmacological and genetic approaches to reduce M5 mAChR function provide consistent results implicating this mAChR subtype in the effects of opioids on DA neurochemistry and related behaviors.

Based on these findings, a hypothesis that discovery of selective M5 mAChRs antagonists may provide novel pharmacotherapeutics that act to decrease the activity of VTA DA projections to NA, thereby reducing the reinforcing effects of substances with high

abuse liability. Previously, described a 1,2,5,6-tetrahydropyridine-3-carboxylic acid scaffold from which several M5 mAChRs antagonists were found (Zheng et al., 2013). From this series of compounds, compound **11** (Fig. 5.1) exhibited the greatest selectivity (11-fold) at M5 over M1 mAChRs; however, **11** provided only modest affinity (Ki = 2.24 μM) for M5 mAChRs.29 Compound **11** inhibited oxotremorine-induced DA release from superfused rat striatal slices, revealing an antagonist action at M5 mAChRs (Zheng et al., 2013).

The 3,4-dimethoxyphenethyl group in compound **11** was suggested to play an important role in its binding preference at the M5 subtype (Zheng et al., 2013). Quinuclidinyl carbamate mAChR antagonists such as SVT-40776 (Fig. 5.1) and its analogs also have high affinity at mAChRs (Prat et al., 2011). In an effort to improve analog affinity and selectivity at M5 mAChRs, herein the synthesis and evaluation of hybrid compounds, **12a** and **13a** (Fig. 5.1), and a series of related analogs (Tables 2 and 3).

5.2 Method

5.2.1 [^{3}H]NMS Binding Assay

[^{3}H]NMS binding assays were performed using 96-well plates. Membrane aliquots containing 10 μg of protein per well for M1 or M5 mAChRs subtypes, 20 μg of protein per well for M2 or M4 mAChRs subtypes, and 3 μg of protein per well for M3 mAChRs were added to wells containing 1 nM to 100 μM of analog, 0.3 nM [^{3}H]NMS (specific activity 84.2 Ci/mmol; Perkin-Elmer/NEN, Boston, MA) and buffer (50 mM Tris–HCl, pH 7.4) and then incubated for 2 h at 25 ºC. Nonspecific binding of [^{3}H]NMS was determined in the presence of 1 μM atropine. Reactions were terminated by rapid filtration onto GF/B filters

using a Filtermate harvester (PerkinElmer Life and Analytical Sciences, Boston, MA) and washed three times with 50 mM Tris–HCl buffer (pH 7.4). Subsequently, 40 µL of MicroScint 20 (PerkinElmer Life and Analytical Sciences, Waltham, MA) was added to each well and radioactivity bound determined using liquid scintillation spectrometry.

5.2.2 [^{3}H]DA Release Assay

Rat striata were dissected and sliced (500 µm, 4-6 mg). Striatal slices were incubated in Krebs' buffer (108 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 1 mM NaH_2PO_4, 1.3 mM $CaCl_2$, 11.1 mM glucose, 25 mM $NaHCO_3$, 0.11 mM L-ascorbic acid and 0.004 disodium EDTA, pH7.4) for 60 min at 34 °C. During the latter 30 min of the incubation, [^{3}H]DA (0.1 µM) was added to the incubation buffer. Each striatal slice was placed in a superfusion chamber after incubation, and then superfused (0.6 mL/min) with Krebs' buffer for 60 min in the presence of nomifensine (a DA transporter inhibitor; 10 µM) and pargyline (a monoamine oxidase inhibitor; 10 µM). Samples were collected every 5 min for a total of 75 min. Samples collected during the first 15 min were used to determine [^{3}H]DA overflow. Samples were collected for 35 min in the absence or presence of scopolamine or **13c**, followed by 25 min in absence or presence of oxotremorine (100 µM) in the superfusion buffer. [^{3}H]DA overflow was determined as the summation of radioactivity in superfusate samples during exposure to compound following the subtraction of basal outflow across the same time period. Radioactivity was determined using liquid scintillation spectroscopy.

5.3 Results and Discussion

Initially, affinity at M5 mAChRs was determined for all analogs. Since M5 mAChRs has relatively high sequence homology with M3 > M1 > M4 > M2 mAChRs (85%, 79%, 73% and 68%, respectively) (Bonner et al., 1988), thus, next affinity of all analogs at M3 and M1 mAChRs were determined. Analogs with high affinity (Ki < 10 nM) were evaluated also at M2 and M4 mAChRs to assess subtype selectivity. CHO cell lines individually expressing each of the human M1-M5 mAChRs were generously provided by Dr. Tom Bonner, National Institute of Mental Health. Analog-induced inhibition of [^{3}H] NMS binding was used to determine affinity at each of mAChR subtypes (Lee et al., 2015). Amount of [^{3}H]NMS bound after a 60-min incubation in the absence and presence of a range of analog concentrations was plotted as a function of analog concentration to obtain IC_{50} values. IC_{50} values were used to calculate compound affinity (inhibition constant, Ki) using the Cheng and Prusoff equation (Cheng and Prusoff, 1973). Maximal inhibition (Imax) of specific [^{3}H]NMS binding was represented as a percent of control (absence of compound). Atropine was used as the positive control, and its Ki values at M1, M2, M3 and M5 were 0.44, 0.90, 0.53, and 0.60 nM, respectively, in good agreement with literature values (Moriya et al., 1999; Hirose et al., 2001).

The Ki and Imax values of analogs with general structure **2** (Fig. 5.1) at M1, M2, M3, and M5 mAChRs are provided in Table 2. Compound **12a**, in which the R group is 3,4-dimethoxy-phenethyl, is the prototypic analog in this series. Compared with parent compound **11**, **12a** retained affinity at M5 mAChRs, however, selectivity over other mAChR subtypes was diminished. Substituents on the phenyl ring of the R group and the length between the phenyl ring and O atom had important influences on binding affinity. Thus, a wide range of Ki values (19 nM - 6.98 μM) was obtained, whereas the majority of the compounds completely inhibited [^{3}H]NMS binding (Imax = 85.9 - 100% of control).

Overall, analogs with a one-carbon linker (**12b**, **12d**, **12j**, **12l**, **12o**, and **12q**) had the highest affinity within each subgroup of compounds having the same substituents on the phenyl ring. However, analogs with a longer linker generally exhibited higher preference for M5 compared with the corresponding one-carbon linker counterparts. Among the analogs in this series, **12m**, in which the R group is 3,4-dichlorophenethyl, exhibited relatively high affinity (Ki = 80 nM) and the greatest selectivity (4.5-fold) for M5 over M1 mAChRs.

Transposition of the phenylalkyl group and the quinuclidin-3-yl group in **12** resulted in analogs **13a**, **13b**, and **13c** as enantiomeric mixtures (Table 3). The design of this group of "rearranged" analogs was based on hypotheses that spatial rearrangement or reorientation of the pharmacophore elements in mAChR ligands would alter affinity and selectivity profiles (Hirose et al., 2001). Compound **13c** with an N-4-fluorophenethyl group exhibited the highest affinity at M5, M1 and M3 mAChRs (Ki = 1.8, 2.0 and 2.6 nM, respectively). Thus, **13c** is ~5-fold and 32 to 48-fold, respectively, higher than **13b** having a 3-methoxyphenethyl, and **13a** having a 3,4-dimethoxyphenethyl group. These findings indicate that substituents on the phenyl ring have major impact on affinity and that an electron-withdrawing group may be favorable for receptor binding. Replacement of the quinuclidin-3-yl group in **13** with an N-methylpiperidin-3-yl, an N-methylpiperidin-4-yl, or a tropan-4-yl provided analogs **14a/b/c**, **15a/b/c**, and **16a/b**, respectively (Table 3). Affinity at M5, M1 and M3 mAChRs for these analogs was lower than their corresponding quinuclidine-containing analogs **13a/b/c**. Despite the overall increase in affinity in this series of analogs compared to analogs in the **12** series, none of these compounds exhibited preference for M5 mAChRs. Of note, analogs in the **12**, **13**, and **14** series were racemic.

As in previous studies (Zheng et al., 2013), the lead analog **13c** was evaluated using a functional assay determining inhibition of oxotremorine-induced DA release from superfused rat striatal slices. Oxotremorine is a nonselective agonist at mAChRs (Barocelli et al., 2000). If analog-induced inhibition is observed, then this suggests that the analog acts via an antagonist action at mAChRs. For the [^{3}H]DA release assay, rat striatal slices were incubated with 0.1 μM [^{3}H]DA for 30 min, and then, were superfused with buffer for 60 min to obtain stable efflux of [^{3}H]DA (Prat et al., 2011; Zheng et al., 2013). Samples were collected for 15 min to determine basal [^{3}H]DA outflow. Superfusion continued for 35 min in the absence and presence scopolamine (1 and 10 μM, positive control) or **13c** (0.1, 1, and 10 μM) added to the superfusion buffer. Then, oxotremorine (100 μM) (Steidl and Yeomans, 2009) was added to the buffer for 25 min. The ability of scopolamine and **13c** to inhibit oxotremorine-evoked [^{3}H]DA overflow was determined. In agreement with previous findings, oxotremorine increased [^{3}H]DA overflow compared to control, and scopolamine (1 and 10 μM) inhibited (51% and 59%, respectively) oxotremorine-evoked [^{3}H]DA overflow ([$F_{3,35}$ = 6.13], $p < 0.005$, one-way ANOVA followed by Tukey's test; Fig. 5.2). The results are consistent with the predicted outcome that scopolamine (positive control) inhibits oxotremorine-evoked DA release from rat striatum. The ability of lead compound **13c** to inhibit oxotremorine-evoked [^{3}H]DA overflow from superfused rat striatal slices was determined (Fig. 5.3). Scopolamine (10 μM) was included as a positive control in these experiments. Similar to the results illustrated in Fig. 5.2, oxotremorine increased [^{3}H]DA overflow and scopolamine decreased the stimulatory effect of oxotremorine. In contrast to expectations, **13c** did not inhibit oxotremorine-evoked [^{3}H]DA overflow, but rather, augmented oxotremorine-evoked [^{3}H]DA overflow. One-way ANOVA followed by Tukey's test revealed that **13c** (10 μM) increased oxotremorine-evoked [^{3}H]DA overflow relative to oxotremorine alone (scopolamine 0 μM; [$F_{3,11}$=6.83], $p < 0.005$; Fig. 5.3).

Augmentation of the effect of oxotremorine by **13c** suggests that **13c** acts as an agonist at mAChRs.

To further determine if **13c** acts as a mAChR agonist, the ability of scopolamine to inhibit **13c**-evoked [^{3}H]DA overflow was determined. One-way ANOVA followed by Tukey's test revealed that the 3c-evoked increase in [^{3}H]DA overflow was inhibited by scopolamine (10 μM) ([$F_{5,42}$=6.92], $p < 0.0001$; Fig. 5.4). Since the effect of **13c** was inhibited by a nonselective mAChR antagonist, scopolamine, these results support the interpretation that **13c** acts as an agonist at mAChRs to increase [^{3}H]DA overflow from rat striatal slices.

Whereas oxotremorine (1, 10, and 100 μM)-evoked striatal [^{3}H]DA overflow was not altered in M1 or M2 mAChR KO mice, oxotremorine-evoked [^{3}H]DA overflow was increased in M3 KO mice, abolished in M4 KO mice, and decreased by 50% in M5 KO mice (Zhang et al., 2002). Thus, M1 or M2 mAChRs do not appear to have a role in mediating striatal DA release in mice. Also, M3, M4 and M5 mAChRs appear to play differing roles in mediating striatal DA release in mice. In contrast to VTA where M5 mAChR subtype expression predominates, M3, M4 and M5 mAChRs mediate oxotremorine-evoked [^{3}H]DA overflow from striatal slices in mice. If allowed to extrapolate from these results obtained using striatal slices from KO mice, the current results suggest that oxotremorine increases striatal DA release via stimulation of M4 and/or M5 mAChRs, but not via M3 mAChRs. Based on the results from the KO studies (Zhang et al., 2002), stimulation of M3 mAChRs would be expected to decrease DA release. Assuming mAChR modulation of DA release is similar in rats and mice, the lead compound **13c** appears to evoke DA release from rat striatal slices either though an agonist action at M4 and/or M5 mAChRs, or through an antagonist action at M3. In any case, **13c** increases striatal DA release via an agonist action at mAChRs, since this effect is inhibited by scopolamine.

Another important finding from the present study is that the two structural scaffolds provided analogs that exhibit selectivity for M3 over M2. Out of the 30 analogs evaluated, 9 analogs (**12d**, **12j**, **12l**, **12n**, **12o**, **12q**, **13a**, **13b** and **13c**) exhibit relatively high affinity (Ki < 100 nM) at M3 mAChRs. For these 9 analogs, selectivity at M3 over M2 mAChRs was determined in consideration of their potential efficacy in treating COPD. Of note, compound **13b** exhibited 17-fold selectivity for M3 over M2 mAChRs. Selectivity between M3 and M2 subtypes is important because antagonists at M3 mAChRs decrease airway smooth muscle contraction and decrease mucus secretion, which would be beneficial in the treatment of COPD; whereas, antagonism at M2 mAChRs increases acetylcholine release from parasympathetic nerves innervating the airway smooth muscle and submucosal glands, ultimately stimulating M3 mAChRs and counteracting the beneficial M3 antagonism produced by therapeutic agents targeting M3 (Gosens et al., 2006; Buels and Fryer, 2012). Using the [^{3}H]NMS binding assay, several compounds have been reported previously to be highly potent (low nM range) and selective for M3 over M2 mAChRs (Dowling and Charlton, 2006). Specifically, (2R)-1-((2S,4R)-4-hydroxy-1-[3,3,3-tris(4-fluoro-phenyl)propanoyl]-pyrrolidine-2-yl)carbonyl-N-(4-piperidinylmethyl)pyrolidine-2-carboxamide (compound **14A**) and (2R)-N-[1-(6-aminopyridin-2-ylmethyl)piperidin-4-yl]-2-[(1R)-3,3-difluorocyclopentyl]-2-hydroxy-2-phenylacetamide (compound **A**) exhibit 1600- and 193-fold selectivity, respectively, for M3 over M2 (Sagara et al., 2006; Sykes et al., 2012). Compound **14A** and **A** act as M3 mAChR antagonists as indicated by inhibition of carbachol-induced contraction of the isolated rat tracheal muscle (Sagara et al., 2006; Sykes et al., 2012). Interestingly, tiotropium and umeclidinium, exhibit only 2 to 3-fold selectivity at M3 relative to M2 mAChRs, as indicated from [^{3}H]NMS binding assays (Salmon et al., 1999; Dowling and Charlton, 2006). Despite, marginal selectivity for M3, tiotropium and umeclidinium have kinetic selectivity for M3 and long dissociation half-life (Salmon et al., 1999). Moreover, both compounds are FDA-approved COPD inhalation

therapeutics. Thus, the current findings that 3b has high affinity (Ki = 7.6 nM) and 17-fold selectivity for M3 over M2 mAChRs suggest that this compound may have beneficial therapeutic effects over currently available medications for COPD.

5.4 Conclusion

Through the synthesis and evaluation of two series of carbamates, **13c** was identified as the most potent (Ki =1.8 nM) analog interacting at M5 mAChRs. Although **13c** exhibited 1,200-fold higher affinity for M5 mAChRs compared to compound **11**, **13c** lacked selectivity for M5 mAChRs. Interestingly, compound **11** inhibited oxotremorine-evoked DA release from rat striatal slices, whereas **13c** augmented oxotremorine-evoked DA release, and moreover, itself increased DA release, indicating that **13c** acts as a mAChR agonist. Future structure activity relationship studies using **13c** as the lead compound will be needed to identify analogs with high affinity and selectivity for M5. Additionally, **13b** exhibited high affinity and selectivity for M3 over M2 mAChRs and may have potential to be developed as a COPD treatment.

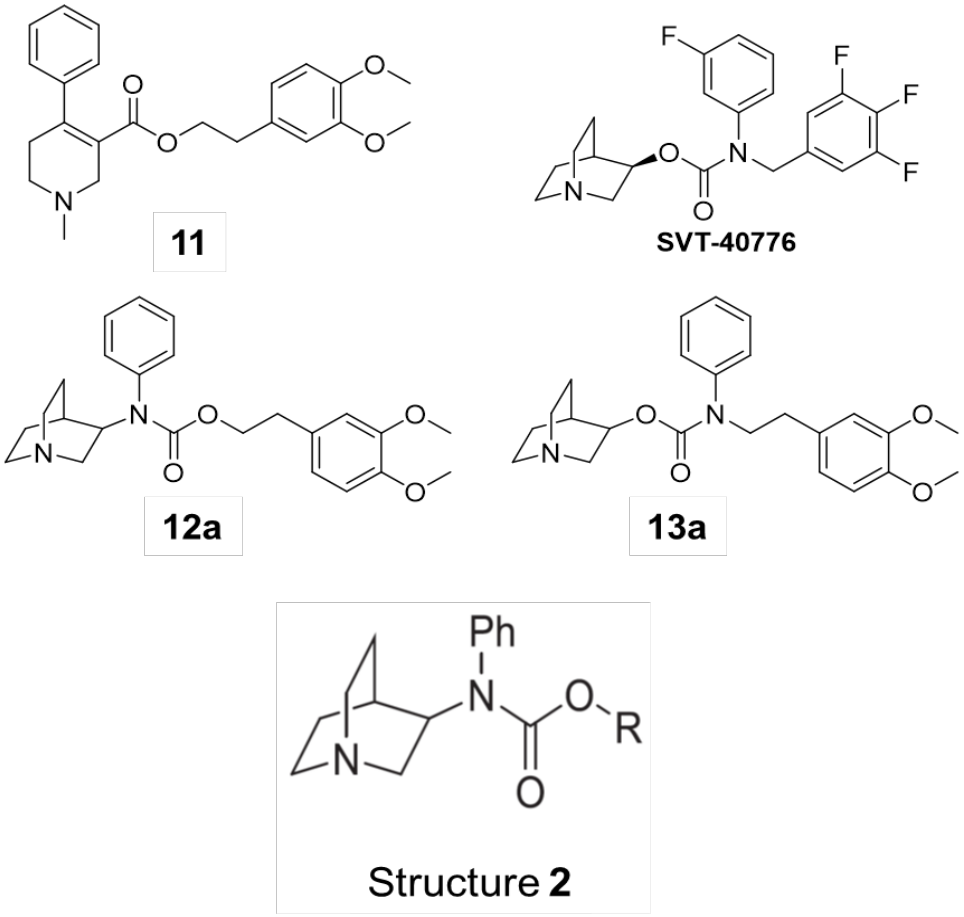
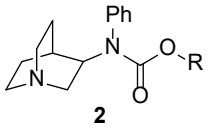
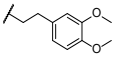
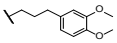
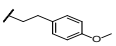
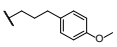
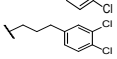
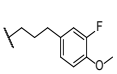
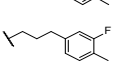
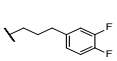


Figure 5.1. Structures of compound 11, SVT-40776, 12a, 13a, and structure 2.

Table 2. Structures and binding affinity at M1, M2, M3, and M5 mAChRs for atropine and analogs with general structure **2**[a]

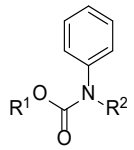


2

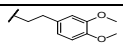
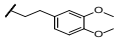
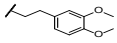
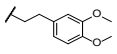
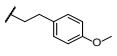
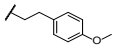
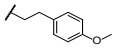
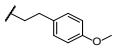
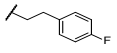
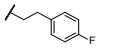
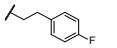
Compd	R	[^{3}H]NMS binding Ki ± SEM (μM) (Imax ± SEM, % inhibition)				Selectivity	
		M1	M2	M3	M5	M1/M5	M2/M3
Atropine	-	0.00044 ± 0.0001[a] (99.5 ± 0.28)	0.0009 ± 0.00005 (99.4 ± 0.74)	0.00053 ± 0.00006 (99.3 ± 0.63)	0.0006 ± 0.00003 (96.7 ± 0.50)	1.7	0.7
11[b]	-	25.3	>100	>100	2.24	11	-
12a		6.98 ± 0.65 (90.6 ± 0.61)	ND[c]	2.76 ± 0.23 (85.9 ± 1.55)[d]	3.49 ± 0.09 (94.0 ± 0.63)	2.0	-
12b		0.77 ± 0.03 (96.8 ± 0.24)	ND	1.02 ± 0.11 (95.4 ± 0.45)	0.55 ± 0.02 (98.7 ± 0.41)	1.4	-
12c		1.46 ± 0.14 (97.1 ± 0.33)	ND	1.56 ± 0.22 (96.3 ± 0.58)	1.61 ± 0.09 (99.0 ± 1.9)	0.9	-
12d		0.19 ± 0.02 (99.5 ± 0.15)	0.12 ± 0.01 (99.4 ± 0.28)	0.09 ± 0.004 (98.6 ± 0.42)	0.15 ± 0.03 (97.2 ± 0.24)	1.3	1.2
12e		0.79 ± 0.08 (88.5 ± 1.37)	ND	0.30 ± 0.01 (99.1 ± 0.504)	0.26 ± 0.03 (96.9 ± 4.4)	3.0	-
12f		0.78 ± 0.05 (99.1 ± 0.28)	ND	0.55 ± 0.03 (98.9 ± 0.42)	0.26 ± 0.008 (95.7 ± 0.17)	3.0	-
12g		0.71 ± 0.06 (97.9 ± 0.21)	ND	1.15 ± 0.06 (95.7 ± 0.52)	0.47 ± 0.05 (97.1 ± 0.89)	1.5	-
12h		0.76 ± 0.02 (98.4 ± 0.08)	ND	0.42 ± 0.05 (99.3 ± 0.22)	0.23 ± 0.005 (98.9 ± 0.09)	3.3	-
12i		0.59 ± 0.02 (98.5 ± 0.19)	ND	0.39 ± 0.04 (96.8 ± 1.25)	0.64 ± 0.10 (99.5 ± 3.5)	0.9	-
12j		0.03 ± 0.003 (100 ± 0.18)	0.03 ± 0.005 (99.4 ± 0.87)	0.019 ± 0.002 (99.9 ± 0.72)	0.04 ± 0.003 (99.7 ± 0.37)	0.8	1.7
12k		0.63 ± 0.10 (99.6 ± 0.40)	ND	0.29 ± 0.03 (98.0 ± 0.53)	0.24 ± 0.06 (97.1 ± 4.8)	2.6	-
12l		0.06 ± 0.002 (100 ± 0.24)	0.22 ± 0.009 (97.5 ± 1.76)	0.07 ± 0.009 (95.8 ± 0.78)	0.06 ± 0.001 (98.6 ± 4.75)	0.9	3.3
12m		0.37 ± 0.02 (99.9 ± 0.36)	ND	0.26 ± 0.02 (99.8 ± 0.27)	0.08 ± 0.02 (98.8 ± 0.30)	4.5	-
12n		0.17 ± 0.01 (100 ± 0.10)	0.30 ± 0.02 (99.9 ± 0.063)	0.06 ± 0.003 (100 ± 0.25)	0.13 ± 0.003 (99.4 ± 0.30)	1.3	4.7
12o		0.07 ± 0.003 (99.5 ± 0.99)	0.10 ± 0.01 (98.8 ± 0.64)	0.05 ± 0.003 (100 ± 0.022)	0.04 ± 0.01 (96.7 ± 0.50)	1.5	1.8
12p		0.43 ± 0.03 (100 ± 0.14)	ND	0.25 ± 0.03 (99.8 ± 1.08)	0.26 ± 0.004 (99.4 ± 0.22)	1.7	
12q		0.04 ± 0.001 (100 ± 0.14)	0.03 ± 0.001 (99.7 ± 0.60)	0.02 ± 0.0003 (99.0 ± 0.56)	0.03 ± 0.003 (97.4 ± 0.13)	1.2	1.3
12r		0.20 ± 0.004 (100 ± 0.25)	ND	0.13 ± 0.009 (100 ± 0.29)	0.13 ± 0.004 (99.9 ± 0.45)	1.5	
12s		0.30 ± 0.02 (100 ± 0.25)	ND	0.22 ± 0.008 (99.5 ± 1.09)	0.10 ± 0.003 (99.4 ± 0.30)	3.0	-

[a] Three independent experiments, each with duplicate samples, were performed to obtain Ki values (Mean ± SEM)
[b] Data from Zheng et al., 2013
[c] Not determined
[d] No plateau for the mean inhibition curve was obtained; Imax % inhibition at 100 μM analogs was reported

Table 3. Structures and binding affinity at M1, M2, M3, M4, and M5 mAChRs for analogs **13-16**[a]



3-6

Compd	R^1	R^2	[^{3}H]NMS binding Ki ± SEM, μM (Imax ± SEM, % inhibition)					Selectivity	
			M1	M2	M3	M4	M5	M1/M5	M2/M3
13a			0.095 ± 0.0037 (100 ± 0.14)	0.35 ± 0.02 (98.6 ± 1.57)	0.06 ± 0.006 (99.7 ± 0.91)	ND[b]	0.065 ± 0.002 (99.8 ± 0.56)	1.5	5.7
14a			4.30 ± 0.49 (94.3 ± 0.61)	ND	4.36 ± 0.90 (85.6 ± 0.85)[c]	ND	4.49 ± 0.32 (89.2 ± 0.87)	1.0	
15a			0.77 ± 0.082 (99.0 ± 0.16)	ND	1.28 ± 0.10 (96.6 ± 0.58)	ND	0.38 ± 0.03 (96.5 ± 2.81)	2.0	
16a			1.87 ± 0.17 (95.3 ± 0.25)	ND	2.80 ± 0.03 (88.8 ± 0.49)[c]	ND	1.37 ± 0.07 (95.9 ± 0.91)	1.4	
13b			0.01 ± 0.0003 (100 ± 0.16)	0.13 ± 0.025 (99.7 ± 0.48)	0.008 ± 0.0003 (99.9 ± 0.41)	0.025 ± 0.0002 (99.9 ± 0.37)	0.01 ± 0.0005 (100 ± 0.68)	1.0	17
14b			0.69 ± 0.092 (98.6 ± 0.36)	ND	1.32 ± 0.069 (96.2 ± 0.28)	ND	1.05 ± 0.02 (97.7 ± 0.45)	0.7	-
15b			0.28 ± 0.038 (99.8 ± 0.33)		0.57 ± 0.092 (99.7 ± 0.43)	ND	0.19 ± 0.01 (99.7 ± 0.82)	1.5	-
16b			0.35 ± 0.02 (99.7 ± 0.05)	ND	0.27 ± 0.01 (98.9 ± 0.35)	ND	0.24 ± 0.02 (97.9 ± 2.0)	1.5	-
13c			0.002 ± 0.0001 (99.9 ± 0.11)	0.013 ± 0.003 (100 ± 0.71)	0.0026 ±0.0001 (100 ± 1.39)	0.0022 ± 0.0001 (99.8 ± 0.33)	0.0018 ± 0.0001 (99.8 ± 0.71)	1.1	5.0
14c			0.12 ± 0.006 (99.9 ± 0.07)	ND	0.35 ± 0.05 (99 ± 1.37)	ND	0.15 ± 0.01 (99.9 ± 1.64)	0.8	-
15c			0.07 ± 0.002 (100 ± 0.14)	ND	0.14 ± 0.019 (100 ± 0.50)	ND	0.06 ± 0.002 (100 ± 0.35)	1.2	-

[a] Three independent experiments, each with duplicate samples, were performed to obtain Ki values (Mean ± SEM)
[b] Not determined
[c] No plateau for the mean inhibition curve was obtained; Imax % inhibition at 100 μM analogs was reported

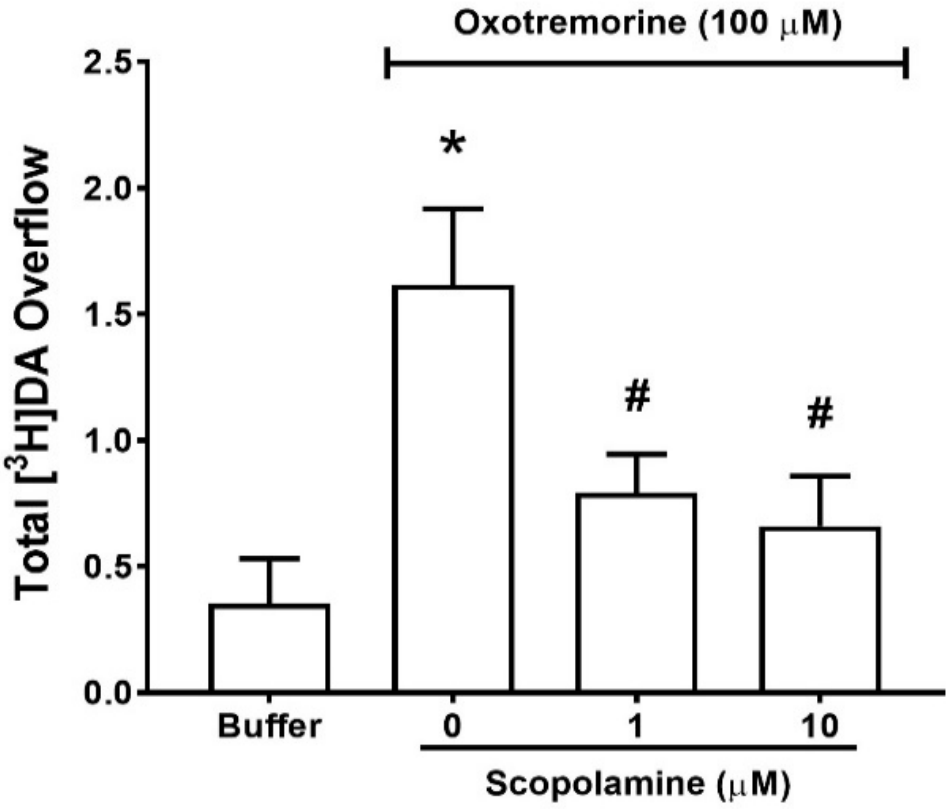


Figure 5.2. Scopolamine (1 and 10 μM, positive control) inhibits oxotremorine (100 μM)-evoked [^{3}H]DA overflow from superfused rat striatal slices. Data are presented as mean ± SEM. *p < 0.05 compared to buffer; #p < 0.05 compared to oxotremorine alone (scopolamine 0 μM), n=10.

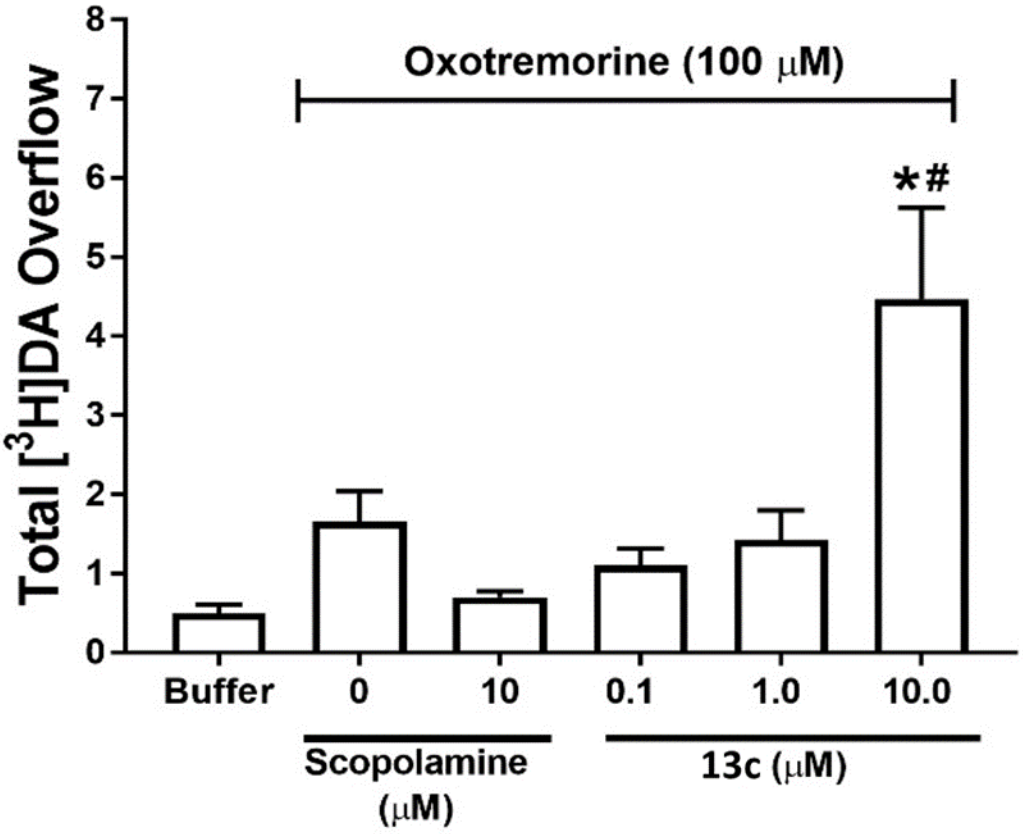


Figure 5.3. Compound 13c increases oxotremorine (100 μM)-evoked [^{3}H]DA overflow from rat striatal slices. A range of concentrations (0.1, 1, and 10 μM) of 13c were evaluated. Scopolamine (10 μM) was used as the positive control. * $p < 0.05$ compared to buffer, # $p < 0.05$ compared to oxotremorine alone (scopolamine 0 μM), n=4-10.

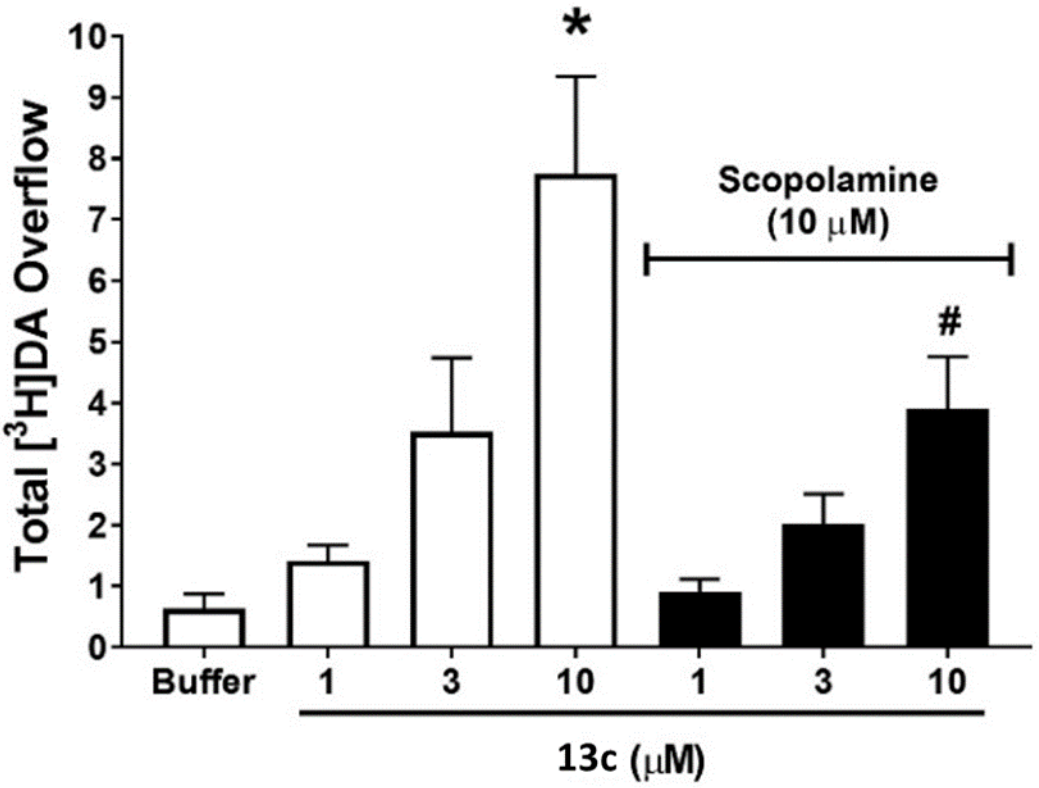


Figure 5. 4. Scopolamine (10 μM) inhibits 13c-evoked [^{3}H]DA overflow from superfused rat striatal slices. * $p < 0.05$ compared to buffer (in the absence of **13c**), # $p < 0.05$ compared to **13c** (10 μM) in the absence of scopolamine, n=6.

6. CHAPTER SIX: OVERALL DISCUSSION

6.1 Review

Substance use disorders are a growing health concern in the US. In 2016, there were 28.6 million Americans ages 12 or older reporting, within the past month, illegal substance use and prescription drug misuse including opioids, and psychostimulants (i.e., cocaine and METH), which has increased compared to 19.9 million in 2007 (SAMHSA, 2017; CDC, 2018). One out of ten (10.6%) Americans (ages 12 or older) used illegal substances in 2016. Among ED visits, the number of individuals who were diagnosed with substance-related disorders increased by 73.7% between 2006 and 2014 (Moore et al., 2017). Also, in 2016, the number of nationwide substance overdose-induced deaths was the highest number on record, and was increased by 86.1% and 21% compared to 2006 and 2015, respectively (UNODC, 2017, 2018). Especially, the number of opioid-induced (excluding methadone) deaths was doubled in 2016 compared to 2015, indicating a recent rapid growth in the opioids epidemic in the US (UNODC, 2018). The second most popular substance among the overdose-induced deaths following opioids was cocaine-involved overdose deaths in 2016 (DEA, 2018). In addition, in 2016, the number of cocaine-induced overdose deaths was doubled compared to 2014 (5,415 to 10, 375) (DEA, 2018). The third highest population was psychostimulants-involved overdose deaths. Importantly, 80% of psychostimulants-involved overdose deaths were due to METH. Reports commonly indicate rapid growth in illegal substance use and prescribed substance misuse in the US. The rapid increase in ED visits related to substances and the number of overdose-induced deaths reflect the severity of the substance use-induced health concerns in the US.

Moreover, according to the report from the UNODC in 2018, the two second most popular substances used worldwide by populations 15-64 years old were amphetamines,

including METH (34 million past-year users) and opioids (34 million past-year users); the most popular being cannabis (192 million past-year users). Cocaine (18 million past-year users) was the fourth most popular substance worldwide (UNODC, 2018). Also, based on the number of countries reporting substance-related seizures during 2012 and 2016, cocaine (146 countries) was the second most popular substance following cannabis (151 countries), followed by opioids (139 countries) and amphetamines, including METH (131 countries) (UNODC, 2018). Worldwide, there were 31 million people who were diagnosed as having substance use disorders in 2016, indicating the high need of pharmacotherapeutics for substance use disorders (UNODC, 2018). However, there are no pharmacotherapeutics that have been approved by FDA for cocaine or METH use disorders. For opioids use disorders, there are FDA-approved medications including methadone, buprenorphine, and naltrexone. However, currently in the US and worldwide, the opioid epidemic is rapidly growing, with a large population of individuals using opioids. Thereby, discovering novel pharmacotherapeutics for opioid use disorders may help to accelerate the fight to combat the opioids epidemic.

To explain the high abuse liability of substances such as METH, cocaine, and opioids, there are three steps of underlying neurobehavioral mechanisms. Initially, these substances activate a rewarding process in the brain by increasing extracellular DA concentration in the NA (Koob and Volkow, 2016; Volkow et al., 2016). The increased amount and duration of extracellular DA after substance administration is greater and longer, respectively, than natural rewards (i.e., food)-induced increases in DA in the NA, resulting in a stronger motivation for substance intake (Di Chiara and Imperato, 1988a). The strong motivation for substance intake leads to repeated substance use. The repeated activation of the reward circuitry following substance administration strengthens the association between cues that lead to the anticipation of substance intake and substance-

induced reward. Also, the repeated substance-induced rewarding effects result in neuronal adaptations, which induces a transition from voluntary to compulsive substance seeking and taking (Black et al., 2010; Volkow and Morales, 2015; Yager et al., 2015). Since repeated substance self-administration behaviors result in long-lasting neuronal adaptation, the relapse rate of substance use behavior is high (40-60%) (McLellan et al., 2000).

As indicated above, there are no FDA-approved pharmacotherapeutics for METH and cocaine use disorders, as there are for opioid use disorders. According to clinical data from opioid use disorders, patients who received pharmacotherapeutics (i.e., buprenorphine, methadone, naltrexone) showed a longer duration of opioid-abstinence and higher adherence to a therapeutic program than the placebo group (Kakko et al., 2003; Mattick et al., 2014; Nunes et al., 2015). Thus, multiple clinical findings in opioid use disorders support the importance of pharmacotherapeutics for substance use disorders.

Importantly, substance use disorders show high relapse rate (40-60%) (McLellan et al., 2000). Accordingly, PET brain imaging studies in humans and monkeys proved that repeated substance use results in long-lasting neuronal adaptations after substance abstinence periods (i.e., 3-4 months in human and 1.5 years in monkeys) (Volkow et al., 1992; Letchworth et al., 2001). These observations indicate that substance use disorders require long-term follow-up care, and that pharmacotherapeutics are vital for substance use disorder treatment. Thus, this dissertation is focused on developing pharmacotherapeutics for METH (Project 1 in Chapters 2 and 3), cocaine, and opioid (Project 2 in Chapters 4 and 5) use disorders via classical drug discovery approaches using novel lead scaffolds.

6.2 Project 2 Review: Discovery Pharmacotherapeutics for Cocaine and Opioid Use Disorders

The second aim of this dissertation is to discover selective and potent antagonists of M5 mAChRs as pharmacotherapeutics to treat cocaine and opioid use disorders. Currently, opioids with METH are the second most popular substance in the world, followed by cocaine (UNODC, United Nations Office on Drugs and Crime, 2018). However, there are no pharmacotherapeutics for cocaine use disorders. Also, recently, a rapid increase has been observed in the opioid epidemic, indicating an increasing need for treatments for this population. Thus, additional novel therapeutics would provide further options to combat the opioid epidemic. Moreover, co-use of cocaine and opioids are often reported. Perhaps novel therapeutics could decrease the use of both substances, which would more efficiently rescue patients from substance use disorders (NIDA, National Institute on Drug Abuse, 2015).

6.2.1 M5 mAChRs Antagonists as Pharmacotherapeutics for Cocaine and Opioids Use Disorders

Multiple substances Including cocaine and opioids exhibit high abuse liability due to their rewarding effects as a result of increasing extracellular DA in the NA (Di Chiara and Imperato, 1988a; Weiss, Paulus, et al., 1992; Basile et al., 2002). Cocaine, a DAT inhibitor, inhibits DA re-uptake from synaptic cleft into cytosol, which results in high extracellular concentration of DA that then interacts with DA receptors expressed on post-synaptic membranes, stimulating downstream signaling (Di Chiara and Imperato, 1988a; Weiss, Paulus, et al., 1992). Opioids, including morphine and heroin, increase extracellular DA in the NA by downregulating GABAergic neuronal activation (an inhibitory

pathway including VTA DA neurons) through binding at MOR expressed on GABAergic neuronal membranes innervating VTA (Gysling and Wang, 1983; Leite-Morris et al., 2004). Moreover, lesion of DA neurons in NA by bilaterally microinfusing neurotoxins, 6-OHDA or kainic acid, resulted in decreased cocaine and heroin self-administration (Zito et al., 1985). Altogether, these findings indicate the crucial role of DA in neurons projecting into NA in the expression of self-administration behavior for opioids and cocaine.

Interestingly, a high density of expression of M5 receptors, a subtype of mAChRs, was found on DA containing neurons in VTA, but rarely in other brain regions or peripheral nerve systems (Yasuda et al., 1993; Lein et al., 2007; Yeomans, 2012). In contrast to most of mAChRs subtypes (M1-M4) being expressed on cholinergic neurons, M5 is expressed on postsynaptic DA neurons in VTA (Weiner et al., 1990). M5 mAChRs modulate DA projection from VTA to NA (Weiner et al., 1990). M5 is a GPCR coupled receptor that signals through Gq protein. Thus, activation of M5 increases intracellular calcium ion concentrations in DA VTA neurons that project to NA (Felder, 1995; Forster and Blaha, 2000; Forster et al., 2002). M5 KO mice showed decreased cocaine-induced hyperlocomotion, preference for cocaine-paired place in CPP, and cocaine self-administration compared to wild-type mice (Fink-Jensen et al., 2003; Thomsen et al., 2005). Also, morphine-evoked DA release in the NA and hyperlocomotion, and preference for morphine-paired place in CPP was decreased in M5 KO mice compared to wild-type mice (Basile et al., 2002; Steidl and Yeomans, 2009).

In addition to the studies using genetic approaches, pharmacological approaches also provide information emphasizing the crucial role of M5 mAChRs in the action of cocaine and morphine. Due to regional localization of M5 expression in the VTA, microinfusion of mAChR antagonists, including scopolamine and atropine, into the VTA allows quasi-selective inhibition of M5. Unilateral microinfusion of scopolamine into the

VTA abolished morphine-induced increase in extracellular DA in the NA (Steidl et al., 2011). Also, atropine, but not the mecamylamine (a nAChR antagonist), decreased morphine-induced hyperlocomotion (Steidl and Yeomans, 2009). Both scopolamine and mecamylamine significantly decreased cocaine-evoked extracellular DA increases in NA in rats never trained for cocaine self-administration (cocaine naïve rats), and cocaine-seeking behavior following 3 days of cocaine extinction in cocaine self-administering rats (Solecki et al., 2013). Thus, M5 mAChRs highly expressed in VTA DA neurons serve crucial roles in opioid- and cocaine-induced rewarding and reinforcing effects.

An M5 selective antagonist (compound **1**) containing 1,2,5,6,-tetrahydropyridine-3-carboxylic acid scaffold, exhibited 11-fold selectivity for M5 over M1 mAChRs: however, affinity of compound **1** for M5 mAChRs was modest (Ki = 2.24 μM) (Zheng et al., 2013). Thus, this dissertation (Chapters 4 and 5) conducted further SAR studies to test the hypothesis that novel M5 selective antagonists would provide pharmacotherapeutics for opioids and cocaine use disorders.

6.2.2 Affinity and Selectivity of Pethidine Analogs for M5 mAChRs over M1 and M3 mAChRs

SAR studies on compound **1** were conducted with the aim of improving affinity and selectivity of analogs for M5 mAChRs over M1-M4 mAChRs. Repositioning of carboxylate group in compound **1** resulted in a series of pethidine analogs. The repositioning of the carboxylate group of compound **1** from C-3 to C-4 of the piperidine ring resulted in pethidine, which exhibited antagonistic properties at mAChRs (Hustveit and Setekleiv, 1993). Also, removing the meta-methoxy group of compound **1** resulted in a 75-fold increase in binding affinity for M5 mAChRs (Zheng et al., 2013). Initially, the carboxylate

group of compound **1** was repositioned from C-3 to C-4 of the piperidine ring to generate the pethidine scaffold. Subsequently, esters (analogs **4** and **6** series), amides (**5** and **7** series), carbamates (**8** and **9** series), or carbamides (**10** series) were added to the pethidine scaffold. Additionally, each analog series contained a phenyl ring with meta- and para-methoxy groups (analog **a** series; di-methoxy substitution in Table 1), and para-methoxy group (analog **b** series; mono-methoxy substitution in Table 1). Affinities of analogs for mAChRs were evaluated to generate SARs. As a first approach, the affinity of analogs for M1, M3, and M5 mAChRs were determined because M1 and M3 were the top two subtypes exhibiting the highest amino acid homology (79% and 85%, respectively) with M5 mAChRs (Bonner et al., 1988). [^{3}H]NMS binding assays revealed that ester-containing pethidine analogs (**4** and **6**) exhibited the highest affinity for M1, M3, and M5 mAChRs. Replacing ester (**4a**/**b** and **6a**/**b**) to amide (**5a**/**b** and **7a**/**b**, respectively) reduced affinity at M1, M3, and M5 (2- to 7-fold). Analogs containing carbamate (**8** and **9**) and carbamide (**10**) exhibited lower affinity for M1, M3, and M5 up to 8-fold compared to ester analogs (**4** and **6**). In addition, reversed ester in **6a**/**b** exhibited higher affinity at M1, M3, and M5 mAChRs compared to **4a**/**b**. Also, in agreement with the previously published literature (Zheng et al., 2013), the analogs containing the mono-methoxy substitution (**4b**, **5b**, **6b**, **7b**, and **10b**) exhibited higher affinity for M5 (2- to 8-fold), M1 (2- to 9-fold), and M3 (3- to 19-fold) in comparison with their corresponding di-methoxy substitution contained analogs. In summary, the pethidine scaffold exhibited up to a 6-fold greater affinity for M5 mAChRs compared to compound **1**, but low selectivity for M5 over M1 or M3. Among the pethidine analogs, compound **6b** showed the highest affinity at M1, M3, and M5 mAChRs (Ki = 0.67, 0.35, and 0.38 μM, respectively) with low selectivity for M5 over M1 and M3 mAChRs. Based on the current findings, further SAR is required to improve selectivity for M5 over M1-M4 mAChRs. Also, due to the pharmacological property of pethidine, it exhibited a weak MOR agonistic activity (Bryant et al., 2010). Thus,

when the pethidine structure-based analogs exhibit high affinity for M5 mAChRs, selectivity for M5 over MOR needs to be evaluated.

6.2.3 Affinity and Selectivity of Quinuclidinyl Carbamate Analogs for M5 mAChRs over M1-M4 mAChRs

SAR studies using another approach employing a methyl phenylcarbamate scaffold were conducted with the goal of improving affinity and selectivity of compound **1** for M5 over M1-M4 mAChRs. According to previous reports, quinuclidinyl carbamate-containing analogs including SVT-40776 exhibited mAChRs antagonism with high affinity (Prat et al., 2011). Thus, a new scaffold containing a 3,4-dimethoxyphenethyl group as in compound **1** and a quinuclidinyl carbamate was employed at the methyl-1-methyl-4-phenyl-1,2,5,6-tetrahydro-3-pyridinecarboxylate position to the structure of compound **1**. Initially compound **12a** and its analogs were synthesized (Table 2). Then, reorientation of carbamate structure in **12a** generated compound **13a** and its analogs (Table 3). The affinity and selectivity of analogs of quinuclidinyl carbamate analogs (compound **12a**-**16b**) were evaluated using [^{3}H]NMS binding assay. Also, a functional assay was conducted using the analog that exhibited the highest affinity at mAChRs among the quinuclidinyl carbamate scaffold analogs to determine its action as an antagonist at mAChRs. Among analogs evaluated, **12j**/**m**/**q**, **13a**/**b**/**c**, and **15c** exhibited high affinity (Ki<100 nM) for M5. However, the highest selectivity for M5 over M1 in the quinuclidinyl carbamate analogs was 4.5-fold (compound **12m**). The analog exhibiting the highest affinity for M5 was **13c** (Ki = 1.8 nM).

6.2.4 Effect of the Most Potent Quinuclidinyl Carbamate Analog at M5 mAChRs on Functional Assay Using Rat Striatal Slices

To further determine if **13c** acted as an antagonist at mAChRs, a functional assay, [^{3}H]DA release using rat striatal slices was conducted. Unexpectedly, **13c** did not inhibit oxotremorine (an agonist of mAChRs)-evoked DA overflow. Further, scopolamine (an antagonist of mAChRs) inhibited **13c**-evoked DA overflow, indicating an agonist action of **13c** on mAChRs. In contrast to VTA containing predominant M5 mAChRs expression, genetic modification studies using mice revealed that M3, M4, and M5 mAChRs mediate oxotremorine-evoked DA overflow from striatal slices (Zhang et al., 2002; Yeomans, 2012). With the assumption that mAChRs modulation of DA release is similar in rats and mice, **13c** appears to evoke DA release from rat striatal slices either through agonist action at M4 and/or M5 mAChRs, or through antagonist action at M3.

Thus, through evaluation of quinuclidinyl carbamate scaffold containing analogs, **13c** was identified as the most potent (Ki = 1.8 nM) analog interacting with M5 mAChRs. Compound **13c** achieved 1200-fold greater affinity for M5 compared to compound **1**: however, **13c** lacked selectivity for M5 mAChRs over M1-M4 mAChRs. Also, compound **1** inhibited oxotremorine-evoked DA release from striatal slices, and **13c** augmented oxotremorine-evoked DA release, which was inhibited by scopolamine, indicating an agonistic action of **13c** on mAChRs.

6.3 Beneficial of M5 mAChRs Selective Antagonists as Therapeutics for Cocaine and Opioid Use Disorders

Expression of M5 mAChRs is highly concentrated in the brain, specifically on VTA DA neurons, indicating low side effects of M5 specific antagonist. Additionally, genetic modification studies and pharmacological approaches showed M5 mAChRs as a strong therapeutic target for cocaine and opioid use disorders, especially since there is rapid increase on co-use of cocaine and synthetic opioids including fentanyl. Between 2015 and 2016, there was a 300% increase in fentanyl seizures also containing cocaine, known as a "speedball" (DEA, 2018). Thus, targeting M5 mAChRs mediating multiple substance-induced rewarding effects may be an effective treatment approach, which would be beneficial for multi-substance users, including cocaine and opioid co-users. However, mAChRs contain high amino acids sequence identities among M1-M5 subtypes. Thus, there are alternative approaches to discover each subtype selective antagonists. Notable achievements were reported from research teams uncovering allosteric antagonists of mAChRs instead of orthostatic antagonists.

6.4 Potential Therapeutic Uses of M3 over M2 mAChRs Selective Antagonists

Another interesting finding from the quinuclidinyl carbamate scaffold analogs is that there were 9 analogs (**12d/j/l/n/o/q** and **13a/b/c**) out of 30 analogs that exhibited high affinity (Ki<100 nM) at M3 mAChRs. Among them, **13b** exhibited highest selectivity (17-fold) for M3 over M2 mAChRs. Since antagonism of M3 mAChRs, which is highly expressed in the respiratory system results in contraction of smooth muscles and reduced mucus secretion, while M2 antagonism induces acetylcholine release from parasympathetic nerves. Thus, the selectivity for M3 over M2 is important to be a COPD

medication. However, FDA-approved COPD medications targeting M3 including tiotropium and umeclidinium exhibit only 2 to 3-fold selectivity at M3 over M2 based on [^{3}H]NMS binding assay. Thus, 17-fold selectivity of **13b** for M3 over M2 indicated a potential of **13b** as a COPD medication. Thus, **13b** was identified as an analog exhibiting high affinity and selectivity for M3 over M2 mAChRs, and may have potential to be developed as a COPD treatment.

6.5 Project 1 Review: Discovery Pharmacotherapeutics for METH Use Disorder

The first aim of this dissertation is to discover pharmacotherapeutics that treat METH use disorders by identifying selective and potent inhibitors of VMAT2 (Chapters 2 and 3).

6.5.1 VMAT2 Function is Critical to the Mechanism of Action of METH

Currently, METH is the second most popular misused substance in the world following cannabis (UNODC, 2018). METH is a psychostimulant exhibiting high abuse liability (Anglin et al., 2000; Vearrier et al., 2012). Due to the high lipophilicity (logP = 2.10) of METH, it is able to penetrate the blood-brain barrier and neuronal cell membranes (Gulaboski et al., 2007). Accordingly, in rats, 9.7-fold higher concentrations of METH were found in the brain compared to serum 3 h following METH (i.v.) administration (Riviere et al., 2000). METH enters into the CNS and inhibits DA uptake at DAT by acting as a substrate, indicating that METH can be taken up via DAT (Zaczek et al., 1991). Also, due to the high lipophilicity of METH, it can pass through the plasmalemmal membrane. In the cytosol, METH inhibits MAO activity and inhibits DA uptake at VMAT2, resulting in

increased cytosolic DA concentrations. METH also is able to pass through vesicular membranes within neuronal cells. Once METH enters the vesicles, it interrupts the pH gradient between the inside and outside of the vesicles by its weak base properties (Sulzer and Rayport, 1990). Additionally, in the presence of VMAT2 inhibitors such as dihydrotetrabenazene and lobeline, METH-evoked DA release from human DAT and VMAT2 co-expressing cells and from rat striatal brain slices was decreased, suggesting that METH induces vesicular DA release by interacting with VMAT2 (Wilhelm et al., 2004; Nickell et al., 2010). Consequently, METH inhibits DA uptake at VMAT2 through pharmacological inhibition of its DA uptake function and interrupting pH gradient, the driving force of DA uptake at VMAT2 (Sulzer and Rayport, 1990; Wilhelm et al., 2004). Overall, METH increases the cytosolic DA concentration, which is released via reversing DAT function (Goodwin et al., 2009). Thus, the increased cytosolic DA is released into the extracellular space cleft via reversal of DAT, which contributes to METH-induced rewarding effects. In contrast to cocaine (a DAT inhibitor), METH exhibited reduced DA release in the cells expressing DAT, but not VMAT2, compared to cells co-expressing DAT and VMAT2, indicating a critical contribution of VMAT2 on METH-evoked DA release (Pifl et al., 1995).

6.5.2 VMAT2 Inhibitors as a Pharmacotherapeutic for METH Use Disorders

Lobeline (a natural alkaloid extracted from Lobelia inflata) inhibits VMAT2 function (Ki = 470 nM) and decreased METH-evokes DA release from rat striatal slices (DK Miller et al., 2001; Nickell et al., 2010). Also, lobeline reduced responding for METH i.v. infusion in the rat METH self-administration model (Harrod et al., 2001). Accumulated findings suggested that VMAT2 as a viable therapeutic target to treat METH use disorders

(Dwoskin and Crooks, 2002; Zheng et al., 2006). In accordance with neuropharmacological studies, heterozygotes VMAT2 KO mice exhibiting 60% reduced VMAT2 expression, measured by the [^{3}H]DTBZ binding assay, demonstrated significantly less preference for amphetamine-paired place in the CPP model compared to wild-type control mice. No difference was found for cocaine-paired place in CPP (Takahashi et al., 1997). Certainly, lobeline completed Clinical Trial Phase Ib and was found to be safe in active METH users (Jones, 2007). However, lobeline also interacts with nAChRs, which likely contributes to the presentation of undesired side effects such as nausea during the clinical trials (Cahill et al., 2007; Glover et al., 2010; Swan et al., 2012). Thus, a series of structure-activity relationship (SAR) studies were initiated to discover VMAT2 selective compound as a potential METH use disorder treatment.

Through SAR studies, a lobeline analog containing a de-functionalized linker region was identified. Lobelane was exhibited 10.4-fold greater affinity for VMAT2 (Ki = 45 nM) and greater than 40-fold higher selectivity for VMAT2 over nAChRs compared to lobeline (Miller et al., 2004; Nickell et al., 2010). Lobelane also showed ability to decrease METH-evoked DA release from rat striatal slices, METH-induced hyperlocomotion activity, and METH self-administration (Neugebauer et al., 2007; Nickell et al., 2010). However, tolerance developed to the effect of lobelane on METH self-administration (reducing response for METH) following repeated lobelane administration (Neugebauer et al., 2007).

With further SAR studies, GZ-793A, an analog of lobelane containing a substituted N-1,2-dihydroxypropyl moiety at N-methyl position was discovered and exhibited comparable affinity for VMAT2 (Ki =29 nM) with no affinity for nAChRs over a range of concentrations (up to 30 μM) (Nickell et al., 2017). GZ-793A inhibited METH-evoked DA release from rat striatal slices and from synaptic vesicular preparation. Also, GZ-793A inhibited METH-induced DA release in the NA (Meyer et al., 2013). Finally, GZ-793A

decreased METH self-administration without developing tolerance (Alvers et al., 2012). However, GZ-793A exhibited affinity for hERG channels, indicating the possibility of GZ-793A having cardiotoxicity (Nickell et al., 2017).

Therefore, this dissertation conducted further SAR studies with the aim of eliminating hERG affinity and proposed a new amphetamine-like scaffold as VMAT2 inhibitors and potential pharmacotherapeutics for METH use disorders. Throughout Chapters 2 and 3, two enantiomers in this scaffold inhibiting VMAT2 function were evaluated. The new scaffold provided viable candidates as pharmacotherapeutics for METH use disorder. In the new scaffold, the N-1,2-dihydroxypropyl piperidine in GZ-793A structure was replaced with a phenylalkyl moiety to provide 3-(4-methoxyphenyl)-N-(1-phenylpropan-2-yl)propan-1-amine. This scaffold contains one chiral center. However, racemic mixtures are less likely to be approved by the FDA as medications. Thus, the two enantiomers, (R)- and (S)-enantiomer, of the new scaffold were synthesized separately and evaluated with findings presented in Chapters 2 and 3, respectively.

On the other hand, there are VMAT2 inhibitors, reserpine and tetrabenazine, known as classic VMAT2 inhibitors (Partilla et al., 2006; summary in Table 4). Tetrabenazine and its deuterated form (deutetrabenazine) are FDA-approved pharmacotherapeutics to treat Huntington's disease and tardive dyskinesia associated chorea (Yero and Rey, 2008; DeWitt and Maryanoff, 2018). Tetrabenazine inhibits VMAT2 with high affinity (Ki = 70-97 nM) (Erickson et al., 1996; Meyer et al., 2011). However, tetrabenazine showed no selectivity for VMAT2 over DAT (Table 4), which would predict high abuse liability of tetrabenazine (Seeman and Lee, 1975; Stathis et al., 1995; Meyer et al., 2011). Tetrabenazine (1 mg/kg, s.c.) decreased METH self-administration, but at a lower dose (0.1 mg/kg, s.c.) increased responding for METH (Meyer et al., 2011). Furthermore, tetrabenazine (1 mg/kg, s.c.) decreased food-maintained responding,

indicating non-selective effects of tetrabenazine. Tolerance did not develop to the decrease in responding for food, such that nonselective effects were maintained across repeated administration. Also, tetrabenazine depleted vesicular DA content and exacerbated METH-induced striatal DA depletion (Reches et al., 1983; German et al., 2015). Since METH use disorder has an 87% chance of relapse within 5 years (Brecht and Herbeck, 2014), it might be anticipated that neurotoxicity would be a potential outcome during a METH relapsing event if a patient is being treated by tetrabenazine as a therapeutic.

Another VMAT2 inhibitor, reserpine (Ki = 12 nM) was also approved by the FDA as an antihypertensive (Erickson et al., 1996). However, reserpine also exhibits no selectivity for VMAT2 over DAT (IC_{50} = 10 nM) (Metzger et al., 2002). Also, reserpine depletes striatal DA and exacerbates METH-induced striatal DA depletion, which limits its therapeutic use (Smith, 1956; Macarthur, 1957; Brookhart et al., 1987). Thus, classic VMAT2 inhibitors, tetrabenazine and reserpine, have several characteristics that prevent them from being considered as therapeutics to treat METH use disorder.

6.5.3 VMAT2 Inhibitors Containing a New Amphetamine-like Scaffold as Therapeutics for METH Use Disorder

The (R)-enantiomer, GZ-11610, exhibited high affinity (Ki = 8.7 nM) for VMAT2 with high selectivity (288- to >3450-fold) for VMAT2 over DAT, SERT, nAChRs, and hERG channels. Also, METH-induced hyperlocomotion in METH-sensitized animals was decreased following GZ-11610 (s.c. and oral). Although s.c. GZ-11610 decreased locomotor activity nonspecifically, orally administered GZ-11610 specifically decreased METH-induced hyperlocomotion by 50% of control. The effective dose of oral GZ-11610

was 20-fold higher compared to the s.c. GZ-11610, which suggests low oral bioavailability. Thus, further formulation studies are needed to achieve greater oral bioavailability.

The (S)-enantiomer, GZ-11608 exhibited high affinity (Ki = 25 nM) for VMAT2 with high selectivity (92- to >1180-fold) for VMAT2 over DAT, SERT, nAChRs, and hERG channels. GZ-11608 (s.c. and oral) specifically decreased METH sensitization. Oral GZ-11608 tended to increase locomotor activity in the saline control group in a dose-dependent manner, which may indicate the potential for GZ-11608 to act like a psychostimulant. However, the self-administration study using i.v. GZ-11608 showed that GZ-11608 does not serve as a reinforcer in drug naïve animals. Also, GZ-11608 was not able to substitute for METH. GZ-11608 had low affinity (Ki = 6 μM) for DAT and high selectivity (241-fold) for VMAT2 over DAT. Thus, neurochemical and behavioral studies support a low abuse liability of GZ-11608. The METH sensitization study also suggested that oral GZ-11608 decreased METH sensitization at 30-fold higher dose compared to s.c. GZ-11608, indicating low oral bioavailability of GZ-11608; however, efficacy with s.c. administration was observed.

GZ-11608 and GZ-793A inhibited METH-evoked DA release by competitive and allosteric mechanisms, respectively (Summary Table 4; Nickell et al., 2017). Tetrabenazine competitively inhibited METH-evoked DA release, like GZ-11608, but tetrabenazine showed a biphasic effect on METH self-administration (Meyer et al., 2011). Tetrabenazine increased responding for METH at a low dose (0.1 mg/kg, s.c.) and was not selective (~2-fold) for VMAT2 over DAT (Erickson et al., 1996; Meyer et al., 2011). DAT inhibitory potency is correlated positively with abuse liability (Seeman and Lee, 1975; Stathis et al., 1995). Thus, low doses of tetrabenazine may be reinforcing due to its ability to inhibit DAT. GZ-11608 exhibited high selectivity (241-fold) for VMAT2 over DAT, and GZ-11608 did not show reinforcing effects. The current findings suggest that GZ-11608

and the new amphetamine-like scaffold have different underlying mechanisms to decrease responding for METH, providing advantages of GZ-11608 as a therapeutic for METH use disorder relative to classic VMAT2 inhibitors and previous lead compounds (i.e., lobeline, lobelane, and GZ-793A).

GZ-11608 also has considerable advantages relative to the previous lead compounds (lobeline, lobelane, and GZ-793A) emanating from our iterative drug discovery program. VMAT2 affinity increased across the successive progression from lead to lead, resulting in Ki values in the low nM range, and selectivity for VMAT2 over DAT was consistent across the leads (Horton et al., 2011; Summary Table 4). Furthermore, low cardiotoxicity is expected with GZ-11608 due to the demonstration of greater than 30-fold selectivity for VMAT2 over hERG, which is improved over our previous lead compounds (Nickell et al., 2017; Summary Table 4). Several positive properties of the previous lead compounds (lobeline, lobelane, and GZ-793A) as therapeutics for METH use disorder were retained in the GZ-11608 molecule: 1) GZ-11608 did not exacerbate METH-evoked striatal depletion, which is consistent with earlier molecules (Eyerman and Yamamoto, 2005; Horton et al., 2013) and 2) the efficacy of GZ-11608 to decrease METH self-administration was comparable with previous molecules. Furthermore, greater specificity was observed with GZ-11608 to decrease METH reinforcement relative to previous molecules (Harrod et al., 2001; Neugebauer et al., 2007; Meyer et al., 2011; Beckmann et al., 2012). With respect to the decrease in METH self-administration, tolerance developed to the efficacy produced by lobelane following repeated administration, whereas, repeated GZ-11608, lobeline and GZ-793A, did not result in tolerance (Harrod et al., 2001; Neugebauer et al., 2007; Beckmann et al., 2012). Like lobeline and GZ-793A (Harrod et al., 2003; Beckmann et al., 2012), GZ-11608 exhibited low abuse liability. Importantly, GZ-11608 decreased cue- and METH-induced METH-seeking behavior. GZ-793A blocked

cue-induced reinstatement of METH-seeking, but GZ-793A and lobeline only marginally reduced (30%), and had no effect on METH-induced reinstatement of METH-seeking, respectively. This supports the greater potential of GZ-11608 as a METH use disorder pharmacotherapeutic relative to lobeline and GZ-793A (Harrod et al., 2003; Alvers et al., 2012). Thus, the current preclinical research provides evidence supporting the pharmacotherapeutic advantages of GZ-11608 for METH use disorder relative to previous lead compounds.

GZ-11608 resulted in a downward and rightward shift in the in the METH self-administration dose-response curve, whereas lobeline and GZ-793A resulted in only a downward shift of the dose-response curve (Harrod et al., 2001; Alvers et al., 2012), suggesting different underlying mechanisms in altering behavior. Schild regression on METH-evoked vesicular DA release also revealed different underlying mechanisms between GZ-11608 and GZ-793A, which may have contributed to the different behavioral outcomes (current findings and Horton et al., 2013).

Altogether, the current findings identified GZ-11608 and GZ-11610, constituting a new scaffold that acts as VMAT2 inhibitors. High selectivity of GZ-11608 and GZ-11610 for VMAT2 over nAChRs, DAT, and hERG indicates a low potential for side effects including nausea, abuse liability, and cardiotoxicity. GZ-11610 (s.c.) and GZ-11608 (s.c. and oral) specifically decreased METH sensitization. Importantly, GZ-11608 decreased METH self-administration and cue- and METH-induced reinstatement of METH-seeking behavior without altering food-maintained response and without having abuse liability. Also, tolerance did not develop to the efficacy of GZ-11608 to decrease METH self-administration following repeated GZ-11608 administration. Increasing the unit dose of METH was not able to surmount the ability of GZ-11608 to decrease response for METH.

Thus, the current study reveals high potential for GZ-11608 as a therapeutic for METH use disorder.

Table 4. Summary of preclinical findings of VMAT2 inhibitors.

VMAT2 inhibitors	Affinity, Ki (μM)					Inhibition of METH-evoked vesicular DA release (Schild slope)	Inhibition of METH self-administration dose-response curve	Effects on METH self-administration (effect on food-maintained response)
	VMAT2	DAT	nAChRs		hERG			
			α4β2	α7				
Reserpine	0.012[a]	0.01[e]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Tetrabenazine	0.07[b]-0.097[a]	0.2[a] (~2-fold)	n.d.	n.d.	n.d.	Competitive[g] (not different from unity)	n.d.	Decrease, no tolerance[b] (decrease, no tolerance)[b]
Lobeline	1.27[c]-0.47[d]	28.2[c]-32[d] (~44-fold)	4.0[f] (~6-fold)	6.3[f] (~9-fold)	0.2[f] (no selec-tivity)	n.d.	Allosteric inhibition[h] (downward shift)	Decrease, no tolerance[h] (decrease, tolerance development)
Lobelane	0.045[d]-0.067[c]	1.1[c]-1.6[d] (~25-fold)	15[f] (~280-fold)	26[f] (~480-fold)	1.4[f] (~26-fold)	n.d.	n.d.	Decrease, tolerance development[j] (no alteration[j])
GZ-793A	0.029[c]	1.4[b] (29-fold)	>30[f] (>1030-fold)	>30[f] (>1030-fold)	0.7[f] (24-fold)	Allosteric[g] (different from unity)	Allosteric inhibition[i] (downward shift)	Decrease, no tolerance[i] (acutely no alteration[i], repeated GZ-793A increase food response[i])
GZ-11610	0.0087[k]	2.51[k] (288-fold)	>30[k] (>3480-fold)	>30[k] (>3480-fold)	9.50[k] (1090-fold)	n.d.	n.d.	n.d.
GZ-11608	0.025[l]	6.1[l] (241-fold)	>30[l] (>1180-fold)	>30[l] (>1180-fold)	4.16[l] (163-fold)	Competitive[l] (not different from unity)	Combination of competitive and allosteric inhibition[l] (downward and rightward shift)	Decrease, no tolerance[l] (acutely no alteration[l], repeated GZ-11608 decrease food[l], and then tolerance develop[l])

n.d., not determined, [a] Erickson et al., 1996, [b] Meyer et al., 2011, [c] Horton et al., 2011, [d] Nickell et al., 2009, [e] Metzger et al., 2002 (IC_{50} value), [f] Nickell et al., 2017, [g] Horton et al., 2013, [h] Harrod et al., 2001, [i] Beckmann et al., 2012, [j] Neugebauer et al., 2007, [k] Lee et al., 2018, [l] Lee et al., 2019 (under revision)

6.6 Discussions on New Amphetamine-like Scaffold VMAT2 Inhibitors

VMAT2 inhibitors containing the new amphetamine-like scaffold include a chiral center in the (R)- and (S)-enantiomers (Chapter 2 and 3,). The effect of each enantiomer was evaluated using neurochemical and behavioral approaches and is discussed in the current section.

6.6.1 Neurochemical Properties of (R)- and (S)-Enantiomers

Two enantiomers exhibited comparable affinity for VMAT2, DAT, SERT, hERG and nAChRs. The (R)-enantiomer, GZ-11610 (Ki = 8.7 nM), exhibited 2.9-fold higher affinity for VMAT2 relative to (S)-enantiomer, GZ-11608 (Ki = 25 nM). Affinity of GZ-11610 and GZ-11608 for DAT (Ki = 2.5 and 2.4 μM, respectively), SERT (Ki = 5.6 and 2.4 μM, respectively), hERG (Ki = 9.5 and 4.2 μM, respectively), and both enantiomers exhibited low affinity (Ki >30 μM) for nAChRs. These data indicate that stereochemistry at the chiral center of amphetamine-like scaffold has a minor impact on interactions of enantiomers with monoamine transporters, hERG channel, and nAChRs.

In comparisons of selectivity of enantiomers for VMAT2 over off-target proteins, effect of stereochemistry of these two enantiomers were notable relative to affinity comparisons. Selectivity of GZ-11610 for VMAT2 over hERG and SERT was 6.7- and 6.9-fold, respectively, higher than GZ-11608. While the selectivity of GZ-11610 for VMAT2 over DAT and nAChRs were comparable with GZ-11608. These results indicate that the (S)-enantiomer, GZ-11608 would exhibit greater potential for cardiotoxicity than GZ-11610 at effective dose for decreasing METH effects, but comparable abuse liability would expected from both enantiomers, based on selectivity for VMAT2 over DAT (Seeman and

Lee, 1975; Abbott et al., 1999; Sanguinetti and Tristani-Firouzi, 2006). Also, low potential of nAChRs-related side effects including nausea (Cahill et al., 2007; Swan et al., 2012) would be expected from both enantiomers. GZ-11608 exhibited 6.9-fold lower selectivity for VMAT2 over SERT relative to GZ-11610, indicating that GZ-11608 may have greater potential than GZ-11610 to inhibit 5-HT uptake at SERT at doses effective at decreasing METH effects via interacting with VMAT2. However, SERT inhibition reduces anxiety, depression, and schizophrenia-related behaviors, which are symptoms often experienced by chronic METH users (~40%) (Heisler et al., 1998; Ramboz et al., 1998; van den Buuse et al., 2011). Furthermore, psychotic symptoms in chronic METH users may contribute to high relapse rate (Glasner-Edwards et al., 2009, 2010; McKetin et al., 2010). Thus, despite the fact that GZ-11608 exhibited 6.7-fold lower selectivity for VMAT2 over hERG than GZ-11610, which may indicate higher cardiotoxic potential of GZ-11608 than GZ-11610, both enantiomer exhibited high affinity and selectivity (92- to 3450-fold) for VMAT2 over DAT, SERT, nAChRs, and hERG. These findings support the potential of both enantiomers as candidates for METH use disorder treatments.

6.6.2 Behavioral Properties of (R)- and (S)-Enantiomers

The (R)-enantiomer, GZ-11610 decreased METH sensitized locomotor activity at lower dose (3 mg/kg, s.c. and 56 mg/kg, oral) than the (S)-enantiomer (10 mg/kg, s.c. and 300 mg/kg, oral). GZ-11610 (s.c.) resulted in a nonspecific decrease on locomotor activity in the saline control group (Lee et al., 2018). Likely, a decrease in locomotor activity could result from sedation or motor impairment (Castagné et al., 2014). Together, the s.c. GZ-11610 effect was not specific for METH sensitization. Thus, there is a need to determine if tolerance develops to repeated s.c. administration of GZ-11610 on METH sensitization

with respect to its nonspecific effects. Perhaps the nonspecific effect of GZ-11610 to decrease locomotion develops tolerance, which would enhance the ability to use the compound as a therapeutic use. Oral administration of GZ-11610 exhibited specificity for decreasing METH sensitized locomotor activity. But, GZ-11610 significantly decreased (by 50% of control) METH sensitization at 56 mg/kg (oral), but 5.4-fold higher dose (300 mg/kg, oral) also decreased 50% of control METH sensitization. These observations suggest GZ-11610 was able to penetrate blood-brain barrier, and achieve sufficient oral bioavailability. However, the maximal effect of oral GZ-11610 was about 50% of control, for which there are several possible reasons, including low solubility decreasing absorption, high metabolism and/or high plasma protein binding (Chung et al., 2004).

In contrast, the (S)-enantiomer, GZ-11608, decreased METH sensitized locomotor activity specifically and in a dose-dependent manner at dose ranges from 10-30 mg/kg (s.c.). Also, oral GZ-11608 specifically decreased METH sensitized locomotor activity. The effective dose of oral GZ-11608 was 300 mg/kg, which is 5.4-fold higher than effective dose of oral GZ-11610, but the efficacy of oral GZ-11608 was higher (60% of control) than oral GZ-11610 (<50%) (Lee et al., 2018). These observations support the potential of GZ-11608 for therapeutics use to treat METH use disorder.

Interestingly, oral GZ-11608 showed the tendency, but not statistically significant, to dose-dependently increase locomotor activity in the saline control group between 56-300 mg/kg. Oral GZ-11608 (300 mg/kg) resulted in locomotor activity (~ 40 meters) during the 45 min session, which is about 20% of METH sensitized locomotor activity. This pattern of an increase in locomotor activity in the saline control group suggests a potential psychostimulant effect of GZ-11608, and taken one step further, might suggest abuse liability. However, SERT inhibitors commonly used to treat depression also increase locomotor activity. Inhibitors of SERT including fluoxetine (10 and 30 mg/kg, i.p.),

escitalopram (0.3-10 mg/kg, i.p.), and sertraline (2.6-10.3 mg/kg), increase locomotor activity in a dose-dependent manner in rodents. However, an inhibitor of SERT and NET, duloxetine (0.3-30 mg/kg, i.p.) and a NET inhibitor, reboxetine (1-30 mg/kg, i.p.) did not alter locomotor activity in rodents (Geyer, 1995; Prinssen et al., 2006). Thus, the tendency of GZ-11608 to increase locomotion may indicate that GZ-11608 has psychostimulant effects and/or GZ-11608 inhibits SERT at high oral doses. However, the GZ-11608 self-administration and substitution studies revealed that GZ-11608 has low abuse liability.

6.6.3 Oral Bioavailability of (R)- and (S)-Enantiomers

Both (R)- and (S)-enantiomers showed 20- to 30-fold higher effective dose with s.c. route than oral administration to decrease METH sensitization, which would indicate low oral bioavailability of enantiomers. However, orally available therapeutics are preferred. Thus, pharmacokinetic studies with an aim of improving oral bioavailability would be essential to determine if GZ-11608 and GZ-11610 are orally bioavailable pharmacotherapeutics for METH use disorder.

There are several research approaches to achieve greater oral bioavailability for compounds exhibiting high potential as therapeutics or for existing medications with low oral bioavailability. For instance, research on nobiletin (2-(3,4-dimethoxyphenyl)-5,6,7,8-tetramethoxychromen-4-one), a compound exhibiting anti-inflammatory effect and rescuing rats from amyloid beta-induced memory impairment, showed 13-fold improvement in oral bioavailability by generating nanosized nobiletin as a high-energy amorphous solid dispersion (using wet-milled technique) (Onoue et al., 2011). Also, 6.5-fold greater bioavailability in agomelatine (an antidepressant approved by European Union in 2009) was reported (Prajapati et al., 2018). Agomelatine exhibited 1% bioavailability

previously, but loading agomelatine into nanostructured lipid carriers resulted in greater bioavailability. In the other research, since 90% of agomelatine was metabolized by a cytochrome P450 1A2, co-administration of fluvoxamine (an inhibitor of cytochrome P450 1A2 and an antidepressant) slowed down the metabolism of agomelatine (Sansone and Sansone, 2011). According to a report of a company, Servier Laboratories Limited, fluvoxamine inhibited metabolism of agomelatine, which 60-fold (range 12 to 412) increased agomelatine exposure (Servier Laboratories Limited, 2018). Also, gabapentin (therapeutic utility in multiple disease such as epilepsy, neuropathic pain, and anxiety disorder) exhibited 17- and 34-fold higher exposure of gabapentin in rats and monkeys, respectively, by using a prodrug form. The prodrug, XP13512 (±)-1-([(α-isobutanoyloxyethoxy)varbonyl]aminomethyl)-1-cyclohexane acetic acid) was designed to be recognized by monocarboxylate transporter type 1 and sodium-dependent multivitamin transporter have responsible for the absorption of small-chain fatty acids and small-chain fatty acids, respectively (Cundy et al., 2004). Thus, the high potential of GZ-11608 as therapeutics for METH use disorder in the current study may be improved further with additional formulation and pharmacokinetic studies in the future.

6.6.4 Alternative Administration Routes for (R)- and (S)-Enantiomers

Data in Chapter 3 support a high potential of GZ-11608 as a therapeutic for METH use disorder with s.c. administration. As alternative approaches, the current section introduces the potential of combination therapy and extended-release injectable suspension. Based on the chemical structure of GZ-11608, it contains multiple moiety preferentially metabolized by P450 hepatic enzymes (Vasanthanathan et al., 2010). Especially, multiple P450 ligands contain similar chemical structures with GZ-11608 and

an antidepressant, fluvoxamine inhibits P450 (Wagner et al., 1994; van Harten, 1995). A case study using combination therapy with fluvoxamine and escitalopram to inhibit metabolism of escitalopram showed improved symptoms of the patient (Tarutani et al., 2016). However, another combination therapy using fluvoxamine and clomipramine administered to patients resulted greatly elevated serum clomipramine levels. Most of patients tolerated pharmacokinetic interactions of the combination therapy, but there were several patients exhibiting intracardiac conductance due to the increased serum clomipramine level (Szegedi et al., 1996). Currently, there are no pharmacotherapeutics for METH use disorder. Since GZ-11608 showed efficacy to reduce METH self-administration and reinstatement of METH-seeking behavior, combination therapy with fluvoxamine may provide an alternative way to improve oral bioavailability of GZ-11608.

Another potential approach would be to use an intramuscular extended-release injectable suspension. A therapeutic for opioids use disorder, naltrexone was initially discovered as orally available therapeutic with once a day or once two or three days administration. However, naltrexone resulted in gastrointestinal disorders in patients, which may contribute to low adherence rate of patients to the treatment program (Ploesser et al., 2010). Indeed, 28% of patients were retained with oral naltrexone treatment based on 13 studies, whereas extended-release injectable naltrexone required once a month injection and showed greater treatment adherence (62% of patients) for 12 months of treatment period (Krupitsky et al., 2013). Also, 51% of the remaining patients achieved 12 months of opioid abstinence, indicating advantages of the extended-release injectable suspension as a therapeutic for substance use disorders. The extended-release intramuscular naltrexone consisted of biodegradable polylactidecoglycolide polymer microspheres and 34% weight/weight naltrexone. The microspheres containing naltrexone were reconstructed in an aqueous suspension immediately before the

intramuscular injection, which would be expected to be released over 28-day (Swainston Harrison et al., 2006). Currently, GZ-11608 has low oral bioavailability. Accumulated data suggest that not only the oral administration, but also the extended-release suspension form of therapeutics showed >50% of treatment adherence rate in opioids use disorders. Thus, the extended-release intramuscular formulation might be an alternative approach for GZ-11608.

6.6.5 Additional Potential for Therapeutic Uses of VMAT2 Selective Inhibitors

There is another potential therapeutic use of VMAT2 selective inhibitors including (R)- and (S)-enantiomers containing the new amphetamine-like scaffold. If GZ-11608 and GZ-11610 can be conjugated with radioactive agent, these can contribute to an improved diagnosis for idiopathic Parkinson's disease. Recent research reported VMAT2 selective compound based brain imaging improved diagnosis in uncertain Parkinsonian syndromes, which led use of appropriate medication, and improved patient outcomes (Arena and Stoessl, 2016; Alexander et al., 2017).

6.6.6 VMAT2 Inhibiter Effects on Other Neurotransmitter Systems

VMAT2 is expressed on the vesicular membrane in neurons expressing DA, NE, and 5-HT, which uptakes DA, NE, and 5-HT from cytosol to vesicles (Eiden and Weihe, 2011). Since DA, NE, and 5-HT interact at the same binding site on the VMAT2 (Erickson and Eiden, 1993; Schütz et al., 1998), compounds that inhibit DA uptake at the VMAT2 may also interact with NE and 5-HT uptake. For instance, reserpine exhibiting slow off-rate from VMAT2, which results in quasi-irreversible inhibition of VMAT2 function,

decreased the uptake of DA at the VMAT2 (Erickson et al., 1996). It also decreased 5-HT uptake according to a cell-based study overexpressing human VMAT2 (Rilstone et al., 2013). Tetrabenazine inhibiting DA uptake at the VMAT2 depleted not only DA, but also 5-HT and NE in the brain (Guay, 2010; Meyer et al., 2011), which allows tetrabenazine to treat several diseases such as Huntington's and Parkinson's disease (Suzuki et al., 2001; Yero and Rey, 2008; German et al., 2015). However, individuals treated with tetrabenazine for their chorea symptoms of Huntington's disease reported depressed mood and suicidal thoughts (Dorsey et al., 2013). Thus, a VMAT2 inhibitor, GZ-11608 inhibiting DA uptake at the VMAT2, may have potential to interrupt NE and 5-HT uptakes at the VMAT2 and alter brain function mediated by NE and 5-HT, such as mood control.

Based on a lobeline study using striatal slides, lobeline-induced DA overflow was observed only at the highest concentration: however, a metabolite of DA, such as dihydroxyphenylacetic acid (DOPAC), overflow was increased in a dose dependent manner (Teng et al., 1997). This observation indicates lobeline induces an increase in cytosolic DA. Though GZ-11608 has potential to increase cytosolic DA, NE, and 5-HT, it may increase cytosolic monoamine neurotransmitter concentration acutely: however, they will be metabolized without significant alteration on their transmission. When VMAT2 expression levels were low, cytosolic DA levels were high, but extracellular DA levels (following activation of the neuron) were low (Lohr et al., 2017). Thus, GZ-11608 potentially decreases DA, NE, and 5-HT mediated functions, such as locomotor activity, mood control, cognition and memory. However, GZ-11608 did not decrease locomotor activity in the control group (Fig. 3.4). Additionally, GZ-11608 did not alter DA content by itself and did not exacerbate METH-induced striatal DA depletion (Fig. 3.5.), which, also supports the claim that GZ-11608 does not have significant effects on the monoamine neurotransmitter system. Sedative studies such as the rotarod test using rotating cylinders

or the righting reflex would provide additional information for GZ-11608 effects on mood disorders. Based on the current study in Chapter 3, GZ-11608 did not alter locomotor activity in the control group, which would indicate that GZ-11608 has low potential to cause depression. It is likely that sedation and compound-induced motor impairment would result in decreased locomotor activity (Castagné et al., 2014). In contrast, tetrabenazine and reserpine deplete neurotransmitters, which are associated often with depression (Guay, 2010; German et al., 2015).

6.6.7 Surmountability

GZ-11608 resulted in a downward and rightward shift in the METH self-administration dose-response curve (Fig. 3.8, Table 4), indicating that GZ-11608 decreased METH self-administration via a combination of competitive and allosteric inhibitory mechanisms. In contrast, lobeline and GZ-793A resulted in only a downward shift at the dose-response curve, indicating an allosteric inhibitory mechanism (Harrod et al., 2001; Alvers et al., 2012). The underlying mechanisms were not identical between GZ-11608 and previous VMAT2 inhibitors, based on this observation (Fig. 3.8). However, the peak of response for METH in the GZ-11608 administered group was lower than the no-treatment group. Since GZ-11608 resulted in not only a rightward shift of METH self-administration dose-response curve (a typical competitive inhibitory mechanism), but it also resulted in a downward shift of the curve. Thus, response for METH in GZ-11608 the treatment group was lower than the control group across the various unit dosage of METH (0.01-0.25 mg/kg, i.v.). If GZ-11608 effects were surmountable, then the peak of response for METH in GZ-11608 administered groups should be comparable with the no-treatment group when a higher unit dose of METH was available: however, this was not found in the

current study. Thus, effects of GZ-11608 to decrease METH self-administration are not surmountable.

6.6.8 GZ-11608 and GZ-11610 as Pharmacotherapeutics for Cocaine and Opioids Use Disorders

In current study, VMAT2 inhibitors have been considered as therapeutics for METH use disorders, but not for other substance use disorders (i.e., cocaine or opioids use disorder). Specifically, in cells expressing VMAT2 and DAT, METH showed increased DA efflux compared to cells expressing only DAT, indicating that VMAT2 serves an important role on METH-evoked DA release (Pifl et al., 1995). Dihydrotetrabenazine (DHTB), a VMAT2 inhibitor, decreased METH-evoked DA efflux from 60% to 20% of preloaded [^{3}H]DA (Wilhelm et al., 2004). Additionally, lobeline and GZ-793A (VMAT2 inhibitors) decreased METH self-administration. Interestingly, the dose of GZ-793A decreasing METH self-administration >90% of vehicle treated control decreased cocaine self-administration by 10% to 20% of control in rats (Beckmann et al., 2012). Also, reserpine was not able to decrease cocaine self-administration in monkeys (Wilson and Schuster, 1974). Additionally, cocaine did not inhibit DHTB binding at the VMAT2 (Hiranita, 2015). Together, previously reported VMAT2 inhibitors showed low potential as a therapeutic for cocaine use disorder.

On the other hand, lobeline showed potential as a pharmacotherapeutic for opioids use disorder by exhibiting affinity at MOR (Miller et al., 2007). Also, due to the respiratory stimulant effects of lobeline, it has been used to treat morphine poisoning (King et al., 1928; Dwoskin and Crooks, 2002). Reserpine, which depletes monoamines, inhibited development of morphine tolerance (Langwinski and Fidecka, 1981). Thus, previously

reported VMAT2 inhibitors have shown potential as therapeutics for opioids overdose poisoning and development of tolerance. However, research has yet to be conducted on the potential of VMAT2 inhibitors as therapeutics for opioid use disorders.

In this respect, GZ-11608 and GZ-11610 show high affinity for VMAT2. GZ-11608 specifically inhibits VMAT2 via a competitive mechanism of action. Tetrabenazine also competitively inhibit VMAT2 function, but it has nonspecific actions, in that it decreased food-maintained responding (Meyer et al., 2011). GZ-11608 administration did not produce similar nonspecific behavioral effects. Importantly, tetrabenzine and reserpine deplete DA content (Kenney and Jankovic, 2006; Guay, 2010). Again, GZ-11608 did not alter DA content; and thus, untoward consequences are not anticipated with this novel compound. Thus, the potential of GZ-11608 and GZ-11610, VMAT2 inhibitors containing a new scaffold, as therapeutics for cocaine and opioids use disorders should be investigated in future studies.

6.7 Limitations

6.7.1 Project 1: Discovery Pharmacotherapeutics for METH Use Disorder

In the current study, effects of GZ-11608 and GZ-11610 on DA, but not NE and 5-HT uptake from cytosol to vesicles via VMAT2 were studied. VMAT2 translocates DA, NE, and 5-HT when expressed in neurons containing DA, NE, and 5-HT (Erickson et al., 1996; Eiden et al., 2004). Although, effects of GZ-11608 and GZ-11610 on NE and 5-HT uptake at VMAT2 were not investigated in current study, DA, NE, and 5-HT interact with VMAT2 at identical binding site and comparable affinity (IC_{50}=1.4, 3.4, and 0.9 µM, respectively; Gasnier et al., 1994; Wimalasena, 2011). Thus, effects of GZ-11608 and GZ-11610 on the

DA system similarly are predicted for the NE and 5-HT neuronal systems. Furthermore, behavioral pharmacological studies in the current dissertation using GZ-11608 and GZ-11610 potentially reflect combined effects of GZ-11608 and GZ-11610 on DA, NE, and 5-HT neuronal systems.

Abuse liability of GZ-11608 was studied using i.v. GZ-11608 self-administration and substitution using rats METH self-administered. However, GZ-11608 oral administration specifically exhibited pattern of increase on locomotor activity. Since s.c. GZ-11608 did not exhibit same pattern on saline control group locomotor activity, metabolite of oral GZ-11608 may have psychostimulant effects. To evaluate the rewarding effect of oral GZ-11608, CPP could have been employed.

Binding site of GZ-11608 at VMAT2 was not studied. Although GZ-11608 inhibits DA uptake at VMAT2, and decreased neurochemical and behavioral METH effect, the binding site for GZ-11608 was not revealed. GZ-11608 exhibits different neurochemical and behavioral effects compared to previous lead compounds. However, the binding site of GZ-11608 at VMAT2 was not studied, which limits understanding of mechanism of action of GZ-11608.

Effect of GZ-11608, a VMAT2 inhibitor, on cognition and memory function were not evaluated. GZ-11608 inhibits DA uptake at the VMAT2, which would ~~may~~ contribute to GZ-11608-induced decreases in responding for METH (i.e., cue- and METH-induced reinstatement of METH seeking behavior) (Lohr et al., 2017). This current study provided limited evidence, but GZ-11608 showed no significant effect on DA content at the dose decreasing responding for METH. This observation would predict that GZ-11608 would have no significant depleting effects on other monoaminergic neurons expressing VMAT2 including NE and 5-HT. However, effects of GZ-11608 on cognition or memory function

have not been evaluated. Thus, further study on the effect of GZ-11608 on cognition and memory could be evaluated by employing the Morris water maze, radial arm maze, multiple choice serial reaction task, and the go/no-go test (Rodriguiz and Wetsel, 2006; Vorhees and Williams, 2014).

6.7.2 Project 2: Discovery Pharmacotherapeutics for Cocaine and Opioid Use Disorders

The [^{3}H]NMS binding assay provides binding affinity of analogs at each subtype of mAChRs, but determination of analogs' effect on function of mAChRs to determine if analogs act as antagonist or agonist was not available for each subtype. CHO cell calcium concentration based colorimetric assay would provide information if analogs are antagonists or agonists. Since antagonists but not agonists can be a therapeutic for substance use disorders, the functional property of the analogs is important. Thus, separate functional analyses were conducted.

M5 mAChRs are highly expressed in the VTA, but slices of striatum were used to evaluate effect of analogs on functional assay. To understand the functional assay data, KO mice studies were used. KO mice studies revealed that M1 or M2 KO mice did not alter oxotremorine-induced DA release from the striatal slices, different effects were found in M3, M4, or M5 KO mice on oxotremorine-induced DA release (Zhang et al., 2002). Thus, observed DA release from rat striatal slices is mediated by M3, M4, and M5 mAChRs. Micro infusion of compound **3c** into VTA may provide more informative data regarding function of compound **3c** on M5 mAChRs specifically. Also, cell-based functional assays would be able to provide the characteristics of compound **3c** for each subtype.

6.8 Future directions

The current dissertation focused on discovering candidate pharmacotherapeutics to treat METH, cocaine, and opioids use disorders. Based on Project 1, a VMAT2 inhibitor, GZ-11608 exhibited high potential as a therapeutic for METH use disorder. However, behavioral studies revealed limited oral bioavailability of GZ-11608. Project 2 revealed informative SAR to discover analogs exhibiting high affinity for M5 mAChRs, but the analogs showed a lack of selectivity for M5 over M1-M4 mAChRs. As such, evaluation of M5 mAChRs antagonists to decrease cocaine and opioids effects was difficult.

Both Project 1 and 2 are at the preclinical level (Fig. 20). Project 1 needs information on drug metabolism (DM) and pharmacokinetics (PK) to determine the Absorption, Distribution, Metabolism, and Excretion (ADME) of GZ-11608. Also, studies determining GZ-11608 safety and dose translation to clinical studies are needed (Chung et al., 2004). For drug metabolism, in vitro assays such as microsome stability, permeability, plasma stability, plasma protein binding, CYP450 inhibition need to be evaluated. In vivo pharmacokinetic (i.e., Cmax, distribution, clearance, half-life) analysis, bioavailability, linearity, metabolism, and routes of excretion need to be evaluated. Also, pharmacokinetics at high dose of GZ-11608 (as known as toxicokinetics) needs to be evaluated. For toxicokinetic assays, biological matrices need to be collected including blood, urine, fat, muscle, liver, kidneys, and brain (the target organ) (Andrade et al., 2016). Species-specific pharmacokinetics should be conducted using rodents and another species (i.e., dog, monkeys) having similar metabolism with humans. Safety pharmacology studies are needed to determine if GZ-1108 presents critical safety issues such as high affinity for hERG. After completing preclinical studies and prior to starting clinical studies, the FDA requires Investigational New Drug (IND) Application for the new

substance, in this case, GZ-11608. After submission of IND application, the FDA reviews it and provides permission to administer the new substance to human.

After authorization from the FDA, clinical trials are initiated. Phase 1 aims to evaluate safety of the new substance (GZ-11608) in human healthy volunteers (Phase 1a) and in patients with the target disease (Phase 1b). Once safety is determined, then Phase 2 is initiated with an aim of determining efficacy with short duration of study with the new substance in a small number of patients (Phase 2a). Whether the new substance exhibits efficacy, then efficacy of GZ-11608 would be determined with longer duration in a larger number of patients (Phase 2b). Whether the new substance exhibit efficacy compared to placebo control group in Phase 2b, then Phase 3 is initiated. The Phase 3 clinical trial evaluates efficacy of the new substance in a large number of patients. Overall risk-benefit ratio of the new substance is determined in Phase 3 (Andrade et al., 2016; Van Norman, 2016). Then, New Drug Application (NDA) is submitted to FDA to be approved for marketing the new substance. After reviews of the NDA by the FDA, Phase 4 clinical trials may be conducted to evaluate efficacy of the new substance and new indications of the substance.

On the other hand, the Project 2 would need to do further SAR studies to discover M5 mAChRs selective antagonist, and then determine effects of analogs in behavioral models. After that, the identified M5 selective analogs would follow the new pharmacotherapeutics approval process as described above and in Fig. 20.

Figure 6.1 New Pharmacotherapeutics Approval Process. IND: Investigational New Drug Application, NDA: New Drug Application.

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Zheng G, Smith AM, Huang X, Subramanian KL, Siripurapu KB, Deaciuc A, Zhan CG, and Dwoskin LP (2013) Structural modifications to tetrahydropyridine-3-carboxylate esters en route to the discovery of M5-preferring muscarinic receptor orthosteric antagonists. *Journal of medicinal chemistry* **56**:1693–703.

Zheng X, Zhou Z, Zhang T, Jin Z, Chen X, Deng J, Zhan C-G, and Zheng F (2017) Effectiveness of a Cocaine Hydrolase for Cocaine Toxicity Treatment in Male and Female Rats. *AAPS J* **20**:3.

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Zunkler BJ, and Wos-Maganga M (2010) Comparison of the effects of methadone and heroin on human ether-a-go-go-related gene channels. *Cardiovasc Toxicol* **10**:161–165.

VITA

Na-Ra Lee

College of Pharmacy, University of Kentucky

A. EDUCATION

2012 - Present **Ph.D. Candidate** in Department of Pharmaceutical Sciences,

College of Pharmacy, University of Kentucky, Lexington, KY, U.S.A.

Dissertation title: Discovery Novel Pharmacotherapeutics for Substance Use Disorders (Expected Graduation in August 2019)

Advisor: Linda P. Dwoskin, Ph.D.

2007 - 2009 **M.S.** in Department of Bio and Nanochemistry, Biochemistry Major

College of Nature Science, Kookmin University, Seoul, South Korea

Thesis title: Enzymatic Properties of Severe Acute Respiratory Syndrome (SARS) Coronavirus Helicase,

Adviser: Yong-Joo Jeong, Ph.D.

2003 - 2007 **B.S.** in Department of Bio and Nanochemistry

College of Natural Science, Kookmin University, Seoul, South Korea

B. RESEARCH AND PROFESSIONAL POSITIONS

2012 - Present **Graduate Student and Research Assistant (Ph.D. Candidate)**

Research Project 1: Discover M5 muscarinic acetylcholine receptor as a novel treatment target for substance use disorders

Research Project 2: Discover vesicular monoamine transporter-2 (VMAT2) selective inhibitors as pharmacological treatments for methamphetamine use disorders

Research Project 3: The underlying mechanism on tolerance development following repeated VMAT2 administration

Department of Pharmaceutical Sciences, University of Kentucky, Lexington, KY, U.S.A., Advisor: Linda P. Dwoskin, Ph.D.

2010 - 2012 **Research Assistant**

Research Project: Discover immunoproteasome subtype selective inhibitors as a cancer treatment

Department of Pharmaceutical Sciences, University of Kentucky, Lexington, KY, U.S.A., Research Advisor: Kyung Bo Kim, Ph.D.

2009 - 2010 **Research Assistant**

Research Project: Characterize helicase enzyme working mechanism of severe acute respiratory syndrome (SARS) coronavirus

Department of Bioscience and Biotechnology, Konkuk University, Seoul, South Korea, Research Advisor: Dong-Eun Kim, Ph.D.

2007 - 2009 **Graduate Student and Research Assistant**

Research Project 1: Enzymatic properties of severe acute respiratory syndrome (SARS) coronavirus helicase

Research Project 2: Discover inhibitors of SARS helicase as anti-viral materials

Department of Bio and Nano-Chemistry, Kookmin University, Seoul, South Korea, Research Advisor: Yong-Joo Jeong, Ph.D.

C. PUBLICATIONS

1. Jang KJ, **Lee N-R**, Yeo WS, Jeong YJ, Kim DE. (2008) *Biochem Biophys Res Comm* 366(3):738–44. PMID: 18082623
2. Lee C, Lee JM, **Lee N-R**, Jin BS, Jang KJ, Kim DE, Jeong YJ, Chong Y. (2009) *Bioorg Med Chem Lett* 19(6):1636–38. PMID: 19233643
3. **Lee N-R**, Lee AR, Lee B, Kim DE, Jeong YJ. (2009) *Bull Korean Chem Soc* 30(8):1724-28
4. Lee C, Lee JM, **Lee N-R**, Kim DE, Jeong YJ, Chong Y. (2009) *Bioorg Med Chem Lett* 19(16):4538–41. PMID: 19625187
5. **Lee N-R**, Kwon HM, Park K, Jeong YJ, Kim DE. (2010) *Nucleic Acids Res* 38(21):7626–36. PMID: 20671029
6. Carmony KC, Lee DM, Wu Y, **Lee N-R**, Wehenkel M, Lee J, Zhan CG, Kim KB. (2012) *Bioorg Med Chem* 20(2):607–13. PMID: 21741845
7. Sharma LK, **Lee N-R**, Jang ER, Lei B, Zhan CG, Lee W, Kim KB. (2012) *Chembiochem* 13(13):1899-903. PMID: 22807337

8. Jang ER, **Lee N-R**, Han S, Wu Y, Sharma LK, Carmony KC, Marks J, Lee DM, Ban JO, Wehenkel M, Hong JT, Kim KB, Lee W. (2012) *Mol Biosyst* 8(9):2295–302. PMID: 22722901

9. Kasam V, **Lee N-R**, Kim KB, Zhan CG. (2014) *Bioorg Med Chem Lett* 24(15):3514-7. PMID: 24913713

10. Miller Z, Kim KS, Lee DM, Kasam V, Baek SE, Lee KH, Zhang YY, Ao L, Carmony K, **Lee N-R**, Zhou S, Zhao Q, Jang Y, Jeong HY, Zhan CG, Lee W, Kim DE, Kim KB. (2015) *J Med Chem* 58(4):2036-41. PMID: 25658656

11. **Lee N-R**, Zhang X, Darna M, Dwoskin LP, Zheng G. (2015) *Bioorg Med Chem Lett* 25(22):5032-5. PMID: 26494260

12. Bommagani S, **Lee N-R**, Zhang X, Dwoskin LP, Zheng G. (2015) *Tetrahedron Letters* 56(46):6472-4

13. **Lee N-R**, Zheng G, Crooks PA, Bardo MT, Dwoskin LP. (2018) *AAPS J* 20(2):29

14. **Lee N-R**, Gujarathi S, Bommagani S, Siripurapu S, Zheng G and Dwoskin LP. (2019) *Bioorg Med Chem Lett* 29(3):471-476

15. **Lee N-R**, Zheng G, Nickell JR, Janganati V, Crooks PA, Bardo MT, Dwoskin LP. *J Pharmacol Exp Ther*, under revision, 2019

D. PATENT

1. U.S. Patent No. 9,586,946: Zhan, C.-G.; Kim, K.-B.; Kasam, V.; **Lee, N.-R.** "Selective Immunoproteasome Inhibitors with Non-peptide Scaffolds". Date of Patent issued: March 7, **2017**.

2. U.S. Patent Application No. 2017/0304227 A1: Dwoskin, L.P.; Crooks, P.A.; Nickell, J.R.; Zheng, G; Zheng, C; **Lee, N.-R.** "Vesicular Monoamine Transporter-2 Ligands and Their Use in the Treatment of Psychostimulant Abuse". Date of Patent Application Published: October 26th, **2017**.

E. FELLOWSHIPS AND AWARDS

2017 **Poster Presentation Award Winner Student Second Place**

The Drug Discovery and Development Colloquium at Little Rock, AR, Sponsored by AAPS (June, 17)

2017 **Oral Presentation Certification Awarded**, The Drug Development and Discovery Colloquium 2017, Sponsored by American Association of Pharmaceutical Scientists (AAPS), Little Rock, Arkansas (June 15-17)

2015 **Outstanding Presentation Award**

2015 KSEA-KY Winter Conference at Lexington, KY, Sponsored by KSEA (December, 19)

2014 **Center of Membrane Sciences (CMS) Graduate Student Mentoring Fellowship Merit Award**, Lexington, KY (May, 2)

2012 - 2013 **Teaching Assistantship**
University of Kentucky, Lexington, Kentucky, USA
(August 2012 – July 2013)

F. LEADERSHIP ROLES

2016 Jan. – 2016 Dec. **Drug Discovery Representative**

American Association of Pharmaceutical Scientists (AAPS) Student Chapter at the University of Kentucky, Lexington, KY

2016 Aug. – 2017 Jul. **Vice President**

Korean Scholars Association at University of Kentucky (KSAUK) in Korean-American Scientists and Engineers Association (KASEA)-Kentucky Chapter, Lexington, KY

2015 Aug. – 2016 Jul. **Vice President**

Korean Bio-Scientists Association at University of Kentucky (KBAUK) in Korean-American Scientists and Engineers Association (KASEA)-Kentucky Chapter, Lexington, KY

2015 Aug. – 2016 Dec. **2015 Winter Conference Organizing Committee**

Korean-American Scientists and Engineers Association (KASEA)-Kentucky Chapter, Lexington, KY

G. TEACHING EXPERIENCES

2017 **Invited Lecturer**

Behavioral Pharmacology Class, Transylvania University,

Lexington, KY, USA, May 17

2012 - 2013 **Teaching Assistant for Accommodations**

Department of Pharmaceutical Sciences, University of Kentucky, Lexington, KY, U.S.A.

2008 - 2009 **Teaching Assistant/ Lecturer for Biochemistry Lab**

Department of Bio and Nanochemistry, College of Natural Science, Kookmin University, Seoul, South Korea

2007 - 2009 **Teaching Assistant/ Lecturer for General Chemistry Lab**

Department of Bio and Nanochemistry, College of Natural Science, Kookmin University, Seoul, South Korea

H. ORAL PRESENTATIONS

National

2016 Enantiomers of (±)GZ-888 Potently and Selectively Inhibit Vesicular Monoamine Transporter-2 Function and Methamphetamine-stimulated Locomotor Activity, College on Problems of Drug Dependence (CPDD), **oral presentation**, Palm Springs, CA, USA, Jun. 11-16

2017 The potent and selective vesicular monoamine transporter-2 inhibitor, GZ-11608, Decreases behavioral response to methamphetamine, The Drug Discovery and Development Colloquium 2017 (DDDC 2017), **oral presentation**, Little Rock, AR, USA, Jun. 15-17

Local

2016 Enantiomers of (±)GZ-888 Potently and Selectively Inhibit Vesicular Monoamine Transporter-2 Function and Methamphetamine-stimulated Locomotor Activity, 2016 Ashland Inc. Distinguished Lectures & Symposium on Drug Discovery & Development (DDD), **oral presentation**, Lexington, KY, USA, Nov. 4

I. POSTER PRESENTATIONS

International

2007 Enzymatic properties of Severe Acute Respiratory Syndrome (SARS) Coronavirus helicase, II33P165, The 100th National Meeting of the Korean Chemical Society, Daegu, South Korea, Oct. 18-19

2008 The study of steady state ATP hydrolysis by SARS Coronavirus helicase, The 101st National Meeting of the Korean Chemical Society, Ilsan, South Korea, Apr. 17-18

2008 ATP hydrolysis assays of SARS Coronavirus helicase in the presence of single-stranded nucleic acids, The 102nd National Meeting of the Korean Chemical Society, Jeju, South Korea, Oct. 16-17

2009 Characterization of Severe Acute Respiratory Syndrome (SARS) Coronavirus helicase, The 103rd National Meeting of the Korean Chemical Society, Seoul, South Korea, Apr. 16-17

2009 Biochemical analysis of ATP hydrolysis and DNA unwinding activity of the Severe Acute Respiratory Syndrome Coronavirus (SARS CoV) helicase, H-12-22, The 66th KSBMB annual meeting 2009, Seoul, South Korea, May 12-13

2009 Kinetic measurement of nucleic acid unwinding by Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) helicase, II36P179, The 104th National Meeting of the Korean Chemical Society, Daejeon, South Korea, Oct. 28-30

2010 The effects of substrate composition on helicase unwinding activity of SARS coronavirus nsP13, II36P226, The 105th National Meeting of the Korean Chemical Society, Incheon, South Korea, Apr. 29-30

National

2013 Discovery of M5-preferring muscarinic receptor antagonists: evaluation of a series of 3-phenyl propyl 1,2,5,6-tetrahydropyridine-3-carboxylates, Pharmaceutics Graduate Student Research Meeting (PGSRM), Iowa City, IA, USA, Jun. 6-8

2015 Discovery of M5 muscarinic acetylcholine receptor antagonists: 1-methyl-4-phenylpiperidine analogs, Experimental Biology, Boston, MA, USA, Mar.28-Apr.1

2015 Discovery of vesicular monoamine transporter-2 inhibitors as potential treatment for methamphetamine abuse: N-Butyl(1-methyl-2-phenylethyl)amine isomers, Pharmaceutics Graduate Student Research Meeting (PGSRM), Lexington, KY, USA, Jun. 11-13

2017 The potent and selective vesicular monoamine transporter-2 inhibitor, GZ-11608, Decreases behavioral response to methamphetamine, The Drug Discovery and Development Colloquium 2017 (DDDC 2017), poster presentation, Little Rock, AR, USA, Jun. 15-17

Local

2015 Discovery of M5 muscarinic acetylcholine receptor antagonists: 1-methyl-4-phenylpiperidine analogs, Blue Grass Society for Neuroscience (BGSFN) Spring Neuroscience Day, Lexington, KY, USA, Mar. 25

2015 Discovery of M5 muscarinic acetylcholine receptor antagonists: 1-methyl-4-phenylpiperidine analogs, Rho Chi Alpha Xi chapter of the University of Kentucky hosts Research Day, Lexington, KY, USA, Apr. 17

2017 (S)-GZ-11608, a Vesicular Monoamine Transporter-2 Inhibitor, Specifically and Potently Decreases the Behavioral Response to Methamphetamine, Blue Grass Society for Neuroscience (BGSFN) Spring Neuroscience Day, Lexington KY, USA, Apr. 21