TARGETING THE TRACE AMINE-ASSOCIATED RECEPTOR 1 IN PSYCHOMOTOR STIMULANT ADDICTION

A thesis submitted in partial fulfilment of the

requirements for the Degree of

Doctor of Philosophy in Psychology

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ABSTRACT

Psychomotor stimulant drugs, such as cocaine and amphetamine-type substances, including amphetamine and methamphetamine (METH), are potent central nervous system (CNS) stimulants with highly addictive potential. They are widely used and abused around the world, generating a staggering burden on society and the individual's family, yet no specific medications have been found to safely facilitate detoxification and promote quicker recovery from chronic stimulant abuse. The mainstay pharmacological approach for stimulant addiction has largely relied on directly altering neurotransmission of the classic monoamines, especially dopamine (DA), which is a key mediator of psychostimulant's effects. However, progress has been hindered by the limited efficaciousness and potential non-specific side effects associated with direct manipulation of the DA system. The newly discovered trace amine-associated receptor 1 (TAAR1) has gained increasing attention as a novel target for the pharmacological development of new addiction treatments. TAAR1 belongs to a family of G protein-coupled receptors (GPCRs) and is activated by trace amines (TAs), a group of endogenous amines that are intimately related to the classic monoamines. TAAR1 shares overlapping CNS distribution with the major monoaminergic pathways and is directly activated by some psychostimulants including METH. Early evidence from *in vitro* preparations and *in vivo* transgenic studies indicated an important role of TAAR1 in the regulation of DA transmission and psychostimulant action, leading to the hypothesis that pharmacological targeting of TAAR1 may present a promising avenue for addiction treatment. However, due to the unavailability of highly selective TAAR1 agonists until very recently, a direct assessment of this hypothesis has been difficult. The goal of the present thesis is to investigate systematically the potential therapeutic effectiveness of TAAR1-based agents in clinically-relevant animal models of addiction. Specifically, we will examine the ability of newly generated TAAR1 selective partial and full agonists to modulate key abuse-related behavioural and neurochemical effects of cocaine and METH. This is accomplished across three major sets of experiments, with 10 experiments in total. The

findings obtained from the current work provide evidence of highly favourable properties of TAAR1 agonists, consistent with an efficacious anti-addiction medication, and support the candidacy of TAAR1 as a pharmacological target for the treatment of stimulant addiction.

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LIST OF FIGURES

ABBREVIATIONS

5-HT	serotonin
AADC	aromatic L-amino acid decarboxylase
aCSF	artificial cerebrospinal fluid
ADHD	attention deficit hyperactivity disorder
AKT	protein kinase B
ANOVA	analysis of variance
arc	activity-regulated cytoskeleton-associated protein
BLA	basolateral amygdala
BP	breaking point
BZT	benztropine
CeA	central nucleus of the amygdala
CNS	central nervous system
CRF	corticotropin-releasing factor
DA	dopamine
DAB	3,3'-diaminobenzidine
DAT	dopamine transporter
DSM-V	Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition
EaCSF	experimental aCSF
FDA	Food and Drug Administration
FR	fixed ratio
FSCV	fast-scan cyclic voltammetry
GABA	γ-aminobutyric acid
GIRK	G protein-coupled inwardly-rectifying K^+ channel
GPCR	G protein-coupled receptor
GSK3	glycogen synthase kinase 3
i.p.	intraperitoneal
i.v.	intravenous
ICSS	intracranial self-stimulation
IEG	immediate early gene
KO	knockout
LSD	lysergic acid diethylamide
MAO	monoamine oxidase
MDMA	3,4-methylenedioxymethamphetamine

MeCP2	methyl CpG binding protein 2
METH	methamphetamine
MFB	medial forebrain bundle
mGluR5	metabotropic glutamate 5 receptor
NAc	nucleus accumbens
NE	norepinephrine
N-K	Newman-Keuls
PB	phosphate buffer
PBS	phosphate buffered saline
PE-50	polyethylene-50
PFC	prefrontal cortex
PR	progressive ratio
SaCSF	sodium-free slicing aCSF
SNpc	substantia nigra pars compacta
SNr	substantia nigra
TA	trace amine
TAAR	trace amine-associated receptor
TAAR1	trace amine-associated receptor 1
UNODC	United Nations Office on Drugs and Crime
VTA	ventral tegmental area
WHO	World Health Organization
β-PEA	β-phenylethylamine

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***Pei Y**, Lee J, Leo D, Gainetdinov RR, Hoener MC, Canales JJ (2014) Activation of the trace amine-associated receptor 1 prevents relapse to cocaine seeking. Neuropsychopharmacology 39:2299-2308. doi: 10.1038/npp.2014.88.*

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***Pei Y**, Asif-Malik A, Hoener M, Canales JJ (2016) A partial trace amine-associated receptor 1 agonist exhibits properties consistent with a methamphetamine substitution treatment. Addiction Biology, doi:10.1111/adb.12410.*

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Chapter One

1 Psychostimulant Addiction

1.1 Psychomotor Stimulant and Its Addiction - Concept and Definition

Psychomotor stimulant drugs, such as cocaine and amphetamine-type substances including amphetamine and methamphetamine (METH), are central nervous system (CNS) stimulants characterized by their ability to increase alertness and awareness, enhance motivation and endurance, stimulate motor activity, and suppress fatigue and sleepiness, as well as reduce appetite. In the peripheral nervous system, they produce sympathomimetic effects such as increased heart rate and blood pressure, increased respiration rate, and raised body temperature (Van Rossum, 1970, Koob and Le Moal, 2005). Acute exposure to these substances typically leads to an instant and intense feeling of well-being, self-confidence, and euphoria (especially when injected or smoked) (American Psychiatric Association, 2013), as well as decreased anxiety, accompanied by enhanced interaction with the environment and improved performance (Gawin and Ellinwood Jr, 1989, Drevets et al., 2001). Due to the initial positive effects on mental functions, some stimulants have been used clinically at relatively low doses for medical purpose. For example, amphetamine has been found to be effective in treating attention deficit hyperactivity disorder (ADHD), narcolepsy, obesity, chronic fatigue syndrome, and anhedonia associated with refractory depression (Van Rossum, 1970, Arnsten, 2006, Heal et al., 2013). Likewise, METH is prescribed in the USA, but not in Europe, for the treatment of ADHD and obesity (Favrod-Coune and Broers, 2010).

One key feature of psychomotor stimulants is that they serve as rewards or reinforcers, mainly due to their hedonic effects as well other positive influence on mental and physical activities, which leads to the initiation of drug taking and continuation of repeated drug use (Schuster, 1981, Koob et al., 1998, Everitt and Robbins, 2005). Repeated exposure to these drugs can produce neuroadaptive changes that underlie the development of addiction or substance dependence (Koob et al., 1998, Koob, 2000). These two terms will be used

interchangeably in this thesis to refer to the same chronically relapsing brain disorder, substance use disorder (as currently defined by the DSM-V), that is characterized by persistent and compulsive use of drugs, loss of control over drug intake, and continued drug use despite significant negative consequences associated with drug use (American Psychiatric Association, 2013). The occasional and limited use of an abusable drug is clinically distinct from escalated drug use and the emergence of chronic compulsive drug seeking that indicates a drug-dependent state (Koob and Volkow, 2010). Tolerance and withdrawal symptoms typically occur along the addiction process. Tolerance refers to the need for higher doses of the stimulant to achieve intoxication or desired effects, which reflects adjustments to the drug-induced homeostatic disturbances and can lead to escalation of drug use (Stewart and Badiani, 1993, Koob and Le Moal, 1997, Koob and Volkow, 2010). Withdrawal involves the experience of adverse symptoms due to a reduction of drug concentrations in the blood or tissue after the cessation of prolonged heavy use of the drug. Opposite to intoxication, withdrawal state is characterized by the development of dysphoric mood with concomitant physiological changes such as fatigue, insomnia or hypersomnia, increased appetite, and psychomotor retardation or agitation. These symptoms can lead to drug craving and re-administration aimed at reversing withdrawal reactions (American Psychiatric Association, 2013). The recurrent compulsive drug seeking and taking aggravate the addiction cycle causing further long-lasting neuroadaptations that persist even after protracted abstinence and lead to chronic relapse, which is the hallmark of addiction (Koob and Volkow, 2010). In addition, a variety of mental problems may develop during the course of acute or long-term stimulant use and withdrawal, such as neurotoxicity, psychosis, anxiety, depression, and long-lasting neurocognitive disorders (Kalechstein et al., 2003, Kita et al., 2003, Curran et al., 2004, Favrod-Coune and Broers, 2010).

1.2 A Worldview of Psychomotor Stimulant Use - Prevalence and Impact

Psychomotor stimulants are the most widely used and abused psychotropic substance around the world with cocaine and amphetamine-type drugs, due to their powerful inherent reinforcing properties, featuring among the most dangerous and addictive ones (Nutt et al.,

2007, Favrod-Coune and Broers, 2010). The recent New Zealand Health Survey (Ministry of Health, 2014) indicated that the prevalence of having used amphetamines-type substances, including METH, in 2013/2014 was 1.1 percent among New Zealand population aged 16-64 years, which equates to about 30600 New Zealanders, although the use of cocaine in New Zealand appeared to be less problematic mainly due to its increasingly high price and the ready availability of METH as a close substitute. At the global level, the United Nations Office on Drugs and Crime (UNODC) estimated that 1.4 percent of the world population aged 15-64 used amphetamine-type stimulants and 0.7 percent used cocaine in 2013 (UNODC, 2015).

The high magnitude of worldwide stimulant use has become one of the foremost public concerns that harm society on multiple levels, given that 1 out of 10 drug users are problem drug users (UNODC, 2015). It has been estimated that the social costs due to substance abuse exceed $484 billion per year in the USA, which includes health care expenditures, loss of earnings, and costs associated with crime and accidents (Rice et al., 1991, Harwood, 2000, Bouchery et al., 2001). Data from the World Health Organization (WHO) showed that, in 1990, mental illness and addiction accounted for almost 11 percent of the total global burden of human disease, and this is expected to rise to around 15 percent by 2020 (Mental Health Commission, 2011). Many top medical problems have been linked to substance abuse such as cancer, heart disease, and HIV/AIDS transmission; and a considerable proportion of deaths from these illnesses were attributed to drug use (Rodriguez et al., 1996, Ockene and Miller, 1997, McGinnis and Foege, 1999, Halkitis et al., 2001). Moreover, the requirement for special education and support service is more frequent for children with prenatal drug exposure, who are more likely to have low birth weight, low child IQ, and other developmental deficits (Lester et al., 2003, Arendt et al., 2004, Singer et al., 2004, Levine et al., 2008). Other significant social outcomes related to drug use and misuse include drugged driving, homelessness, child abuse, violence, and crime, all of which take a tremendous toll on society (McCarty et al., 1991, Rivara et al., 1997, Kelly et al., 2004, Gustavsen et al., 2006).

1.3 Behavioural Theories of Addiction

1.3.1 A conceptual framework

In the psychiatric-motivational framework proposed by Koob and Le Moal (2008a), drug addiction is conceptualized as having aspects of both impulse control disorders and compulsive disorders. Impulsivity is defined as 'a predisposition toward rapid, unplanned reactions to internal and external stimuli without regard for the negative consequences of these reactions to themselves or others' (Moeller et al., 2001). In impulse control disorders, the committing of an impulsive act is preceded by an increasing sense of tension or arousal, accompanied by pleasure, gratification, or relief, and may or may not be followed by regret, self-approach, or guilt (American Psychiatric Association, 1994). Positive reinforcement plays a critical role in impulse control disorders (Koob, 2004, Koob and Volkow, 2010). On the other hand, compulsivity is characterized by perseveration of a behavioural response that is inappropriate to the situation; often produces undesirable consequences; and has no obvious relationship to the overall goal (Dalley et al., 2011). Anxiety and stress signal the emitting of a compulsive repetitive behaviour, and performance of the compulsive behaviour relieves the stress and anxiety. Thus, compulsive behaviour is largely driven by negative reinforcement and automaticity (i.e., occurrence of behaviour without conscious intentionality) (Figure 1.1) (Koob and Volkow, 2010). Impulsivity, compulsivity, and addiction are intertwined in such a way that drug addiction is conceptualized as a composite spiral cycle composed of three stages - binge/intoxication, withdrawal/negative affect, preoccupation/anticipation - with the early stages dominated by impulsivity and the later stages dominated by compulsivity combined with impulsivity (Koob, 2004, Koob and Volkow, 2010). The transition from impulsivity to compulsivity is accompanied by a concurrent shift from positive reinforcement to negative reinforcement as the source of drive for the motivated behaviour (Koob, 2004). The three stages are not separate, instead they progressively feed into each other and intensify each other, forming a spiral that increases in amplitude, ultimately leading to the final pathological state of addiction (Figure 1.2) (Koob and Le Moal, 1997). Neuroplasticity occurs at all the three stages, and may begin from the

top of the spiral where the first drug use is initiated in susceptible individuals, and progresses with increased engagement with drugs (Koob and Volkow, 2010). Section 1.4, which reviews research on the neurobiology of addiction, will further elaborate on these neurological changes.

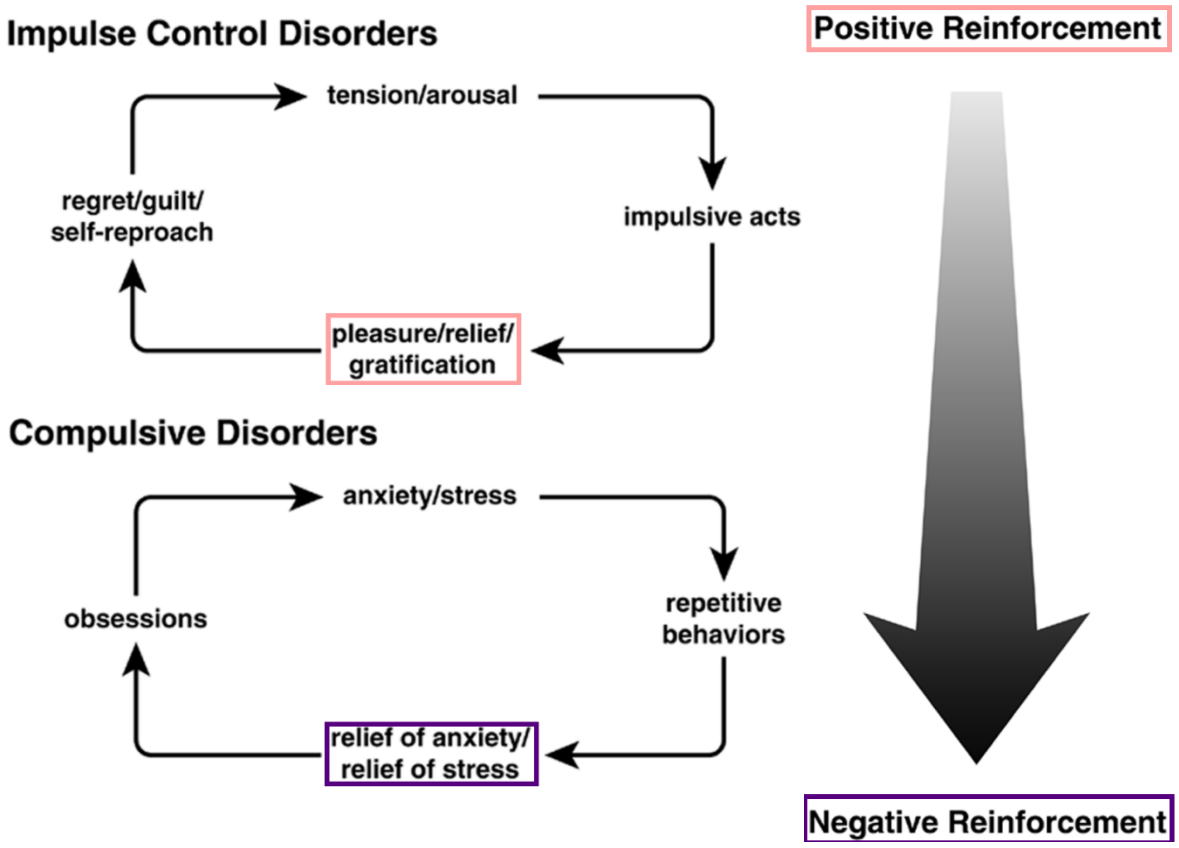

Figure 1.1 Stages of impulse control disorder and compulsive disorder related to the sources of reinforcement
Positive reinforcement (pleasure/gratification) is more closely associated with impulse control disorders. Negative reinforcement (relief of anxiety or stress) plays a more critical role in driving compulsivity. (Adapted from Koob, 2004).

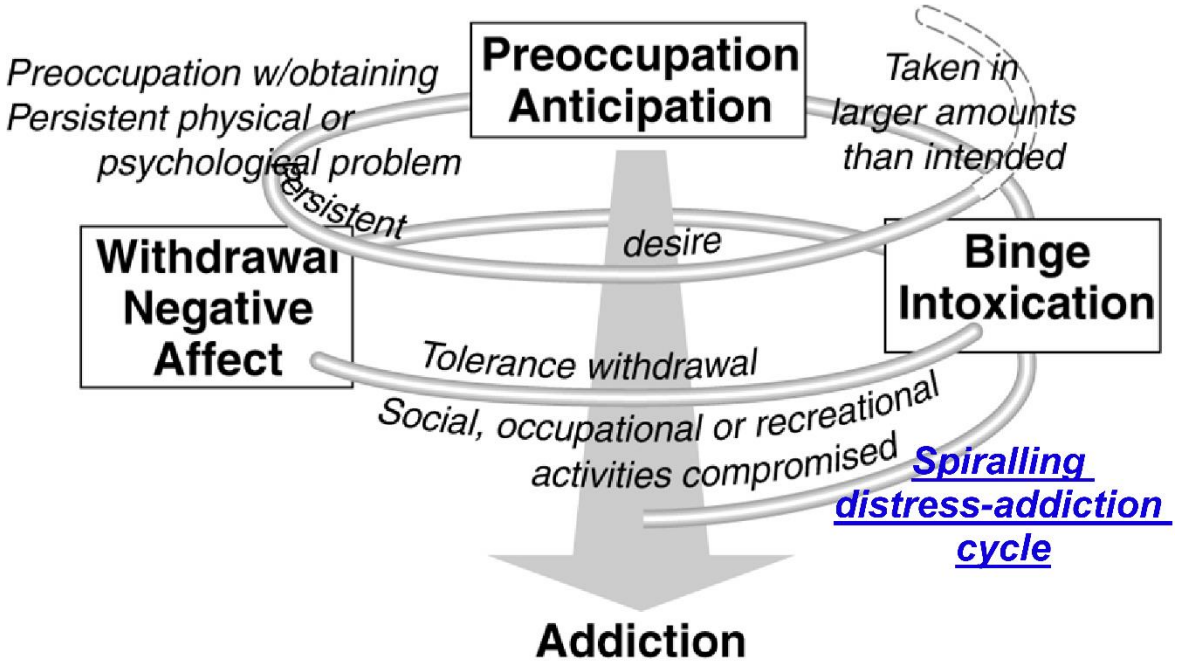

Figure 1.2 The spiralling addiction cycle from the psychiatric-motivational perspective
Drug addiction is conceptualized as a spiral cycle composed of three stages - binge/intoxication, withdrawal/negative affect, preoccupation/anticipation - that progressively feed into each other and intensify each other, growing in amplitude and ultimately leading to the pathological state of addiction. (Adapted from Koob and Le Moal, 2001).

1.3.2 Motivation and opponent process theory

Motivational mechanisms are the key to conceptualize drug addiction as a chronic compulsive disorder involving loss of control over excessive drug intake (Koob and Volkow, 2010). Motivation, defined as a 'tendency of the whole animal to produce organized activity' (Hebb and Donderi, 2013), is an internal state that varies over time and functions to guide behaviour according to changes in the internal and external environment (Koob and Le Moal, 2008a). Early work by Wikler (1952) addressed the importance of changes in the motivational state as the foundation of drug dependence, and this idea has been incorporated into the opponent process theory of motivation proposed by Solomon and Corbit (1974), where motivation has been inextricably linked with hedonic, affective, or emotional states in the transition to addiction. According to Solomon and Corbit (1974), there is an affect control system that automatically inhibits any departures from hedonic neutrality, forming a single

negative feedback or opponent loop. Any stimulus-evoked affective state is opposed or counteracted by CNS-mediated mechanisms to reduce the intensity of these feelings. The opponent process is hypothesized to consist of two processes, the a-process and the b-process. The a-process represents either positive or negative hedonic responses, varies with the duration, intensity, and quality of the stimulus, and takes place immediately after stimulus presentation. The b-process, which is hypothesized to oppose the a-process, occurs following the termination of the a-process and has a sluggish onset, a slow accumulating rate to reach asymptote, and a slow decay rate. While the a-process develops tolerance with repeated drug exposure, the b-process gets larger over time, defining the affective dynamics of the opponent process where new motives and opportunities are born that drive behaviour (Figure 1.3) (Solomon and Corbit, 1974). In the context of drug addiction, the a-process represents the positive hedonic response to the initial acute drug effect (euphoria) and is opposed by the b-process, manifested as the acute withdrawal state involving aversive negative emotions (dysphoria). The initial drug-taking phase is characterized by a strong a-process and a weak b-process and drug-taking behaviour is driven by the positive reinforcement of drug-induced euphoria. But with repeated drug exposure, the intensity of the a-process diminishes while that of the b-process escalates, generating powerful motivation mediated by negative reinforcement from exacerbated withdrawal state that leads to compulsive drug use and dependence (Figure 1.4).

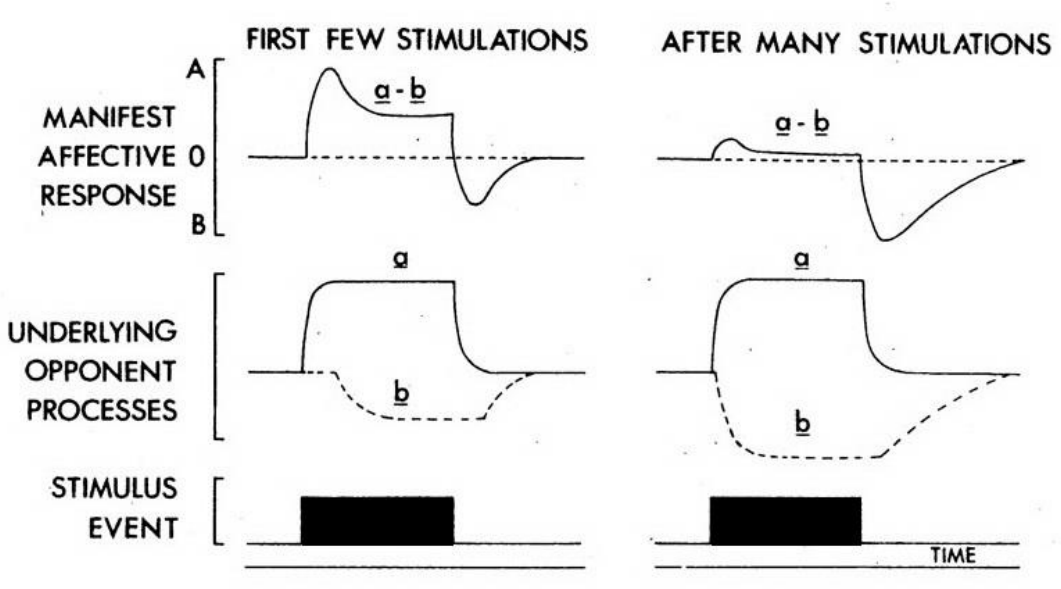

Figure 1.3 The affective dynamics of the opponent process
The left panel shows the manifestation of the affective response resulting from the summation of the underlying opponent processes, a and b, for the first few stimulations. The right panel shows the operation of the summing device after many repeated stimulus exposures. (Adapted from Solomon and Corbit, 1974).

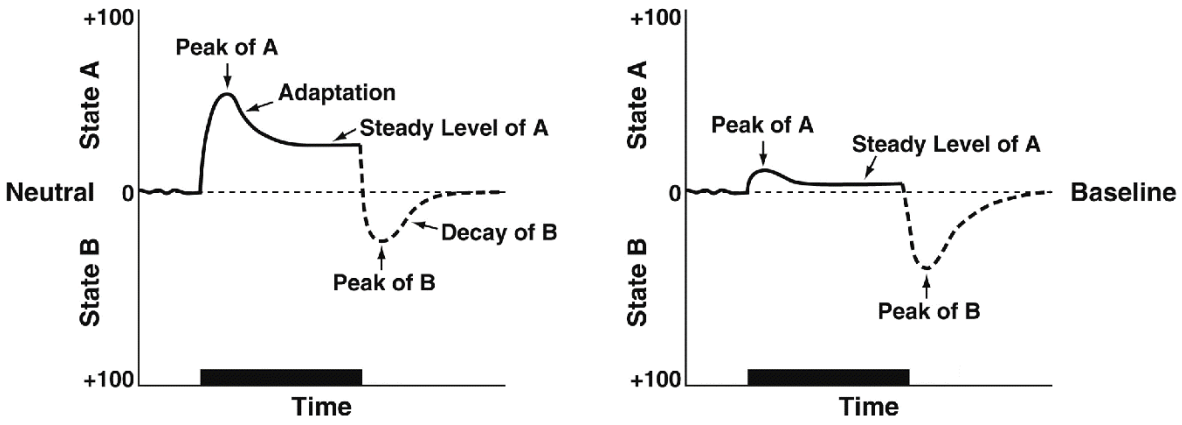

Figure 1.4 The opponent process theory relevant to addiction
The left panel shows the standard pattern of affective dynamics for the first few drug stimulations that are characterized by a strong a-process and a weak b-process, where positive reinforcement drives drug-taking. The right panel shows the standard pattern of affective dynamics after frequent, repeated drug exposures, where drug-taking is driven by negative reinforcement resulting from a compromised a-process and a strengthened b-process. (Adapted from Koob and Le Moal, 2008b).

1.3.3 Allostatic model

More recently, an allostatic model of the brain motivational system has been proposed as an extension of the opponent process theory to further explain the persistent changes in motivation in the transition to addiction. This model takes a physiological perspective and addresses neurobiological adaptations at a neurocircuitry level that underlie addiction (Koob and Le Moal, 2001, 2008a). A concept of an antireward system was postulated as a brain mechanism to oppose excessive activity of the reward system. Both systems are increasingly dysregulated, forming a spiralling cycle resulting in negative hedonic states that lead to compulsive drug use and long-term vulnerability to relapse (Koob and Le Moal, 2008a). Allostasis is a process of re-establishing homeostasis to achieve a more dynamic balance (Sterling and Eyer, 1988) and, from the addiction perspective, is designed to maintain the apparent stability of reward function through changes in brain mechanisms (Koob and Le Moal, 2001). Counteradaptive processes, such as the b-process, which originate as a normal homeostatic regulation of reward function, are nevertheless never able to return to the normal homeostatic range, resulting in a chronic progressive deviation from the normal operating reward set point (Figure 1.5) (Koob and Le Moal, 2001).

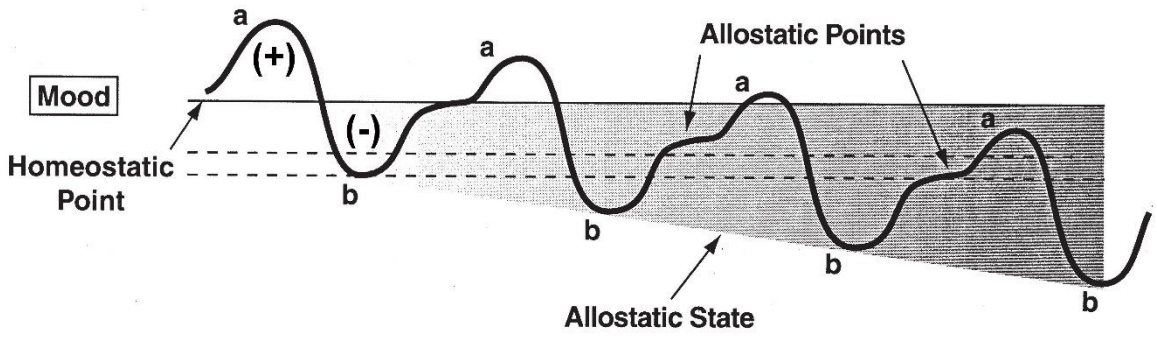

Figure 1.5 The allostatic model of drug addiction
As an extension of the opponent process theory, the allostatic model of drug addiction proposes that both the a- and b-process are increasingly dysregulated with frequent, repeated drug use, such that the b-process is never able to return to the original homeostatic level before drug-taking begins again. This creates a greater and greater allostatic state in the brain reward system and may represent a transition to compulsivity of addiction. (Adapted from Koob and Le Moal, 2001).

Allostatic change is hypothesized to be mediated by neuroadaptations that occur at two neurocircuitry levels: within-system and between-system (Koob and Bloom, 1988). The within-system opposing process involves molecular or cellular changes within a given reward circuitry, that is in place for natural rewards to shape survival and counteract excessive hedonic processing induced by drugs. It is the same cellular and molecular elements that are involved in the primary response to drugs that undergo adaptation to neutralize the drug's effect. The opposing effects persist after the drug terminates, leading to a rebound aftereffect of cellular overadaptation, contributing to the withdrawal state and decreased reward function. In the between-system opposing process, a separable circuitry, that is different from the primary response entity directly involved in the positive reinforcing effects of drugs, is recruited to provide inhibitory feedback to the reward system, defined as the antireward system (Koob and Le Moal, 2001, 2008a). Particularly, the brain stress/emotional circuit is triggered by overactivity of the reward system and produces an additional force to reduce reward function. With repeated drug exposure, this antireward system becomes dysregulated by chronic activation of the reward system. In this fashion, a functional decrement in brain reward system and the recruitment of antireward system form the addiction cycle that worsens over time, continuously deviating the allostatic state from the normal homeostatic level, resulting in increasingly powerful negative reinforcement that drives compulsivity and addiction (Koob and Le Moal, 2008a).

1.3.4 Incentive-sensitization theory

The incentive-sensitization theory was proposed by Robinson and Berridge (1993) to capture the subjective feelings of obsessive drug-wanting and drug-craving that are considered as fundamental to addiction and lead to compulsive drug seeking and taking. Robinson and Berridge (1993) argue that the motivation that drives compulsive drug taking does not come from either the positive reinforcement associated with the hedonic effects of drugs or the negative reinforcement linked to removal of withdrawal symptom; instead such a motivation is explained in terms of incentive salience.

The basic thesis of the incentive-sensitization theory is that all potentially abusable drugs have the common ability to induce persistent organizational changes in brain regions that are involved in processing incentive motivation and reward (Robinson and Berridge, 1993). As a result, the brain regions become sensitized and are hypersensitive to drugs and drug-associated stimuli. It is further postulated that two distinct psychological components are involved in reward: "liking" and "wanting", which are mediated by different neural systems. In this formulation, "wanting" refers to incentive salience, which is a type of incentive motivation that determines the value of incentives and the degree of their attractiveness, and thus their ability to promote behaviour directed to the goal (Robinson and Berridge, 1993). In contrast, "liking" represents the pleasurable or euphoric effects of drugs, and should be neurologically and psychologically dissociated from "wanting". According to Robinson and Berridge (1993), the neural systems that are sensitized by drugs are those specifically involved in the "wanting", but not the "liking", component of reward. Thus, with repeated drug use, the incentive salience of drugs and associated stimuli becomes more and more enhanced, accompanied with an increasing narrowing of attentional focus towards drug-related stimuli, at the expense of natural rewards, through associative pairing of the stimuli with drugs' effects. As a result, drugs become more and more pathologically wanted or desired, and the drug-related stimuli gain increasing power to induce craving and trigger compulsive drug seeking and taking (Robinson and Berridge, 1993, Robinson and Berridge, 2000). In contrast, the neural systems mediating "liking" are not sensitized, which may account for the increasing "wanting - liking" dissociation as addiction develops, such that drugs are often more and more "wanted" even when they are less and less "liked" (Robinson and Berridge, 2001). In addition, the sensitization-related neuroadaptations are long-lasting so that the drug-associated stimuli retain incentive salience and remain effective in precipitating drug-seeking long after drug use is ceased, which explains the persistent propensity to relapse (Robinson and Berridge, 1993, Robinson and Berridge, 2001).

1.4 Neurobiology of Addiction and the Role of Dopamine (DA)

Drug addiction has been conceptualized as the endpoint of a transitional process evolving from initial voluntary drug use through gradual loss of control over this behaviour and ending in habitual and compulsive drug use (Everitt et al., 2008). Psychostimulants produce physiological and subjective changes that mediate their initial reinforcing and rewarding effects, which act as a major motivational factor that initiates first drug use and maintains drug-taking in the early phase of addiction. As drug-taking continues and escalates, increasingly broader neural circuits become involved that display ongoing neuroadaptations and dysregulation, progressively leading to the final compulsive state (Koob and Le Moal, 2005, Everitt et al., 2008). This section will briefly review the neurobiological mechanisms that underlie acute psychostimulant reinforcement and the protracted transitional process. Among the various brain neurotransmitter systems, the DA system is widely believed to play the most pivotal role in psychostimulant addiction, although the importance of other neurotransmitters, in particular glutamate, has also gained increasing recognition. Because the present thesis is specially centred on TAAR1, whose functional interaction with the DA system has been most extensively researched in the past 15 years, the following review will focus on the role of DA. Nonetheless, as emerging evidence begins to implicate TAAR1 in glutamatergic function, the involvement of the glutamate system in stimulant addiction will be also discussed in relation to the current findings in later experimental chapters.

1.4.1 Acute reinforcement/reward of psychostimulants

Attempts to explore the neurological substrates underlying the reinforcing and rewarding effects of drugs of abuse have gained insight from the discovery of electrical brain stimulation reward through the method of intracranial self-stimulation (ICSS). ICSS involves delivering of electrical stimuli to a specific brain area through chronically implanted electrodes that is contingent on lever-press produced by the animal. This procedure allows the determination of an ICSS threshold for a certain brain region, defined as the minimal stimulation voltage that is required to sustain an ICSS response (Olds and Milner, 1954). Varied and widespread neuronal sites have been identified to sustain ICSS, but the most

sensitive neural elements yielding the lowest ICSS thresholds are embedded in the medial forebrain bundle (MFB) (Olds and Milner, 1954, Bielajew and Shizgal, 1986, Wise and Rompré, 1989, Wise, 2005). The MFB is a complex fibre system that traverses through lateral preoptic area and lateral hypothalamus, consisting of both ascending and descending fibre pathways of different origins and terminals (Veening et al., 1982), among which the trajectories connecting the ventral tegmental area (VTA) and the lateral hypothalamus as well as the basal forebrain are most strongly implicated in brain stimulation reward (Olds and Milner, 1954, Wise, 2005, Koob and Volkow, 2010). Acute administration of virtually all addictive drugs decreases ICSS thresholds indicating an elevation in brain reward function, which may in turn facilitate the acquisition and maintenance of drug-taking behaviours due to an enhanced sensitivity to the reinforcing effects of drugs (Kornetsky et al., 1979, Kenny and Markou, 2006). On the contrary, extended access to drugs gradually increases ICSS thresholds which correlates with escalation of intake, suggesting a downward shift from hedonic homeostasis that possibly contributes to the development of addiction (Jang et al., 2013). Increases in ICSS thresholds are also seen during withdrawal from chronic drug exposure, reflecting a reduced reward function and a negative emotional and motivational state that is believed to lead to craving and drug-taking (Schulteis et al., 1995, Epping-Jordan et al., 1998, Ahmed et al., 2002, Miyata et al., 2011).

Although various monoamine pathways, including norepinephrinergic, serotonergic, and dopaminergic pathways, are contained in the MFB, the mesolimbic and mesocortical DA pathways, which are together termed the mesocorticolimbic pathway, are most critical for brain reward and the acute reinforcing effects of drugs of abuse, especially psychostimulants (Koob et al., 1998, Everitt et al., 2008, Carlson, 2010). DA neurons in the mesolimbic system originate in the VTA of the midbrain and project their axons to various forebrain regions including the nucleus accumbens (NAc), olfactory tubercle, amygdala, and hippocampus. The mesocortical system also begins in the VTA but projects to the prefrontal cortex (PFC), the limbic cortex, and the hippocampus (Figure 1.6) (Koob et al., 1998, Carlson, 2010). It has been well established that the primary reinforcing effects of psychostimulants reside in

dopaminergic innervation of the NAc, especially the shell subregion (Everitt and Wolf, 2002, Koob and Le Moal, 2005). Cocaine and *d*-amphetamine, when self-administered intravenously, increased extracellular DA concentration in the NAc (Hurd et al., 1989, Pettit and Justice Jr, 1989, Di Ciano et al., 1995, Pontieri et al., 1995, Wise et al., 1995, Ranaldi et al., 1999), and the increase in DA-dependent signal was time-locked to each drug infusion (Pettit and Justice Jr, 1989, Kiyatkin and Stein, 1995). Selective destruction of the mesolimbic DA pathway, either at the dopaminergic terminals in the NAc or at DA cell bodies in the VTA, abolished previously established self-administration of cocaine (Roberts et al., 1980, Roberts and Koob, 1982), and selective lesion of DA neurons in the NAc impaired both the acquisition and maintenance of amphetamine self-administration in rats (Lyness et al., 1979). Moreover, rats readily self-infused *d*-amphetamine directly into the NAc (Hoebel et al., 1983, Phillips et al., 1994a, Phillips et al., 1994b), and selective antagonism of either D1 or D2 DA receptor in the NAc increased response rate, indicating that *d*-amphetamine reinforcement requires activation of NAc DA receptors (Phillips et al., 1994b). Accordingly, cocaine sustains intracranial self-administration into the NAc, VTA, and medial PFC of rats (Goeders and Smith, 1983, McKinzie et al., 1999, Rodd et al., 2005). In addition, *d*-amphetamine, when microinjected directly into the NAc, produced conditioned place preference in rats (Carr and White, 1983, 1986). These findings support the critical role of NAc DA in the reinforcing and rewarding effects of psychostimulants.

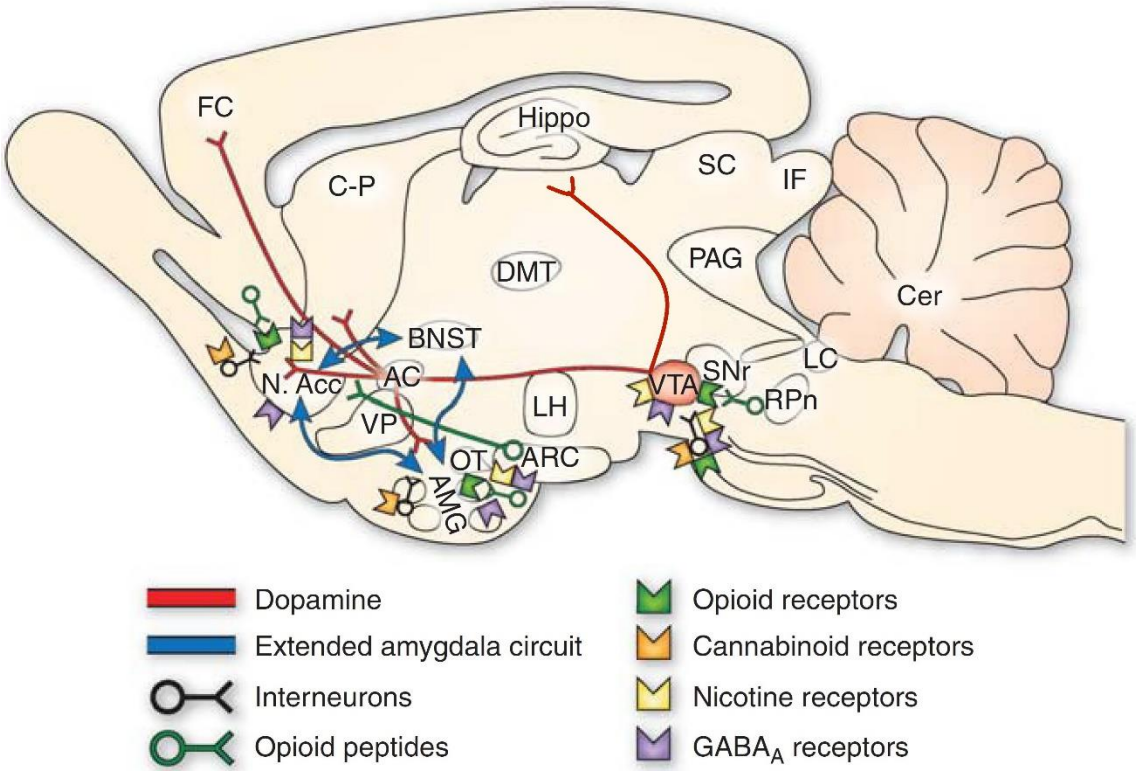

Figure 1.6 The mesocorticolimbic dopaminergic pathways
Sagittal section of the rodent brain illustrating the mesolimbic and mesocortical dopaminergic pathways. Red arrows represent the mesolimbic pathway (originating in the VTA and projecting to various forebrain regions including the NAc, olfactory tubercle, amygdala, and hippocampus) and the mesocortical pathway (originating also from the VTA and projecting to the PFC, the limbic cortex, and the hippocampus). AC, anterior commissure; AMG, amygdala; ARC, arcuate nucleus; BNST, bed nucleus of the stria terminalis; Cer, cerebellum; C-P, caudate-putamen; DMT, dorsomedial thalamus; FC, frontal cortex; Hippo, hippocampus; IF, inferior colliculus; LC, locus coeruleus; LH, lateral hypothalamus; N Acc., nucleus accumbens; OT, olfactory tract; PAG, periaqueductal gray; RPn, reticular pontine nucleus; SC, superior colliculus; SNr, substantia nigra pars reticulata; VP, ventral pallidum; VTA, ventral tegmental area. (Adapted from Koob and Volkow, 2010).

Several other forebrain regions, possibly through their connections with the NAc, are also important contributors to the acute reinforcing effects of psychostimulants. In particular, the central nucleus of the amygdala (CeA), which together with the NAc shell constitutes part of a neural continuum called the “extended amygdala”, is one of these regions (Robledo and Koob, 1993, Koob and Volkow, 2010). Rats readily self-administered *d*-amphetamine

directly into the CeA in a dose-dependent fashion (Chevrette et al., 2002), and blockade of the D1 DA receptors in the CeA increased cocaine self-administration in rats (McGregor and Roberts, 1993, Caine et al., 1995). In addition, the ventral pallidum is a major output region for the NAc (Heimer and Wilson, 1975). Lesions of the ventral pallidum impaired cocaine self-administration (Hubner and Koob, 1990, Robledo and Koob, 1993), and microinjection of cocaine or *d*-amphetamine into the ventral pallidum produced conditioned place preference in rats (Gong et al., 1996). Finally, the olfactory tubercle, extending ventrally to the NAc shell, has been implicated in drug reward (Ikemoto, 2007). Cocaine is readily self-administered into the olfactory tubercle by rats, and selective blockade of D1 or D2 DA receptors disrupted intra-tubercle self-administration of cocaine. Further, injection of cocaine into this region induced conditioned place preference (Ikemoto, 2003). Together these findings suggest the functional importance of the CeA, ventral pallidum, and olfactory tubercle in mediating psychostimulant reinforcement and reward, in addition to the NAc.

1.4.2 Voluntary drug-seeking

Through Pavlovian conditioning and Pavlovian-to-instrumental transfer, an environmental stimulus that has been repeatedly paired with the drug may become conditioned and its presentation subsequently induces drug craving and seeking (Everitt et al., 2001). The acquisition and early performance of this initial drug-seeking behaviour are believed to be voluntary and goal-directed, that is, it is still under the control of response-outcome contingencies and sensitive to outcome devaluation (Everitt et al., 2008). At this stage, the NAc core and its afferent input from the basolateral amygdala (BLA) are critically involved (Everitt et al., 2008). Selective lesion of either the BLA or the NAc core impaired the acquisition of cocaine seeking, assessed with a second-order schedule of self-administration in which an initial response on a drug-seeking chain is required for the access to the drug-taking chain (Whitelaw et al., 1996, Ito et al., 2004). In contrast, simple drug-taking, maintained by a standard schedule of continuous reinforcement, was not affected when neural activity in the BLA or NAc core was disrupted (Whitelaw et al., 1996, Ito et al., 2004). Importantly, neuropharmacological disconnection of the BLA and NAc core by way of

unilateral antagonist treatments reduced cocaine seeking (Di Ciano and Everitt, 2004). These findings support the notion that the initial voluntary drug-seeking maintained by response-outcome contingencies requires the integrity of the NAc core and its afferent from the BLA (Di Ciano and Everitt, 2004, Everitt et al., 2008).

1.4.3 Compulsive drug-seeking

1.4.3.1 The striato-nigro-striatal loop

With repeated drug exposure, drug-seeking behaviour becomes increasingly automatic and habitual, and is triggered and maintained by stimulus-response contingencies (Everitt et al., 2001, Everitt et al., 2008). Collective evidence has shown that the transition to the habitual drug taking represents a shift in the locus of control from ventral to dorsal striatum. This is believed to occur through a striato-nigro-striatal loop in which the ventral tiers of the striatum influence the dopaminergic innervation of more dorsal tiers by way of a series of ascending spiralling connections with the midbrain (Figure 1.7) (Everitt et al., 2008). In particular, DA neurons in the NAc shell project to the VTA, which projects both back to the NAc shell and more dorsally to the NAc core. The NAc core projects DA neurons in the substantia nigra (SNr), which innervates not only the NAc core itself but also the dorsal striatum, including the dorsomedial caudate-putamen and the dorsolateral striatum (Haber et al., 2000). Behavioural studies have investigated the functional significance of this ventral-dorsal striatal organization in the development of habit-like compulsive drug seeking. In rats that have established a habit of cocaine seeking, the presentation of a non-response contingent cocaine-associated stimulus increased DA level selectively in the NAc core, while the presentation of the same stimulus, when made contingent on cocaine-seeking response, increased DA only in the dorsal striatum. These findings suggest that the development of compulsive or habitual drug seeking is associated with a gradually weakened role of the NAc core and a progressive engagement of the dorsal striatum in the control over behaviour (Ito et al., 2000, Ito et al., 2002). Moreover, in the elegant study by Belin and Everitt (2008), ventral-dorsal striatal disconnection, by unilateral lesion of the NAc core combined with contralateral blockade of the DA receptors in the dorsolateral striatum, reduced habitual

cocaine seeking in rats, but did not affect normal sucrose taking. Again, these results reveal a shift from ventral to dorsal striatum in the control over drug-seeking behaviour as compulsivity develops (Belin and Everitt, 2008). On the other hand, given the potent DA-releasing ability of psychostimulant drugs in the striatum, chronic drug exposure may reciprocally accelerate or consolidate the ventral-to-dorsal shift (Everitt and Wolf, 2002, Everitt et al., 2008).

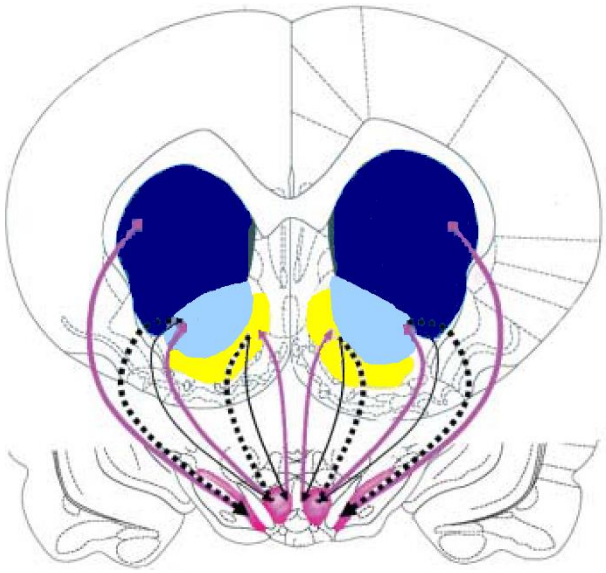

Figure 1.7 The striato-nigro-striatal loop for the transition to compulsive drug seeking
The alternation of pink and black arrows between the midbrain and ventral and dorsal striatum represents the DA-dependent spiralling circuitry that functionally connects the ventral with more dorsal regions. The loop starts from the NAc shell (yellow) that projects (black arrow) to regions of the VTA (pink) which send DA projections back to the NAc shell (pink arrow) and to more dorsal NAc core (soft blue) (bold dotted arrow). Similarly, the NAc core projects to areas of the VTA which innervate not only to the NAc core but also to the dorsomedial striatum. The spiral continues to encompass more dorsal striatal regions (dark blue) innervated by the more lateral SNr (pink). (Adapted from Everitt et al., 2008).

1.4.3.2 The frontostriatal pathway

In addition to the subregional transition within the striatum, a gradual shift in balance from prefrontal cortical to striatal control over drug-taking and seeking has been implicated in the

development of compulsive drug use and addiction (Robbins and Everitt, 1999, Everitt et al., 2008). The PFC is the central source for executive function and inhibitory response control, which is important for outcome evaluation and withholding inappropriate, pre-potent (i.e., previously conditioned) responses (Dias et al., 1996, Dias et al., 1997, Jentsch and Taylor, 1999, Bechara et al., 2000, Ridderinkhof et al., 2004). Increasing evidence has linked impaired prefrontal cognitive functions with a reduced ability to inhibit drug-seeking impulses and make decisions about the drug in human addicts (Dom et al., 2005, Schoenbaum et al., 2006, Olausson et al., 2007, Goldstein and Volkow, 2011). Impulsivity, a trait closely associated with prefrontal dysfunction, has been considered as a vulnerability marker for escalation of drug intake, relapse after abstinence, and compulsive drug use (Dalley et al., 2007, Economidou et al., 2007, Belin et al., 2008, De Wit, 2009). Meanwhile, chronic drug use was shown to significantly affect prefrontal cognitive functions and increase impulsive behaviour, which promotes uncontrolled drug intake and addiction. Indeed, prefrontal defects and impulsivity have been postulated as both predisposing factors for addiction and consequences of chronic drug exposure (Jentsch and Taylor, 1999, De Wit, 2009).

Prefrontal dysregulation has been show to affect striatal processing through corticostriatal projections from medial PFC to the caudate nucleus and NAc (Jentsch and Taylor, 1999). It has been hypothesized that subcortical DA activity is under tonic inhibitory control by cortical DA, whose loss of function may lead to upregulated DA transmission in the striatum (Deutch, 1992, Prasad et al., 1999). Thus, a reduced frontal-striatal inhibitory modulation may result in an augmentation of striatal DA-mediated drug reinforcement and drug-seeking (Jentsch and Taylor, 1999). Evidence shows that rats with DA depletion in the medial PFC were supersensitive to the acute reinforcing effects of cocaine (Schenk et al., 1991) and were hyper-motivated to seek cocaine in a progressive ratio (PR) schedule of reinforcement (McGregor et al., 1996), supporting the notion that impaired prefrontal function underlies a frontal-to-striatal shift in the control over behaviour during the transition to compulsive drug use.

Moreover, the incentive-sensitization theory postulates that chronic drug exposure sensitizes the DA innervation of the NAc, which is responsible for attributing incentive salience to reward-related stimuli. These sensitized DA neurons become over-activated when presented with drug-associated cues, leading to an elevated incentive motivational state of drug craving/wanting (Robinson and Berridge, 2001, 2008). It has been shown that repeated amphetamine treatment sensitized amphetamine-stimulated DA release in the NAc in rats (Robinson et al., 1988, Wolf et al., 1993) and in healthy human (Boileau et al., 2006), which may provide a neural basis for incentive motivational process (Robinson and Berridge, 2008).

Collectively, the transition to addiction occurs as a result of an impaired frontal function combined with upregulated striatal responses. This frontal-striatal neuroadaptation may lead to poor decision-making and loss of inhibitory impulse control, accompanied by sensitized incentive motivational states that drive compulsive drug seeking and taking (Olausson et al., 2007, Everitt et al., 2008).

1.4.4 Negative withdrawal state

The negative emotional state associated with drug withdrawal is believed to be an important factor that drives drug craving and seeking via a negative reinforcement mechanism (Koob, 2013). The opponent process theory and the allostatic model postulate that the negative withdrawal state progressively worsens with chronic drug exposure, which involves neuroadaptations at both a within-system and a between-system level (Koob and Le Moal, 2008a). In the within-system process, acute withdrawal from psychostimulants leads to decreased function of the mesolimbic DA system which is critically implicated in the acute reinforcing and rewarding effects of drugs. Reduced basal level of DA in the ventral striatum, including the NAc, has been found in rats withdrawn from cocaine or amphetamine (Robertson et al., 1991, Rossetti et al., 1992, Weiss et al., 1992, Kuhar and Pilotte, 1996). Moreover, human addicts displayed decreased DA D2 receptors in striatal neurons, indicating hypodopaminergic function (Volkow et al., 2003). The downregulated mesolimbic DA function is hypothesized to contribute to the negative emotional state that drives resumption

of drug use after acute withdrawal as well as chronic relapse following protracted abstinence (Koob and Le Moal, 2008a). On the other hand, the between-system process recruits additional brain circuits involved in modulating stress and anxiety-like effects, which constitutes the antireward circuitry, which is hypothetically to be localized in connections of the extended amygdala and recruit neurotransmitter systems such as the corticotropin-releasing factor (CRF), norepinephrine (NE), and dynorphin (Koob and Le Moal, 2008a). Rats that were withdrawn from chronic cocaine administration showed intense anxiety-like behaviour, accompanied by altered concentration of CRF in the hypothalamus, amygdala, and basal forebrain structures; and CRF antagonism completely prevented withdrawal-induced anxiety (Sarnyai et al., 1995, Basso et al., 1999). Similarly, blockade of dynorphin receptors attenuated cocaine withdrawal-induced anhedonia measured as increased ICSS thresholds (Chartoff et al., 2012). Therefore, a compromised mesolimbic DA function and a recruitment of extended amygdala-dependent stress system together contribute to the negative hedonic effects of withdrawal and provide a powerful drive for the development of addiction (Koob and Le Moal, 2008a).

1.4.5 Summary

Taking together, while the initial reinforcing and rewarding effects of psychostimulants depend primarily on the NAc shell, the integrity of the NAc core and its afferents from the BLA is also required for the acquisition and early maintenance of drug seeking driven by response-outcome contingencies. However, as drug use escalates, drug-seeking behaviour becomes more and more habitual and stimulus-response-driven, which ultimately leads to compulsive drug seeking and addiction. The neural mechanism underlying this switch involves a gradual transition from ventral to dorsal striatal control over drug-seeking and taking as well as a progressive control shift from the PFC to the striatum, through DA-dependent processes. In addition, the negative emotional states associated with withdrawal, resulting from deficient mesolimbic DA transmission and dysregulated stress modulation mediated by the extended amygdala, also promote compulsive drug seeking through negative reinforcement mechanism (Figure 1.8).

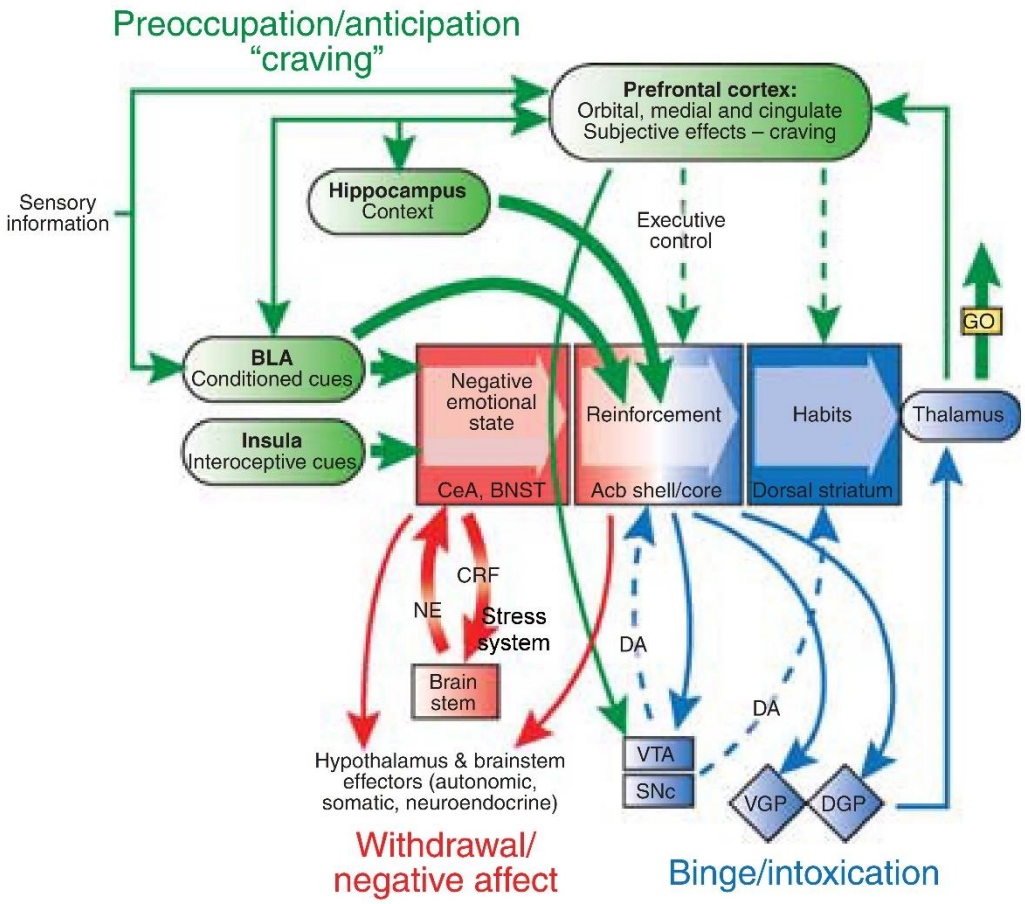

Figure 1.8 Neuroadaptations in the brain circuitry for the three stages of addiction cycle
The NAc integrates with the BLA to process conditioned reinforcement and with hippocampus to recruit contextual information. The transition to compulsive drug use depends on the striato-nigro-striatal loop that connects DA neurons with ventral and dorsal striatum through spiralling loops. Transition to addiction also involves frontal-striatal neuroadaptations where PFC function is compromised and striatal DA is sensitized. The negative withdrawal state is driven by an altered DA system and an increased recruitment of the brain stress system including CRF and NE. Acb, nucleus accumbens; BNST, bed nucleus of the stria terminalis; SNc, substantia nigra pars compacta; GP, globus pallidus (D, dorsal; V, ventral). (Adapted from Koob and Volkow, 2010).

1.5 Current Treatment and Problems

Psychostimulant addiction is recognized for its treatment challenges. Currently there are no medications available that are approved by the US Food and Drug Administration (FDA) specifically for psychostimulant dependence (Taylor et al., 2013). The mainstay of treatment has been behavioural interventions, including Cognitive-Behavioural Therapy, the Community Reinforcement Approach, contingency management, combinations of these, and

others (Ciccarone, 2011). These programs are critical for promoting recovery and preventing recidivism, but their success has only been limited thus far (Dutra et al., 2008, Phillips et al., 2014). Whereas several forms of non-specific pharmacology are currently in use, none have shown conclusive efficacy in safely facilitating detoxification and preventing long-term relapse with tolerable side effects (Ciccarone, 2011, Phillips et al., 2014). The following sections will review some major drug classes that have been explored in preclinical models and clinical settings which include antidepressants, γ-aminobutyric acid (GABA) agents, and dopaminergic drugs such as DA receptor ligands and DA replacement agonists, including DA releasers and DA reuptake inhibitors (Taylor et al., 2013). For the sake of brevity, drugs in other classes will not be detailed here as no concrete evidence exists for their superiority in treating stimulant addiction.

1.5.1 Antidepressants

The use of antidepressants in treating psychostimulant dependence is based on the notion that negative symptoms experienced during withdrawal and abstinence are associated with deficits in monoamine transmission, which can be rescued by antidepressants that are known to augment monoamine levels. Thus antidepressants are expected to alleviate abstinence symptomatology, relieve dysphoria, and reduce craving (Margolin et al., 1995). However, current evidence of antidepressant pharmacotherapies, including heterocyclic, selective serotonin reuptake inhibitors (SSRIs), monoamine oxidase (MAO) inhibitors and others, does not generally support their efficacy in treating stimulant abuse (Shoptaw et al., 2008b, Pani et al., 2011). A study that evaluated the efficacy of bupropion in treating METH dependence found no significant effects for bupropion relative to placebo in reducing METH use, increasing retention, or reducing METH craving (Shoptaw et al., 2008b). Another study by Pani et al. (2011) reviewed 37 randomized controlled clinical trials on METH dependence, including 3551 patients and a range of antidepressant agents, reporting disappointing results. Although partially positive outcomes on mood were obtained, consistent with the primary effect of antidepressants, they were not judged promising in treating stimulant dependence (Pani et al., 2011).

1.5.2 DA replacement agonists - releasers

Agonist replacement therapy involves replacing the abused drug with one that has similar positive reinforcing effects but with less addictive potential to treat stimulant dependence. One type of such pharmacological therapy is monoamine releasers, such as amphetamine analogues, which have shown inconsistent efficacy in treating cocaine or amphetamine dependence. Although some human laboratory studies reported a reduction in cocaine's reinforcing effects during *d*-amphetamine maintenance treatment (Greenwald et al., 2010, Rush et al., 2010), others found no effect of *d*-amphetamine on amphetamine self-administration (Stoops et al., 2007). Moreover, a large-scale review study revealed no significant effects of psychostimulants, including *d*-amphetamine and METH, on improving cocaine use, maintaining abstinence, or promoting treatment retention in clinical trials (Castells et al., 2010), although the opposite conclusion was reached elsewhere (Stoops and Rush, 2013). Also, given the inherent risks of misuse and potential abuse of these substitute substances, further studies are required to fully assess their therapeutic efficacy for stimulant addiction (Mariani and Levin, 2012).

1.5.3 DA replacement agonists - uptake inhibitors

Apart from monoamine releasers, replacement agonists also include uptake inhibitors. Methylphenidate and bupropion, both of which are dual NE-DA uptake inhibitors, are currently available for use in humans. One human laboratory study showed that maintenance on methylphenidate decreased the reinforcing effects of cocaine (Collins et al., 2006). However, results from clinical trials generally failed to demonstrate the effectiveness of methylphenidate in treating cocaine dependence (Grabowski et al., 1997, Schubiner et al., 2002, Levin et al., 2006). Moreover, studies have shown no effectiveness of methylphenidate in reducing amphetamine use, although it has been argued that the negative outcome could be attributed to a floor effect in the percentage of basal amphetamine-positive urines in the sample (Tiihonen et al., 2007, Konstenius et al., 2010, Stoops and Rush, 2013).

Similar controversial results have been obtained for bupropion. One laboratory study found that acute bupropion attenuated cocaine's rewarding effects in human participants, but increased subject-rated positive experience of cocaine (Stoops et al., 2012). Clinical trials generally indicated a lack of major effects of bupropion in managing cocaine dependence (Margolin et al., 1995, Levin et al., 2006, Shoptaw et al., 2008a), yet suggested that combination with cognitive behavioural therapy may increase its efficacy. Moreover, the ability of bupropion in reducing METH dependence has been argued to be limited to light users and those able to achieve abstinence early in treatment (Elkashef et al., 2008, Shoptaw et al., 2008b, Brensilver et al., 2012, McCann and Li, 2012).

1.5.4 DA replacement agonists - atypical DA uptake inhibitors

Because of the limited clinical efficacy and the significant inherent abuse potential of typical monoamine reuptake inhibitors and releasers, considerable efforts have been directed towards identifying alternative replacing agonists with less abuse liability and increased efficacy to treat psychostimulant dependence. Modafinil (Provigil ®) is an atypical dopamine transporter (DAT) inhibitor that binds to the same site on the DAT as cocaine but in a distinct manner (Federici et al., 2013, Okunola-Bakare et al., 2014, Reith et al., 2015). The behavioural effects of modafinil overlap to some extent with those of prototypical stimulants, producing discriminative-stimulus effects that partially substitute cocaine in rhesus monkeys and humans (Rush et al., 2002a, Newman et al., 2010). It is FDA approved for the treatment of narcolepsy and may also be used for ADHD (Turner, 2006, Kumar, 2008). Nonetheless, modafinil appears to have minimal abuse potential as it did not support conditioned place preference in rats (Deroche-Gamonet et al., 2002) and was not deemed as being positively reinforcing in humans (Rush et al., 2002b, Vosburg et al., 2010). Due to these characteristics, modafinil has been suggested as a putative alternative agonist replacement therapy. Human laboratory studies found that, in frequent drug users, modafinil maintenance reduced the reinforcing effect of cocaine (Hart et al., 2008) but not that of METH (De La Garza Ii et al., 2010). Clinical trials reported limited efficacy of modafinil in cocaine users, preferentially in non-alcohol dependent males (Anderson et al., 2009, Dackis et al., 2012), and in METH users

without other substance dependence and those attending counselling (Shearer et al., 2009). Taken together, although modafinil possesses a neuropharmacological profile consistent with an anti-addiction medication, it produced only modest efficacy in select populations and more clinical trials identifying those subpopulations with positive responses are needed.

Another possible DA replacement approach is based on the benztropine (BZT) class of DAT inhibitors. Some of these BZT analogues have high affinity and selectivity for the DAT (Newman and Agoston, 1998), exhibit slower rates of DAT occupancy compared with cocaine, and provoke long-lasting striatal DA release (Desai et al., 2005, Raje et al., 2005, Tanda et al., 2009). Most importantly, several of these compounds appear to be devoid of classical cocaine-like stimulating effects such that they did not evoke vigorous hyperlocomotion, did not produce conditioned place preference, only partially substituted for cocaine in cocaine discrimination tasks, and were less likely to sustain self-administration than cocaine (Katz et al., 2001, Desai et al., 2005, Hiranita et al., 2009, Velázquez-Sánchez et al., 2009), suggesting low abuse liability. Studies have demonstrated their ability to counteract critical abuse-related effects of cocaine and amphetamine-type stimulants in animal models. For example, AHN-1055, a *N*-substituted BZT analogue, was able to block cocaine self-administration without affecting responding for natural reward (Ferragud et al., 2009), reduced cocaine-stimulated locomotor activity, and prevented cocaine-conditioned place preference and associated early gene expression in the NAc and dorsomedial striatum (Velázquez-Sánchez et al., 2009). Another *N*-substituted BZT analogue, JHW 007, has also been shown to block cocaine-induced conditioned place preference, locomotor hyperactivity, and behavioural sensitization (Velázquez-Sánchez et al., 2010). In addition, these two compounds were effective in antagonizing hyperlocomotion and self-administration produced by amphetamine or METH (Velazquez-Sanchez et al., 2011, Ferragud et al., 2014) and in blocking the rewarding and sensitizing effects of amphetamine (Velazquez-Sanchez et al., 2011). Together, these findings support the novel generation of BZT analogues as lead compounds for pharmacological development to treat stimulant addiction.

1.5.5 DA receptor ligands

The direct focus on the DA receptors themselves is based on the well-established central role of DA in brain reward regulation and psychostimulant action. Extensive research has been carried out in this area, yielding complex results. While D1-like full and partial agonists, as well as antagonists, all have been found to attenuate cocaine-induced reinstatement of drug seeking (Barrett-Larimore and Spealman, 1996, Self et al., 1996, Barrett-Larimore and Spealman, 1997), the discriminative-stimulus effects of cocaine were decreased by a D1-like antagonist and a partial agonist but accentuated by full agonists in monkeys (Spealman et al., 1997). Moreover, several D1-like full and partial agonists were shown to maintain self-administration in monkeys and rats (Self and Stein, 1992, Weed and Woolverton, 1995, Grech et al., 1996), suggesting that they possess positive reinforcing properties. Although the ability of D1-like antagonists to block cocaine self-administration has been demonstrated in monkeys and rats, the specificity of this effect has been questioned (Woolverton, 1986, Corrigall and Coen, 1991, Caine and Koob, 1994a). Human trials did not produce more optimistic results. In spite of the finding that acute pretreatment with a D1-like antagonist, ecopipam (SCH 39166), reduced subject-reported euphoria and the desire to take cocaine (Romach et al., 1999), a regimen of chronic maintenance on this compound enhanced smoked cocaine self-administration and amplified the subjective effects of cocaine, questioning the viability of treating cocaine abuse through antagonism of the DA D1 receptors (Haney et al., 2001).

Studies on D2-like ligands reported even more complex findings. While D2-like DA receptor antagonists have been shown to attenuate cocaine-primed reinstatement of drug seeking in monkeys (Spealman et al., 1999, Khroyan et al., 2000), they failed to affect cocaine use or cocaine-induced subjective effects including euphoria and post-use craving in humans. In addition, they produced clinically significant adverse effects, including dysphoria, anxiety, and abnormal movement (Ohuoha et al., 1997, Grabowski et al., 2000).

The DA D3 receptor may represent the most promising DA receptor target for anti-addiction drug development. Studies have shown that the D3 receptor partial agonist, BP 897, inhibited cue-evoked cocaine seeking in rats (Pilla et al., 1999), reduced the discriminative-stimulus effect of cocaine and *d*-amphetamine in mice (Beardsley et al., 2001), and was not self-administered by rhesus monkeys (Beardsley et al., 2001). However, extrapyramidal side effects have been associated with BP 897 as it produced catalepsy in rats (Pilla et al., 1999) and induced a return of MPTP-induced parkinsonian symptoms in monkeys treated with L-DOPA (Hadjtahar et al., 1999). In addition, BP 897 produced sedative effects and, at high doses, induced ptosis and lethargy in monkeys (Beardsley, 1999). Thus a full assessment of its psychopharmacological and side effect profiles is needed and its therapeutic window must be carefully monitored (Garcia‐Ladona and Cox, 2003).

More recent attention has been drawn to D3 receptor antagonists, some of which were demonstrated to reverse the elevation of brain stimulation reward produced by cocaine and METH without altering reward function by themselves (Vorel et al., 2002, Spiller et al., 2008), block cocaine-conditioned place preference without producing place conditioning of their own (Macdonald et al., 2003, Cervo et al., 2005), and reduce self-administration response under PR but not fixed ratio (FR) schedule of reinforcement (Xi et al., 2005, Xi and Gardner, 2007), as well as attenuate reinstatement of cocaine seeking induced by cocaine challenge (Vorel et al., 2002, Xi et al., 2006, Xi and Gardner, 2007), cocaine-associated cues (Gilbert et al., 2005, Gál and Gyertyán, 2006), or stress (Xi et al., 2004). In addition, studies have precluded any untoward side effects of D3 receptor antagonists, as these compounds did not induce impairments in locomotor, cognitive, and affective function nor displayed abuse liability (Heidbreder and Newman, 2010). These preclinical studies have provided incentives to advance the discovery and development of novel D3 antagonists with improved pharmacokinetics and bioavailability in humans, but such progress appears to be challenging and has not yet materialized (Heidbreder and Newman, 2010, Searle et al., 2010, Newman et al., 2012). Much more human studies are necessary to determine the complete

pharmacological profile of candidate compounds, which will ultimately lead to human clinical trials to assess their therapeutic efficacy for stimulant dependence.

1.5.6 GABAergic agents

Evidence has suggested the possibility that GABAergic drugs may be effective in the treatment of stimulant addiction (Brebner et al., 2002, Cousins et al., 2002). Several $GABA_B$ agonists have been shown to reduce cocaine self-administration in rodents, but the degree of effectiveness seems dependent on the reinforcement schedule and the unit dose of cocaine (Roberts et al., 1996, Shoaib et al., 1998, Brebner et al., 2000, Roberts, 2005). Among these compounds, baclofen (Lioresal®) is the only one available for testing in humans and has been found to reduce self-reported craving and cocaine use in human cocaine users (Gudeman et al., 1996, Ling et al., 1998, Shoptaw, 2000). However, the short-lived action (3-4 h) of baclofen, which requires administration four times per day, poses an obstacle that limits outpatient compliance (Brebner et al., 2002). Moreover, baclofen may induce untoward side effects such as sedation and motor impairment due to its muscle relaxant and sedative properties (Brebner et al., 2002, Roberts, 2005). Together, although there are preclinical and clinical data supporting the usefulness of baclofen in addiction pharmacotherapy, problems exist that may compromise its efficacy and must be addressed by future investigations.

1.5.7 Other medications

The DA system closely interacts with, and is regulated by, various other neurotransmitter systems; hence a drug development strategy may be to target these non-dopaminergic circuits to indirectly modulate DA activity. These include serotonergic, opioid, glutamatergic, GABAergic (as abovementioned), endocannabinoid, and neuropeptide systems. Despite the extensive efforts that have been devoted in this field, no compound has yet been shown to be definitely effective in addiction management with tolerable side effects.

1.5.8 Summary

Currently there is no specific pharmacological therapy with established effectiveness in the treatment of stimulant dependence. The mainstay approach remains behavioural

interventions, which have been proven to be only partially successful. The progress of preclinical and clinical research into potential anti-addiction medications was largely hampered by their limited efficacy or noticeable adverse side effects. Numerous compounds have been explored but a very small portion of them demonstrated favourable therapeutic properties. DA D3 receptor antagonists and atypical DA reuptake inhibitors hold the most promise. However, direct manipulation of the DA system for treatment purposes is in some instances associated with long-term side effects, as evidenced by the debilitating motor complications induced by chronic dopaminergic therapies in Parkinson's disease and schizophrenia. In addition, DAT-based replacement medications may have an increased abuse liability, although preclinical data has preliminarily precluded this potential risk for several promising *N*-substituted BZT analogues.

Chapter Two[1]

2 Targeting Trace Amine-Associated Receptor 1 (TAAR1) to Treat Psychostimulant Addiction

Addiction to psychomotor stimulants is a chronic, relapsing disease of the brain for which an effective medication is yet to be found. Given the central role of DA in brain reward and psychostimulant action, the mainstream pharmacotherapeutic strategies have been to either directly or indirectly target the DA system. These approaches have generally been hindered by limited efficacy or potential non-specific side effects, although some promising results have been reported (see chapter one). During the on-going search for more efficacious medications in stimulant addiction, the trace amine (TA) transmitter system, a secondary amine system intimately related to the DA system, has attracted increased attention in recent years. TAs belong to a group of endogenous amines whose neurobiological functions were largely neglected until the breakthrough discovery of a group of G protein-coupled receptors (GPCRs), the so-called trace amine-associated receptors (TAARs), by two independent research groups in 2001 (Borowsky et al., 2001, Bunzow et al., 2001). Research conducted in the past 15 years has concentrated primarily on TAAR1, as the only member in this receptor family that is both responsive to TAs and phylogenetically conserved in the mammalian brain (Borowsky et al., 2001, Lindemann et al., 2005). TAAR1 has proved to be an important modulator of DA and has been implicated in psychostimulant action, giving rise to the suggestion that it may serve as a target for anti-addiction medicinal development. The general aim of the present thesis is to systematically investigate the therapeutic potential of TAAR1-based agents in stimulant addiction. This chapter will firstly summarize the latest advancements in understanding the pharmacological and neurochemical role of the TA system in mammalian CNS followed by a detailed review of *in vitro* and *in vivo* evidence

[1] Published paper. This chapter is based on the published paper: Pei Y, Asif-Malik A, Canales JJ (2016) Trace Amines and the Trace Amine-Associated Receptor 1: Pharmacology, Neurochemistry, and Clinical Implications. Frontiers in Neuroscience 10:148. doi:10.3389/fnins.2016.00148.

demonstrating the important implication of TAAR1 in psychostimulant addiction. Next, the rationale and the specific objectives of the current work will be stated, incorporating an outline of the subsequent experimental chapters (chapters three-five).

2.1 Trace Amines

TAs, including *ρ*-tyramine, *m*-tyramine, *β*-phenylethylamine (*β*-PEA), *m*-octopamine, *ρ*-octopamine, tryptamine, and synephrine, are a group of endogenous amines found in both invertebrate and vertebrate species (Berry, 2004). TAs have well-documented roles in invertebrates as major neurotransmitters, with octopamine believed to be the sympathetic nervous system counterpart of NE in vertebrates (Robertson and Juorio, 1976, Evans and O'Shea, 1977, Roeder, 1999). By contrast, although the existence of TAs in the vertebrate central and peripheral nervous systems has long been recognized, their functions were largely unknown due to the apparent lack of identified receptors specific for TAs, which prompted their description as "false neurotransmitters" (Berry, 2004). TAs are structurally similar to, and share same biosynthetic and metabolic pathways with, the classic monoamines (Berry, 2004, Ledonne et al., 2011). Initial studies postulated that TAs exerted sympathomimetic actions in the vertebrate peripheral nervous system, linking them to blood pressure regulation and electrolyte homeostasis (Barger and Dale, 1910, Podder et al., 1979). This notion can be traced back to the clinical observation of the so-called "cheese reaction", a hypertensive crisis experienced by sensitive patients treated with MAO inhibitor class of antidepressant drugs and exposed to aged cheese and other types of processed food enriched in *ρ*-tyramine produced through bacteria decarboxylation during fermentation (Blackwell and Mabbitt, 1965, Boulton et al., 1970, Rice et al., 1976, Stratton et al., 1991, Anderson et al., 1993). It has been shown that peripheral tyramine releases endogenous NE from peripheral stores, which in turn stimulates adrenergic nerves, a process responsible for its indirect sympathetic action (Crout et al., 1962, Tapper et al., 1981).

In the CNS, TAs are present at low nanomolar concentrations at a range that is several hundred-fold below that of the classical neurotransmitters, which can be linked to their

extremely rapid turnover rate and a very short half-life of around 30 seconds (Burden and Philips, 1980, Berry, 2004). TAs exhibit a heterogeneous distribution that closely parallels the classic monoaminergic projection pathways, with enhanced expression in the nigrostriatal and mesolimbic dopaminergic pathways (Philips, 1984). Evidence suggests that some TAs, including *ρ*-tyramine, *β*-PEA, and tryptamine, are synthesized within nigrostriatal DA neurons while *ρ*-octopamine is synthesized within adrenergic neurons (Berry, 2004). However, as indicated previously, TAs were regarded for a considerable time as mere metabolic by-products of other neurotransmitters and having little neurophysiological significance in their own right. In subsequent studies, TAs were classified as endogenous neuromodulators that regulated, and were themselves susceptible to regulation by, co-existing neurotransmitters (Berry, 2004). Indeed, ample evidence has demonstrated an intimate functional inter-regulation between TAs and the classic monoamines, especially DA. First, changes in monoamine activity were able to alter TA levels. For example, while reductions in striatal levels of *β*-PEA (Juorio et al., 1991b) and *ρ*-tyramine (Jones et al., 1983) were found to follow an increased DA release triggered by electrical stimulation of the SNr, inhibition of DA neurotransmission led to an elevated accumulation rate of *β*-PEA in the striatum (Juorio et al., 1991a). Reciprocally, TAs appeared to potentiate the efficacy of synaptic transmission of these monoamines (Philips, 1984, Burchett and Hicks, 2006). For instance, administration of MAO-B inhibitors, which increased *β*-PEA levels above their physiological range, enhanced striatal neuronal response to DA (Berry et al., 1994) and DA agonists (Paterson et al., 1991). Moreover, iontophoretic application of *β*-PEA elicited a potentiated cortical neuronal response to NE (Paterson and Boulton, 1988, Paterson, 1993). Similarly, iontophoretic administration of *ρ*-tyramine, *m*-tyramine, and *β*-PEA applied at weak currents increased cortical neuronal response to DA (Jones and Boulton, 1980). Likewise, application of octopamine through weak iontophoretic currents enhanced both inhibitory and excitatory neuronal responses mediated by NE (Jones, 1982a). The effect of tryptamine on serotonin (5-HT) neurotransmission appeared to be more complex as both a depression and potentiation of 5-HT-mediated neuronal effects were observed, which might be accounted for by the

biphasic effects of 5-HT itself on cortical neuron activity (Jones, 1982b). Taken together, these findings suggest that TAs are likely to serve as a fine-tuning mechanism that keeps a balanced monoaminergic tone by responding to endogenous- or exogenous-induced monoamine fluctuations, a process that may be partly mediated through interaction with specific receptors for TAs (Berry, 2004).

Given the abovementioned reciprocal relationship between TAs and monoamines, it is not surprising that dysfunction of TA signalling has been historically associated with a broad spectrum of neurological pathologies that involve changes in monoamine function, including ADHD (Baker et al., 1991), depression (Sandler et al., 1979, Wolf and Mosnaim, 1983, Sabelli et al., 1995), bipolar affective disorder (Karoum et al., 1982), and schizophrenia (Potkin et al., 1979, Buckland et al., 1997). Most importantly, of particular interest to the present thesis, ample evidence has implicated TAs in psychostimulant action and brain reward (Greenshaw, 1984, Janssen et al., 1999), suggesting their potential role in drug addiction.

Firstly, *β*-PEA has been found to bear close structural and pharmacological similarity with amphetamine (Tinklenberg et al., 1978, Janssen et al., 1999). At concentrations that were several orders of magnitude above its normal physiological range, *β*-PEA induced amphetamine-like effects in rodents and monkeys, including hyperactivity (Dourish, 1985) and stereotypic behaviour (Borison et al., 1977, Tinklenberg et al., 1978). This evidence led to the characterization of *β*-PEA as brain "endogenous amphetamine" (Janssen et al., 1999). Moreover, *β*-PEA activity is altered by exogenous application of *d*-amphetamine. Acute application of *d*-amphetamine resulted in an initial decrease and a subsequent increase in brain levels of *β*-PEA in rabbits (Borison et al., 1975). Also, chronic administration of amphetamine in rats downregulated aromatic L-amino acid decarboxylase (AADC) mRNA levels, which could in turn lead to decreased *β*-PEA activity (Buckland et al., 1996). On the other hand, certain behavioural effects of amphetamine appeared to be dependent on *β*-PEA

levels as depletion of brain *β*-PEA blocked the motor-stimulating effect of *d*-amphetamine in mice and rabbits (Borison et al., 1975).

Furthermore, consistent with its hypothesized role as "endogenous amphetamine", *β*-PEA was shown to possess reinforcing properties, a defining feature that underlies the abuse liability of psychostimulants. *β*-PEA was as effective as amphetamine in its ability to produce conditioned place preference in rats (Gilbert and Cooper, 1983) and was readily self-administered by dogs that had a stable history of amphetamine or cocaine self-administration (Risner and Jones, 1977, Shannon and Thompson, 1984). Moreover, high concentrations of *β*-PEA dose-dependently maintained responding in monkeys that were previously trained to self-administer cocaine, and pretreatment with a MAO-B inhibitor, which delayed *β*-PEA deactivation, further increased response rates (Bergman et al., 2001).

On the other hand, more variable influences on brain reward have been reported for other TAs. In rats that responded at threshold levels for ICSS of the lateral hypothalamus, tryptamine antagonists not only caused dose-related increases when given alone but also potentiated the brain reward-facilitating effects of amphetamine (Silveira Filho and Graeff, 1977). Conversely, systematic application of tryptamine decreased ICSS in both the medial raphe nucleus and the lateral hypothalamus (Broadbent and Greenshaw, 1985). Moreover, in sharp contrast to the conditioned place preference produced by *β*-PEA, tryptamine induced conditioned taste aversion to a novel saccharin solution in rats (Fletcher, 1986). These findings suggest an inhibitory regulation of reward function via tryptamine-mediated pathways. In addition, while little is known about the role of octopamine in brain reward in vertebrates, the functional contribution of octopamine to reward-associated learning has been well-noted in insects (Hammer, 1997, Unoki et al., 2006, Perry and Barron, 2013). Also, studies conducted in *Drosophila* suggest that tyramine is essential for the development of cocaine sensitization (McClung and Hirsh, 1999), a phenomenon thought to share similar underlying neuroadaptive mechanisms to those mediating craving and relapse (Kalivas et al., 1998, Vezina, 2004).

In summary, these findings underscore the important implications of TAs in psychostimulants-mediated effects and in brain reward processing, which, together with their widely documented interaction with the classic monoamines, strongly suggest a potential involvement of the TA system in the neurological adaptations underlying drug addiction. However, due to the apparent absence of specialized receptors for TAs and the lack of knowledge of their signalling mechanisms, research interest in their neurobiological functions and neuropathological implications was largely discouraged for a considerable time.

2.2 Identification of TAAR Family

Progress in the characterization of the neurobiological functions of TAs has been hampered by the difficulty in identifying their specific receptor targets. Although saturable high-affinity binding sites distinct from the amine transporters and receptors had been identified in the mammalian brain (Kellar and Cascio, 1982, Brüning and Rommelspacher, 1984, McCormack et al., 1986, Nguyen and Juorio, 1989), it was at the beginning of the twenty-first century that two research groups independently reported the cloning and identification of a novel family of mammalian GPCRs (Borowsky et al., 2001, Bunzow et al., 2001). Such receptors, including several orphan receptors, shared an unusually high degree of sequence homology, and some were directly activated by TAs. The discovery of receptors for TAs supported their role as *bona fide* neurotransmitters, that is, as molecules able to trigger cellular events directly, and led to a renewed interest in the TAs and their biological functions. In subsequent studies, Lindemann and collaborators proposed a uniform nomenclature for this newly discovered GPCR family, together with closely related receptors, as TAARs, acknowledging the fact that some members are unresponsive to TAs (Lindemann et al., 2005). Further work by the same group completed the identification of all members of this GPCR family in rats, mice, chimpanzees, and humans, demonstrating remarkable differences in the number of receptor genes and the proportion of pseudogenes amongst the four species (Lindemann et al., 2005). There are nine *TAAR* genes in human including three pseudogenes; nine genes in chimpanzee including six pseudogenes; 19 and 16 in rat and mouse with two and one being pseudogenes, respectively. In spite of these significant inter-species differences, three TAAR

subfamilies were identified based on phylogenetic relationships and pharmacophore similarities, which remained consistent across all of the four species (Lindemann et al., 2005). The three subfamilies consist of TAARs 1-4, TAAR5, and TAARs 6-9, with each subgroup represented by at least one functional *TAAR* gene. Not surprisingly, the only two receptors that are activated by TAs, namely TAAR1 and TAAR4, both belong to the first subfamily, supporting a functional basis for the classification (Lindemann et al., 2005). While TAAR1 is sensitive to all TAs and the *TAAR1* gene is phylogenetically conserved in all the studied species including human, *TAAR4* is a pseudogene in the human genome and rat TAAR4 responds only to *β*-PEA and tyramine, although to a much lesser degree than TAAR1 (Borowsky et al., 2001, Bunzow et al., 2001, Lindemann et al., 2005). As a result, TAAR1 has received by far the most attention over the past decade and it is the best characterized receptor of the class (Lindemann and Hoener, 2005).

2.3 Expression, Signalling, and Pharmacology of TAAR1

TAAR1 couples to a $G\alpha_s$ G protein and, upon stimulation, triggers accumulation of intracellular cAMP via adenylyl cyclase activation and stimulates G protein-coupled inwardly-rectifying K^+ channel (GIRK) (Borowsky et al., 2001, Bunzow et al., 2001, Miller et al., 2005, Xie et al., 2007, Bradaia et al., 2009). TAAR1 activation can also lead to PKA and PKC phosphorylation and upregulation of the transcription factors, CREB and NFAT (Panas et al., 2012). TAAR1 also signals via a G protein-independent, β-arrestin2-dependent pathway involving the protein kinase B (AKT)/glycogen synthase kinase 3 (GSK3) β signalling cascade, an important player in many DA-mediated actions (Harmeier et al., 2015) (Figure 2.1). Among the TAs, *β*-PEA and tyramine are the most potent activators at TAAR1, with *β*-PEA being more potent than tyramine at human and mouse TAAR1, with the opposite being true at rat TAAR1 (Bunzow et al., 2001, Grandy, 2007, Wainscott et al., 2007). Strikingly, in addition to TAs as principal binding ligands, TAAR1 is also activated by a vast variety of endogenous and exogenous molecules, including the major catecholamines, DA, NE, and 5-HT, and some of their metabolites, amphetamine-like compounds including amphetamine itself, METH, and 3,4-methylenedioxymethamphetamine (MDMA), ergot

derivatives including lysergic acid diethylamide (LSD), and several adrenergic ligands (Bunzow et al., 2001), as well as certain thyroid hormones derivatives (Hart et al., 2006). Studies aiming at determining TAAR1 distribution in the mammalian system have consistently reported a widespread and unique pattern of TAAR1 mRNA or protein expression in the central and peripheral nervous system in human, mouse, rat, and rhesus monkey. In the brain, TAAR1 mRNA has been detected throughout the limbic system and in regions associated with the major monoaminergic pathways, including the VTA, SNr, locus coeruleus, raphe nucleus, caudate nucleus, putamen, NAc, hippocampus, hypothalamus, and amygdala (Borowsky et al., 2001, Bunzow et al., 2001, Miller et al., 2005, Xie et al., 2007, Lindemann et al., 2008, Espinoza et al., 2015a). In addition to subcortical areas, TAAR1 is also expressed in cortical regions, especially in layer V pyramidal neurons of the PFC (Espinoza et al., 2015b). The cellular distribution of TAAR1 is predominantly intracellular, with diffuse expression within the perikaryon and along axonal processes, and sparse membrane-associated neuronal expression (Bunzow et al., 2001, Xie et al., 2007). Therefore, it has been postulated that the intracellular TAAR1 might recruit an accessory protein for translocation to the plasma membrane (Bunzow et al., 2001) or indeed might signal intracellularly, given the accessibility of several TAAR1 endogenous ligands within the cytoplasm and the ability of intracellular GPCRs to exert downstream effects (Xie et al., 2007, Lam et al., 2015). Remarkably, TAAR1 is co-localized with DAT in a subset of DA neurons, and/or expressed in neurons that are in close apposition to DAT-expressing neurons in mouse and rhesus monkey SNr (Xie et al., 2007). There is also evidence suggesting co-expression of TAAR1 with the NE transporter in adrenergic neurons in the rhesus monkey locus coeruleus, as well as co-localization with 5-HT transporter in serotonergic neurons in the mouse dorsal raphe nucleus (Lindemann et al., 2008, Xie et al., 2008). The neuroanatomical distribution of TAAR1 in relation to the major monoamine systems suggests that TAAR1 might be in a position to regulate monoaminergic transmission through direct interactions with monoamine transporters and presynaptic autoreceptors co-expressed with TAAR1 within single neurons, or by way of intercellular communication with nearby

monoaminergic neurons. The next section will review evidence from different research lines in support of such fundamental neurochemical interactions.

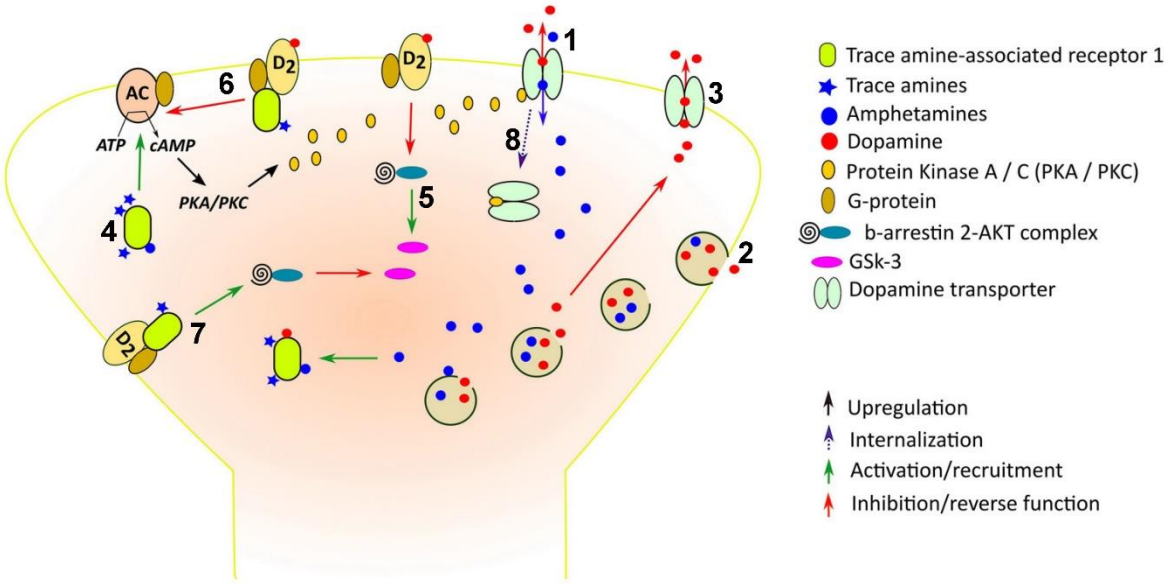

Figure 2.1 Signalling of trace amine-associated receptor 1 (TAAR1) at a DA synapse
Amphetamines enter the presynaptic neurons through competitive reuptake inhibition of the DA transporter (DAT) (1) and by diffusion through presynaptic membranes, causing release of DA by way of vesicular monoamine transporter-mediated exocytosis (2) and reverse transport through the DAT (3). Both amphetamines and endogenous TAs activate TAAR1, leading to adenylyl cyclase activation and downstream stimulation of PKA/PKC (4). TAAR1 also signals via G protein-independent, β-arrestin2-dependent pathway involving the protein kinases B (AKT)/glycogen synthase kinases 3 (GSK3) β signalling cascade. D2 receptor activation through β-arrestin2 dephosphorylates AKT, resulting in AKT inactivation and increased GSK3β signalling (5). TAAR1 interacts with both presynaptic D2s and postsynaptic D2 receptor through formation of heterodimeric complexes, leading to reduced TAAR1-stimulated cAMP accumulation (6, postsynaptic process not show). Interaction of TAAR1 with D2 receptor also shifts β-arrestin2 recruitment from D2 receptor to TAAR1, resulting in reduced GSK3β activation (7). In addition, phosphorylation of the DAT through TAAR1-stimulated activation leads to DAT internalization, which may result in reduced DA uptake (8). (Adapted from Pei et al., 2016a).

2.4 Functional Interaction of TAAR1 with Brain Monoamine Systems

Studies with heterologous expression systems and brain synaptosomes have revealed a complex tripartite relationship between TAAR1, monoamine transporters, and monoamine autoreceptors *in vitro.* First, in such *in vitro* preparations, TAAR1 activation by agonist ligands, including TAs, the classic biogenic amines, and drugs of the amphetamine-class, was shown to be markedly enhanced by co-transfecting TAAR1 with monoamine transporters (Miller et al., 2005, Xie et al., 2007). Since these TAAR1 agonists are also substrates at monoamine transporters, it has been hypothesized that the monoamine transporters might serve as conduits for the entry of TAAR1 agonists into the synaptic terminal such that activation of intracellular TAAR1 can occur (Miller et al., 2005, Xie et al., 2007, Miller, 2011). Alternatively, the transporter-mediated agonist uptake might trigger trafficking of TAAR1 into the plasma membrane (Xie et al., 2007). Additionally, such *in vitro* assays have revealed that TAAR1 activation functionally downregulates the activity of monoamine transporters. In cells co-transfected with both TAAR1 and one of the main aminergic transporters, TAAR1 activation by DA, NE, or 5-HT led to functional inhibition of the co-expressed transporter, reducing uptake and increasing efflux of the associated neurotransmitter (Xie et al., 2008).

Moreover, further evidence suggests that both TAAR1 and monoamine autoreceptor activation modulate monoamine transporter function reciprocally by way of opposing interactions on aminergic transmission (Xie et al., 2008). While TAAR1 activation promotes efflux of monoamines through their transporter proteins, autoreceptor activation leads to increases in the uptake of classical biogenic amines in monkey and wild-type mouse striatal and thalamic synaptosomes, with this effect being absent in synaptosomes from TAAR1 knockout (KO) mouse (Xie et al., 2008). When the DA, NE, and 5-HT transporters were co-transfected with D2s, $\alpha_{2A,}$ or 5-HT_{1B} autoreceptors, uptake of the respective amine transmitter was significantly enhanced. Conversely, NE and 5-HT reduced retention of the preloaded neurotransmitter in the presence of the specific autoreceptor antagonist in both monkey and wild-type mouse, but not in TAAR1 KO, synaptosomes (Xie et al., 2008). This

TAAR1-dependent monoamine efflux has been attributed to reversed transport of monoamines through their corresponding transporters resulting from TAAR1-mediated intracellular cAMP accumulation and substrate phosphorylation (Xie et al., 2008). Together, these findings indicate that the classical biogenic amines interact with both TAAR1 and monoamine autoreceptors to regulate transporter function. Thus, a concept of presynaptic receptor balancing has been proposed whereby TAAR1 and monoamine autoreceptors equilibrate monoamine activity, with the former inhibiting uptake and the latter facilitating it (Xie et al., 2008, Xie and Miller, 2009b).

More recent evidence from both *in vitro* and *in vivo* studies suggests a direct interaction of TAAR1 with monoamine autoreceptors that may underlie the presynaptic receptor balancing previously proposed. First, TAAR1 and D2s receptors, when co-expressed in cells, were able to form constitutive heterodimers in plasma membrane, thus allowing functional regulation of these two GPCRs and/or other cellular substrates (Espinoza et al., 2011). Indeed, both total and membrane expression level of TAAR1 was decreased by co-expressing it with D2s in the same cells (Espinoza et al., 2011). Such interactions between TAAR1 and D2s receptors are in part mediated by receptor heterodimerization. Previously, it was shown that co-transfecting D2s, α_{2A} or α_{2B}, or 5-HT_{1A} or 5-HT_{1B} along with TAAR1 attenuated TAAR1 activation-induced intracellular cAMP in response to DA, NE, or 5-HT, respectively (Xie et al., 2008). Thus, the facilitating effects of autoreceptor activation on monoamine transporter function could result from either direct enhancement or suppression of TAAR1-mediated inhibition of transporter function. Importantly, more recent studies indicated that TAAR1 activation may also potentiate D2s-mediated inhibition of monoamine transmission, an effect that is lacking in TAAR1 KO mice (Leo et al., 2014). Similarly, previous findings indicated that activating TAAR1 with specific agonists increased agonist potency at 5-HT_{1A} receptors whereas selective blockade of TAAR1 produced the opposite effects (Revel et al., 2011). Collectively, these findings suggest that TAAR1 stimulation may have dual effects on monoaminergic activity. While TAAR1's direct inhibition of the monoamine transporter may result in extracellular monoamine accumulation, TAAR1-mediated upregulation of

monoamine autoreceptors may lead to enhanced transporter function and depressed monoamine transmission.

Therefore, it would appear that the relative activation of TAAR1 and D2s receptors by endogenous or exogenous ligands critically determines the net output of monoaminergic systems through key effects on transporter regulation (Xie and Miller, 2009b). Unlike the common biogenic amines, which activate both types of receptors, the endogenous TAs are agonists at TAAR1 only. Consequently, selective TAAR1 stimulation by TAs or specific agonists is distinct from TAAR1 activation by classical monoamines in that these trigger inhibitory modulation of TAAR1 through autoreceptor co-stimulation. Not surprisingly, *in vitro*, the co-expression of monoamine autoreceptors with TAAR1 attenuated TAAR1 signaling in response to common monoamines, but not to *β*-PEA (Xie and Miller, 2008). In agreement with these findings, while the common biogenic amines significantly enhanced uptake in cells co-transfected with the respective monoamine autoreceptors and transporters, *β*-PEA did not (Xie and Miller, 2008).

Further accentuating the complexity of TAAR1 molecular interactions at monoamine synapses, recent studies have unveiled regulatory effects of TAAR1 signaling on postsynaptic D2 receptors. While TAAR1 KO mice had impaired striatal presynaptic D2s-mediated autoinhibition (Leo et al., 2014), they also exhibited upregulation of striatal postsynaptic D2 receptors mRNA and overactivity of D2 receptor-mediated G protein/cAMP-independent, β-arrestin2-dependent signaling pathway (Espinoza et al., 2015a). D2 receptor activation through β-arrestin2 has been shown to dephosphorylate AKT and its downstream target GSK3β, leading to inhibited AKT activity and subsequent increase in GSK3β signaling (Beaulieu et al., 2011). Decreased phosphorylation of AKT with concomitant elevation of GSK3β is associated with excessive dopaminergic stimulation, as produced either by indirect DA agonists including psychostimulants, such as amphetamine and cocaine, or by DAT deletion (Beaulieu et al., 2004, Li and Gao, 2011). Conversely, pharmacological or genetic inhibition of GSK3β reversed DA-stimulated behaviors and reduced the effects of

psychostimulants (Beaulieu et al., 2004, Beaulieu et al., 2005, Li and Gao, 2011). Therefore, the finding that striatal D2 receptors and associated AKT/GSK3β signaling pathway are upregulated in TAAR1 KO mice suggests that TAAR1 activation may counteract DA signaling at postsynaptic sites, although this effect is likely to be indirect. A recent study reported that the formation of heteromeric complexes between TAAR1 and D2 receptors not only reduces TAAR1-stimulated cAMP accumulation, but also shifts β-arrestin2 recruitment from activated D2 receptors to activated TAAR1 (Harmeier et al., 2015). Interestingly, TAAR1-mediated β-arrestin2 signaling leads to increased phosphorylation of AKT and GSK3β, indicating an enhanced AKT activation and a silencing of GSK3β activity, opposite to the known effect of β-arrestin2 on its downstream target (Harmeier et al., 2015) (Figure 2.1).

This complementary evidence suggests that TAAR1 may be able to downregulate DA transmission not only by potentiating D2s-mediated presynaptic autoinhibition but also through inhibiting D2 receptor-mediated postsynaptic signaling. In support of this notion, mice with TAAR1 depletion exhibited greater locomotor activity compared to wild-type counterparts when challenged with quinpirole, a D2-like receptor agonist that is known to inhibit locomotion at low doses via stimulating presynaptic D2s and enhance locomotion at high doses by activating postsynaptic D2-like receptors, suggesting that TAAR1 deletion may induce supersensitivity of postsynaptic D2-like receptors (Espinoza et al., 2015a).

Taken together, these findings suggest that multidirectional interactions occur between TAAR1 and monoamine molecular targets at both pre- and post-synaptic sites. However, knowledge derived from analysis at cellular and molecular levels raises the question as to the ultimate functional outcome of these complex interactions and the exact neurophysiological role of TAAR1. Inspection of the neurological and behavioral adaptations exhibited by transgenic mice with TAAR1 modifications provides additional insight into this question. Compared to wild-type littermates, TAAR1 KO mice exhibited no differences in general health indicators as well as general motor function (Wolinsky et al., 2007, Lindemann et al.,

2008), but displayed deficits in pre-pulse inhibition of acoustic startle, a DA-dependent response, indicating impaired sensorimotor gating (Wolinsky et al., 2007). Moreover, these KO mice had elevated spontaneous firing frequency and depolarized resting membrane potential of DA neurons in the VTA (Lindemann et al., 2008), amplified spontaneous spike rate of 5-HT neurons in the dorsal raphe nucleus (Revel et al., 2011), and increased extracellular DA in the NAc (Leo et al., 2014). Moreover, they showed enhanced sensitivity to amphetamine-induced locomotor activity and increase in extracellular DA, NE, and 5-HT levels in the striatum (Wolinsky et al., 2007, Lindemann et al., 2008), and METH-induced conditioned place preference (Achat-Mendes et al., 2012). When challenged with MDMA, the wild-type mice displayed dose-dependent, biphasic thermoregulatory responses with early hypothermia followed by hyperthermia, but TAAR1 KO mice only showed long-lasting hyperthermia accompanied by supersensitivity to MDMA-stimulated locomotor activity and release of DA and 5-HT in the NAc and dorsal striatum, and of DA in the frontal cortex (Di Cara et al., 2011). By contrast, mice engineered to overexpress TAAR1 showed unaltered spontaneous locomotor activity but hyposensitivity to amphetamine-induced psychomotor activity and catecholamine release in the NAc (Revel et al., 2012a). Taken together, these findings suggest that TAAR1 may be constitutively active or tonically activated by ambient levels of endogenous amines to exert an inhibitory influence on monoaminergic neurotransmission; and that TAAR1 activation may restrain the potentiation of monoamine transmission elicited by stimulant drugs. Actually, it has been suggested that TAAR1 may be recruited by amphetamine-class drugs, such as MDMA and METH, which are themselves agonists at TAAR1, to modulate their neurochemical and behavioural actions (Lindemann et al., 2008, Di Cara et al., 2011, Miller, 2011).

2.5 Target TAAR1 to Treat Stimulant Addiction

The very recent engineering of the highly selective TAAR1 agonists and antagonists has provided the first direct tools to investigate the functional role of this receptor in monoamine transmission. It has been shown that the selective TAAR1 antagonist, EPPTB, increased the firing rate of DA neurons in mouse VTA (Bradaia et al., 2009), which is in agreement with

the idea that TAAR1 is constitutively active or tonically activated by ambient levels of amines to downregulate DA tone. Moreover, while the selective TAAR1 full agonists, RO5166017 and RO5256390, decreased the firing frequency of DA neurons in the VTA and of 5-HT neurons in the dorsal raphe nucleus, the partial agonists, RO5203648 and RO5263397, enhanced the firing rate of these same neurons, acting in the similar manner as the selective antagonist, further confirming the constitutive activity or tonic activation status of TAAR1 such that partial agonism results in an antagonistic-like effect (Revel et al., 2011, Revel et al., 2012b, Revel et al., 2013). Further, the full agonist, RO5166017, has been recently reported to reduce electrically evoked DA release in both the dorsal striatum and NAc in slices of mouse brain, whereas the antagonist, EPPTB, increased DA release in the NAc (Leo et al., 2014), demonstrating homogenous inhibitory effects of TAAR1 at dopaminergic cell bodies and terminals. In addition, these findings provide evidence that full activation of TAAR1 is capable of actively suppressing DA signalling and DA release.

As a result, the critical hypothesis arises that targeting TAAR1 pharmacologically may provide a therapeutic means to modulate the characteristic aberrant DA transmission that hallmarks the addictive process, and may present a novel avenue for medicinal development in psychostimulant addiction. Early *in vivo* studies with the highly selective TAAR1 agonists have offered preliminary evidence supporting this possibility. Firstly, an important behavioural manifestation of psychostimulant-induced increase in mesocorticolimbic DA transmission in rodents is locomotor hyperactivity and, at high doses, stereotypies, which are repetitive, invariant behavioural patterns with no obvious goal or function, consisting of repetitive head bobbing, gnawing, sniffing, licking, and biting (Fog, 1969, Schuster, 1981, Mason, 1991, Nakagawa et al., 2011). It has been shown that, the full agonists, RO5256390 and RO5166017, and the partial agonists, RO5263397 and RO5203648, reduced cocaine- or *d*-amphetamine-stimulated hyperlocomotion or stereotypies in mice or rats, with little or no effect on locomotor activity when administered alone (Revel et al., 2011, Revel et al., 2012b, Revel et al., 2013). In addition, mutant mice that lack the *Slc6a3* gene encoding the DAT exhibited spontaneously elevated locomotor activity. Likewise, the full agonist, RO5166017,

and the partial agonist, RO5203648, reduced the spontaneous hyperactivity in these mice (Revel et al., 2011, Revel et al., 2012b). Together, these observations suggest that full or partial TAAR1 activation is sufficient to counteract behavioural abnormalities induced by excessive DA transmission, and highlight the remarkable potential of TAAR1-based compounds to modulate the pathological neuroadaptations that abused drugs produce on the DA system.

In particular, the use of partial agonists may be more advantageous than full agonists in situations where drug-induced neurochemical imbalance leads to insufficient or excessive TAAR1 stimulation, providing a means to "stabilize" TAAR1 activity. In turn, this bidirectional regulation on TAAR1 activation level through partial agonism allows a state-dependent modulation of DA neurotransmission which fluctuates across different stages in the addiction cycle. As previously described, the acute euphoria effect of the drug that positively reinforces drug taking at early stages is mediated by large increases in DA transmission in the NAc, while the negative hedonic affective state marking the withdrawal stage is caused by a reduced mesolimbic DA function that progressively worsens with chronic drug use (Koob and Le Moal, 2008a, b). In addition, an activation of DA transmission is involved in the reinstatement of drug seeking induced by re-exposure to drug-associated cues (Ito et al., 2000, Weiss et al., 2000) or drug itself (Stewart, 2000, Di Ciano et al., 2001). For instance, in a typical cocaine or *d*-amphetamine self-administration session, response during the initial "loading" phase, which is characterized by rapid accumulation of drug infusions, is accompanied by elevation in extracellular DA concentration in the NAc until a peak DA level is reached. During the subsequent "maintenance" phase where response is well-spaced with relatively uniform inter-response intervals, DA level remains tonically elevated but displays phasic fluctuations that are time-locked to the periodic lever pressing (Kiyatkin and Stein, 1995, Wise et al., 1995, Ranaldi et al., 1999). By contrast, DA concentration gradually declines in an acute extinction session despite increased lever pressing, with the period of highest responding rate marked by the greatest DA decline. Moreover, when response subsides during later phase of extinction, a

single non-contingent drug infusion or drug-paired stimulus causes an immediate increase in DA level in the NAc and a recovery of lever pressing (Ranaldi et al., 1999, Weiss et al., 2000).

Thus, partial activation of TAAR1 may attenuate acute drug reward and drug-taking by suppressing drug-stimulated DA overactivity, reduce withdrawal-associated anhedonia and drug-craving by compensating for diminished DA, and prevent cue- or drug-induced reinstatement of drug seeking through inhibiting hyperdopaminergic response to the cue or drug itself. On the other hand, full activation of TAAR1 may have a more general inhibitory influence on the DA system, which is more beneficial in situations where DA transmission is potentiated by psychostimulants such as during drug intoxication and relapse. In the long term, the disrupted DA system is expected to gradually become stabilized and "normalized" under TAAR1 regulation, allowing for a longer period of or even lifetime abstinence, which is the final goal of an efficacious anti-addiction treatment.

However, due to the unavailability of highly selective TAAR1 agonists until very recently, early attempts to directly test the hypothesis that TAAR1 activation may be effective in reducing neurochemical and behavioural markers of psychostimulant addiction are extremely scarce. The abovementioned studies where several TAAR1 agonists reversed cocaine- or *d*-amphetamine-induced motor response are informative because an increase in extracellular DA levels in the mesolimbic pathway, especially the NAc, has been postulated as a common mechanism for the acute motor-stimulating and reinforcing effects of psychostimulants (Vezina, 2004). A recent study assessed the effects of the partial TAAR1 agonist, RO5203648, on cocaine self-administration in rats. The results showed a dose-dependent reduction of response rate for cocaine (Revel et al., 2012b). Recent neurochemical data has showed that RO5203648 effectively reduced cocaine-induced DA overflow in the NAc in rat brain slices (Pei et al., 2014), which may at least partially account for TAAR1's regulation of cocaine-induced behavioural effects. Together, these findings provided preliminary

confirmation of the anti-addiction properties of TAAR1 agonists and paved the way for further investigations[2].

2.6 The Present Work

The general aim of the present thesis was to test the hypothesis that TAAR1 may present a novel target for therapeutics in stimulant addiction. This was achieved by means of a systematic investigation of the functional regulation by TAAR1 of key behavioural aspects associated with psychostimulant action as well the physiological mechanisms underlying the effects of TAAR1. We utilized well-validated animal models of addiction that are associated with specific elements of the addiction process. The selective partial agonists, RO5203648, RO5263397, and compound M, were used as the primary pharmacological tools for this investigation. The full agonist, RO5256390, was also tested in some models for comparison purposes. The overarching goal was to provide a step-by-step assessment of the potential therapeutic-like effectiveness of TAAR1 agonists in relevant models of stimulant addiction, and to understand the underlying physiological mechanisms. This was accomplished across three sets of experiments (10 experiments in total described in chapters three to five). The below sections describe the rationale and objectives of each experiment.

2.6.1 Experiment set one

The first set of experiments (experiments 1-2) investigated the effect of TAAR1 partial agonism on the locomotor-activating effects of METH and explored the neurobiological basis for this effect (chapter three). Acute cocaine or amphetamine produces hyperlocomotion in rodents, and repeated exposure to these drugs leads to behavioural sensitization, which is a progressive increase in their ability to elicit locomotor activity. When established, behavioural sensitization is enduring as animals may remain hypersensitive to the motor-activating effects of drugs for months or years after the discontinuation of drug treatment (Robinson and Berridge, 1993, Robinson and Berridge, 2001, Vezina, 2004).

[2] During the three-year PhD, new findings have been reported with those selective TAAR1 ligands. To avoid confusions about the presented rationale which is based on earlier evidence, these newer findings will be considered in the discussion section in the following experimental chapters in relation to the present data.

Although hyperlocomotion and behavioural sensitization are not common phenomena in human addicts, they are of the most typical behavioural effects observed in rodents and, as previously mentioned, both have been related to addiction processes because the neural substrates underlying these effects at least partially overlap with those responsible for drug reward and the transition to addiction (Wise and Bozarth, 1987, Robinson and Berridge, 2001). In particular, the acute ability of psychostimulants to increase extracellular levels of DA in the NAc has been critically associated with both their locomotor-activating effects and reinforcing efficacy (Robinson and Berridge, 2001, Vezina, 2004). Moreover, the long-term neuroadaptations produced by repeated drug exposure that underlie behavioural sensitization and escalation of drug use both involve sensitized dopaminergic responses in the NAc to the drug (Vezina, 2004). Also, the long-lasting potentiation of DA neuronal responsiveness caused by chronic drug exposure has been linked to prolonged relapse vulnerability after cessation of drug use (Shaham and Hope, 2005). For these reasons, one of the pharmacotherapeutic strategies for stimulant addiction is to develop medications that are able to reverse neurobiological changes induced by acute and chronic drug exposure, which can be assessed in models that produce hyperactivity and behavioural sensitization after specific drug treatment regimens. Therefore, one of the objectives of the present work is to study the ability of TAAR1 agonists to modulate psychostimulant-induced locomotor changes as an initial step in the evaluation of their therapeutic potential in stimulant abuse.

Although this question has been addressed to some degree previously (Revel et al., 2011, Revel et al., 2012b, Revel et al., 2013), these studies did not provide a full picture on the ability of TAAR1 to regulate the psychomotor-activating effects of stimulants. Thus, experiments 1-2 further explored this issue. Briefly, the ability of TAAR1 partial activation to modulate locomotor activity produced by acute (experiment 1) and repeated (experiment 2) METH was assessed with the selective TAAR1 partial agonists, RO5203648 (experiment 1) and compound M (experiment 2). Experiment 2 also examined how compound M influenced the effects of chronic METH on the inducibility of the immediate early genes (IEGs), c-fos and activity-regulated cytoskeleton-associated protein (arc), and the transcriptional regulator,

methyl CpG binding protein 2 (MeCP2), in brain areas important for addiction in order to gain insight into the underlying neurobiological mechanisms.

2.6.2 Experiment set two

The second set of experiments (experiments 3-6) examined the effect of partial and full TAAR1 activation on the reinforcing efficacy of psychostimulants (chapter four). Psychostimulants are characterized by their powerful intrinsic reinforcing and rewarding properties, which is one of the major contributors to the initiation and maintenance of drug taking in human addicts and critically underlies their ability to sustain self-administration in animals. The reinforcing efficacy of a drug has been suggested to correlate positively with its actual abuse liability (Schuster, 1981). Thus, the ability of a medication to decrease the reinforcing effects of psychostimulants may be indicative of anti-addiction potential (Mello and Negus, 1996). In this regard, animal models of self-administration, which closely mimic human drug taking, afford a valuable approach for the preclinical evaluation of new pharmacotherapies for addiction (Mello and Negus, 1996, Pierce and Kumaresan, 2006).

There is only one existing study that has examined the influence of a TAAR1 agonist on cocaine self-administration behaviour in rats, which showed a decrease in cocaine responding (Revel et al., 2012b). However, only a single dose of cocaine (0.5 mg/kg/infusion) was examined in this study, making interpretation of the results problematic. It has been consistently reported that, within the range of doses that sustain self-administration, animals adjust their response as a function of the unit injection dose of the drug. For drugs like cocaine and METH, the dose-response function typically displays an inverted U-shaped curve characterised by an ascending limb, where increases in unit doses of the drug result in increased self-administration until a peak level is reached, and a descending limb, where further increases of drug dose lead to decreased self-administration (Nader and Reboussin, 1994, Mello and Negus, 1996, Shippenberg and Koob, 2002, Clemens et al., 2006, Sharpe et al., 2014). A treatment medication may affect the dose-response curve in three possible ways, causing a rightward, leftward, or downward shift of the curve, which, respectively, suggests

an antagonism, potentiation, or complete blockade of the reinforcing effects of the drug. The ideal therapeutic outcome is a downward shift which results in decreased drug-taking across all of the drug doses. Shifting the curve to the right is less satisfactory because it decreases responding rate at some unit doses but simultaneously increases responding at other doses, representing a simple change in drug potency that can be compensated by self-adjusting the amount and frequency of drug intake (Mello and Negus, 1996). Therefore, a complete dose-effect function for drug self-administration should be constructed to evaluate the treatment effectiveness of a medication (Mello and Negus, 1996, Shippenberg and Koob, 2002). Hence, in order to extend the previous study that examined a single dose of cocaine, experiment 3 explored the effect of full and partial TAAR1 activation on the dose-response function for cocaine self-administration, with the use of the full agonist, RO5256390, and the partial agonist, RO5203648.

In addition, the PR schedule has been widely adopted by self-administration studies to investigate the reinforcing property of a drug and to assess the effectiveness of pharmacological pretreatment (Arnold and Roberts, 1997). Under the PR schedule, the number of responses required to obtain each subsequent drug infusion systematically increases, and the final response ratio in the series at which responding ceases is defined as the breaking point (BP), which reflects the maximum effort that an animal will expend to earn a drug infusion and thus provides a measure of motivation to take the drug (Richardson and Roberts, 1996, Arnold and Roberts, 1997). Here, to gain further insight into TAAR1's regulation of the reinforcing efficacy of psychostimulants, we tested the effects of the selective partial TAAR1 agonists, RO5203648 (experiment 4) and RO5263397 (experiment 5), on the self-administration of cocaine or METH, respectively, under a PR schedule of reinforcement. Besides, to control for potential side effects of TAAR1 agonists on non-drug related behaviour, such as motor function and motivation for natural reinforcers, the influence of these two partial agonists on the motivation for food was examined using the same PR schedule.

The last experiment in this chapter aimed to gain insight into the neuronal mechanism involved in TAAR1's regulation of psychostimulant action. It was recently reported that RO5203648 blocked cocaine-induced DA release in the NAc in rat brain slice as measured by fast-scan cyclic voltammetry (FSCV) (Pei et al., 2014), which is consistent with the hypothesized counteracting effects of TAAR1 on DA transmission. Experiment 6 extended this finding by measuring the influence of RO5263397 on METH-evoked DA overflow in the NAc core using FSCV. The NAc core was chosen because it is a major action site for psychostimulants and critically mediates the transition to compulsive drug use and addiction. Given the rich expression of TAAR1 in the NAc, local application of RO5263397 within this region was expected to attenuate the DA augmentation produced by METH.

2.6.3 Experiment set three

As will be described in detail in the following experimental chapters, the results obtained from experiment sets one and two provided evidence on the ability of TAAR1 to modulate several important effects of psychostimulants. The third set of experiments was designed to further explore TAAR1's anti-addiction properties by assessing the therapeutic-like potential of TAAR1 agonists in relapse, which is one of the core features of addiction and represents a major obstacle in addiction treatment (chapter five). Experiment 7 employed a model of context-induced renewal of drug-seeking after prolonged abstinence without extinction, which approximates human situations where re-experiencing the environment associated with past drug-taking behaviour triggers relapse of drug seeking after a long drug-free period (Fuchs et al., 2006, Ferragud et al., 2009). It has been shown that DA transmission in the mesolimbic pathway plays a role in context-induced reinstatement (Crombag et al., 2008), and elevation of DA concentrations by drug-associated stimuli has been reported in the dorsal striatum (Volkow et al., 2006) and the NAc (Fontana et al., 1993). Thus, given the known ability of TAAR1 to dampen DA activity, pharmacological activation of TAAR1 was expected to attenuate context-induced renewal of drug-seeking. Experiment 7 tested this hypothesis in cocaine relapse with the full agonist, RO5256390, and the partial agonist, RO5203648. In addition, the effect of the two agonists on food self-administration was

examined in order to control for their non-specific effects on general motoric and appetitive functions.

Another model that has been widely used to study relapse is the extinction-reinstatement model, in which animals undergo extinction training until response rate decreases to a set criterion and reinstate drug seeking when challenged with the drug itself, drug-associated cues, or stressors (Shalev et al., 2002, Shaham and Hope, 2005). The neurobiological mechanisms underlying the three types of reinstatement have been found to be partially dissociable. Whereas both drug- and cue-induced reinstatement largely involve DA-mediated processes that differ to some degree in pharmacology and neuroanatomy, stress-induced reinstatement seems to rely more on the CRF and the NE systems (Shalev et al., 2002). Because the functional modulation by TAAR1 has been much better characterized for DA compared to other neurotransmitter systems, stress-induced reinstatement was not assessed in the current thesis. Moreover, the effects of re-exposure to drug-paired cues after extinction may be to certain degree analogous to the effects of re-exposure to drug-associated context after abstinence (experiment 7), as both processes involve learned associations between drug-associated cues, including both discrete cues and contextual cues, and the drug's pharmacological effects (Bossert et al., 2013). For this reason, cue-induced reinstatement was not examined in the present thesis, but further studies are warranted given the limited overlap in the neuronal substrates for drug seeking after extinction versus abstinence (Fuchs et al., 2006). Therefore, experiments 8 and 9 investigated the ability of TAAR1 activation to suppress cocaine (experiment 8) or METH (experiment 9) reinstatement induced by a prime injection of the previously self-administered drug. As our findings (from experiment 7) indicated a more desirable profile of the partial TAAR1 agonist than that of the full agonist, only the partial agonists, RO5203648 (experiment 8) and RO5263397 (experiment 9), were tested in this model.

The last experiment (experiment 10) aimed to determine the potential abuse liability of TAAR1 agonists, which is an important aspect to consider in drug development. The

self-administration paradigm, due to its high level of face and predictive validity, has been regarded as the gold standard for preclinical abuse liability screening of novel medications (Haney and Spealman, 2008, Carter and Griffiths, 2009). A typical method is the substitution procedure where a dose of the test compound is substituted for a dose of a reinforcing drug that is already maintaining a high level of self-administration. The response rate for the test compound obtained at different doses is compared with that of the initial reinforcing drug and vehicle, which serve as a positive and negative control, respectively (Ator and Griffiths, 2003, Carter and Griffiths, 2009). In experiment 10, the partial agonist, RO5263397, was substituted at two different doses for METH in rats with a stable METH self-administration history.

Chapter Three

3 TAAR1 Activation Modulates Psychostimulant-Induced Locomotor Activity and Neuronal Plasticity

3.1 Introduction

It is well-established that acute psychostimulant treatment produces locomotor hyperactivity, an effect that is mediated by an increased mesolimbic DA transmission. With repeated exposure, the mesolimbic DA pathway is gradually sensitized, displaying augmented responsiveness to further drug challenges. This translates into behavioural sensitization, which is a progressively enhanced ability of psychostimulants to elicit locomotor activity (Robinson and Berridge, 2001, Vezina, 2004). It has been suggested that the short- and long-term neurobiological changes involved in the locomotor-activating effects of psychostimulants are also involved in drug reward, escalation of drug use, and propensity to relapse (Robinson and Berridge, 2001, Vezina, 2004, Shaham and Hope, 2005, Pierce and Kumaresan, 2006). Thus, pharmacological manipulations that counteract the locomotor effects of psychostimulants could have therapeutic implications in addiction.

Previous studies examining the influence of TAAR1 selective agonists on the acute locomotor-enhancing effects of psychostimulants have generally reported a blockade of cocaine- or *d*-amphetamine-induced hyperactivity in rats or mice (Revel et al., 2011, Revel et al., 2012b, Revel et al., 2013). However, in these studies, locomotor activity was monitored only for a short period (30 min) and reported as total distance travelled, providing limited data as to the magnitude and duration of TAAR1's effects. Thus, experiment 1 aimed to investigate TAAR1's regulation of acute METH-induced locomotor activity over an extended period of time (3-h) during which locomotor fluctuations were examined across time[3].

[3] Published paper. Experiment 1 has been published in the following paper: Cotter R, Pei Y, Mus L, Harmeier A, Gainetdinov RR, Hoener MC, Canales JJ (2015) The trace amine-associated receptor 1 modulates

Because the effects of METH on locomotion and DA activity may subside in the later phase of the prolonged test, a partial agonist (RO5203648) was chosen given its potential ability to bidirectionally influence TAAR1 activation and therefore state-dependently regulate DA transmission.

In addition, the effects of selective TAAR1 agonists on METH-induced behavioural sensitization have not yet been tested. Thus, experiment 2 explored this question with another partial agonist, compound M. In order to gain insight into the underlying neuronal mechanisms of TAAR1's modulatory effects, the associated changes in the brain expression of c-Fos, Arc, and MeCP2 were examined following the last METH challenge in the final probe test.

3.2 Materials and Methods

3.2.1 Subjects

Male Long Evans rats (*n* = 35, for experiment 1) and male Lister hooded rats (*n* = 32, for experiment 2) were sourced from the University of Canterbury and the University of Leicester, respectively. Rats were around 8 weeks-old when experiments began and were housed in a temperature and humidity controlled colony room with a 12-h light/dark cycle (lights off at 8 a.m.). Water and standard laboratory rat chow was given *ad libitum* at all times. Experiments were conducted in compliance with the New Zealand Animal Welfare Act 1999, and approved by the University of Canterbury Animal Ethics Committee, and with appropriate project and personal license authority granted by the UK Home Office under the Animals (Scientific Procedures) Act 1986.

3.2.2 Pharmacological agents

METH hydrochloride was obtained from BDG Synthesis (Wellington, New Zealand) and Sigma-Aldrich (UK) and dissolved in 0.9% physiological saline for intraperitoneal (i.p.) injection. RO5203648 and compound M were synthesized at F. Hoffmann-La Roche Ltd.

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methamphetamine's neurochemical and behavioral effects. Frontiers in Neuroscience 9:39. doi: 10.3389/fnins.2015.00039.

(Switzerland) and dissolved in 10% dimethylsulfoxide in 0.9% physiological saline or 2% Tween20 in distilled water for i.p. injection. All drugs were prepared fresh on the day.

3.2.3 Apparatus

Locomotion test was conducted in a set of four open field boxes made of black Perspex (50 × 40 × 35 cm for experiment 1; 50 × 50 × 30 cm for experiment 2). Locomotor activity was monitored and measured with a video tracking system and image analysis software (Viewpoint 2.5, Champagne au Mont D'Or, France for experiment 1; ANYMAZE software for experiment 2) that provided automatic measures of travelled distance, trajectory, and velocity of the subjects.

3.2.4 Behavioural procedures (experiments 1 and 2)

In experiment 1, six groups of rats (n = 5-6 per group) were habituated in the open field for 10 min for two consecutive days. During the test, rats were given a pretreatment of RO5203648 (0, 5, or 10 mg/kg, i.p.) followed 15 min after by METH (0 or 0.75 mg/kg, i.p.). 10 min after METH treatment, rats were placed into the open field and locomotor activity was measured for 3 h. Four rats were tested concurrently in four separate open fields. Locomotor activity was estimated as distance travelled and recorded in 20 min bins.

In experiment 2, six groups of rats were habituated in the open field for 20 min for two consecutive days. During the 10-day sensitization period, rats received daily treatment of compound M (0, 10, or 20 mg/kg, i.p.) followed 15 min after by METH (0 or 1.25 mg/kg, i.p.) and were allowed to freely explore the open field for 60 min. Four rats were tested concurrently in four separate open fields. Locomotor activity was estimated as distance travelled and recorded in 10 min bins. After the sensitization period, rats underwent withdrawal from all pharmacological treatments for ten consecutive days. The group of rats that received vehicle pretreatment followed by saline injection was further divided into a Vehicle-Saline group and a control group with similar mean locomotor measures. This resulted in seven groups in total (n = 4-5 per group). On day 21, all rats received a challenge with a low dose of METH (1 mg/kg, i.p.) to probe for sensitization, except for the control

group which received a saline injection. Locomotor activity was monitored for 60 min and recorded in 10 min bins in the probe test.

3.2.5 Immunohistochemistry and microscopy (experiment 2 cont.)

All rats from experiment 2 were deeply anesthetized with sodium pentobarbitone (100 mg/kg) 3 h after the challenge injection of METH or saline in the final probe test. Rats were perfused transcardially first with 0.9% saline followed by 4% paraformaldehyde (pH 7.4, dissolved in 0.1 M phosphate buffer, PB). Brains were removed and submerged in 4% paraformaldehyde for 24 h at 4 °C, and then transferred into 15% sucrose (in 0.1 M phosphate buffered saline, PBS) for 48 h, and finally to 30% sucrose (in 0.1 M PBS) at 4 °C until tissue sank. Brains were then fast frozen with isopentane and dry ice and stored at -80 °C until sectioning. Brains were coronally sectioned at 30 µm thickness using a cryostat. Sections were collected through the PFC to the striatum, six parallel series per brain, and stored in 0.1 M PB containing 0.1% sodium azide at 4 °C prior to immunohistochemical staining.

The first three parallel series of each brain were selected for immunohistochemical staining of c-Fos, Arc, and MeCP2, respectively. For c-Fos staining, free floating sections were rinsed three times with 0.1 M PBS for 5 min, treated 10 min with 3% H_2O_2 in PBS, and washed three times with PBS containing 1% Triton X-100 (PBS-Tx) for 5 min. The sections were then incubated with 5% normal goat serum (Biorbyt, UK) in PBS for 30 min, washed three times with PBS for 5 min, and incubated for 48 h with gentle rotation at 4 °C in a rabbit polyclonal anti-Fos primary antibody (Insight Biotechnology, UK) at 1:2000 in PBS-Tx with 1% normal goat serum. After three rinses in PBS for 5 min, sections were incubated with secondary biotinylated Goat Anti-Rabbit IgG Antibody (Vector Laboratories, UK) at 1:500 in PBS with 1% normal goat serum, and washed in PBS for 5 min three times. This was followed by 1-h incubation in ABC mix from VECTASTAIN Elite ABC Kit (Standard*) (Vector Laboratories, UK) at 1:200 in PBS and three times wash in 0.1M PB for 5 min. Sections were then reacted with nickel-enhanced 3,3’-diaminobenzidine (DAB, composition 0.25 mg/ml DAB, 0.8 mg/ml nickel (II) sulfate hexahydride, 0.0035% H_2O_2 in 0.1 M PB) for

5 min and washed three times in 0.1 M PB for 5 min. The process for staining MeCP2 and Arc was identical to that of c-Fos, except that sections were incubated for 48 h at 4 °C in a rabbit polyclonal anti-MeCP2 primary antibody (Merck Millipore, UK) or a rabbit polyclonal anti-Arc primary antibody (SYSY, Germany), respectively, at 1:2000 in PBS-Tx with 1% normal goat serum.

The brain regions of the prelimbic cortex, the NAc core, and the NAc shell were selected for the quantification of immunoreactivity of c-Fos, Arc, and MeCP2 protein as an index of expression of each protein. Photomicrographs containing one of the selected areas (4-6 photographs per site) were taken at 40 × magnification with a digital Panasonic camera (17 megapixels) attached to a Zeiss microscope. The number of immunoreactive neurons for each protein was counted manually and averaged for each selected area. Results were expressed as cell density (number of stained neurons per mm^2).

3.2.6 Statistical analysis

Data were analysed by analysis of variance (ANOVA) with repeated measures when a within-subjects design was in use. *Post hoc* comparisons were conducted with the method of Newman-Keuls (N-K) using the sampling error from the overall ANOVA as denominator or with Fisher's PLSD test. Statistical significance was set at $\alpha = 0.05$. Statistical analyses were performed using StatView 5.0 (SAS Institute, NC, USA).

3.3 Results and Summary

3.3.1 Experiment 1 results

RO5203648 altered METH-induced locomotor activity in a time-dependent fashion, producing an early attenuation followed by a striking late potentiation. A repeated measure ANOVA for locomotor activity in 20 min bins across the 3-h test showed a significant effect of treatment ($F_{5, 29} = 15.80$, $p < .0001$) and time ($F_{8, 232} = 15.94$, $p < .0001$), as well as a significant interaction between these factors ($F_{40, 232} = 8.35$, $p < .0001$) (Figure 3.1). METH produced high levels of locomotor activity in the first 20 min that decreased gradually over the rest of the test. RO5203648 significantly attenuated METH-induced hyperlocomotion

during the first h at both the low and the high doses ($p < .01$ for the 1-3rd bins, by N-K tests). However, as the effect of METH treatment alone slowly subsided, RO5203648 potentiated METH's effect by maintaining locomotor activity at moderately high levels, which were significantly higher than that produced by METH alone at both the low dose ($p < .05$ for the 6th bin, $p < .01$ for the 7-9th bins, by N-K tests) and the high dose ($p < .05$ for the 5th bin, $p < .01$ for the 6-9th bins, by N-K tests) of RO5203648.

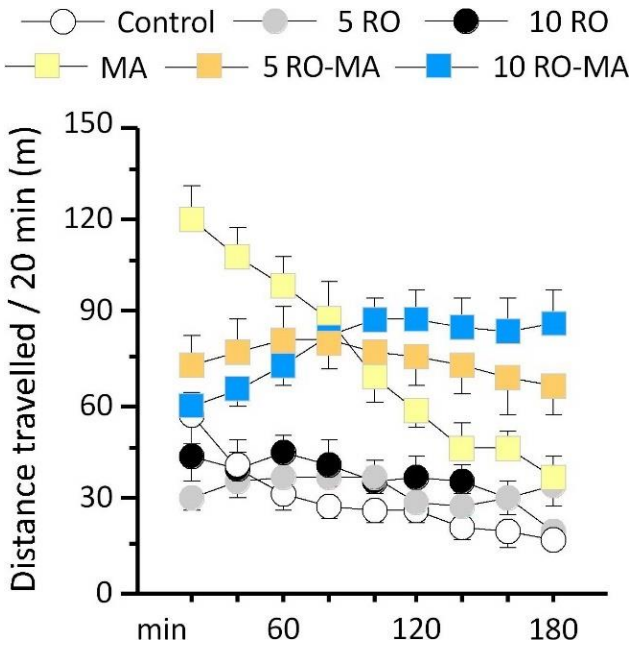

Figure 3.1 RO5203648 time-dependently modulated METH-induced locomotion
METH produced robust increases in locomotor activity compared with control treatment. RO5203648 time-dependently modulated METH-stimulated locomotor response characterized by an early attenuation and a late potentiation when METH's effect began to subside. 5 RO = 5 mg/kg RO5203648, 10 RO = 10 mg/kg RO5203648, MA = methamphetamine.

3.3.2 Experiment 1 summary

To examine TAAR1 modulation of the locomotor-activating effects of acute METH, RO5203648 (0, 5, or 10 mg/kg, i.p.) was given 15 min prior to METH (0 or 0.75 mg/kg, i.p.) and locomotor activity was examined for 3 h. RO5203648 significantly attenuated

METH-induced hyperlocomotion in the early phase of the test, but potentiated it in the later phase when METH's effects began to decay, indicating a time-dependent interaction between METH and the partial agonist.

3.3.3 Experiment 2 results

During the 10-day sensitization phase, compound M administered 15 min prior to METH had no significant effects on METH-induced hyperlocomotion at either the low or the high dose. When given on its own, compound M did not alter baseline locomotor behaviour at either dose compared with the saline treatment.

Following the sensitization phase, rats underwent a 10-day withdrawal period with no pharmacological exposure. Behavioural sensitization was tested by giving a METH challenge on day 21 to all the rats except for the control group which received saline. A repeated measure ANOVA for locomotor activity from the 2nd 10 min bins revealed a significant main effect of treatment ($F_{6, 25} = 4.57$, $p < .005$) and time ($F_{4, 100} = 4.46$, $p < .005$). *Post hoc* comparisons showed that previous repeated METH treatment significantly enhanced the locomotor response to the METH challenge compared with repeated saline treatment ($p < .05$, by Fisher's PLSD), demonstrating the expression of behavioural sensitization, which persisted after a prolonged withdrawal period. This expression of METH sensitization was not altered by previous compound M pretreatment at either dose. Repeated treatment with compound M alone had no significant effects on subsequent METH-induced locomotor response relative to saline treatment, but the high dose group showed a significantly higher level of locomotion compared with the low dose group ($p < .05$, by Fisher's PLSD), with the average for the saline group being between these two groups (Figure 3.2).

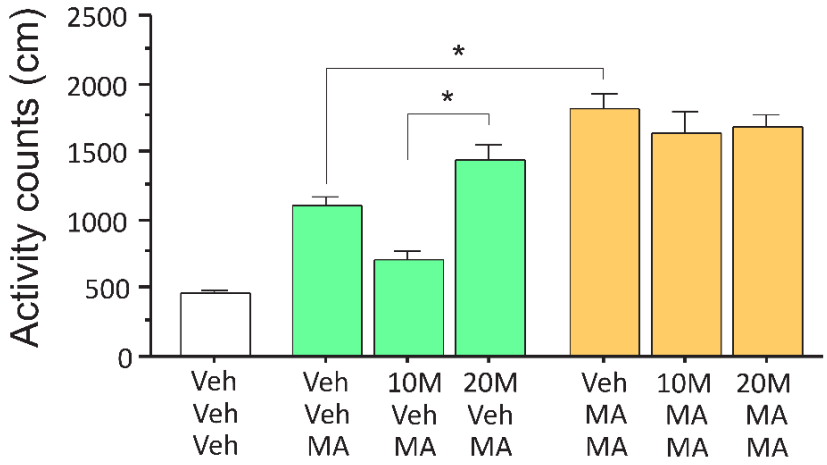

Figure 3.2 Compound M altered subsequent locomotor response to a METH probe
On the final probe test, previous chronic METH treatment significantly increased METH-probed locomotor response comparing to previous saline treatment, which was not altered by compound M concurrent administration with METH. However, previous compound M treatment alone produced a dose-dependent effect on locomotor response to the METH challenge. * $p < .05$. Veh = vehicle, 10M = 10 mg/kg compound M, 20M = 20 mg/kg compound M, MA = methamphetamine.

Immunohistochemistry was performed to examine the effects of compound M on the expression of c-Fos, Arc, and MeCP2 in the NAc core and shell, and the prelimbic cortex after the final METH challenge. One-way ANOVA revealed a significant effect of treatment on the expression of c-Fos in the NAc core ($F_{6, 25} = 29.99$, $p < .0001$) and shell ($F_{6, 25} = 13.89$, $p < .0001$), and the prelimbic cortex ($F_{6, 25} = 12.74$, $p < .0001$); on the induction of MeCP2 in the NAc core ($F_{6, 25} = 4.69$, $p < .005$) and shell ($F_{6, 25} = 6.15$, $p < .001$); and on Arc expression in the NAc core ($F_{6, 25} = 7.64$, $p < .0001$) and shell ($F_{6, 25} = 5.16$, $p < .005$), and the prelimbic cortex ($F_{6, 25} = 5.75$, $p < .001$). *Post hoc* comparisons with Fisher's PLSD test showed that a history of repeated METH exposure significantly increased METH-probed expression of c-Fos, Arc, and MeCP2 in the NAc core and shell, but not in the prelimbic cortex, relative to previous saline treatment ($p < .05$). Compound M, at the high dose, when co-administered with METH, enhanced chronic METH-induced c-Fos elevations in the NAc core ($p < .05$) and the prelimbic cortex ($p < .05$). When administered on its own, compound M increased

c-Fos inducibility in the NAc core at both the low and the high doses relative to saline treatment ($p < .05$) (Figure 3.3).

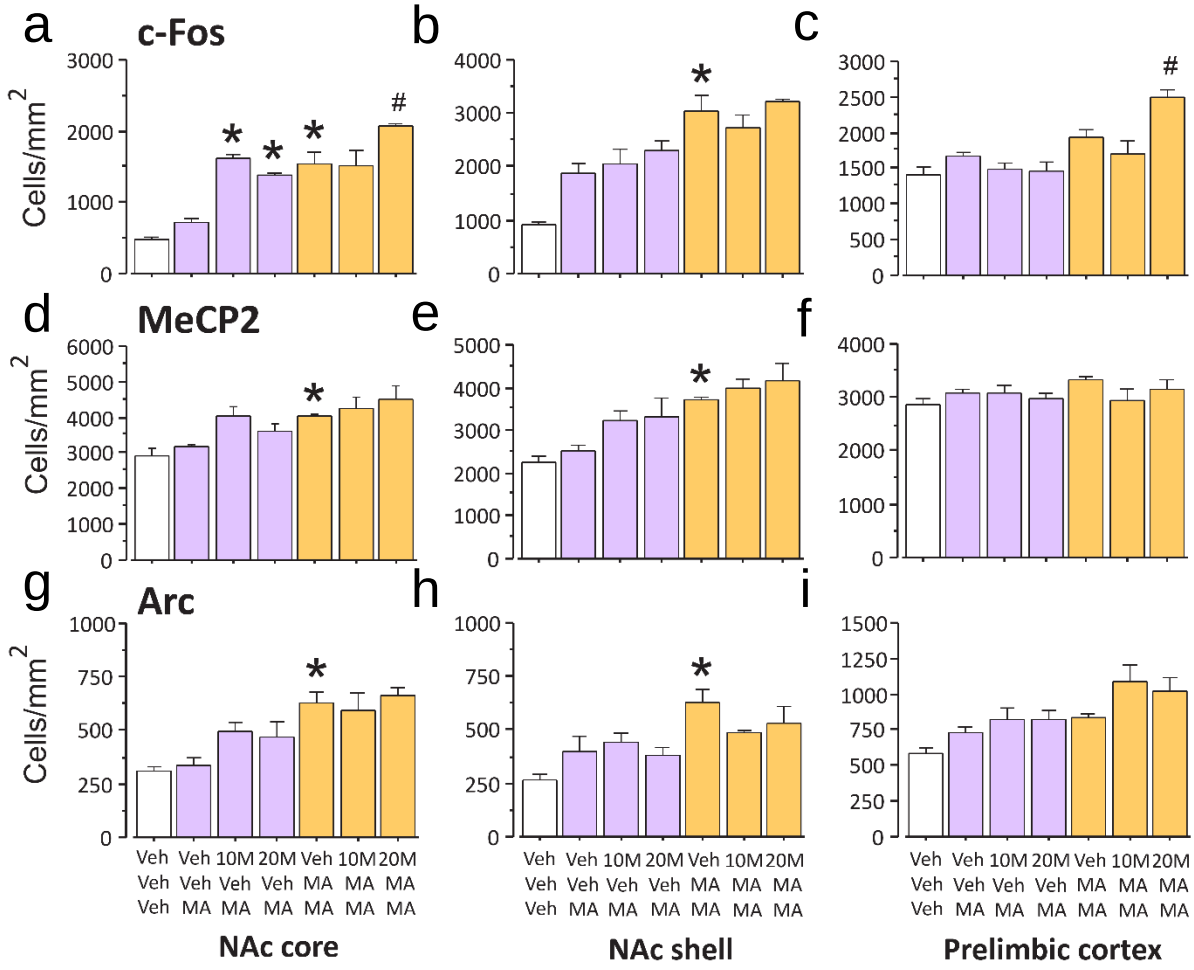

Figure 3.3 Compound M modulated the effects of repeated METH on brain gene inducibility A history of repeated METH exposure significantly elevated subsequent METH-inducible c-Fos (a and b), MeCP2 (d and e), and Arc (g and h) in the NAc core and shell. Compound M at the high dose potentiated METH-induced increase in c-Fos inducibility in the NAc core (a) and prelimbic cortex (c). Previous compound M treatment alone also increased METH-probed c-Fos expression in the NAc core at both doses (a). * $p < .05$ significantly different from previous saline treatment; # $p < .05$ significantly different from previous repeated METH treatment. Veh = vehicle, 10M = 10 mg/kg compound M, 20M = 20 mg/kg compound M, MA = methamphetamine.

3.3.4 Experiment 2 summary

Compound M pretreatment had no effect on METH-induced locomotor activity during the sensitization phase or on the expression of METH behavioural sensitization on the probe test at either the high or the low dose. When applied singly, compound M at both doses did not affect locomotor behaviour during the acquisition phase or on the probe test after METH challenge. However, compound M alone produced a dose-dependent effect on METH-induced locomotion in the final test, with the saline group between the high and the low dose groups.

Immunohistochemistry showed that previous chronic METH treatment significantly increased METH-evoked expression of c-Fos, Arc, and MeCP2 in the NAc core and shell, but not in the prelimbic cortex. Previous daily pretreatment with compound M potentiated METH-induced changes in c-Fos inducibility in the NAc core and prelimbic cortex. Repeated exposure to compound M alone also increased METH-induced c-Fos in the NAc core.

3.4 Discussion

METH is well known for its ability to potently increase extracellular DA levels by acting at the DAT, where it competes with DA for reuptake, causes transporter internalization, and promotes transporter-mediated efflux (reverse transport) (Sandoval et al., 2001, Elliott and Beveridge, 2005). METH also interferes with vesicular monoamine transporter-2, depleting DA vesicular storage and increasing cytosolic DA availability for reverse transport by the DAT (Sulzer et al., 2005, Fleckenstein et al., 2009). The potent DA-releasing capacity of METH is thought to critically underlie its motor-stimulating and rewarding effects, and contribute to its abuse liability (Howell and Kimmel, 2008). In addition, as abovementioned, METH is a potent full agonist at TAAR1. TAAR1 stimulation by METH triggers a series of cellular phosphorylation cascades, leading to reduced DA uptake, enhanced DA efflux, and DAT internalization, that partially contribute to the known DA-releasing effects of METH, although these findings were obtained *in vitro* (Xie and Miller, 2009a). Therefore, it has been suggested that pharmacological targeting of TAAR1 may have the potential to modulate

abuse-related behavioural and neurophysiological effects of METH. The findings described in chapter one provided preliminary evidence for this concept by demonstrating complex interacting effects between METH and two TAAR1 selective partial agonists, RO5203648 and compound M, on locomotor activity and brain gene expression.

Experiment 1 showed that RO5203648 pretreatment time-dependently modulated the locomotor-activating effects of acute METH, characterized by an early attenuation followed by a late potentiation during a 3-h extended test session. This bidirectional modulation of METH's effects by TAAR1 appears to be consistent with the previously proposed state-dependent regulation of DA transmission by TAAR1 partial agonism. The onset of METH's effects is rapid and extracellular DA levels are expected to rise sharply at the beginning of the test, which corresponded with the period of strongest locomotor activity. In this phase the effect of RO5203648 was to increase TAAR1 activation, potentially leading to reduced DA transmission and suppressed locomotion. On the contrary, as METH's effects started to decay in the late phase of the test, which may have been accompanied by a gradual return of extracellular DA concentrations, RO5203648 may have become more antagonistic-like at TAAR1, maintaining DA and locomotion at moderately high levels. Alternatively, or complementarily, the attenuation effect at the early phase may be explained by a direct competition of RO5203648 with METH, which blocked the TAAR1-dependent pathway of METH-induced DA release. Indeed, the present study could be linked to an *in vivo* microdialysis study by Cotter et al. (2015) that measured the regulation of METH-stimulated DA overflow in the NAc by the same partial agonist. This study tested the effects of TAAR1 activation on DA overflow in the NAc during a 3-h test period and reported a similar temporal biphasic modulation whereby METH-induced DA accumulation was transiently depressed early in the session but elevated afterwards, although the late potentiation effect did not reach statistical significance (Cotter et al., 2015). This suggests that mechanisms other than net DA transmission at NAc synapses are likely to contribute to the complex regulation of METH-induced behavioural hyperactivity by TAAR1. In addition, the same authors showed that, in striatal synaptosomes, RO5203648 did not affect

METH-mediated DA efflux and uptake inhibition, suggesting that RO5203648's influence on METH's behavioural effects is unlikely to depend exclusively on direct, local actions at the DAT. As those authors suggested, the discrepancy between the findings from their *in vitro* synaptosomal preparations and *in vivo* microdialysis measures could be explained by the different concentrations used in each experiment and a broader network effect of systemic TAAR1 activation (Cotter et al., 2015). As described previously, the VTA appears a critical action site for TAAR1 where TAAR1 full agonists inhibit the firing rate of DA neurons and partial agonists produce the opposite effect. It is possible that TAAR1's regulation on the terminal release of DA in the NAc derives from TAAR1-induced alterations in the discharge rate of midbrain DA neurons that send projections to the NAc.

Moreover, apart from DA as the chief mediator, the glutamate system also appears to be importantly engaged in psychostimulants actions, partially via glutamate-DA interactions within the mesocorticolimbic circuits (Burns et al., 1994, Wang and McGINTY, 1999, Tzschentke, 2001). For example, while blockade of NMDA receptors in the medial PFC facilitated the locomotor-stimulating effect of METH (Han et al., 2012), intra-cerebroventricular injection of NMDA receptor agonists prevented it (Atsushi et al., 1991), suggesting a potential involvement of cortical glutamatergic hypofunction in METH-induced hyperactivity. Critically, studies have established a regulatory role of TAAR1 in glutamate transmission. Mice with TAAR1 depletion exhibited altered subunit composition and deficient functionality of the NMDA receptors in the PFC (Espinoza et al., 2015b). Furthermore, the selective TAAR1 full agonists, RO5256390 and RO5166017, and the partial agonist, RO5263397, were shown to inhibit hyperlocomotion induced by NMDA receptor blockers (Revel et al., 2011, Revel et al., 2013). Therefore, it may be reasonable to assume that TAAR1 modulation of the motor-stimulating effects of METH also involves interference with the glutamatergic system, possibly through an upregulation of glutamate activity, whose hypofunction has been implicated in METH-induced hyperlocomotion. However, these mechanisms are novel and require further investigations.

Following repeated administration, METH produces long-lasting neuroadaptations in the mesolimbic DA system, characterized by a progressive augmentation of METH-induced DA efflux, which translates into an increased locomotor response to METH treatment, known as behavioural sensitization. To examine the effects of TAAR1 on METH-induced long-term behavioural and neuronal adaptations, compound M, another selective partial TAAR1 agonist, was applied 15 min prior to METH for ten consecutive days. Following a 10-day withdrawal period, locomotor response and brain gene expression induced by a METH challenge were examined. Our results showed that daily pretreatment with compound M had no effects on METH-induced hyperlocomotion during the acquisition phase or the subsequent expression of METH behavioural sensitization on the probe test. This finding is inconsistent with a recent study by Cotter et al. (2015) where daily treatment of RO5203648 prior to METH blocked both the early induction and the later expression of METH-induced behavioural sensitization. Actually, as experiment 1 indicated, RO5203648 effectively blocked the locomotor-stimulating effects of acute METH before METH's effect subsided, and produced a decrease in METH-stimulated DA overflow. Thus, the inhibition by RO5203648 of METH behavioural sensitization could be explained by the ability of RO5203648 to attenuate the increase in DA transmission elicited by METH exposure (Cotter et al., 2015). Moreover, in a similar paradigm, another partial TAAR1 agonist, RO5263397, attenuated the development and expression of cocaine sensitization (Thorn et al., 2014b). Thus the lack of effects of compound M on METH behavioural sensitization is an unexpected finding and might be explained by a different pharmacological profile of compound M. No existing published study has used compound M and information about its pharmacology is scarce. In addition, our choice of 10 and 20 mg/kg dosage of compound M might have fallen outside its therapeutic window. It is possible that an inhibition by compound M of METH's effects would become evident at its therapeutically effective doses. Moreover, we know that this compound has been developed by Hoffmann-La Roche Ltd for human clinical trials, and therefore its pharmacodynamics/kinetic profile may not be ideal for rat studies.

An interesting finding is that, while daily treatment of compound M alone did not affect baseline locomotor activity during the acquisition phase, it appeared to have an impact on the subsequent response to METH. On the probe test, previous repeated compound M exposure produced a dose-dependent effect such that METH-probed locomotor response was significantly higher for the high dose group than the low dose group, although the differences between saline and the two compound M groups did not reach statistical significance. This dose-dependent effect of compound M might have important functional implications because it suggests that chronic TAAR1 partial agonism with compound M might produce long-lasting neuroadaptations resulting in altered sensitivity to METH with the high dose enhancing it and the low dose reducing it. One possibility is that, through regulating the spontaneous firing rate of midbrain DA neurons, repeated compound M may induce persistent structural and functional plasticity of these neurons that influence their sensitivity to DA agonists, leading to increased or decreased DA releasability upon exposure to METH. Alternatively, intermittent compound M stimulation of TAAR1 may produce neuroadaptations of this receptor, resulting in altered responsiveness of TAAR1 to endogenous and exogenous ligands. Thus, given that METH direct interaction with TAAR1 partially contributes to METH's DA-releasing properties, compound M might regulate METH-induced DA release and the ensuing locomotor response by interfering with this TAAR1-mediated pathway.

Actually, this dose-dependent effect of compound M could be linked to Cotter et al. (2015)'s finding that RO5203648 at the high dose cross-sensitized with METH, suggesting that chronic TAAR1 partial activation has the potential to cause neuroadaptive changes in a manner similar to METH. On the other hand, the low dose of the TAAR1 partial agonist did not cross-sensitize with METH in the Cotter et al. (2015)'s study, and a low dose of compound M showed a tendency to desensitize rats to METH treatment. These data, together with the acute interaction between METH and RO5203648 in experiment 1, further emphasize the complex regulation of psychostimulant actions by TAAR1. Moreover, these findings also suggest that relatively lower doses of these TAAR1 agonists should be used for

chronic administration in clinical settings due to the risk of developing long-term METH-like neuroadaptations. In this regard, one direction for future research is to characterize the pharmacological and behavioural effects of chronic compound M treatment, as is the characterization of other selective TAAR1 agonists, to determine the optimal dosages that may be used in human.

To gain insight into the underlying neurological process that may mediate the effects of compound M, the expression of c-fos, arc and MeCP2 was examined following the last METH challenge. The induction of c-Fos protein has been considered as an immediate marker of neuronal activation and is believed to play a role in stimulant-induced structural plasticity (Zhang et al., 2006, Jedynak et al., 2012). Similarly, Arc is rapidly activated by plasticity-producing stimulation and is specifically localized in the stimulated synaptic sites (Plath et al., 2006). Arc expression has been critically linked to the induction and maintenance of the neuronal plasticity that underlies learning and memory (Kodama et al., 1998, Czerniawski et al., 2011). Studies have shown that the induction of c-Fos and Arc by stimulants, including cocaine and METH, is at least partially dependent on the NMDA receptor (Torres and Rivier, 1993, Ohno et al., 1994, Kodama et al., 1998), consistent with the central role of NMDA receptor-dependent long-term potentiation in synaptic adaptations that critically underlie reward-related learning and behavioural modifications associated with drug addiction (Malenka and Bear, 2004, Jones and Bonci, 2005, Zweifel et al., 2008). On the other hand, MeCP2 acts as a key transcriptional repressor and activator by binding to methylated DNA to exert epigenetic control over gene expression (Chahrour et al., 2008, Cohen et al., 2008). MeCP2 is important for neuronal maturation and the functional regulation of synaptic activity such as the formation of long-term potentiation and synaptic plasticity (Asaka et al., 2006, Moretti et al., 2006, Smrt et al., 2007). Evidence has demonstrated the involvement of MeCP2 in psychostimulant-induced behavioural and neuronal effects, including self-administration, conditioned place preference, locomotor hyperactivity and sensitization, and changes in neuronal excitability, as well as the expression of selective IEGs (Deng et al., 2010, Im et al., 2010, Su et al., 2012, Schmidt et al., 2013,

Deng et al., 2014). Therefore, alterations in the activity of these genes or proteins might be part of the long-lasting neuroadaptations that underlie psychostimulant sensitization and, more broadly, the development of compulsivity of drug addiction.

The present experiment showed that a history of repeated METH exposure significantly altered the inducibility of all the three genes upon a METH challenge, which is consistent with the notion that chronic psychostimulants induce enduring synaptic plastic changes. In particular, we found an enhanced METH-probed expression of c-Fos, Arc, and MeCP2 in the NAc core and shell, but not in the prelimbic cortex, in rats that were previously treated with METH compared to those treated with saline. Previous studies examining sensitization-associated changes in c-Fos and Arc have yielded inconsistent results. Some studies showed a blunting effect of repeated psychostimulants treatment on IEG expression in the PFC, striatum, and NAc (Hope et al., 1992, Persico et al., 1993, Renthal et al., 2008, McCoy et al., 2011). However, other studies have reported an increase in drug-probed c-Fos in the NAc following repeated amphetamine or cocaine exposure and withdrawal (Crombag et al., 2002b, Mattson et al., 2007). Yet, evidence elsewhere showed a region-specific sensitization of c-Fos inducibility after chronic METH exposure, which was observed in the dorsal striatum but not in the NAc (Jedynak et al., 2012). In the case of Arc, previous studies showed that chronic METH pre-exposure had no effect on METH challenge-induced Arc expression in the striatum, orbital cortex, and cingulate cortex, but increased it in the frontal cortex and the parietal cortex (Kodama et al., 1998, McCoy et al., 2011). On the other hand, Arc expression in the frontal cortex was downregulated following repeated METH treatment without withdrawal (Cheng et al., 2015) but increased by repeated cocaine administration (Freeman et al., 2002). Several factors might contribute to the different results found in those previous and the present studies including the withdrawal duration, the environmental context, the dose of the challenge drug, and the specific subregions that were studied. For example, it has been suggested that cocaine sensitization is associated with time-dependent anatomical neuroadaptations such that repeated cocaine administration decreased c-Fos expression in the medial PFC, the lateral zone of the NAc shell, and the rostral pole of the NAc when

challenged after a 2-day withdrawal, but had no effect on these areas when the challenge was given after a 2-week withdrawal, instead increasing c-Fos protein in the intermediate zone of the NAc shell (Todtenkopf et al., 2002, Brenhouse and Stellar, 2006). Moreover, Renthal et al. (2008) examined the influence of chronic amphetamine on subsequent c-Fos inducibility in the striatum across a 1-10 day withdrawal period and found a suppressant effect only during the first five days of withdrawal, suggesting that varying the withdrawal length can affect the results. Furthermore, it has been shown that the experimental context (i.e., the home cage or a novel environment) and drug history interact to regulate c-Fos expression induced by amphetamine challenge (Ostrander et al., 2003), and that the environmental context modulates the effects of acute amphetamine on Arc expression in multiple brain regions especially the NAc shell (Klebaur et al., 2002). Finally, Crombag et al. (2002b) found that the ability of past cocaine exposure to enhance subsequent c-Fos induction in the NAc was critically dependent on the dose of the cocaine challenge. Therefore, our finding of a sensitized c-Fos and Arc response in the NAc core and shell by repeated METH treatment might reflect effects specific to the treatment procedures adopted in the present experiment.

The observation that the inducibility of MeCP2 was simultaneously enhanced by previous METH treatment suggests that MeCP2-mediated transcriptional regulation of new gene products may contribute to the altered activity of c-Fos and Arc. Studies have shown that psychostimulant induces MeCP2 phosphorylation in a specific neuronal subset within the NAc and disruption of MeCP2 phosphorylation led to enhanced sensitivity to amphetamine-induced behavioural sensitization and the associated changes in Fos response in those NAc neurons (Deng et al., 2010, Deng et al., 2014). Similarly, mice with dysfunctional MeCP2 gene were hypersensitive to cocaine-induced locomotor hyperactivity and exhibited enhanced inducibility of Arc in the striatum (Su et al., 2012). These observations led to the suggestion that MeCP2 might be recruited by psychostimulants to limit their effects on the neuronal and behavioural plasticity that is associated with the development of psychostimulant addiction (Deng et al., 2014). The present experiment showed that repeated METH-induced MeCP2 upregulation in the NAc upon a METH challenge was accompanied

by a sensitized reactiveness of c-Fos and Arc, which paralleled the expression of behavioural sensitization, implying a rather complex mechanism of psychostimulant-triggered transcriptional regulation of IEGs that might involve additional regulatory events beyond MeCP2-mediated epigenetic modifications.

We showed that daily concurrent administration of compound M with METH potentiated METH-induced increase in the subsequent inducibility of c-Fos in a region- and dose-specific manner, suggesting that partial TAAR1 agonism with compound M is able to modulate the long-term neuronal plasticity produced by repeated METH exposure. The expression of c-Fos was enhanced by compound M in both the prelimbic cortex and the NAc core, suggesting that compound M's regulation of METH occurs at both striatal and prefrontal cortical levels. This process may involve TAAR1's modulation of both DA- and glutamate-mediated pathways because evidence has suggested a role for both neurotransmitters in psychostimulant-induced IEG expression (Graybiel et al., 1990, Young et al., 1991, Torres and Rivier, 1993, Drago† et al., 1996, Carta et al., 2000, Amano et al., 2002, Ujike et al., 2002, Zhang et al., 2004, Deng et al., 2010, Mao et al., 2011). In addition, these data also suggest that the regulation of METH-induced behavioural and neuronal adaptations by compound M may be dissociable given the lack of effects of compound M on METH locomotor sensitization.

Moreover, our results showed that repeated compound M treatment alone changed subsequent METH-inducible c-Fos expression in the NAc core, further supporting the idea that chronic partial TAAR1 activation may cause neuroadaptations, mainly in the striatum, that alter neuronal sensitivity to psychostimulants. This TAAR1-mediated regulation of brain early gene activity highlights some of the neural mechanisms that may underlie the dose-dependent effect of compound M on METH-probed locomotor behaviour in the current experiment and the cross-sensitization of RO5203648 with METH in the Cotter et al. (2015)'s study.

In summary, the findings from the first set of experiments demonstrated complex interactions between METH and selective TAAR1 partial agonists on locomotor behaviour and brain

gene expression. These data suggest that partial TAAR1 activation has the ability to modulate acute and repeated METH-induced behavioural and neuronal plasticity that is associated with the development of compulsive drug use, and that TAAR1 may serve as a potential pharmacological target for the treatment of METH addiction.

Chapter Four

4 TAAR1 Activation Reduces the Reinforcing Efficacy of Psychostimulants

4.1 Introduction

Psychostimulants act as reinforcers, which by definition increase the likelihood of repeated drug taking, a precondition necessary for the escalation of drug intake, the transition to compulsive drug use, and the ultimate development of addiction (Breiter et al., 1997). Indeed, the addictive potential of a drug has been positively related to its reinforcing efficacy (Schuster, 1981). The powerful reinforcing effects of psychostimulants rely primarily on their ability to potently increase mesolimbic DA transmission (Marona-Lewicka et al., 1996, Howell and Kimmel, 2008). As previously described, amphetamine-like drugs, such as METH, are known as DA releasers through interference with the DAT and the vesicular monoamine transporter-2, causing DA accumulation at the synapse (Heal et al., 2013). The magnitude of amphetamine-induced DA release in the ventral striatum has been positively correlated with subjective experience of euphoria (Drevets et al., 2001, Oswald et al., 2005) and self-reported drug wanting in humans (Leyton et al., 2002). Likewise, although via a different mechanism, cocaine acts as a DAT blocker that inhibits reuptake of DA from the extracellular fluid into presynaptic terminals through the DAT, which also leads to augmented extracellular concentrations of DA (Pettit and Justice Jr, 1991). In human cocaine abusers, a greater degree of DAT occupancy by cocaine was associated with a higher rating of self-reported “high” produced by intravenous (i.v.) cocaine (Volkow et al., 1997). In animals, the reinforcing efficacy of a drug is best characterized in the self-administration paradigm which has been widely used to model key features and patterns of human drug abuse (Richardson and Roberts, 1996). Studies have consistently demonstrated an increased level of extracellular DA in the striatum, including the NAc, in rats that self-administered cocaine or *d*-amphetamine (Hurd et al., 1989, Di Ciano et al., 1995, Wise et al., 1995), and

DA depletion in the NAc markedly decreased self-administration of these drugs (Lyness et al., 1979, Roberts et al., 1980, Caine and Koob, 1994b). Moreover, in rats that were trained to self-administer cocaine, increases in the unit dose of cocaine led to proportional increases in both total amount of cocaine intake and extracellular level of DA in the NAc that was maintained throughout the self-administration session, suggesting that the stronger reinforcing efficacy of a larger unit dose of cocaine is associated with a greater ability to elevate DA levels in the NAc (Pettit and Justice Jr, 1991). Therefore, given the hypothesized ability of TAAR1 to prevent DA hyperactivity, TAAR1 activation is expected to downregulate self-administration of psychostimulants by reducing their reinforcing efficacy through counteracting their potentiating effects on mesolimbic DA transmission.

As mentioned before, the only study (by the time the current thesis started) that tested TAAR1 selective agonists in psychostimulants self-administration used a FR1 schedule of reinforcement and a single unit dose of the drug (Revel et al., 2012b). The present chapter aimed to more comprehensively investigate TAAR1 regulation of the reinforcing properties of psychostimulants by assessing the ability of the partial agonist, RO5203648, and the full agonist, RO5256390, to shift the dose-response function of cocaine self-administration (experiment 3)[4], and the effects of RO5203648 (experiment 4)[5] and another partial agonist, RO5263397 (experiment 5)[6], on the BP for cocaine or METH self-administration, respectively, under a PR schedule of reinforcement. Additionally, experiments 4 and 5 also examined the influence of the two partial agonists on food self-administration under the same

[4] Published paper. Experiment 3 has been published in the following paper: Pei Y, Mortas P, Hoener MC, Canales JJ (2015) Selective activation of the trace amine-associated receptor 1 decreases cocaine's reinforcing efficacy and prevents cocaine-induced changes in brain reward thresholds. Progress in Neuro-Psychopharmacology and Biological Psychiatry 63:70-75.

[5] Published paper. Experiment 4 has been published in the following paper: Pei Y, Lee J, Leo D, Gainetdinov RR, Hoener MC, Canales JJ (2014) Activation of the trace amine-associated receptor 1 prevents relapse to cocaine seeking. Neuropsychopharmacology 39:2299-2308. doi: 10.1038/npp.2014.88.

[6] Published paper. Experiment 5 and 6 have been published in the following paper: Pei Y, Asif-Malik A, Hoener M, Canales JJ (2016) A partial trace amine-associated receptor 1 agonist exhibits properties consistent with a methamphetamine substitution treatment. Addiction Biology, doi: 10.1111/adb.12410.

PR schedule in order to control for their potential non-specific effects. Finally, in order to account for TAAR1's behavioural regulation at the physiological level, experiment 6 explored the effects of the partial agonist, RO5263397, on METH-induced DA overflow in the NAc core in rat brain slices by way of FSCV[6]. A recent report indicated that RO5203648 effectively blocked cocaine-stimulated DA release in the NAc (Pei et al., 2014). A similar result was expected for experiment 6.

4.2 Materials and Methods

4.2.1 Subjects

Male Long Evans rats were sourced from the University of Canterbury and the University of Leicester and were 10-12 weeks-old when experiments began. All animals were housed in temperature and humidity controlled colony rooms with a 12-h light/dark cycle (lights off at 8 a.m.). Rats were given a maintenance diet and kept at 100% of their weight seven days' post-surgery. Water was given *ad libitum* at all times. All procedures were approved by the Animal Ethics Committee of the University of Canterbury or the University of Leicester.

4.2.2 Pharmacological agents

Cocaine was obtained from the National Institute of Drug Abuse (USA) and dissolved in 0.9% physiological saline for i.v. self-administration. METH hydrochloride was obtained from BDG Synthesis (Wellington, New Zealand) and Sigma-Aldrich (UK) and dissolved in 0.9% physiological saline for i.v. self-administration or artificial cerebrospinal fluid (aCSF) for the voltammetry experiments. RO5256390, RO5203648, and RO5263397 were synthesized at F. Hoffmann-La Roche Ltd. (Switzerland) and dissolved in 10% dimethylsulfoxide in 0.9% physiological saline for i.p. injections or in aCSF for the voltammetry assays.

4.2.3 Catheter implantation surgery

Rats assigned to cocaine or METH self-administration experiments were operated on to implant i.v. catheters. In experiment 4 and 6, animals were anesthetized with ketamine (85 mg/kg, i.p.) and domitor (medetomidine, 0.35 mg/kg, i.p.). The analgesic carprofen was administered before surgery (5 mg/kg, i.p.). Catheters (O/D 0.63 mm, I/D 0.30 mm,

Camcaths, Cambridge, UK) were implanted into the right jugular vein, existing dorsally between the scapulae. Analgesic and antiseptic cream was applied to the back and neck incision areas following suturing. On completion of surgery, rats were given Antisedan (atipamezole, 1.0 mg/kg, i.p.) to reverse the anesthesia. To prevent infection, rats were treated with antibiotic (cephalexin, 50 mg/kg, s.c.) 90 min before surgery. Catheters were flushed with heparinized saline (0.1 ml, 70 IU/ml) before and after each self-administration session to prolong patency. Animals in experiment 5 were anesthetized with 2,2,2-tribromoethanol solution (12.5 mg/ml solution in 2.5% tertiary amyl alcohol, 2 ml/100g of body weight) and received carprofen (5 mg/kg, i.p.) immediately after surgery. The antibiotic (cephalexin, 25 mg/kg, s.c.) was given daily pre and post-surgery for seven days. The rest of the surgical procedure remained the same as described above.

4.2.4 Self-administration apparatus

Twelve self-administration chambers (Med Associates, VT, USA) controlled by software (Med-PC IV®) were used. Chambers had two response levers designated as active and inactive. Active lever presses resulted in activation of the infusion pump and delivery of cocaine or METH, and illumination of a light stimulus for 5 seconds. Presses on the inactive lever were recorded but had no programmed consequences. Each experimental chamber was enclosed in a sound-attenuating box. The house light was on throughout training and test sessions. Rats were connected to a liquid swivel with polyethylene-50 (PE-50) tubing protected by a metal spring. In the food self-administration assay, active lever presses resulted in delivery of a chocolate-flavored pellet (45 mg, Bio-Serve, NJ, USA) into a food magazine situated between the two levers, and illumination of the stimulus light for 5 seconds.

4.2.5 Cocaine dose-response training and test (experiment 3)

Rats (n = 36) were firstly trained to lever press to self-administer cocaine (0.45 mg/kg/infusion in 100 µl, over 5 seconds) under a FR1 schedule of reinforcement in daily 60 min sessions. After the responses met the stability criterion (number infusions per session ≥

12 for three consecutive days with less than 20% variance in the last three days), rats were randomly assigned to five groups (n = 7-8 per group) that self-administered cocaine either at the same dose (0.45 mg/kg/infusion) or at a substitute dose (1.0, 0.2, 0.1, or 0.03 mg/kg/infusion). The new dose of cocaine was maintained until a criterion of stability was met (less than 10% variation in the last three days). The amount of cocaine was limited throughout the experiment to a maximum of 20 mg/kg per session to prevent overdose.

Two experiments were performed that respectively examined the effects of RO5203648 and RO5256390 on cocaine dose-response functions. After training, each rat was subjected to five cocaine intake tests in which a pretreatment of RO5203648 (3 or 6 mg/kg, i.p.), RO5256390 (3 or 6 mg/kg, i.p.), or vehicle was administered 15 min before the cocaine self-administration session (60 min). The five cocaine intake tests were administered in randomized order. The test days were separated by at least two days during which rats undertook regular cocaine self-administration sessions until response returned to their original response level. The same values for the vehicle pretreatment condition were used in the two experiments as control.

4.2.6 Cocaine PR test (experiment 4)

Rats (n = 9) were trained to lever press for cocaine (0.5 mg/kg/infusion, 100 µl), being sequentially exposed to FR1, FR2, and finally FR3 reinforcement schedules during long-access sessions. Schedule progression was dependent upon meeting a criterion (number infusions per session ≥ 30). Rats were then transferred to daily FR3 90 min sessions until a criterion of stability and consistency was met (number infusions per session ≥ 15 for three consecutive days with less than 20% variability). Cocaine infusions were limited to a maximum of 40 (20 mg/kg) per session to prevent overdosing. On completion of training, each rat was subjected to three PR tests in which RO5203648 (0, 3, or 10 mg/kg, i.p.) was administered in counterbalanced fashion 15 min before the start of the session. The PR tests were conducted on three different days separated by at least one regular 90 min FR3 cocaine self-administration session. The PR test lasted for 6 h during which the number of active

lever responses required for each subsequent infusion increased as follows: 1, 2, 4, 6, 9, 12, 15, 20, etc., following the exponential equation:

$NP\ (n) = [5e^{0.2n}]-5,$

with *n* representing the injection rank (Richardson and Roberts, 1996). After the first FR1 was achieved, the BP was defined as the largest ratio completed prior to a period lasting for 60 min or more during which no infusions were obtained.

4.2.7 METH PR test (experiment 5)

The procedures for METH self-administration training and PR test were the same as those described above for the cocaine PR experiment. Briefly, rats (n = 9) were trained on METH self-administration (0.05 mg/kg/infusion, 100 µl) under FR3 reinforcement schedules in 90 min daily sessions until they achieved a stability criterion (number infusions per session ≥ 17 for five consecutive days with less than 20% variance in the last three days). METH infusions were limited to a maximum of 40 (2 mg/kg) per session to prevent overdosing. The PR tests were conducted on three different days during which RO5263397 (0, 3, or 10 mg/kg, i.p.) was administered in counterbalanced manner 15 min before the start of the session.

4.2.8 Food PR test (experiments 4 and 5 cont.)

The food PR experiment with each of the two partial agonists was respectively conducted following the cocaine or METH PR experiment with the same partial agonist used in the drug PR test. After the cocaine or METH PR test, an additional group of rats (n = 8) was trained to lever press for chocolate-flavored pellets under FR1 reinforcement schedule in daily 60 min sessions until a stability criterion was met (number of pellets per session ≥ 20 for three consecutive days). Each rat received three food PR tests in which RO5203648 (0, 3, or 10 mg/kg, i.p.) or RO5263397 (0, 3, or 10 mg/kg, i.p.) was administered 15 min before the start of the session. The PR test lasted for 6 h and had identical response requirement as the cocaine or METH PR test. The PR tests were conducted on different days separated by one standard FR1 60 min food self-administration session.

4.2.9 FSCV equipment (experiment 6)

The FSCV setup was custom built, consisting of a slice chamber, stimulating, recording, and reference electrodes connected to a computer and amplifier. Recording electrodes were manufactured as previously described (Fortin et al., 2015). Reference electrodes were manufactured with a piece of silver wire coated in KCl (Ag/AgCl) and attached to a silver pin. Bipolar stimulating electrodes were obtained from FHC (ME, USA). The recording and reference electrodes were connected to a potentiostat and head-stage circuit (Chemclamp, Dagan Instruments, USA) and a computer running DemonVoltammetry software (Wakeforest Innovations; NC, USA). Two National Instruments data acquisition cards (NI-DAQ; PCI-6711 and PCI-6052e; National Instruments, Austin, TX) were used for interfacing Demon Voltammetry with the potentiostat (Dagan Corporation; Minneapolis, MN). The recording electrode potential was linearly scanned at a rate of 400V/s as a triangular waveform from -0.4 V to 1.3 V and back to -0.4 V vs. the reference electrode. Cyclic voltammograms were recorded at the recording electrode every 100 ms by means of the voltammeter/amperometer (Dagan Instruments, USA). At this waveform, DA oxidizes at ~ 0.6 V and reduces at ~ -0.2 V.

4.2.10 Tissue preparation

Rats (n = 9) were anaesthetized with isoflurane and sacrificed via a Schedule 1 procedure. The brain was rapidly removed and placed in a tube containing pre-carboxygenated (i.e. bubbled with 95% O_2 and 5% CO_2), ice-cold, sodium-free slicing aCSF (SaCSF) consisting of 250 mM sucrose, 2.5 mM KCl, 11 mM d-glucose, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$, 0.4 mM l-ascorbic acid, 0.1 mM $CaCl_2$, and 4 mM $MgCl_2$ adjusted to pH 7.4. The brain was then sectioned in ice-cold carboxygenated aCSF on a Vibratome 1000 Classic vibrating microtome (The Vibratome Company, MO, USA). Coronal slices (400 µM) through the NAc were maintained at room temperature in continuously carboxygenated experimental aCSF (EaCSF), which consisted of 126 mM NaCl, 2.5 mM KCl, 11 mM d-glucose, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$, 0.4 mM l-ascorbic acid, 2.4 mM $CaCl_2$, and 1.2 mM $MgCl_2$ adjusted to pH 7.4. Slices were allowed to recover for at least 30 min at room temperature

before recording. Experimental recordings started 5-10 min after transfer to the FSCV slice chamber (see below) to allow slices to equilibrate in warmed aCSF.

4.2.11 FSCV recordings

For recordings, a slice was placed in the FSCV slice chamber, held in place with a purpose-built grid and superfused with continuously carboxygenated EaCSF at a flow rate of 1.4 ml/min and heated with a purpose-built Peltier to 32-33˚C. The recording electrode was positioned ~75 µm below the surface of the slice in the NAc. DA release was electrically evoked every 5 min by a 4 ms, one-pulse stimulation (monophasic, 300 µA), using the stimulating electrode placed 100 - 200 µm from the recording electrode within the NAc core. Current pulses were generated by the acquisition software and applied via an ISo-Flex stimulus isolator (AMP Instruments; Jerusalem, Israel). DA was confirmed in each recording by observation of the cyclic voltammogram (noting the position of oxidation and reduction peaks) and colour plots permitted the visualization of release dynamics over time (Figure 4.5a and b). Drugs were applied by superfusion at the same time as the recording was initiated. Slices were randomly assigned to the different conditions. Conditions consisted of 20 min runs with an electrical stimulation being passed every 5 min with either no drug, 2 µM METH, 2 or 4 µM RO5263397 followed by 20 min of co-application of the compound (2 or 4 µM) with 2 µM METH. Background subtracted cyclic voltammograms were obtained by subtracting the current obtained in the first 2 min of every experiment, before drug superfusion into the slice chamber. The peak oxidation current (nA) for DA in each voltammogram has been deemed an appropriate measure of DA release (Yorgason et al., 2011). However, these values were also converted into a measure of the DA concentration by pre/post-calibration of the electrode using 1-5 µM DA (Sigma Aldrich, MO, USA). DA calibrations were performed for each electrode in a flow cell designed from a custom-built Y-shaped chamber, as previously described (Sinkala et al., 2012).

4.2.12 Statistical analysis

Data were analysed by ANOVA with repeated measures when a within-subjects design was in use, followed by *post hoc* comparisons with the method of N-K tests using the sampling error from the overall ANOVA as denominator. Statistical significance was set at $\alpha = 0.05$ for all experiments. All statistical analyses were performed using StatView 5.0 (SAS Institute, NC, USA).

4.3 Results and Summary

4.3.1 Experiment 3 results

Rats were firstly trained to self-administer cocaine at a pre-training dose (0.45 mg/kg/infusion) before being subsequently divided into five groups that self-administered cocaine at five different doses (1.0, 0.45, 0.2, 0.1, or 0.03 mg/kg/infusion). Performance in the pre-training phase was matched for the five groups. A repeated measure ANOVA for lever presses during the last three pre-training sessions revealed only a significant effect of lever (active vs. inactive, $F_{1, 33} = 393.04$, $p < .0001$). Similarly, a one-way ANOVA showed no significant differences in the average number infusions obtained by the five groups across the last three pre-training sessions ($p = .9953$). After transfer to the substitute cocaine dose, rats re-stabilized response to the new dose. A one-way ANOVA for the average number of infusions over the last three training sessions revealed a significant effect of cocaine unit dose ($F_{4, 33} = 81.92$, $p < .0001$). The decrease in the number of infusions attained was paralleled by a dose-dependent increase in cocaine intake with increasing unit dose. ANOVA for the average cocaine intake over the last three training sessions showed a significant main effect of cocaine dose ($F_{4, 33} = 9.48$, $p < .0001$).

In the test, RO5203648 (3 or 6 mg/kg, i.p.) and RO5256390 (3 or 6 mg/kg, i.p.) administered 15 min prior to the self-administration session dose-dependently shifted the cocaine dose-response curve downward. A repeated measure ANOVA for the number of cocaine infusions obtained following RO5203648 treatment, with cocaine unit dose as a between-subjects factor and RO5203648 treatment as a within-subjects factor, revealed a

significant main effect of cocaine unit dose ($F_{4, 33} = 54.46$, $p < .0001$) and dose of RO5203648 ($F_{2, 66} = 102.07$, $p < .0001$), as well as a significant interaction between those factors ($F_{8, 66} = 26.83$, $p < .0001$) (Figure 4.1a). *Post hoc* comparisons showed a significant reduction in cocaine infusions for 0.1 mg/kg and 0.03 mg/kg unit dose of cocaine by RO5203648 at both doses ($p < .01$, by N-K tests) and for 0.2 mg/kg cocaine unit dose by RO5203648 at the high dose ($p < .01$, by N-K tests). ANOVA for the effect of RO5203648 on amount intake during the test showed a significant main effect of cocaine unit dose ($F_{4, 33} = 10.19$, $p < .0001$) and dose of RO5203648 ($F_{2, 66} = 144.93$, $p < .0001$) (Figure 4.1c). N-K tests showed that RO5203648 at both doses significantly reduced cocaine intake across all five cocaine doses ($p < .01$).

Similarly, ANOVA for the effect of the full agonist, RO5256390, was conducted with one between-subjects factor, cocaine dose, and one within-subjects factor, dose of RO5256390. ANOVA for the number cocaine infusions revealed a significant main effect of unit cocaine dose ($F_{4, 33} = 43.51$, $p < .0001$) and dose of RO5256390 ($F_{2, 66} = 139.86$, $p < .0001$), as well as a significant interaction between those factors ($F_{8, 66} = 36.41$, $p < .0001$) (Figure 4.1b). Means comparisons showed a significant reduction in cocaine infusions for 0.03, 0.1, and 0.2 mg/kg cocaine unit dose at both the low and the high doses of RO5256390 ($p < .01$, by N-K tests). ANOVA for cocaine intake yielded a significant main effect of cocaine unit dose ($F_{4, 33} = 9.63$, $p < .0001$) and dose of RO5256390 ($F_{2, 66} = 241.96$, $p < .0001$), as well as a significant interaction effect ($F_{8, 66} = 2.75$, $p = .0111$) (Figure 4.1d). *Post hoc* comparisons indicated a significant reduction in cocaine intake across all cocaine unit doses at both doses of RO5256390 ($p < .01$, by N-K tests).

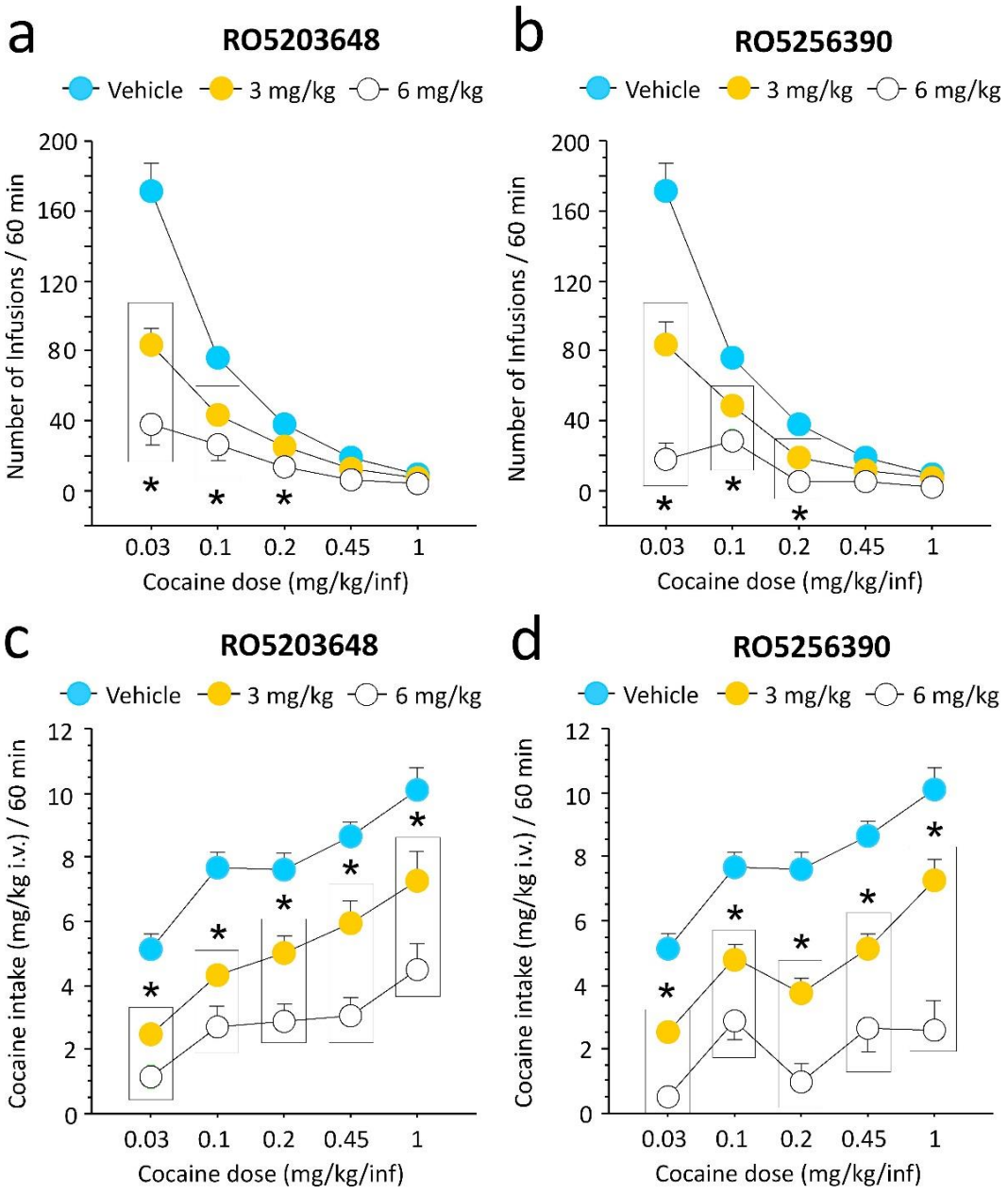

Figure 4.1 RO5203648 and RO5256390 shifted the cocaine dose-response curve downward
The number of cocaine infusions decreased as the cocaine unit dose increased. RO5203648 (a) and RO5256390 (b) dose-dependently reduced number of infusions at all unit doses of cocaine. The decrease in cocaine infusions with increasing cocaine unit dose was paralleled by an increase in total amount of cocaine intake, which was dose-dependently attenuated by RO5203648 (c) and RO5256390 (d) across all unit doses of cocaine. * $p < .01$ significantly different from vehicle treatment at the specific cocaine unit dose.

4.3.2 Experiment 3 summary

Firstly, a dose-response function for cocaine self-administration (0.03, 0.1, 0.2, 0.45, and 1 mg/kg/infusion) was established such that increases in unit injection dose of cocaine led to decreased number of infusions despite an overall increase in the total amount of cocaine intake in the session. Both the selective TAAR1 partial and full agonists, RO5203648 and RO5256390, respectively, produced a marked downward shift in the cocaine dose-response curve, indicating diminished cocaine reinforcement at all doses. These findings demonstrated a clear ability of TAAR1 partial and full activation to effectively block the reinforcing efficacy of cocaine.

4.3.3 Experiment 4 results

After rats were trained to stably self-administer cocaine (0.45 mg/kg/infusion) under a FR3 reinforcement schedule, they received three PR tests during which RO5203648 (0, 3, or 10 mg/kg, i.p.) was given 15 min prior to the start of the PR session. One-way ANOVA failed to reveal a significant effect of RO5203648 treatment on the number of active lever presses made during the test ($F_{2, 16} = 1.16$, $p = .338$) or on the number of infusions obtained ($F_{2, 16} = 0.59$, $p = .564$).

However, the temporal parameters of responding for cocaine under the PR schedule were markedly altered by RO5203648 treatment. Active lever presses in each 1-h bin, cumulative number of cocaine infusions, and time to reach BP were analyzed by ANOVA. RO5203648 dose-dependently reduced active lever pressing in the early phase of the PR session, lengthened the accumulation of cocaine infusions, and delayed the time point to reach BP. A repeated measure ANOVA for active lever presses broken down in 1-h bins showed a significant effect of time ($F_{5, 40} = 13.37$, $p < .001$) and a significant interaction between treatment and time ($F_{10, 80} = 11.08$, $p < .001$). RO5203648 produced a dose-dependent temporal shift to the right in the number of active lever presses made during the session. Response rate was significantly attenuated by RO5203648 in the 1st h at both doses ($p < .01$, by N-K tests) and in the 2nd h at the high dose ($p < .05$, by N-K tests). The high dose of

RO5203648 also significantly increased response rate during the 4th time bin ($p < .05$, by N-K tests) (Figure 4.2a).

RO5203648 dose-dependently delayed the time point at which rats reached each successive cocaine infusion. ANOVA was performed for the cumulative number of infusions obtained by the end of each 1-h bin, which revealed a significant effect of dose of RO5203648 ($F_{2, 16} = 6.74$, $p < .01$) and time ($F_{5, 40} = 122.69$, $p < .0001$), as well as a significant interaction between time and RO5203648 ($F_{10, 80} = 18.72$, $p < .001$) (Figure 4.2b). Rats receiving vehicle injections rapidly obtained the first 10 infusions (FR = 40) by the end of the 1st h bin and achieved BP by the 2nd h bin on average. Time to reach BP was significantly delayed by RO5203648 ($F_{2, 16} = 20.31$, $p < .001$). Means ± SEM were 1.67 ± 0.24 h, 3.56 ± 0.44 h, and 4.56 ± 0.34 h to reach BP for control, low and high doses of RO5203648, respectively, with both doses of the partial agonist being significantly different from control values ($p < .01$, by N-K tests).

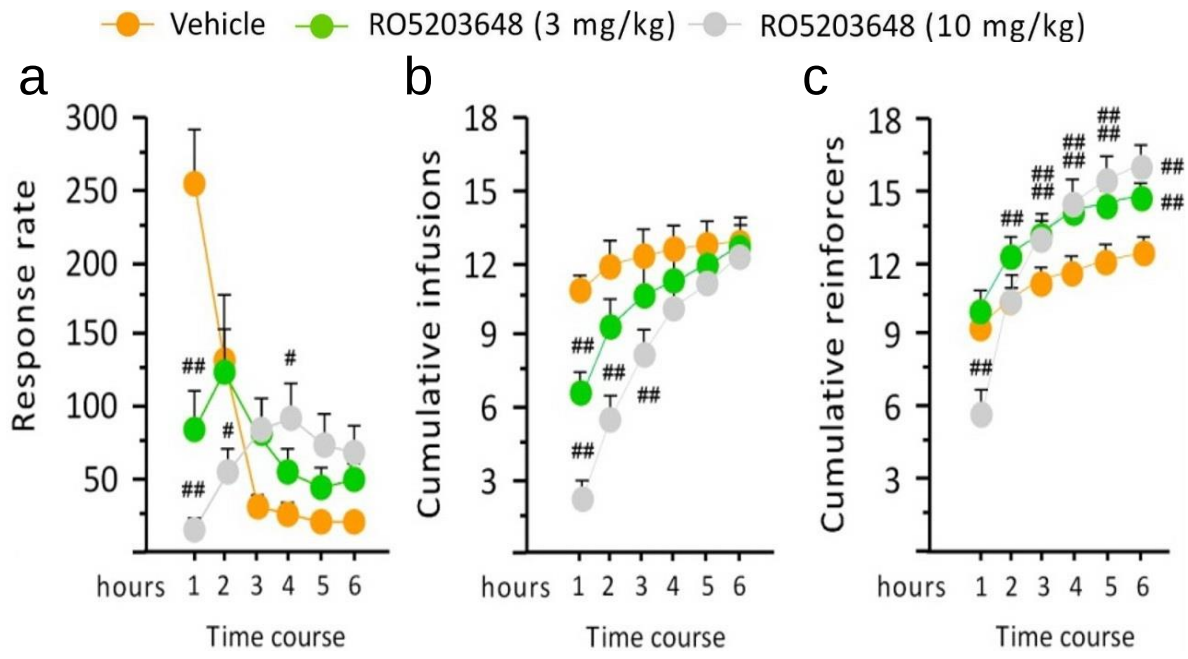

Figure 4.2 RO5203648 differentially regulated the reinforcing efficacy of cocaine and food
In a PR schedule of reinforcement, RO5203648 dose-dependently shifted the cocaine response rate curve rightward (a) and delayed the time to reach BP (b). However, RO5203648 enhanced the reinforcing efficacy of food in the same PR paradigm (c). # $p < .05$, ## $p < .01$ significantly different from vehicle pretreatment.

To examine the potential non-specific effects of RO5203648 on motivation to obtain a natural reward (i.e. food), an additional group working for food under FR1 reinforcement schedule was tested with the same PR procedures. RO5203648 significantly increased the number of active lever presses ($F_{2, 14} = 15.06$, $p = .0003$) and the number of pellets obtained ($F_{2, 14} = 19.23$, $p < .0001$) in the 6-h food PR test, as revealed by one-way ANOVA tests. *Post hoc* comparisons showed that responses on the active lever were significantly increased by RO5203648 at both the low ($p < .05$, by N-K tests) and the high dose ($p < .01$, by N-K tests), and so was the number of pellets obtained during the test at both doses ($p < .01$, by N-K tests). Similarly, the BP achieved in the PR test was significantly increased by RO5203648 ($F_{2, 14} = 16.89$, $p = .0002$), with N-K tests revealing a significant effect for both the low ($p < .05$) and the high doses ($p < .01$).

A temporal breakdown of the 6-h test showed that RO5203648 changed the pattern of responding for food. A repeated measure ANOVA for active lever response in 1-h bins revealed a significant effect of treatment ($F_{2, 14} = 15.06$, $p = .0003$) and time ($F_{5, 35} = 12.54$, $p < .0001$), as well as a significant interaction between time and treatment ($F_{10, 70} = 7.13$, $p < .0001$). Responding under vehicle treatment was high in the 1st h and dropped afterwards. At the low dose, RO5203648 upwardly shifted the response rate across the test, with a significant increase at the 2nd ($p < .01$, by N-K tests) and the 3rd time bin ($p < .05$, by N-K tests). At the high dose, RO5203648 significantly reduced response rate during the 1st h ($p < .01$, by N-K tests) but significantly increased it at the 2nd h and subsequent time bins ($p < .01$ at the 2 - 5th time bins, by N-K tests).

Correspondingly, the cumulative number of pellets obtained by the end of each time bin was also altered by RO5203648. A repeated measure ANOVA yielded a significant effect of treatment ($F_{2, 14} = 4.85$, $p = .025$) and time ($F_{5, 35} = 351.10$, $p < .0001$), as well as a significant interaction between these factors ($F_{10, 70} = 21.23$, $p < .0001$) (Figure 4.2c). RO5203648 at the low dose significantly increased the cumulative number of reinforcers from the 2nd h and onwards ($p < .01$ for 2 - 6th time bins, by N-K tests). The high dose of RO5203648 significantly lowered the number of pellets attained in the 1st h ($p < .01$, by N-K tests) but elevated pellet accumulation from the 3rd time bin to the end of the test ($p < .01$ from 3 - 6th time bins, by N-K tests). Means ± SEM for the total number of pellets obtained at the end of the test were 12.50 ± 0.50, 14.88 ± 0.40, and 16.13 ± 0.83 for control, low and high doses of RO5203648, respectively.

4.3.4 Experiment 4 summary

In the cocaine PR test, RO5203648 treatment did not affect the overall responding rate or the total number of infusions earned during the test. However, it produced a significant rightward shift in the temporal pattern of responding across time bins, manifested as a significant reduction of active lever pressing in the early phase of the test, prolongation of the accumulation of cocaine infusions, and delay of the time to reach BP. In contrast, food

self-administration under the same PR procedure was significantly enhanced by RO5203648 at both doses, achieving a significantly higher BP than the vehicle treatment. These results suggest that the TAAR1 partial agonist is likely to make cocaine less reinforcing and has differential regulation on the motivation for cocaine and food reinforcement.

4.3.5 Experiment 5 results

RO5263397 administered 15 min before the start of the METH PR session dose-dependently reduced the motivation to self-administer METH. A repeated measure ANOVA for active lever presses revealed a significant effect of treatment ($F_{2, 16} = 11.45$, $p < .001$) and lever ($F_{1, 8} = 216.48$, $p < .0001$), as well as a significant interaction between these factors ($F_{2, 16} = 5.88$, $p < .05$). *Post hoc* comparisons showed that responding on the active lever was dose-dependently attenuated by RO5263397 at both the low ($p < .05$) and the high doses ($p < .01$, by N-K tests) (Figure 4.3a). This corresponds to a significant reduction in number of infusions obtained ($F_{2, 16} = 7.12$, $p < .01$) and in the BP achieved ($F_{2, 16} = 7.80$, $p < .01$) (Figure 4.3b), as revealed by additional one-way ANOVA tests. RO5263397, at the high dose, but not the low dose, significantly decreased number of infusions ($p < .01$) and the BP ($p < .01$, by N-K tests).

Active lever responses were also analyzed across 1-h bins for a closer examination of the effects of RO5263397. A repeated measure ANOVA revealed a significant effect of treatment ($F_{2, 16} = 9.21$, $p < .01$) and time ($F_{5, 40} = 10.99$, $p < .0001$), as well as a significant interaction between treatment and time ($F_{10, 80} = 9.53$, $p < .0001$). Under the vehicle treatment, active lever presses reached peak level at the 3rd h and dropped afterwards. RO5263397 dose-dependently reduced response rate in the first three h ($p < .01$ for 1 - 3rd time bins, by N-K tests) and produced a downward and rightward shift of the peak response curve (Figure 4.3c). Similarly, the temporal pattern for the accumulation of infusions was downwardly shifted by RO5263397 (Figure 4.3d).

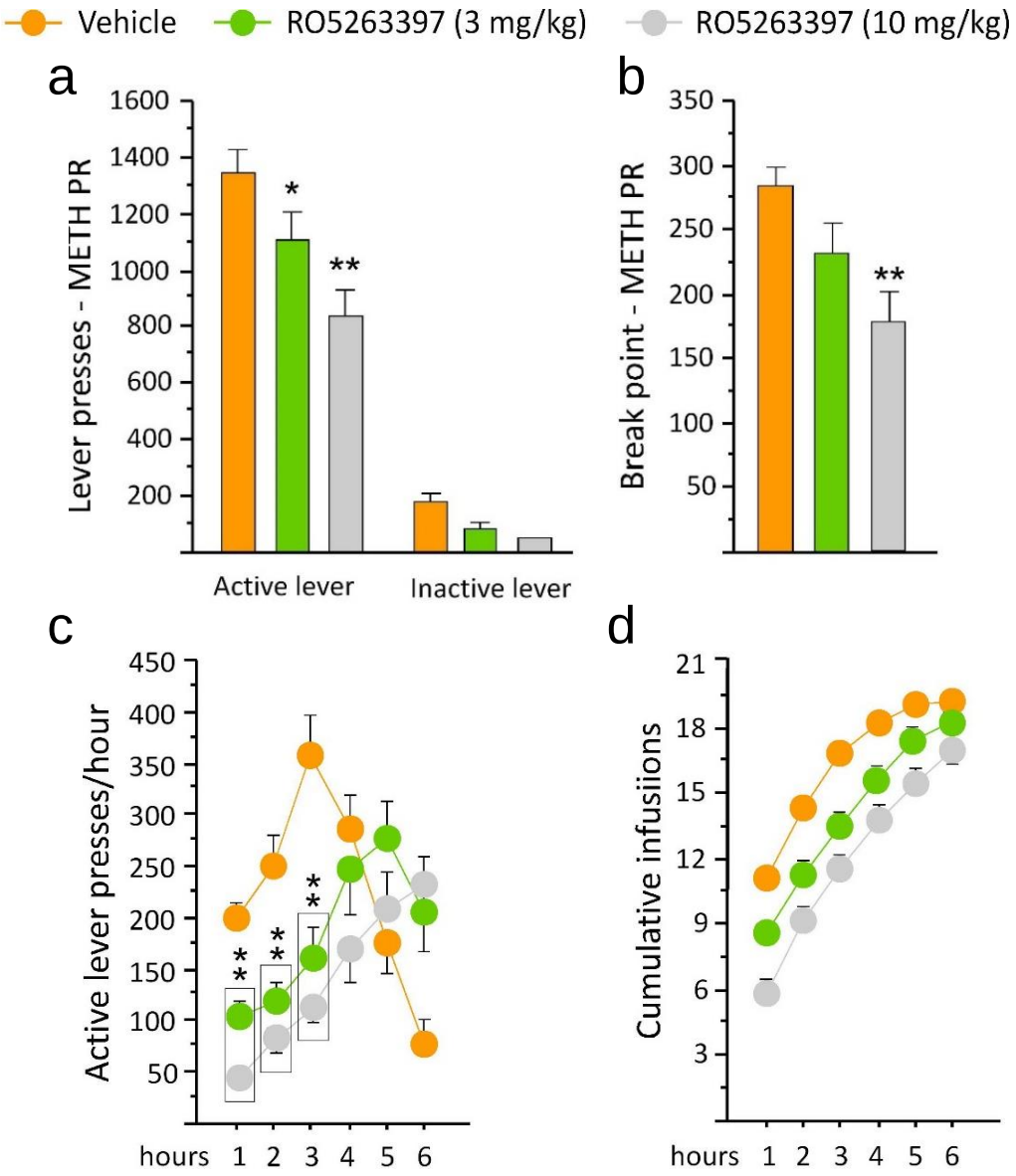

Figure 4.3 RO5263397 reduced the reinforcing efficacy for METH

RO5263397 significantly reduced the total number of active lever presses (a) and BP (b) for METH self-administration under a PR schedule of reinforcement. Time course analysis of active lever response showed that RO5263397 significantly attenuated METH-maintained responding in the first three h and produced a downward and rightward shift of the peak response curve (c), which corresponds with a downward shift of the cumulative number of infusions obtained across the 6-h period (d). * $p < .05$, ** $p < 0.1$ significantly different from vehicle pretreatment.

To control for its non-specific effects, RO5263397 was tested in another group of rats that worked for food under the same PR requirement. In sharp contrast with METH-maintained responding, RO5263397 enhanced the motivation for food. A repeated measure ANOVA for active lever presses revealed a significant effect of treatment ($F_{2, 14} = 3.95$, $p < .05$) and a significant effect of lever ($F_{1, 7} = 63.77$, $p < .0001$). This was accompanied by an increase in the number of pellets earned ($F_{2, 14} = 5.44$, $p < .05$) and in the BP achieved ($F_{2, 14} = 4.86$, $p < .05$), as revealed by additional one-way ANOVA. N-K tests showed that RO5263397 at the high, but not the low, dose significantly increased active lever presses ($p < .01$) (Figure 4.4a) and number of pellets obtained ($p < .05$), as well as the BP ($p < .05$) (Figure 4.4b).

The temporal breakdown of active lever presses during the 6-h test was analyzed. A repeated measure ANOVA showed a significant main effect of treatment ($F_{2, 14} = 3.72$, $p < .05$) and time ($F_{5, 35} = 37.39$, $p < .0001$), as well as a significant interaction between these factors ($F_{10, 70} = 3.51$, $p < .001$). Under the vehicle treatment, response rate was high during the 1st h followed by a rapid decline. RO5263397 significantly increased responding rate during the first two h ($p < .05$ for the low dose at the 1st h; $p < .05$ for the high dose at the 2nd h, by N-K tests) (Figure 4.4c) and the cumulative number of reinforcers (Figure 4.4d).

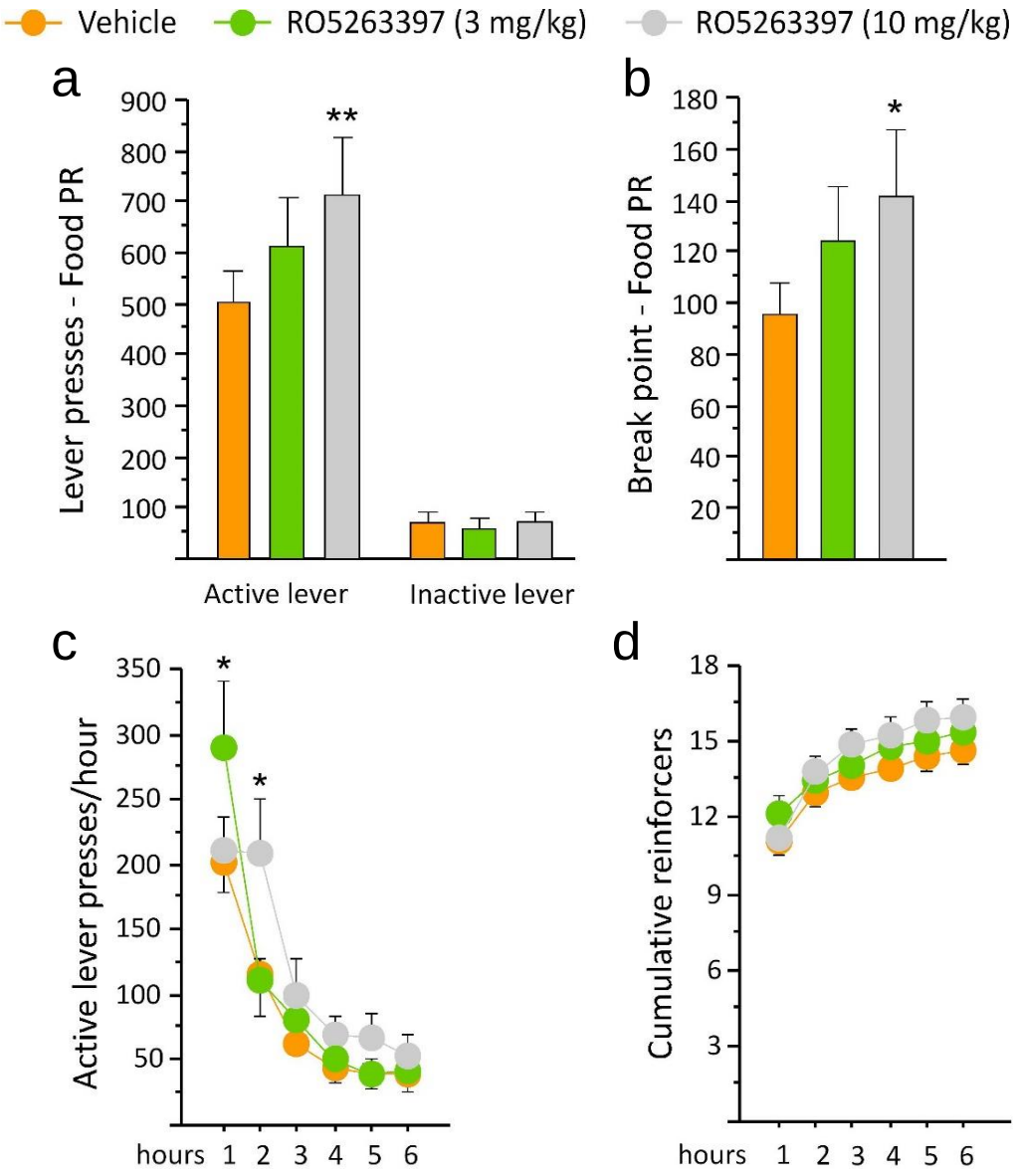

Figure 4.4 RO5263397 increased the reinforcing efficacy for food

Under a PR schedule of reinforcement, RO5263397 significantly increased the response rate on the active lever (a) and the BP (b) maintained by food. Temporal breakdown of the response rate revealed that RO5263397 significantly increased active lever responding during the first two h (c), which corresponds to an upward shift in the cumulative reinforcers obtained over time (d). * $p <$ .05, ** $p <$ .01 significantly different from vehicle pretreatment.

4.3.6 Experiment 5 summary

RO5263397 dose-dependently reduced METH self-administration and the BP under a PR schedule of reinforcement, suggesting that TAAR1 partial activation decreases the reinforcing efficacy of METH and the motivation for METH. On the contrary, RO5263397 significantly enhanced the BP maintained by food in the same PR procedure, indicating that TAAR1's regulation of the motivational mechanisms underlying drug and food rewards can be clearly dissociated.

4.3.7 Experiment 6 results

To gain insight into the underlying mechanisms mediating TAAR1's ability to modulate the effects of METH, METH-stimulated DA overflow was measured in NAc slices in the presence of RO5263397. Brain slices were perfused with either METH (2 µM), RO5263397 (2 µM or 4 µM), or combination thereof for 20 min. A two-way repeated measure ANOVA revealed a significant main effect of treatment ($F_{5, 32} = 9.073$, $p < .0001$) and time ($F_{19, 608} = 15.883$, $p < .0001$), and a significant interaction between them ($F_{95, 608} = 5.353$, $p < .0001$). *Post hoc* comparisons for the main effect of treatment showed that METH produced a robust and significant increase in DA levels ($p < .01$, by N-K tests), which was attenuated by both doses of RO5263397 ($p < .01$). RO5263397, when applied alone, failed to affect DA overflow at any dose (Figure 4.5d). Analysis of DA concentrations across the 20 min tests indicated a rapid and sustained increase following exposure to 2 µM METH ($p < .05$ at min 7; $p < .01$ at min 8 - 20, by N-K tests), reaching a ten-fold elevation relative to baseline after 12 - 14 min exposure. METH-induced DA accumulation was suppressed by RO5263397 at both 2 µM and 4 µM concentrations ($p < .05$ at min 7; $p < .01$ at min 8 - 20, by N-K tests) (Figure 4.5e).

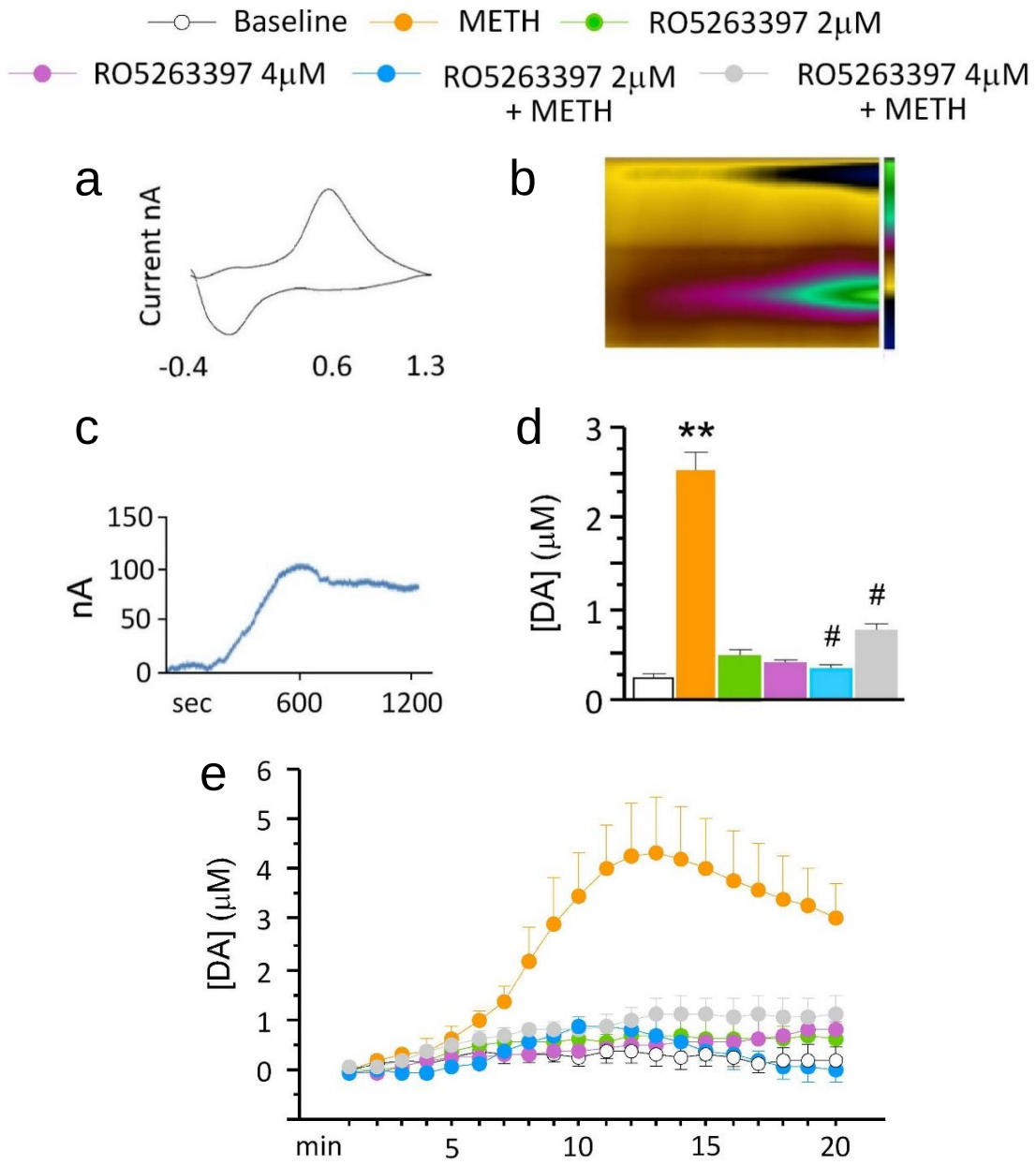

Figure 4.5 RO5263397 reduced METH-induced DA outflow in slices of rat NAc core
DA calibrations indicated a linear relationship between the peak of the raw current and the specific DA concentrations used. The background-subtracted cyclic voltammogram identified the detected analyte as DA (a). The colour plots represent the voltammetric currents (encoded in colour in the z-axis) plotted against the applied potential (y-axis) and time (x-axis) (b). A representative trace of METH demonstrates the effect of METH (2 µM) perfusion on DA outflow in raw current (nA) within a 20-min time period (c). Application of 2 µM METH significantly increased DA outflow, which was significantly attenuated by both 2 µM and 4 µM RO5263397 (d and e). ** $p < .01$ significantly different from baseline; # $p < .01$ significantly different from METH.

4.3.8 Experiment 6 summary

RO5263397 effectively blocked METH-evoked DA overflow in the NAc core measured by FSCV in rat brain slice, supporting the inhibitory effects of TAAR1 on DA overstimulation induced by psychostimulants. When applied on its own, RO5263397 did not affect DA concentrations, suggesting that this partial agonist is devoid of METH-like stimulating properties.

4.4 Discussion

The present chapter described a series of experiments demonstrating that TAAR1 agonists display the ability to reduce cocaine and METH reinforcement and prevent METH-induced neurochemical changes. Firstly, the full and partial agonists, RO5256390 and RO5203648, respectively, shifted the dose-response curve for cocaine self-administration downward, suggesting that full and partial activation of TAAR1 block cocaine reinforcement. Secondly, in a PR schedule of reinforcement, the partial agonists, RO5203648 and RO5263397, delayed the time to reach BP for cocaine self-administration and decreased the BP for METH, respectively, suggesting that TAAR1 partial activation reduces the reinforcing value of cocaine and METH as well as the motivation for them. Moreover, both partial agonists significantly increased food-maintained responding under the same PR requirement, indicating differential regulation by TAAR1 on drug and food reward. Finally, FSCV data showed that RO5263397 effectively blocked METH-induced increase in DA release in the NAc core. Together, these results provided strong evidence for TAAR1 regulation of various abuse-related behavioural and neurochemical effects of psychostimulants, supporting TAAR1 as a potential target for pharmacological development to treat addiction.

Although the TA system has long been implicated in brain reward function and in psychostimulant reinforcement, its functional role in such processes only began to be understood with the recent identification of TAAR1 and the latest development of several highly selective TAAR1 agonists. TAAR1 KO mice displayed hypersensitivity to METH-induced conditioned place preference, showing a faster acquisition rate and a greater

resistance to extinction (Achat-Mendes et al., 2012), suggesting an inhibitory role of TAAR1 in the rewarding effects of psychostimulants. An early study with the partial agonist, RO5203648, showed a reduced responding rate for a single dose of cocaine under the TAAR1 treatment (Revel et al., 2012b), which could be interpreted as either an agonistic or antagonistic action of TAAR1 on cocaine reinforcement. The present finding that TAAR1 partial and full activation produced a downward shift of the dose-response function for cocaine self-administration provided explanation for the previous equivocal result. Unlike a rightward or leftward shift of the dose-response curve, which represents a mere alteration (i.e., decrease or increase, respectively) in the reinforcing potency of the drug, a downward shift is best interpreted as a blockade of cocaine reinforcement because responding was reduced across all cocaine doses. Thus, a downward shift is the most desirable treatment outcome as it cuts the motivation for the drug regardless of doses, which is different from lateral shifts that can be easily compensated by self-regulating drug intake (Mello and Negus, 1996, Veeneman et al., 2012).

This inhibitory effect of TAAR1 on cocaine reinforcement is likely to depend on the ability of TAAR1 to inhibit DA transmission that is potentiated by cocaine. This idea is supported by the recent finding that RO5203648 prevented cocaine-stimulated DA overflow in the NAc (Pei et al., 2014). However, the molecular mechanisms involved in TAAR1's regulation on DA are less clear. RO5203648 did not affect cocaine-induced changes in the tau measure of DA uptake, suggesting that the DAT is not directly involved in such modulation (Pei et al., 2014). The possible routes of action may include TAAR1 interaction with the D2 autoreceptors, known to form a mechanism of presynaptic receptor balancing that regulates DA activity, or TAAR1's indirect influence on the DAT.

In addition to producing augmentation of DA function, cocaine modifies glutamate receptors and causes enduring alterations in both pre- and postsynaptic glutamate transmission in the VTA and NAc (Fitzgerald et al., 1996, Churchill et al., 1999, Carlezon and Nestler, 2002, Kalivas, 2004, Tang et al., 2004, Sarti et al., 2007, Mameli et al., 2011), which is critical for

understanding cocaine reinforcement and relapse. Evidence showed that antagonism of the metabotropic glutamate 5 receptor (mGluR5), which is biochemically and structurally coupled to NMDA receptor, reduced the place-conditioning effects of cocaine (McGeehan and Olive, 2003), decreased cocaine self-administration (Kenny et al., 2005), and attenuated cocaine-induced lowering of ICSS thresholds (Kenny et al., 2005), demonstrating a mediating role of glutamate in the regulation of cocaine reinforcement. Thus, given the previously mentioned implication of TAAR1 in glutamatergic function (Revel et al., 2011, Revel et al., 2013, Espinoza et al., 2015b), the ability of TAAR1 agonists to downwardly shift the cocaine dose-response curve could be linked to TAAR1 modulation of glutamate transmission. However, as the interactions between TAAR1 and glutamate systems are largely unknown, this possibility awaits future research.

Our cocaine PR experiment (experiment 4) added further support for TAAR1's inhibitory actions on cocaine reinforcement by showing that the partial agonist, RO5203648, reduced cocaine-maintained responding early in the PR session and prolonged the time to reach BP. Although the total number of infusions received during the test was not affected by TAAR1 treatment, the temporal pattern of responding was significantly regulated. The TAAR1 partial agonist appeared to have decelerated responding such that responses became more evenly spaced, which might reflect an attenuated urge for the drug. Correspondingly, the time it took to obtain each successive reinforcer was markedly delayed by TAAR1, suggesting a lessened motivation to work for cocaine and a weakened reinforcing power of cocaine.

The clear suppression on cocaine reinforcement by TAAR1 activation observed here is in agreement with reports from more recent behavioural studies examining the same or different TAAR1 selective agonists in other animal models of cocaine reward. Firstly, both the full and partial agonists, RO5256390 and RO5263397, respectively, dose-dependently prevented cocaine-induced lowering of ICSS thresholds in rats (Pei et al., 2015). As mentioned in chapter one, abusable drugs are able to decrease ICSS thresholds by acting themselves as potent rewarding stimuli that potentiate brain stimulation reward (Kornetsky et al., 1979).

Thus, this finding indicates that full or partial activation of TAAR1 renders cocaine less rewarding. Moreover, the study by Thorn et al. (2014a) found that the partial agonist, RO5263397, blocked the expression, but not the development, of cocaine-induced conditioned place preference, suggesting that TAAR1 partial activation may suppress the conditioned rewarding properties attributed to cocaine-associated cues resulting from their repeated pairing with cocaine. However, the lack of effects on the induction of cocaine-conditioned place preference is counterintuitive and may reflect a gradually diminished ability of the partial agonist to oppose cocaine reward as a consequence of chronic stimulation at TAAR1. Finally, using a between-session PR procedure of cocaine self-administration, the same group of researchers showed that RO52633397 increased the elasticity of cocaine demand curve, causing a faster decline rate in cocaine intake as the response requirement increased (Thorn et al., 2014a). According to the authors, their results suggest that RO5263397 reduced the essential value of cocaine and the motivation to take cocaine (Thorn et al., 2014a). However, RO5263397 did not affect cocaine consumption (0.75 mg/kg/infusion) at the minimal price (FR5), which contradicts the findings from the present experiments and is difficult to explain.

The last behavioural experiment in this chapter generalized the reinforcing-decreasing effects of TAAR1 to METH, by showing that, the partial agonist, RO5263397, reduced the BP for METH self-administration under the PR schedule. Data generated from more recent studies reported that METH self-administration under FR schedule of reinforcement was reduced by RO5203648 at a single METH unit injection dose (0.05 mg/kg/infusion) (Cotter et al., 2015) and by RO5263397 across a range of METH doses (0.03 - 0.1 mg/kg/infusion) (Jing et al., 2014). These, and the present, data provide straightforward and comprehensive evidence for the ability of TAAR1 partial activation to reduce the reinforcing efficacy of METH. Indeed, as mentioned previously, TAAR1's involvement in METH reinforcement was firstly noticed in the observation that TAAR1 KO mice were hypersensitive to METH-conditioned place preference (Achat-Mendes et al., 2012). Moreover, newer findings indicate that the *TAAR1* gene is within the specific chromosomal locus identified to account for more than 50% of

genetic difference in voluntary METH intake in mice, suggesting that this gene plays a part in influencing the genetic risk for METH use. Mice with a non-functional allele of the *TAAR1* gene displayed heightened METH drinking, accompanied by hyposensitivity to METH-induced conditioned taste aversion and hypothermia. On the contrary, expression of functional *TAAR1* segregated with low METH consumption and increased sensitivity to METH-related aversive and hypothermic effects (Harkness et al., 2015, Phillips and Shabani, 2015). Thus, it has been suggested that an increased TAAR1 function may protect against METH consumption through amplifying the unpleasant physiological or subjective effects of METH (Harkness et al., 2015, Phillips and Shabani, 2015).

The physiological mechanisms underlying TAAR1's dampening effects on METH reinforcement may partially overlap with those for cocaine, given the common role of DA and glutamate, as well as their interactions, in psychostimulant actions (Wang and McGINTY, 1999, Wang et al., 2006, Kalivas, 2007). Acute METH increased extracellular concentrations of DA and glutamate in the striatum (O'Dell et al., 1991, Nash and Yamamoto, 1992, Ohmori et al., 1996). Moreover, the BP for METH self-administration under PR reinforcement was reduced by selective blockade of the DA D3 receptor (Higley et al., 2011, Chen et al., 2014) or the mGluR5 receptor (Osborne and Olive, 2008, Gass et al., 2009), supporting the importance of both DA and glutamate in the reinforcing efficacy of METH. Although neurochemical studies are yet to measure the effects of TAAR1 on glutamatergic transmission, it might be possible that TAAR1 agonists have the potential to modulate glutamatergic response to METH or interfere with glutamatergic afferent to DA neurons, either directly or indirectly.

Experiment 6 was conducted to test if TAAR1 regulates METH-induced alteration in DA transmission and found that RO5263397 prevented the robust and sustained increases in DA overflow produced by METH treatment. This evidence could, at least in part, account for the marked ability of RO5263397 to reduce the acute reinforcing effect of METH that is responsible for maintaining self-administration and sustaining motivation for the drug.

Moreover, this finding is consistent with the inhibitory regulation by RO5203648 on cocaine-induced DA accumulation in the NAc (Pei et al., 2014), which together support the hypothesis that TAAR1 activation is able to counteract abnormally elevated DA neurotransmission, especially DA surges produced by psychostimulants. Besides, experiment 6 also showed that RO5263397 did not augment DA overflow when applied singly, suggesting that the partial agonist does not share the neurochemical properties of METH, which is a favourable feature for anti-addiction drugs to have.

The exact molecular processes underlying the reduction in METH-induced DA release by TAAR1 are unclear. As previously described, different from cocaine which has no affinity at TAAR1, METH interaction with TAAR1 triggers a series of phosphorylation events to inhibit DA uptake, enhance DA efflux, and trigger DAT internalization (Xie and Miller, 2009a). Thus, given its partial agonist profile, RO5263397 may exert antagonistic action at TAAR1 to reduce the TAAR1-dependent effects of METH on DA release. Meanwhile, the phosphorylation cascades resulting from METH stimulation of TAAR1 may be opposed by D2 autoreceptors, which adds additional inhibitory control over METH-augmented DA concentrations (Miller, 2011). In addition, TAAR1 expression was observed in neurons that did not co-express DAT but were in approximation to DAT neurons in mouse SNr (Xie et al., 2007). Activating TAAR1 located in non-DA neurons by METH or TAAR1 selective agonists may inhibit the firing frequency of the adjacent DA neurons, leading to decrease in terminal DA release (Miller, 2011).

Lastly, we showed that both RO5203648 and RO5263397 increased response rates and the BP for food pellets under the same PR requirement, suggesting that psychostimulant and food reinforcement are mediated by different mechanisms in the brain and that TAAR1 treatment inhibits drug-related behaviours specifically. The remarkable dissociation is not entirely surprising because electrophysiological studies have reported non-overlapping patterned discharge of NAc neurons in response to psychostimulants (i.e., cocaine) and natural rewards (i.e., food and water), suggesting that the neuronal population encoding across different

reward conditions is largely segregated within the NAc (Carelli et al., 2000, Carelli, 2002). It is interesting to explore the mechanisms by which TAAR1 is able to differentially influence such divergent neuronal networks. The fact that stimulant drugs produce more robust enhancement in DA transmission, which is larger in magnitude and longer in duration, relative to natural reinforcers may be part of the cause (Volkow et al., 2004, Ries et al., 2009). It is possible that fluctuations in DA transmission modulate the magnitude and direction of TAAR1-mediated regulation, which is consistent with the previously proposed notion that TAAR1 serves as a stabiliser to "normalize" deviated DA activity, rather than as a simple brake on the DA system.

Taken together, the findings from the present chapter showed that TAAR1 selective agonists reduced the reinforcing efficacy of cocaine and METH and blocked METH-induced DA release in the NAc without affecting DA level when administered alone. These results highlight the therapeutic potential of TAAR1 agonists in influencing abuse-related effects of psychostimulants and support the TAAR1-centered approach in anti-addiction drug development.

Chapter Five

5 TAAR1 Activation Prevents Relapse to Drug Seeking

5.1 Introduction

Results obtained from experiments in chapters three and four strongly demonstrated the ability of TAAR1 to modulate abuse-related behavioural and neurochemical effects of psychostimulants including cocaine- or METH-induced hyperlocomotion, reinforcement, and DA release/overflow. These findings, along with early evidence for the involvement of TAAR1 in DA transmission and psychostimulant action, support TAAR1 as a promising pharmacological target to treat stimulant addiction. Chapter five aimed to further characterize the therapeutic potential of TAAR1-based agents in clinically-relevant models of addiction, in particular relapse models. Relapse to drug seeking is indeed one of the major obstacles addicts encounter on the path to recovery. After a long drug-free period, relapse to drug seeking can be triggered by re-experiencing contexts associated with past drug-taking behaviour, which can be studied in animal model of context-induced renewal of drug-seeking without extinction (Fuchs et al., 2006). Moreover, relapse can be induced by the drug itself following a period of forced extinction training, which is best captured in the extinction-reinstatement model (Shalev et al., 2002, Shaham and Hope, 2005). Studies have implicated an elevated mesolimbic DA transmission in both drug-primed reinstatement and context-induced relapse (Self, 1998, Anderson et al., 2003, Crombag et al., 2008). Both the drug and drug-associated stimuli increase DA concentration in the striatum including the NAc and the dorsal striatum, which is associated with drug craving and drug seeking (Shaham and Hope, 2005, Volkow et al., 2006). Therefore, given the inhibitory control of TAAR1 over dopaminergic function, TAAR1 activation is expected to suppress drug-seeking behaviour in these situations. To test this hypothesis, we firstly examined the effects of RO5203648 and RO5256390, the partial and full TAAR1 selective agonists, respectively, on context-induced renewal of drug seeking in rats previously trained to self-administer cocaine.

The two agonists were also tested against food-taking behaviour in order to control for potential motoric and motivational confounds (experiment 7)[7]. Next, the ability of the partial TAAR1 agonists, RO5203648 and RO5263397, to reduce drug-primed reinstatement of cocaine (experiment 8)[7] and METH (experiment 9)[8] seeking, respectively, was investigated. The last experiment assessed the potential abuse liability of TAAR1 ligands, which is an important issue to consider in evaluating novel anti-addiction medications. A substitution procedure was used to test the degree to which the partial agonist, RO5263397, sustained self-administration that was previously maintained by METH (experiment 10)[8].

5.2 Materials and Methods

5.2.1 Subjects

Long Evans male rats (8-10 weeks-old at the start of the experiments) were obtained from the University of Canterbury and maintained on a reversed 12-h light/dark cycle (lights off at 8:00 a.m.) and standard conditions of temperature and humidity. Rats were given a maintenance diet (i.e., kept at 100% of their weight seven days' post-surgery) and free access to water. All procedures were approved by the Animal Ethics Committee of the University of Canterbury.

5.2.2 Pharmacological agents

Cocaine hydrochloride was obtained from the National Institute on Drug Abuse (USA). METH hydrochloride was obtained from BDG synthesis (Wellington, New Zealand). Both drugs were dissolved in 0.9% physiological saline for i.p. injection and i.v. self-administration. RO5256390, RO5203648, and RO5263397 were synthesized at F.

[7] Published paper. Experiment 7 and 8 have been published in the following paper: Pei Y, Lee J, Leo D, Gainetdinov RR, Hoener MC, Canales JJ (2014) Activation of the trace amine-associated receptor 1 prevents relapse to cocaine seeking. Neuropsychopharmacology 39:2299-2308. doi: 10.1038/npp.2014.88.

[8] Published paper. Experiment 9 and 10 have been published in the following paper: Pei Y, Asif-Malik A, Hoener M, Canales JJ (2016) A partial trace amine-associated receptor 1 agonist exhibits properties consistent with a methamphetamine substitution treatment. Addiction biology, doi: 10.1111/adb.12410.

Hoffmann-La Roche Ltd (Switzerland) and dissolved in 10% dimethylsulfoxide and 0.9% physiological saline for i.p. injection or i.v. self-administration.

5.2.3 Catheter implantation surgery

The surgical procedure was the same as previously described in chapter four.

5.2.4 Apparatus

The self-administration apparatus was the same as described in chapter four. During the drug self-administration training sessions, active lever presses resulted in activation of the infusion pump and delivery of the drug (i.e., cocaine or METH) or saline. In the substitution sessions (experiment 10), active lever presses led to pump activation and infusion of a dose of RO5263397, METH, or saline. In the food assay, response on the active lever resulted in delivery of a chocolate-flavoured pellet (45 mg, Bio-Serve, NJ, USA) into a food magazine situated between the two levers. The delivery of reinforcers was always paired with illumination of a light stimulus for 5 seconds.

5.2.5 Context-induced relapse of cocaine seeking (experiment 7)

Rats were trained to self-administer cocaine (0.5 mg/kg/infusion, 100 µl) or saline, being sequentially exposed to FR1, FR2, and finally FR3 reinforcement schedules during long-access sessions. Schedule progression was dependent upon meeting a criterion (number infusions per session ≥ 30). Rats were then transferred to and maintained at daily FR3 90 min sessions until meeting a criterion of stability and consistency (number infusions per session ≥ 15 for five consecutive days with less than 20% variability). Cocaine infusions were limited to a maximum of 40 to prevent overdosing. After self-administration training, rats underwent withdrawal during which no access was given to the self-administration chambers for 14 days. Relapse to drug seeking was tested by placing the rats back to the drug-taking context (i.e., self-administration chamber with the two levers exposed) for 45 min. Lever presses had no programmed consequence (neither the drug delivery nor the light stimulus). Each rat completed three drug-seeking tests on alternate days on which RO5203648 (0, 3, or 10

mg/kg, i.p.) or RO5256390 (0, 3, or 10 mg/kg, i.p.) was given 15 min before entering the chamber.

5.2.6 Food assay (experiment 7 cont.)

After completion of the context-induced relapse tests, rats were left undisturbed for two weeks and were later trained to lever press for chocolate-flavoured pellets under a FR1 schedule in daily 45 min sessions. The maximal number of food pellets was limited to 40 per session to approximate the response rates obtained in cocaine relapse test. Once a stability criterion was met (number of food pellets ≥ 20 for three consecutive days), each rat was subjected to three food intake tests by receiving RO5203648 (0, 3, or 10 mg/kg, i.p.) (n = 8) or RO5256390 (0, 3, or 10 mg/kg, i.p.) (n = 8) 15 min prior to the test session (45 min) with the different doses administered in counterbalanced order.

5.2.7 Cocaine-primed reinstatement of drug seeking (experiment 8)

Rats were trained to stably self-administer cocaine (0.5 mg/kg/infusion, 100 µl) on daily FR3 90 min sessions. The training procedure and stability criterion were the same as above. The extinction sessions were then introduced during which lever press was not reinforced either by infusions or the stimulus light. The extinction was conducted daily during 90 min sessions until a criterion was met (at least ten days of extinction training and the active lever presses during the last three extinction sessions being ≤ 15% of the average number of active lever presses during the last five drug self-administration sessions). Rats that did not meet this criterion after 21 extinction sessions were excluded from this study. After the extinction phase, each rat underwent two reinstatement tests, receiving cocaine (10 mg/kg, i.p.) or saline in counterbalanced fashion before the start of the sessions. To assess the effects of TAAR1 partial agonists on drug-induced reinstatement, rats were divided into three experimental groups (n = 7-8 per group) receiving a pretreatment of RO5203648 (0, 3, or 10 mg/kg, i.p.) 15 min prior to cocaine or saline injection. The reinstatement sessions were identical to extinction sessions except that they lasted for 3 h. In between the two reinstatement tests rats underwent daily 90 min extinction sessions to ensure that active lever presses returned to

pre-reinstatement levels for two consecutive days. The three experimental groups were matched for performance during the drug self-administration sessions and extinction phase. To control for potential non-specific responding, a separate group of rats (n = 5) responding for saline during the self-administration phase underwent the same extinction procedure and received a pretreatment of vehicle 15 min before cocaine or saline injection in the reinstatement tests.

5.2.8 METH-primed reinstatement of drug seeking (experiment 9)

Rats (n = 27) were trained to lever press for METH (0.05 mg/kg/infusion, 100 µl) in FR3 90 min daily sessions, with the same training procedures as described above. METH infusions were limited to a maximum of 40 per session to prevent overdosing. After response reached stability and consistency (number infusions per session ≥ 17 for five consecutive days with less than 20% variance in the last three days), rats underwent extinction training followed by two reinstatement tests, with the procedure same as that for experiment 8. During the reinstatement test, RO5263397 (0, 3, or 10 mg/kg, i.p.) was administered 15 min prior to a prime injection of METH (1 mg/kg, i.p.) or saline. An additional group (n = 4) responding for saline during the self-administration phase was included to control for non-specific responding. The control rats underwent the same extinction procedures and received vehicle treatment 15 min before METH or saline injection during the reinstatement tests.

5.2.9 Substitution assay (experiment 10)

Rats (n = 26) were trained on METH self-administration (0.05 mg/kg/infusion) under a FR3 reinforcement schedule in daily 90 min sessions until a stability criterion was met, as above. To study the reinforcing property of RO5263397, rats were divided into four groups (n = 6-7 per group) to receive RO5263397 (0.25 or 0.5 mg/kg/infusion), a lower dose of METH (0.017 mg/kg/infusion), or vehicle as the substitute compound. Rats were trained on the same substitute treatment for five consecutive days. The average METH intake and response rate over the last three METH self-administration pre-training sessions were matched between the four groups before introduction of the substitution treatment.

5.2.10 Statistical analysis

Data were analysed by ANOVA with repeated measures when a within-subjects design was in use, followed by *post hoc* comparisons with the method of N-K tests using the sampling error from the overall ANOVA as denominator. Statistical significance was set at $\alpha = 0.05$ for all experiments. All statistical analyses were performed using StatView 5.0 (SAS Institute, NC, USA).

5.3 Results and Summary

5.3.1 Experiment 7 results

Firstly, we tested the ability of the partial TAAR1 agonist, RO5203648, to attenuate context-induced cocaine seeking after a 2-week period of abstinence from cocaine self-administration. Rats were trained to lever press for cocaine (0.05 mg/kg/infusion) ($n = 7$) or saline ($n = 8$) on a FR3 schedule of reinforcement. The ANOVA and *post hoc* comparisons showed that rats responding for cocaine produced a significantly higher number of active lever presses (ANOVA $F_{1, 13} = 116.98$, $p < .001$; $p < .01$, by N-K tests) and obtained more cocaine infusions (ANOVA $F_{1, 13} = 207.95$, $p < .001$; $p < .01$, by N-K tests) than rats responding for saline (Figure 5.1a and inset).

After two weeks of abstinence, rats were placed back into the self-administration chambers with the two levers exposed but lever pressing was not reinforced either by the drug infusion or the light stimulus. Rats previously trained on cocaine self-administration showed robust relapse to drug seeking on the active lever upon re-exposure to the chambers, which was effectively blocked by RO5203648 pretreatment. ANOVA for lever presses revealed significant main effects of drug (cocaine vs. saline, $F_{1, 13} = 44.78$, $p < .001$), lever (active vs. inactive, $F_{1, 13} = 25.86$, $p < .001$), and dose of RO5203648 ($F_{2, 26} = 57.33$, $p < .001$), and a significant interaction between drug and lever ($F_{1, 13} = 27.40$, $p < .001$), dose and drug ($F_{2, 16} = 30.62$, $p < .0001$), lever and dose ($F_{2, 26} = 9.61$, $p < .001$), as well as a significant three-way interaction between these factors ($F_{2, 26} = 7.77$, $p < .002$) (Figure 5.1b). *Post hoc* comparisons showed significant effects of both the low ($p < .01$, by N-K tests) and the high dose ($p < .01$,

by N-K tests) of RO5203648 on active lever presses. There was a comparatively lower but significant increase in the number of inactive lever presses made by cocaine-trained rats. Such an increase was similarly prevented by RO5203648 at both doses ($p < .01$, by N-K tests).

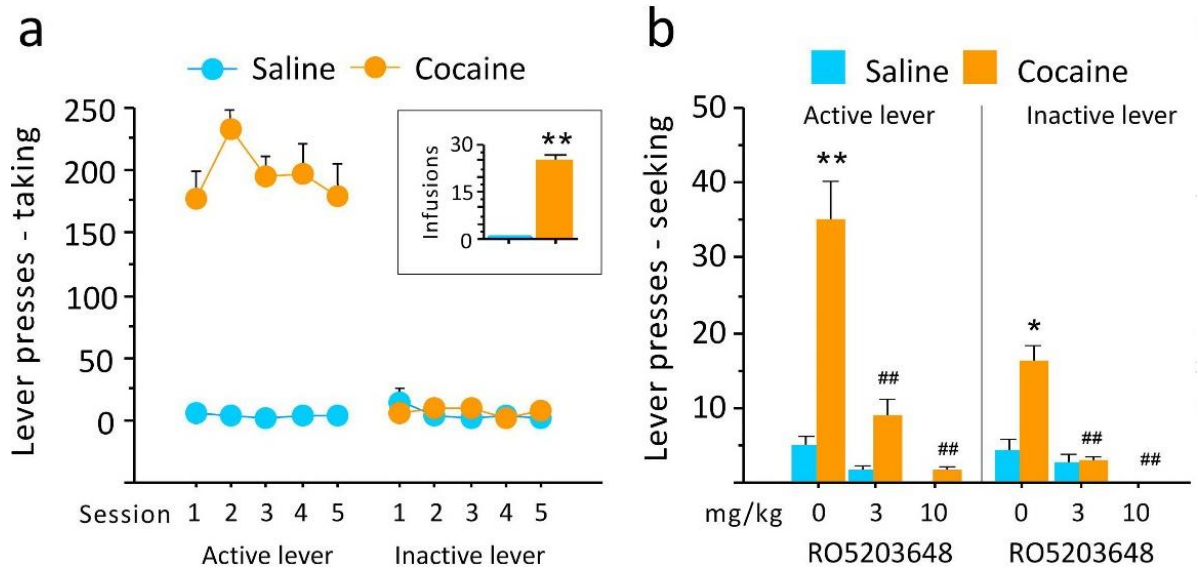

Figure 5.1 RO5203648 suppressed context-induced relapse of cocaine seeking
After consistent cocaine self-administration performance (a) and protracted abstinence, rats showed robust drug-seeking behaviour upon exposure to the self-administration chambers, which was significantly suppressed by RO5203648 (b). * $p < .05$, ** $p < .01$ significantly different from saline value; ## $p < .01$ significantly different from vehicle pretreatment for cocaine value.

To control for potential non-specific effects of RO5203648 on general motivation and motor performance, RO5203648 was tested in rats ($n = 8$) responding for chocolate-flavoured pellets under a FR1 reinforcement schedule. The overall responding rate was not affected by RO5203648 at any dose. ANOVA did not reveal any significant effects of RO5203648 on food-maintained lever presses ($F_{2, 14} = 1.39$, $p = 0.283$). However, analysis of responding rate across time bins revealed a significant interaction between TAAR1 treatment and time ($F_{4, 28} = 6.86$, $p < .001$). Rats treated with the high dose of RO5203648 obtained a similar amount of

food pellets in the 45 min session (Figure 5.2a) but were slower to initiate responding, earning significantly fewer reinforcers in the 1st 15 min ($p < .01$, by N-K tests) (Figure 5.2b).

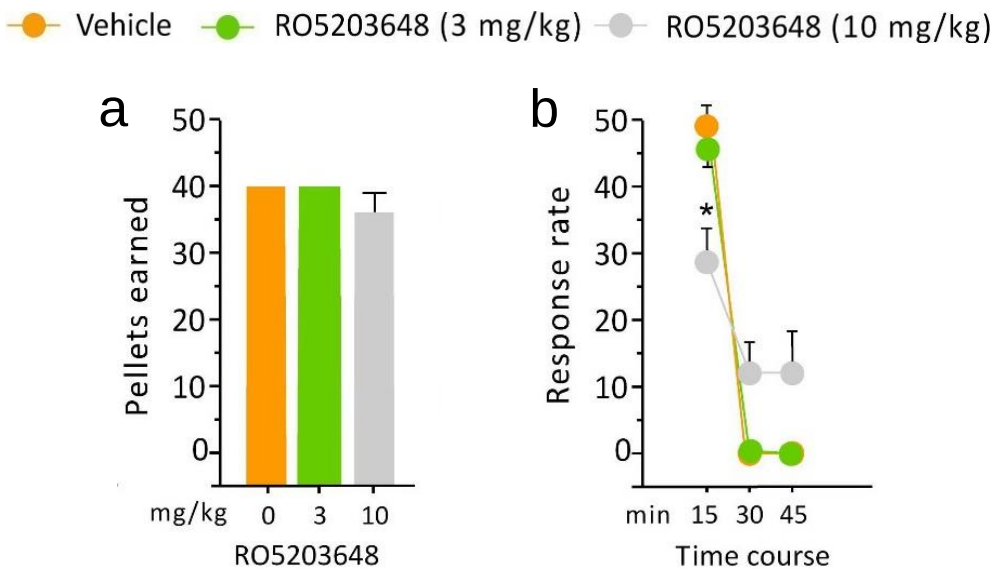

Figure 5.2 RO5203648 delayed food intake only at the high dose
Rats were trained to stably respond for food pellets under FR1 reinforcement schedule. RO5203648 pretreatment did not affect total number of pellets earned (a) but slightly delayed response initiation at the high dose only (b). * $p < .01$ significantly different from vehicle pretreatment.

Next, we examined the effects of full TAAR1 activation on context-induced cocaine relapse by testing RO5256390 under identical conditions. Rats were firstly trained to stably self-administer cocaine ($n = 8$) or saline ($n = 6$) on a FR3 reinforcement schedule. ANOVA revealed a significant effect of the drug (cocaine vs. saline) on active lever presses ($F_{1, 12} = 196.61$, $p < .001$) and on infusions obtained ($F_{1, 12} = 136.00$, $p < .001$), with both parameters being significantly higher for rats responding for cocaine than for saline ($p < .01$, by N-K tests) (Figure 5.3a and inset). In the relapse test, rats previously trained for cocaine showed high level of drug seeking only on the active lever, which was dose-dependently blocked by RO5256390. ANOVA for lever presses revealed significant effects of drug (cocaine vs. saline, $F_{1, 12} = 20.15$, $p < .001$), lever (active vs. inactive, $F_{1, 12} = 32.73$, $p < .001$), and dose of

RO5256390 ($F_{2, 24}$ = 33.73, p < .001), as well as a significant interaction between these factors ($F_{2, 24}$ = 13.83, p < .001). RO5256390 significantly reduced active lever presses at both the low (p < .01, by N-K tests) and the high dose (p < .01, by N-K tests) (Figure 5.3b).

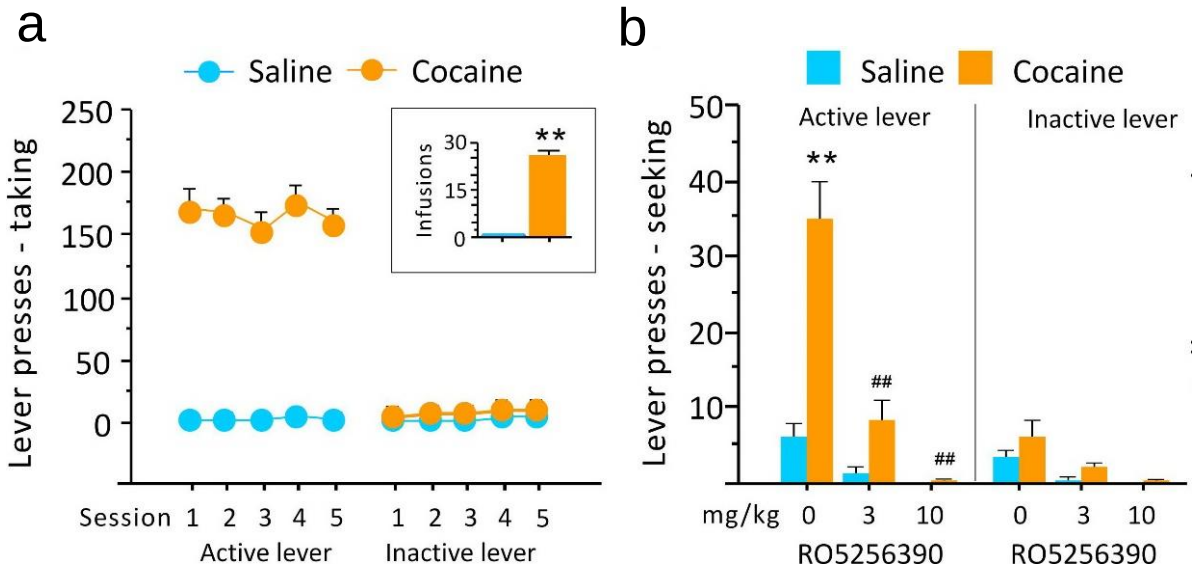

Figure 5.3 RO5256390 suppressed context-induced relapse of cocaine seeking
After stable cocaine self-administration training (a) and abstinence, relapse to drug seeking was induced by re-exposing to the self-administration chambers. RO5256390 pretreatment significantly blocked this context-induced relapse of cocaine seeking (b). ** p < .01, significantly different from saline values; ## p < .01 significantly different from vehicle pretreatment for cocaine values.

In the food assay, RO5256390, at the high, but not low, dose, produced nonspecific effects on food-maintained responding. The overall number of food pellets earned was significantly reduced by the high dose of RO5256390 as revealed by ANOVA ($F_{2, 14}$ = 5.02, p < .05) and *post hoc* comparisons (p < .05, by N-K tests) (Figure 5.4a). Time course analysis showed a significant interaction between RO5256390 and time ($F_{4, 28}$ = 15.07, p < .001). RO5256390 at the high dose delayed response rate and significantly decreased number of reinforcers obtained in the initial 15 min of the session (p < .05, by N-K tests) (Figure 5.4b).

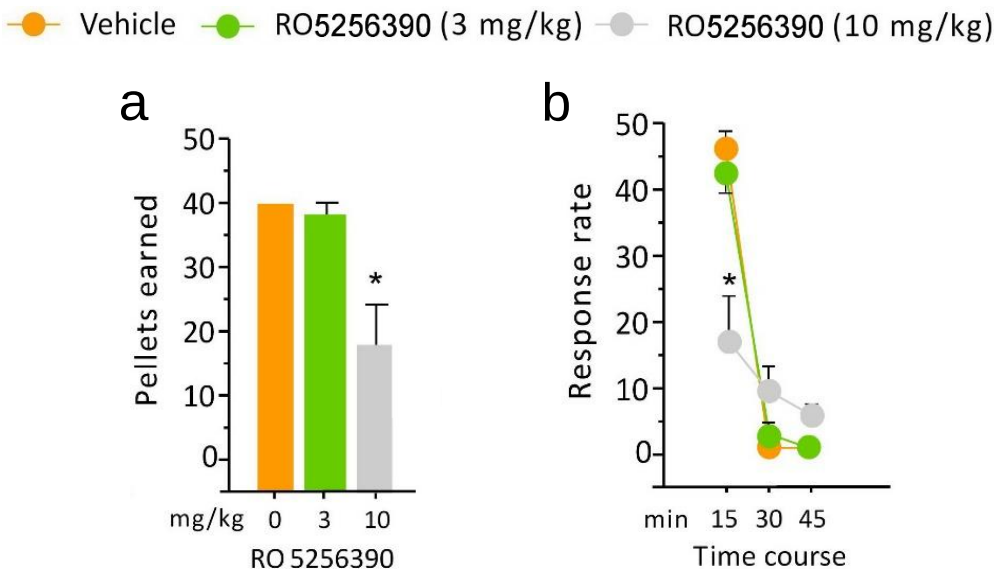

Figure 5.4 RO5256390 affected food intake only at the high dose
Rats were trained to stably respond for food pellets under FR1 reinforcement schedule. RO5256390 pretreatment at the high dose reduced food intake (a) and delayed response (b). * $p < .05$ significantly different from vehicle pretreatment.

5.3.2 Experiment 7 summary

After protracted withdrawal from chronic cocaine self-administration, the partial and full TAAR1 agonists, RO5203648 and RO5256390, respectively, prevented relapse of cocaine seeking induced by re-exposing the rats to the self-administration chambers. In the food assay, RO5203648 at the high dose delayed response initiation without affecting overall number of reinforcers earned. RO5256390 at the high dose delayed response and reduced number of pellets obtained. Both agonists at the low dose, which was still highly effective in attenuating cocaine relapse, did not impair food-maintained behaviour.

5.3.3 Experiment 8 results

To test the ability of RO5203648 to reduce cocaine-primed reinstatement of drug seeking, three groups of rats (n = 7-8 per group) were firstly trained to self-administer cocaine on a FR3 reinforcement schedule. The three groups responded similarly and obtained similar number of infusions in the last five days of cocaine self-administration before extinction was introduced. A control group of rats (n = 5) responding for saline showed very low response

rate. ANOVA for lever presses during the last five cocaine self-administration sessions revealed a significant effect of group (cocaine vs. saline, $F_{3, 23} = 34.56$, $p < .001$) and lever (active vs. inactive, $F_{1, 23} = 390.06$, $p < .001$), and a significant interaction between these factors ($F_{3, 23} = 35.83$, $p < .001$). Mean comparisons showed a significant difference between the control group and each of the three experimental groups in the number of active lever presses ($p < .01$, by N-K tests), but not in the number of inactive lever presses. ANOVA for the average number of infusions over the five days showed a significant effect of group ($F_{3, 23} = 39.32$, $p < .001$), which was due to significantly more infusions obtained by the three cocaine groups compared with the control group ($p < .01$, by N-K tests) (Figure 5.5a and inset).

During extinction, all but seven rats in the experimental groups gradually decreased responding on the active lever and reached the extinction criteria within 21 days. These seven rats were excluded from further analysis. Responding on the inactive lever was low throughout and did not differ between groups. A repeated measure ANOVA for lever presses during the last three extinction sessions showed a significant effect of group ($F_{3, 23} = 4.74$, $p < .01$) and lever ($F_{1, 23} = 40.74$, $p < .001$), and a significant interaction between these factors ($F_{3, 23} = 5.34$, $p < .01$). *Post hoc* comparisons revealed no significant differences between the three groups previously responding for cocaine (Figure 5.5b).

In the reinstatement tests, rats in the experimental groups received RO5203648 (0, 3, or 10 mg/kg, i.p.) followed 15 min after by cocaine (10 mg/kg, i.p.) or saline injection before entering the chambers. The control rats received vehicle pretreatment before cocaine or saline. The reinstatement session lasted for 3 h during which lever presses had no programmed consequences. ANOVA for the total number of lever presses during the test showed a significant main effect of prime injection (saline vs. cocaine, $F_{1, 23} = 18.81$, $p < .001$), lever (active vs. inactive, $F_{1, 23} = 47.69$, $p < .001$), and dose of RO5203648 ($F_{3, 23} = 7.62$, $p < .001$), and a significant interaction between lever and dose of RO5203648 ($F_{3, 23} = 8.25$, $p < .001$), prime injection and RO5203648 ($F_{3, 23} = 6.03$, $p < .01$), lever and prime

injection ($F_{1, 23} = 12.17$, $p < .005$), as well as a significant three-way interaction between these factors ($F_{3, 23} = 4.69$, $p < .01$). *Post hoc* comparisons showed that cocaine priming induced significantly higher response rates on the active lever, but not on the inactive lever, compared with saline treatment ($p < .01$, by N-K tests). This reinstatement of drug seeking was dose-dependently attenuated by RO5203648 at both the low ($p < .01$, by N-K tests) and the high dose ($p < .01$, by N-K tests). RO5203648 did not in its own right (i.e. when given before saline) renew drug seeking at any dose (Figure 5.5c).

ANOVA analysis for active lever presses in 20 min bins across the reinstatement test for the three cocaine groups showed a significant effect of dose of RO5203648 ($F_{2, 12} = 6.33$, $p < .05$), cocaine priming ($F_{1, 12} = 9.28$, $p < .05$), and time ($F_{8, 96} = 8.04$, $p < .001$), and a significant interaction between RO5203648 and cocaine priming ($F_{2, 12} = 6.55$, $p < .05$), time and RO5203648 ($F_{16, 96} = 7.01$, $p < .0001$), cocaine priming and time ($F_{8, 96} = 2.57$, $p < .05$), as well as a significant three-way interaction between these factors ($F_{16, 96} = 5.98$, $p < .001$). N-K tests indicated that cocaine produced significantly higher number of active lever presses in the 1st ($p < .01$) and the 2nd ($p < .01$) 20 min bins, and this effect was significantly attenuated by RO5203648 at both the low ($p < .01$) and the high ($p < .01$) dose (Figure 5.5d).

5.3.4 Experiment 8 summary

After extinction of cocaine self-administration, the partial TAAR1 agonist, RO5203648, effectively blocked reinstatement of cocaine seeking triggered by a prime injection of cocaine. RO5203648 in its own right did not reinstate cocaine seeking.

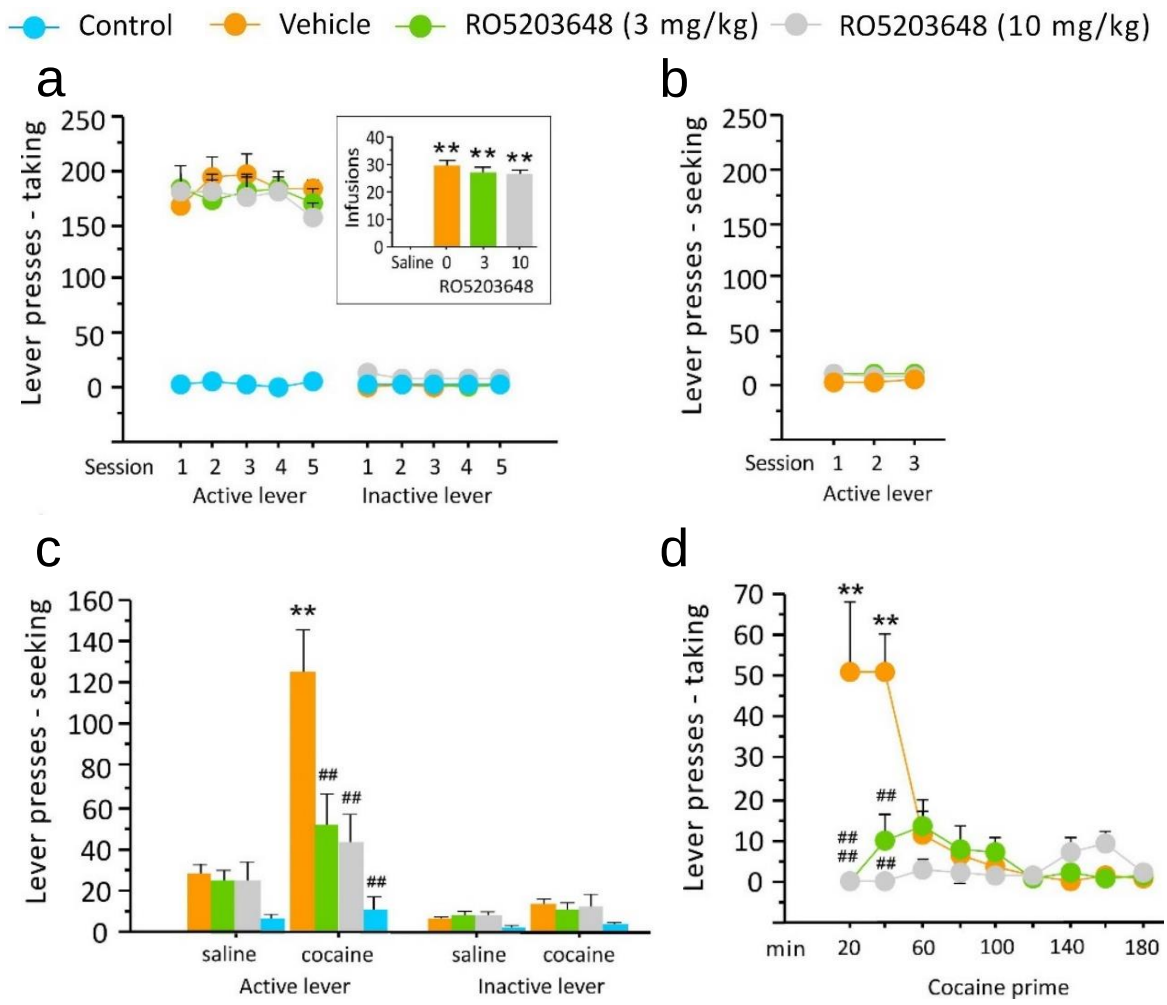

Figure 5.5 RO5203648 prevented cocaine-primed reinstatement of cocaine seeking
Three groups of rats were trained to stably self-administer cocaine, showing significantly higher levels of response than the group self-administering saline (a). This phase was followed by extinction during which response rate dropped to low levels in the last three extinction sessions (b). A prime injection of cocaine generated strong reinstatement of cocaine seeking, which was dose-dependently attenuated by RO5203648 (c and d). ** $p < .01$, significantly different from saline values; ## $p < .01$, significantly different from cocaine values.

5.3.5 Experiment 9 results

To test if partial TAAR1 agonism was also effective in preventing METH-primed reinstatement of METH seeking, the partial agonist, RO5263397, was tested in the same extinction-reinstatement model. During the self-administration phase, the three experimental groups (n = 9 per group) responding for METH (0.05 mg/kg/infusion) showed similar level

of response rate and obtained similar number of infusions during the last five METH self-administration sessions, which were significantly higher than the control group (n = 4) responding for saline. ANOVA for lever presses during the last five METH training sessions showed a significant effect of group (METH vs. saline, $F_{3, 27} = 40.37$, $p < .0001$) and lever (active vs. inactive, $F_{1, 27} = 334.92$, $p < .0001$), and a significant interaction between them ($F_{3, 27} = 24.59$, $p < .0001$). *Post hoc* comparisons showed a significant difference between the control group and each of the three experimental groups in active lever presses ($p < .01$, by N-K tests), but not in inactive lever presses (Figure 5.6a).

During extinction, all the rats in the experimental groups gradually decreased responding on the active lever and met the extinction criterion within 21 days. A repeated measure ANOVA for lever presses during the last three extinction sessions showed a significant effect of lever ($F_{1, 27} = 52.65$, $p < .0001$) and a significant interaction between lever and group ($F_{3, 27} = 3.38$, $p < .05$). This was due to a significantly higher response rate on the active lever than on the inactive lever in the three METH groups during the last three extinction sessions ($p < .05$, by N-K tests). The three METH groups did not differ in active or inactive lever responding in the last three days of extinction training (Figure 5.6b).

After extinction, reinstatement of drug seeking was examined by giving the rats METH (1 mg/kg, i.p.) or saline in counterbalanced fashion. The ANOVA for the total number of lever presses in the reinstatement test showed a significant main effect of dose of RO5263397 ($F_{3, 27} = 10.47$, $p < .0001$), lever ($F_{1, 27} = 38.54$, $p < .0001$), and prime injection ($F_{1, 27} = 29.16$, $p < .0001$), and a significant interaction between lever and RO5263397 ($F_{3, 27} = 10.14$, $p < .0001$), prime injection and RO5263397 ($F_{3, 27} = 9.11$, $p < .001$), lever and prime injection ($F_{1, 27} = 30.99$, $p < .0001$), as well as a significant three-way interaction between those factors ($F_{3, 27} = 10.05$, $p < .001$). *Post hoc* comparisons showed that METH priming induced robust responding on the active, but not inactive, lever compared to the saline injection ($p < .01$, by N-K tests). The METH-primed reinstatement was significantly attenuated by RO5263397 at both the low ($p < .01$) and the high dose ($p < .01$). RO5263397 applied alone did not reinstate

drug seeking at any dose (Figure 5.6c). Active lever presses during the reinstatement test were further analysed in 1 h bins for the three experimental groups. A repeated measure ANOVA showed a significant effect of dose of RO5263397 ($F_{2, 24} = 11.84$, $p < .001$) and prime injection ($F_{1, 24} = 44.97$, $p < .0001$), and a significant interaction between RO5263397 and prime injection ($F_{2, 24} = 11.19$, $p < .001$), time and RO5263397 ($F_{4, 48} = 2.60$, $p < .05$), prime injection and time ($F_{2, 48} = 4.89$, $p < .05$), as well as a significant three-way interaction between RO5263397, prime injection, and time ($F_{4, 48} = 2.99$, $p < .05$). Drug seeking was prevented by RO5263397 at both doses during the 1st ($p < .01$, by N-K tests) and the 2nd ($p < .01$, by N-K tests) h, but not during the last h, as evidenced by a significant increase in active lever presses relative to the preceding time bins ($p < .05$ for both the 1st and the 2nd time bin and for both doses of RO5263397) (Figure 5.6d).

5.3.6 Experiment 9 summary

The partial TAAR1 agonist, RO5263397, suppressed METH-primed reinstatement of METH seeking after extinction from chronic METH exposure and did not renew drug seeking when given alone.

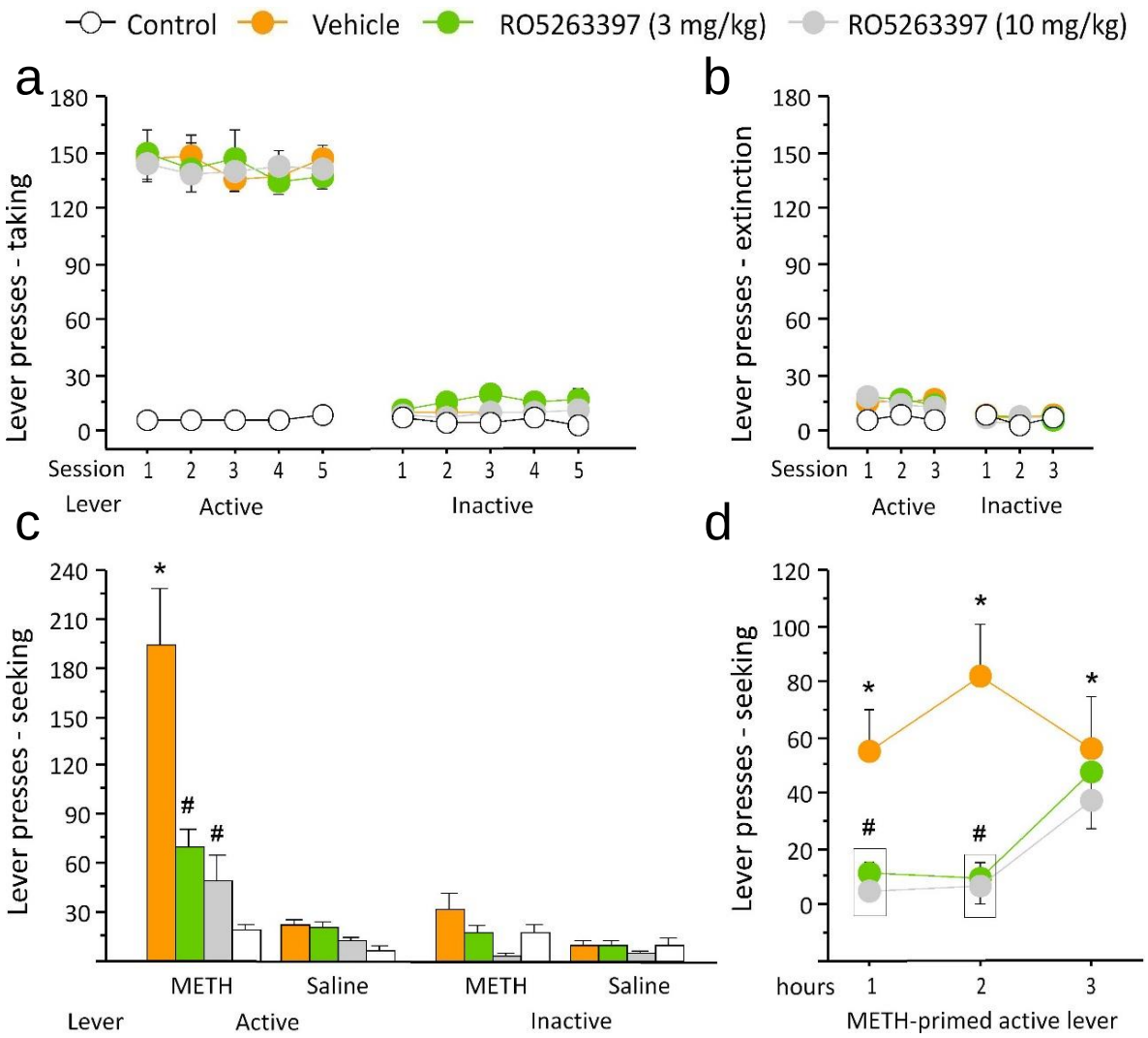

Figure 5.6 RO5263397 prevented METH-primed reinstatement of METH seeking
After rats were trained to stably self-administer METH or saline (a), extinction was introduced during which response decreased to low levels in the last three extinction sessions (b). A challenge dose of METH produced robust reinstatement of drug seeking, which was significantly reduced by RO5263397 (c and d). * $p < .01$ significantly different from saline values; # $p < .01$ significantly different from METH values.

5.3.7 Experiment 10 results

To test the abuse potential of RO5263397, we substituted RO5263397 for METH in the self-administration task. Four experimental groups were trained to stably self-administer METH (n = 6-7 per group; 0.05 mg/kg/infusion) on a FR3 schedule of reinforcement. These four groups responded similarly and obtained similar number of infusions in the last three

METH self-administration training sessions. In the substitution tests, two doses of RO5263397 (0.25 or 0.5 mg/kg/infusion), a lower dose of METH (0.017 mg/kg/infusion), or vehicle were given as the substitute compound for five consecutive days. Unlike the low dose of METH, which elevated the response rate, RO5263397 did not sustain responding at either dose. A repeated measure ANOVA for active lever presses during the five days revealed a significant effect of treatment ($F_{3, 22} = 76.25$, $p < .0001$), lever ($F_{1, 22} = 327.37$, $p < .0001$), and day ($F_{4, 88} = 8.34$, $p < .0001$), and a significant interaction between lever and treatment ($F_{3, 22} = 113.12$, $p < .0001$), lever and day ($F_{4, 88} = 5.71$, $p < .001$), as well as a significant three-way interaction between those three factors ($F_{12, 88} = 3.41$, $p < .001$). Means comparisons showed that the low dose of METH (0.017 mg/kg/infusion) produced the highest active lever responding rate among the four groups in all the five test sessions ($p < .01$ compared with vehicle and both doses of RO5263397, by N-K tests). Response for both doses of RO5263397 was significantly lower than that for vehicle on day 1 ($p < .01$, by N-K tests) (Figure 5.7a). For number of infusions obtained during the five sessions, repeated measure ANOVA revealed a significant main effect of treatment ($F_{3, 22} = 196.05$, $p < .0001$) and day ($F_{4, 88} = 12.77$, $p < .0001$), and a significant interaction between treatment and day ($F_{12, 88} = 3.36$, $p < .001$). *Post hoc* comparisons showed that rats obtained most infusions when given the low dose of METH for all the five test days ($p < .01$ compared with vehicle and the two RO5263397 substitution groups). RO5263397 at both doses, when substituted for METH, produced significantly less infusions than vehicle on the first session ($p < .05$ for the low dose; $p < .01$ for the high dose of RO5263397, by N-K tests) (Figure 5.7b).

5.3.8 Experiment 10 summary

In rats stably self-administering METH, two doses of RO5263397, a lower dose of METH, and vehicle were introduced as the substitute drug through i.v. infusions. Unlike the low dose of METH which increased response, RO5263397 at both doses led to an immediate drop in response rate, which was even lower than that produced by vehicle substitute on the first session. These results suggest that this partial TAAR1 agonist possesses low abuse liability.

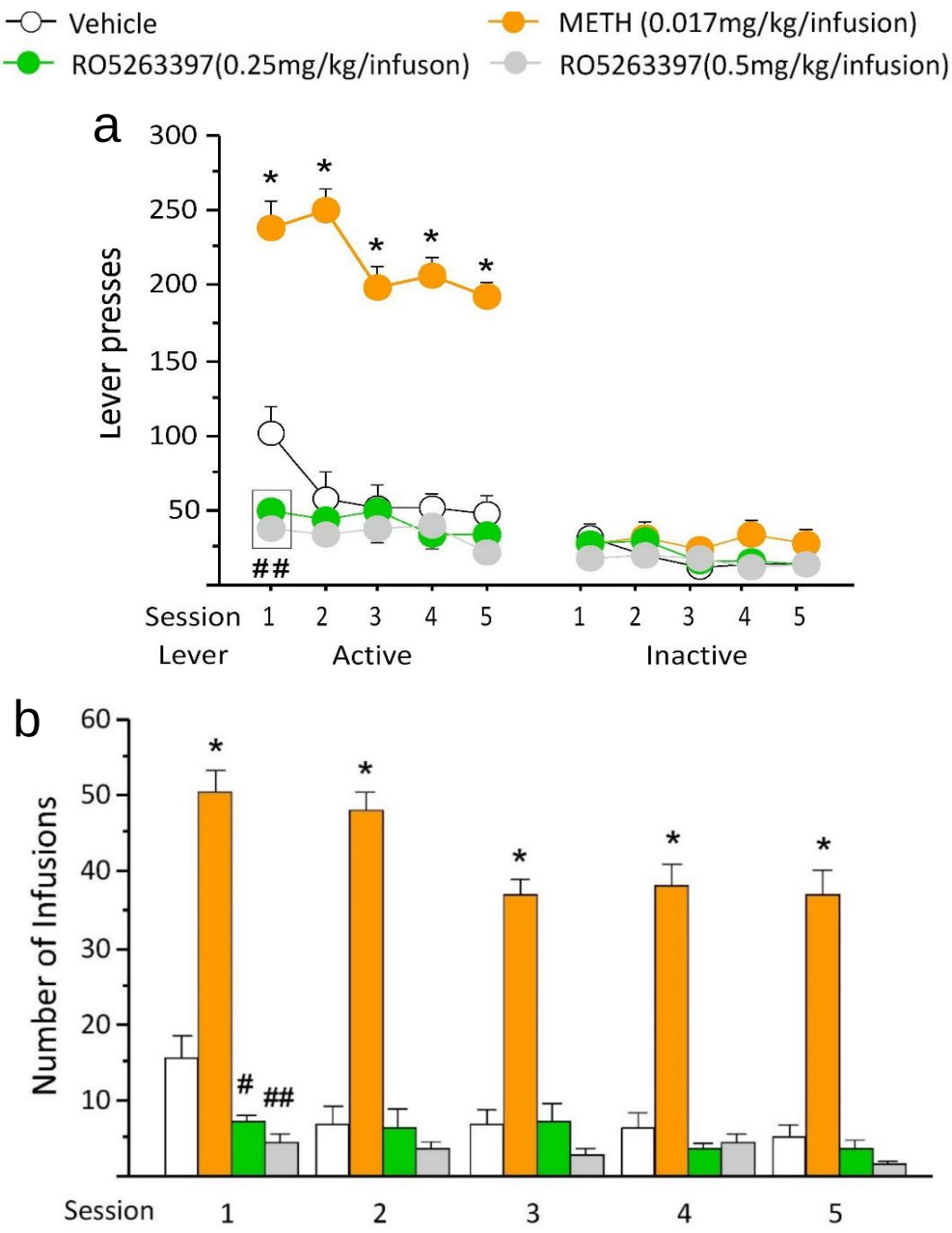

Figure 5.7 RO5263397 did not sustain responding when substituted for METH
After rats were trained to stably self-administer METH, a lower dose of METH, vehicle, or RO5263397 at a high or a low dose was given as substitute infusion for five days. The low dose of METH increased and sustained responding. Lever presses and infusions obtained for RO5263397 remained low at both doses across the five days, and were significantly lower than those for the vehicle substitution on day 1 (a and b). * $p < .01$ significantly different from each of other three groups; # $p < .05$, ## $p < .01$ significantly different from vehicle substitution group.

5.4 Discussion

The final set of experiments provided strong evidence for the effectiveness of selective TAAR1 agonists in two different animal models of relapse, a feature that is highly desirable for an efficacious anti-addiction medication to have. Firstly, we determined that the full and partial TAAR1 agonists, RO5256390 and RO5203648, respectively, prevented context-induced renewal of cocaine seeking in rats after protracted withdrawal from chronic cocaine self-administration. Both agonists were highly effective at doses that did not affect food-maintained responding. Moreover, after extinction from cocaine self-administration, reinstatement of cocaine seeking triggered by a cocaine prime was blocked by the partial agonist, RO5203648. Similarly, METH-primed reinstatement of METH seeking was significantly reduced by partial TAAR1 agonism with RO5263397. Furthermore, RO5263397, when substituted for METH, did not sustain self-administration behaviour, displaying low abuse liability. Collectively, these results support TAAR1 agonists as potential lead compounds for future development of therapeutic interventions for psychostimulant addiction.

Recurrent cycles of abuse, recovery, and relapse are the defining features of psychostimulant addiction, thus developing medications that could aid in recovery and facilitate stable abstinence from the drug would be highly beneficial from the medical standpoint. The recent discovery of TAAR1, which has shown marked ability to regulate DA transmission and psychostimulant action, has generated a great deal of interest in the field of addiction pharmacology research. Based on the existing data showing the ability of several highly selective TAAR1 agonists to modulate cocaine- or METH-induced locomotor activity and inhibit the reinforcing and DA-releasing effects of these drugs, the present work further evaluated the potential utility of targeting TAAR1 to treat stimulant addiction by assessing the effectiveness of TAAR1 agonists in clinically-relevant animal models of relapse and their own abuse liability.

Firstly, in the model of context-induced renewal of drug seeking, relapse is triggered, after a long-term abstinence from cocaine self-administration, by re-entering the self-administration chambers that were previously associated with drug taking. This process may involve the excitatory Pavlovian conditioning mechanisms through which the chambers acquire conditioned stimulus properties and incentive motivational salience during their pairing with drug availability, and thus elicit drug craving and drug seeking in abstinent rats upon re-exposure (Crombag et al., 2008, Hearing et al., 2008b). It is known that drug-related conditioned stimuli are able to produce marked changes in gene expression within the corticostriatal circuits including the dorsal striatum, NAc, anterior cingulate cortex, medial PFC, and orbitofrontal cortex (Hearing et al., 2008a, Hearing et al., 2008b), and induce conditioned increases in mesolimbic DA transmission (Fontana et al., 1993). Studies using FSCV have detected a rapid surge of DA in the NAc that coincided with the initiation of drug-seeking behaviour, and such behaviour could be reproduced by or/and synchronized to electrically evoked DA transients to the NAc (Phillips et al., 2003). Therefore, context-induced drug seeking may crucially rely on mesolimbic DA transmission that is potentiated by re-exposure to the drug self-administration context. Consistent with this, systemic injections of D1-like or D2-like DA receptor antagonists attenuated context-renewed cocaine seeking (Crombag et al., 2002a), and this effect has been attributed to a decrease in DA transmission in the NAc shell (Crombag et al., 2008).

In addition to DA, neuroadaptations of the glutamatergic system, mainly within projections from the PFC to the NAc, are believed to mediate behavioural neuroplasticity associated with relapse (Kalivas, 2004). Changes in both pre- and postsynaptic glutamate transmission in the VTA and NAc also play a role (Kalivas, 2004). These adaptations lead to amplified biological saliency of glutamate release in the NAc upon exposure to stimulus that triggers relapse, which translates into behavioural drive for drug-seeking (Kalivas, 2004). Studies have shown that antagonism of AMPA or NMDA receptors in the NAc attenuated cocaine relapse induced by cocaine-associated cues (Bäckström and Hyytiä, 2007), whereas stimulation of either glutamate receptor subtype within the NAc elicited cocaine seeking

(Cornish et al., 1999), supporting the engagement of an increased glutamate transmission in cocaine relapse. Moreover, it has been suggested that glutamate-mediated effects on drug seeking may involve its local interaction with DA in the VTA and NAc (Crombag et al., 2008), as DA neurons in both regions are controlled in part by glutamatergic input from various brain areas (Voorn et al., 2004, Pierce and Kumaresan, 2006, Geisler et al., 2007).

Therefore, based on the previously mentioned evidence suggesting opposing actions of TAAR1 on the DA system, activation of TAAR1 could attenuate context-induced relapse to cocaine seeking through preventing the conditioned release of DA upon re-exposure to the drug-associated context. TAAR1 may also influence glutamatergic inputs to the NAc, especially those from the PFC given the aforementioned implication of TAAR1 in prefrontal glutamate transmission, although how this interaction eventually leads to reduced cocaine seeking is unknown. Nevertheless, the current results confirmed our prediction of a reduced cocaine relapse. Both full and partial TAAR1 activation with RO5256390 and RO5203648, respectively, significantly diminished cocaine seeking upon re-entering the self-administration chambers. In the experiment in which we tested RO5203648, rats also displayed a moderately high level of responding on the inactive lever during the relapse test, which might reflect a strategic search for cocaine reinforcement and thus could also be interpreted as drug-seeking behaviour. RO5203648 dose-dependently attenuated response not only on the active lever but also on the inactive lever, consistent with its anti-relapse profile.

Besides, both compounds, at the high dose, produced non-specific effects on food intake, characterized by slow response initiation and, in the case of the full agonist, decreased overall intake as well. This effect is unlikely to be caused by an impaired motoric function, at least for RO5203648, as it has been previously shown that 10 mg/kg of this partial agonist did not affect locomotor behaviour in rats (Revel et al., 2012b). Alternatively, TAAR1 agonists may create a physiological or internal “satisfaction” state in the rats, possibly via balancing the activity of neurotransmitters, leading to less interest in external rewards such as food, although this is not likely in light of the evidence presented earlier indicating that TAAR1

activation increases motivation to seek food in the PR schedule of reinforcement. Complementarily, studies have reported the expression of *TAAR1* in peripheral organs involved in food absorption and control of glucose metabolism, suggesting that TAAR1 may regulate metabolic functions (Mitchell et al., 2008, Revel et al., 2013). Although the exact functional contribution of TAAR1 in the peripheral system is unclear, it might be possible that TAAR1 influences food-intake through mechanisms outside the CNS. As mentioned, in the food PR experiments described in the previous chapter, both RO5203648 and RO5263397 enhanced the BP for food self-administration, suggesting an increase in the motivation for food. However, in the early phase of the PR session, characterized by low response requirement, RO5203648 at the high dose delayed responding, mirroring its effects in the present food-intake assay. Such discrepant findings (i.e. differential TAAR1 effects on food-maintained responding under FR1 and PR schedules of reinforcement) may reflect differential regulation by TAAR1 on the appetitive and consummatory aspects of eating, which are separable in terms of the underlying neuronal substrates (Foltin, 2005, Gan et al., 2010, Oleson et al., 2011). Nonetheless, it is important to note that both compounds were highly effective in reducing context-induced cocaine seeking at doses (3 mg/kg) that had no negative impact on food-maintained responding, suggesting that TAAR1 agonists can act within a wide therapeutic window. Due to the propensity of the full agonist to produce a more profound non-specific suppression on food taking than the partial agonist, only partial TAAR1 agonists were tested in subsequent experiments.

We next tested TAAR1 agonists in the extinction-reinstatement model of relapse where drug seeking is triggered by a challenge dose of the previously self-administered drug. Evidence has indicated an important role of both glutamate and DA in drug-induced reinstatement. In rats extinguished from METH self-administration, a METH prime produced concurrent increases of glutamate efflux in both the dorsal medial PFC and the NAc, accompanied by an elevation in DA concentrations only in the dorsal medial PFC (Parsegian and See, 2014). Similarly, increased release of glutamate, but not DA, in the NAc, has been specifically associated with cocaine-primed reinstatement, and this accumbens glutamate rise, along with

cocaine-seeking behaviour, was eliminated by inhibition of the dorsal PFC (McFarland et al., 2003). Moreover, blockade of either D1-like or D2-like DA receptors in the PFC, but not in the NAc, decreased cocaine-induced reinstatement (McFarland and Kalivas, 2001, Sun and Rebec, 2005), whereas antagonism of the AMPA receptor in the NAc blocked cocaine seeking induced by either a systemic injection of cocaine (Cornish and Kalivas, 2000) or intra-medial PFC cocaine infusion (Park et al., 2002). Together, these results suggest that the glutamatergic projection from the PFC to the NAc in part underlies drug-reinstated drug seeking, and that prefrontal DA plays a more enabling role than accumbens DA. In the present experiments, following consistent cocaine or METH self-administration and extinction, a prime injection of the self-administered drug produced robust reinstatement of drug seeking. The TAAR1 partial agonists, RO5203648 and RO5263397, dose-dependently prevented cocaine- and METH-primed reinstatement, respectively, consistent with the inhibitory control of TAAR1 over augmented drug-induced DA transmission and with a potential functional interaction of TAAR1 with the corticoaccumbens glutamatergic pathway activated by the drug prime. In agreement with the present findings, studies conducted while this thesis was in progress reported that RO5263397 prevented cue- and drug-induced reinstatement of cocaine and METH seeking (Jing et al., 2014, Thorn et al., 2014a), which further strengthens the evidence for the ability of TAAR1 agonists to prevent relapse.

However, it is worth noting that the mechanisms involved in TAAR1's regulation of the reinstatement induced by METH may be more complex than that induced by cocaine. While TAAR1 partial agonism produced a complete blockade of cocaine-induced drug seeking throughout the entire 3-h test, this effect was only observed during the first 2 h of the METH reinstatement, with responding recovering in the last part of the session. This later recovery in response was not picked up by Jing et al. (2014)'s study as their reinstatement test stopped after 2 h. Thus, additional direct interaction between METH and RO5263397 may have occurred given the ability of METH, but not cocaine, to stimulate TAAR1 and trigger downstream signalling events. Actually, this time-dependent modulation of TAAR1 over METH-induced effects is not entirely unexpected given the results from experiment 1

showing an early attenuation followed by a later potentiation of METH-induced locomotor activity by RO5203648. However, while in the locomotor test there was a clear biphasic interaction between METH and TAAR1 pretreatment (Figure 3.1), in TAAR1 agonist-treated rats the METH-primed drug seeking in the latter part of the test did not exceed the levels elicited by METH alone. As discussed earlier, the biphasic modulation by TAAR1 of the motor-stimulating effects of METH may fit into the notion of a state-dependent regulation of DA transmission by TAAR1 partial agonists. However, in the METH reinstatement model which involves prolonged METH exposure and extinction, a subsequent METH prime injection may induce a more exaggerated increase in DA release compared with the acute METH treatment in the locomotor assay (Yamada et al., 1988, Kazahaya et al., 1989), thus promoting a sustained agonistic-like action of the partial agonist to suppress DA transmission. In addition, the corticostriatal glutamatergic pathway also undergoes neuroadaptations after chronic METH treatment, displaying enhanced stimulated glutamate transmission in the PFC and striatum (Stephans and Yamamoto, 1995, Nishioku et al., 1999, Bustamante et al., 2002), which did not occur in the acute METH locomotor activity test. Moreover, TAAR1 receptor functionality may be affected by repeated METH stimulation, resulting in altered responsiveness to subsequent exposure to TAAR1 ligands, including METH itself and the selective agonist. Other neuroadaptive consequences following chronic TAAR1 activation may include changes in the coordinated interaction between TAAR1 and D2 receptors, D2 receptor-mediated autoinhibition, and prefrontal glutamatergic transmission. Any of these elements may contribute to the differential TAAR1 modulation of the effects induced by acute- and chronic-METH.

Another important observation in the present reinstatement experiments is that both RO5203648 and RO5263397 failed to reinstate drug seeking when administered alone (i.e., followed by saline injection). This finding demonstrates a psychopharmacological profile of TAAR1-specific compounds that is distinct from some of the psychomotor stimulants and hallucinogenic drugs that bind non-selectively to TAAR1, including amphetamine, MDMA,

and METH, which do produce cross-reinstatement of extinguished drug-seeking behaviour (De Wit and Stewart, 1981, Schenk et al., 2008, Nawata et al., 2015).

The last experiment provided further support for the lack of stimulant-like characteristics of TAAR1-selective agonists. We found that RO5263397, at either the low or the high dose, did not sustain self-administration when substituted for METH. This is in sharp contrast with the elevated responding rate when a lower dose of METH was used as the substitute, which possibly reflects the rats' attempt to maintain an optimal subjective drug effect by self-adjusting response according to the unit drug dosage. Consistently, Cotter et al. (2015) showed that substitution of different concentrations of RO5203648 for METH did not generate varying levels of responding over and above vehicle substitute, while lowering the unit injection dose of METH produced a proportional increase in response rate from that maintained by the training dose. These results strongly suggest that TAAR1 partial agonists do not exhibit METH-like discriminative properties and possess low abuse liability, which is a desirable feature for an addiction pharmacotherapy to have. Moreover, in the present experiment, response level for RO5263397 was even lower than that for vehicle in the first substitution session. This might be an indication that the first couple of RO5263397 self-infusions reduced METH craving and METH seeking, decreasing the high response rate observed early during extinction in animals receiving the vehicle substitution. This speculation is consistent with the inhibitory control by TAAR1 over increased DA activity such that TAAR1 activation with RO5263397 attenuated DA surges mediated by re-exposure to the drug-paired context and cues. In addition, studies found that potentiation of mGluR5 led to facilitated extinction of cocaine-associated contextual memory (Gass and Olive, 2009) and overexpression of GluR1 or GluR2 subunits of AMPA receptors in the NAc shell promoted extinction from chronic cocaine self-administration (Sutton et al., 2003), raising the possibility that RO5263397 regulation over drug seeking during extinction may also involve TAAR1-mediated effects on glutamatergic function in the NAc. In this regard, TAAR1-selective agents may have the potential to aid in extinction training, besides its use in relapse prevention. Promisingly, a new study by Liu et al. (2016) showed that, during

extinction from chronic cocaine self-administration, daily treatment with RO5263397 before each extinction session significantly decreased cocaine seeking, suggesting that TAAR1 activation may inhibit the expression of cocaine reward memory and could be targeted to improve extinction learning.

Taken together, the results obtained from the last set of experiments demonstrated a remarkable ability of TAAR1 agonists to prevent context-induced relapse of cocaine seeking and drug-primed reinstatement of cocaine and METH seeking. Moreover, the partial agonist, RO5263397, was shown to be devoid of METH-like stimulant properties and possess low abuse liability. These data highlight the ideal characteristics of TAAR1 agonist treatment in animal models of cocaine and METH addiction that are highly predictive of therapeutic efficacy, and support the candidacy and clinical development of novel TAAR1-based medications for stimulant addiction.

Chapter Six

6 General Discussion

Each of the previous experimental chapters are self-contained studies with their own discussion, therefore this chapter will briefly summarize the major findings, reiterate highlights and limitations, and address the implications and future research directions.

6.1 Summary of Study

Previous evidence has shown the functional importance of TAAR1, a newly discovered GPCR that is responsive to the TAs, in brain monoamine transmission and psychostimulant action. Consequently, it has been suggested that pharmacological targeting of TAAR1 may present a novel avenue for the treatment of stimulant addiction. The goal of the present thesis was to test this idea with the recently generated highly selective TAAR1 agonists in clinically-relevant animal models of addiction that are predictive of therapeutic effectiveness. The study consisted of three sets of experiments (experiments 1-10) that systematically investigated the ability of TAAR1 selective agonists to regulate a broad spectrum of psychostimulants effects. The aspects we have examined include locomotor-stimulation, behavioural and neuronal sensitization, reinforcement and motivation, neurochemical changes, and relapse/reinstatement, which cover several key behavioural and neuronal markers of addictive disorder, as measured in animal models.

The first set of experiments demonstrated a complex modulatory role of TAAR1 in acute and repeated METH-induced locomotor hyperactivity, behavioural sensitization, and neuronal plasticity. Experiment 1 showed that the selective TAAR1 partial agonist, RO5203648, when administered prior to METH, time-dependently modulated METH-stimulated locomotion, with an early attenuation followed by a late potentiation, in a 3-h extended test session. Experiment 2 involved daily pretreatment with another TAAR1 partial agonist, compound M, prior to METH for ten consecutive days followed by a 10-day withdrawal period and a METH challenge injection on day 21 (no compound M was administered on this day). While

concurrent administration of compound M with METH did not affect the early induction or the late expression of METH-induced behavioural sensitization, repeated treatment of compound M on its own during the acquisition phase produced a dose-dependent effect on the probe test such that the high dose group exhibited a significantly higher level of locomotor response compared with the low dose group, with the average for saline group falling between the two groups receiving compound M alone. At the neuronal level, compound M pretreatment potentiated chronic METH-induced changes in c-Fos inducibility in both striatal and prefrontal cortical structures after METH challenge. When administered alone, compound M also altered subsequent METH-induced expression of c-Fos in the NAc core. These findings demonstrate intricate interactions between TAAR1 and METH at both behavioural and neuronal levels. They also provide evidence for the ability of TAAR1 to regulate some METH-induced neuroadaptations and maladaptive behaviours.

The second set of experiments demonstrated that full and partial activation of TAAR1 effectively reduced the reinforcing efficacy of cocaine and METH. Experiment 3 tested the effects of the full and the partial agonists, RO5256390 and RO5203648, respectively, on the dose-response function of cocaine self-administration. Results suggested a downward shift of the dose-response curve, indicating a substantial blockade of cocaine reinforcement. Experiments 4 and 5 explored the effects of RO5203648 and another partial agonist, RO5263397, on the motivation for cocaine and METH, respectively. While RO5203648 prolonged the latency to reach BP under a PR schedule of cocaine reinforcement, RO5263397 reduced the BP for METH, indicating suppression of the reinforcing and motivational effects of these two drugs by TAAR1 partial agonism. Notably, these effects could not be attributed to TAAR1's non-specific influence on general motoric or motivational function because RO5203648 and RO5263397 elevated the BP maintained by food under the same PR procedure, which additionally suggests differential TAAR1 regulation of drug and food reinforcement. Experiment 6 aimed at examining the physiological mechanisms underlying TAAR1 regulation of psychostimulants' effects through the use of FSCV. Our data showed that RO5263397 completely prevented METH-evoked DA overflow in the NAc,

which extends previous findings with cocaine, confirming the inhibitory control that TAAR1 exerts over DA transmission potentiated by psychostimulants.

The final set of experiments further evaluated the potential clinical utility of TAAR1 agonists in treating stimulant addiction by examining the ability of TAAR1 activation to modify relapse behaviour. Experiment 7 showed that partial and full activation of TAAR1, with RO5203648 and RO5256390, respectively, prevented context-induced relapse of cocaine seeking after protracted abstinence from chronic cocaine self-administration. In parallel experiments in which rats worked for food under a FR1 reinforcement schedule, these two agonists at the high dose delayed response initiation and RO5256390 also reduced the total number of reinforcers earned. Notwithstanding, they did not affect food-maintained responding at the low dose, which was still highly effective in attenuating cocaine relapse, suggesting that TAAR1 agonists may have a wide therapeutic window. Experiments 8 and 9 employed an extinction-reinstatement model and demonstrated that the partial agonists, RO5203648 and RO5263397, reduced drug prime-induced reinstatement of cocaine or METH seeking, respectively, without producing cross-reinstatement when given alone, further strengthening the anti-relapse properties of TAAR1 agonists. Experiment 10 examined the abuse liability of TAAR1-selective compounds using a substitution procedure. Results revealed that RO5263397 did not sustain self-administration when substituted for METH nor evoke extinction-like responses during the substitution sessions, suggesting that this compound is devoid of stimulant-like discriminative properties and has low abuse potential. Together, the present thesis provided compelling evidence for the remarkable ability of TAAR1 to regulate key abuse-related behavioural and physiological effects of cocaine and METH, demonstrating highly desirable properties of TAAR1 agonists that are consistent with those of an efficacious pharmacotherapy for stimulant addiction. The present data, along with others' findings reported simultaneously during the course of the current project, strongly support the development of TAAR1 agonists as a novel generation of anti-addiction medicines.

6.2 Physiology and Regulatory Effects of TAAR1 Activation

A brain slice electrophysiology study on TAAR1 KO mice revealed elevated spontaneous firing rate and depolarized resting membrane potential of DA neurons in the VTA (Lindemann et al., 2008). These mutant mice also showed increased levels of extracellular DA in the NAc in an *in vivo* microdialysis study, and enhanced electrically evoked DA release in NAc brain slices measured by FSCV (Leo et al., 2014). Therefore, it has been suggested that TAAR1 is constitutively active or tonically activated by ambient levels of endogenous amines to maintain a negative control over mesolimbic DA activity (Lindemann et al., 2008). Importantly, TAAR1 depletion did not change Tau or the half-life of released DA in NAc slices, but reduced D2 autoreceptor-mediated autoinhibition in a paired-pulse stimulation FSCV test, suggesting that the augmented DA release in the absence of TAAR1 did not involve alterations in DA reuptake through the DAT, but instead was caused by a less effective autoinhibitory regulation by the D2 autoreceptor (Leo et al., 2014). Previous evidence has suggested that the D2 autoreceptors maintain tonic activation under physiological conditions by basal concentrations of extracellular DA and spontaneous firing of DA neurons, which enables a tonic autoinhibitory control over DA activity (Dugast et al., 1997, Moquin and Michael, 2009). Thus, tonic activation of TAAR1 may support the tonic D2 autoreceptor-mediated autoinhibition, through which TAAR1 acts as a homeostatic regulator of the DA system.

Observations of TAAR1 KO mice led to the hypothesis that pharmacologically increasing TAAR1 activation may amplify its DA-suppressing effects, possibly through promoting the function of D2 autoinhibition; and TAAR1 blockade may produce the opposite effects. In agreement with this, the selective full agonists, RO5166017 and RO5256390, decreased the firing rate of DA neurons in the VTA (Revel et al., 2011, Revel et al., 2013), whereas the selective antagonist, EPPTB, increased it (Bradaia et al., 2009), as measured by brain slice electrophysiological recordings. Moreover, while RO5166017 inhibited evoked DA release in NAc brain slices in FSCV, EPPTB not only increased it but also blocked the effects of RO5166017 (Leo et al., 2014). Notably, the effects of EPPTB also provide further

confirmation for the constitutive activity or tonic activation status of TAAR1 which apparently serves to keep the DA system under control. As expected, none of the treatments changed DA uptake kinetics, suggesting that the DAT is not involved in the action of these TAAR1 selective compounds (Leo et al., 2014). Subsequent investigation supports D2-mediated autoinhibition as a potential alternative mechanism of action by which selective TAAR1 agonism reduces DA release. RO5166017 synergized with quinpirole, a D2 class receptor agonist, to decrease evoked DA overflow in an additive manner in NAc brain slices in a FSCV study (Leo et al., 2014), suggesting the possibility that increasing TAAR1 activation with selective full agonists may facilitate the autoinhibitory function of D2 autoreceptors, which might explain their suppressant effects on somatodendritic and axonal terminal DA activity. However, as previously discussed, activation of TAAR1 and D2 receptors have been demonstrated *in vitro* to regulate DA reuptake through the DAT in opposition, with the former inhibiting it and the latter enhancing it. This has led to the concept of "presynaptic receptor balancing" that equilibrates DA activity (Xie et al., 2008, Xie and Miller, 2009b). This notion appears to be inconsistent with the DAT-independent effects of TAAR1 ligands on DA release. Actually, it is worth re-emphasizing that both physical and functional interactions between TAAR1 and D2 receptors were observed *in vitro* at both pre- and postsynaptic levels such that they mutually modulate the signalling of each other, which is likely to be mediated via receptor heterodimerization (Espinoza et al., 2011, Salahpour et al., 2012, Espinoza et al., 2015a, Harmeier et al., 2015). Thus, the DA-suppressing effects of full TAAR1 agonism might depend preferentially on TAAR1-D2 receptor complex formation, circumventing the DA reuptake machinery via the DAT. Moreover, D2 autoreceptors can regulate DA activity through several mechanisms that do not necessarily involve increasing DAT-mediated uptake. These mechanisms include inhibiting DA exocytosis from the axon terminals (Palij et al., 1990, Benoit-Marand et al., 2001, Phillips et al., 2002), decreasing DA synthesis through suppressing tyrosine hydroxylase (Kehr et al., 1972, Wolf and Roth, 1990), and modulating DA neuronal firing by activating a hyperpolarizing GIRK current at the somatodendritic site (Aghajanian and Bunney, 1977,

Lacey et al., 1987, Courtney et al., 2012). Nevertheless, it has been suggested that the activation of D2 autoreceptor-mediated DAT regulation requires a saturating amount of extracellular DA that is beyond the levels raised by tonic background firing of DA neurons and by stimulations delivered via single electrical pulse (which was used in the above FSCV study) (Kennedy et al., 1992, Benoit‐Marand et al., 2011). Thus, easier detection of changes in DA uptake could be expected in situations where prolonged trains of stimulation are applied that trigger strong D2 autoreceptor activation, thereby supporting the putative role of the DAT in the modulation of DA transmission by selective TAAR1 ligands.

Further complicating the picture are the findings with partial TAAR1 agonists, which could potentially act as net agonists or antagonists depending on the level of receptor stimulation by endogenous ligands. Electrophysiological recordings of mice brain slice revealed that the TAAR1 partial agonists, RO5263397 and RO5203648, increased the firing rate of VTA DA neurons (Revel et al., 2012b, Revel et al., 2013), as did the antagonist, EPPTB (Bradaia et al., 2009). These findings further support the notion that TAAR1 is constitutively active or tonically activated by ambient levels of agonists, whereby partial agonism produces antagonistic-like effects at TAAR1. The resultant reduction in TAAR1 activity liberates DA neuronal firing from the tonic autoinhibitory control by tonic D2 autoreceptor activation that is potentially sustained in part by TAAR1 tonic activity. However, the TAAR1 partial agonism-induced enhancement of somatodendritic DA firing activity did not readily translate into increased terminal DA transmission in the NAc, as RO5203648 had no effects on NAc DA release in an *in vivo* microdialysis experiment (Cotter et al., 2015) or on electrically evoked DA overflow in NAc brain slice in FSCV (Pei et al., 2014). Similarly, in the present FSCV study (experiment 6), RO5263397 did not alter evoked DA outflow in the NAc core when applied by itself. Several factors might contribute to this dissociation. Firstly, the extent to which local TAAR1 regulation observed in brain slice preparations reflects the effects of systemic TAAR1 administration on the VTA is unclear. Apart from being regulated by local VTA D2 autoreceptors, VTA DA activity is subjected to a long-loop negative feedback control from the NAc involving both D1 and D2 receptors (Rahman and McBride, 2000,

2001). Thus TAAR1 regulation of NAc DA may provide an additional source of control over VTA DA by adjusting the signalling of D1 and D2 receptors which determine the activity of the feedback loop. Moreover, the VTA receives a variety of non-DA afferents from other brain areas, which also contribute to the regulation of VTA DA activity, including serotonergic input from dorsal and median raphe nucleus (Moore et al., 1978, Hervé et al., 1987, Broderick and Phelix, 1997, Vertes et al., 1999), noradrenergic projections from the locus coeruleus (Herve et al., 1982, Grenhoff et al., 1993), cholinergic fibres from the pedunculopontine tegmental nucleus and the laterodorsal tegmental nucleus (Oakman et al., 1995, Blaha et al., 1996), and GABAergic input originating from the NAc, ventral pallidum and the pedunculopontine nucleus (Waalas and Fonnum, 1980, Klitenick et al., 1992, Charara et al., 1996). Because TAAR1 has been shown to localize in some of those distant regions and interact with the respective neurotransmitters, TAAR1 activation therein may have an impact on VTA DA through fine-tuning of these non-DA-mediated pathways. Most importantly, glutamatergic projections from the medial PFC form the predominant extrinsic source of excitatory input to the VTA (Johnson and North, 1992, Adell and Artigas, 2004) and stimulation of the PFC causes glutamate release in the VTA and burst firing of VTA DA neurons (Murase et al., 1993, Tong et al., 1996, Rossetti et al., 1998). Given the aforementioned evidence implicating TAAR1 in prefrontal glutamatergic functioning, TAAR1 ligands may influence VTA DA activity through regulating prefrontal glutamatergic output into the VTA. Therefore, taken together, TAAR1 is able to regulate VTA DA activity both directly through interacting with local DA and indirectly by interfering with distant DA or non DA neurotransmitters, which cannot be fully captured in isolated VTA brain slices.

On the other hand, although the synaptic release of DA in the NAc is thought to originate from neuronal firing of DA neurons in the VTA, DA release exhibits a dynamic pattern that is controlled by multiple adaptive processes in the terminal (Montague et al., 2004, Sombers et al., 2009). Factors such as the rate of DA biosynthesis and vesicular packing, inhibition of release through presynaptic D2 autoreceptors, and recycling of released DA through reuptake could modulate extracellular DA (Michael et al., 1987, Phillips et al., 2002, Montague et al.,

2004, Kita et al., 2007). Accordingly, NAc TAAR1 may exert local effects on DA release through interacting with terminal D2 autoreceptors and DAT. In addition, glutamatergic afferents from the PFC and the BLA play an important role in regulating DA output in the NAc, which might or might not depend on activation of DA cell bodies in the VTA (Taber and Fibiger, 1995, Jackson and Moghaddam, 2001, Howland et al., 2002). Thus, TAAR1 expressed in the amygdala and PFC may act on glutamate efferents to the NAc whereby it influences terminal DA activity.

It is, therefore, reasonable to expect differential effects of TAAR1 ligands on DA activity in the somatodendritic and terminal regions. Nevertheless, the fact that the antagonist, EPPTB, did increase evoked DA release in NAc brain slices (Leo et al., 2014), which is consistent with its potentiating effects on VTA DA neuron firing, implies intrinsic difference in the nature of DA regulation by TAAR1 antagonism and partial agonism. While the antagonist in principle abolishes TAAR1 activation and thereby reduces D2 autoreceptor-mediated autoinhibition, the partial agonist maintains TAAR1 activity within a submaximal range that is still sufficient to sustain a functional D2-mediation. Importantly, the partial agonists, RO5203648 and RO5263397, effectively prevented cocaine- or METH-induced DA overflow in the NAc (Pei et al., 2014, Pei et al., 2016b), demonstrating their ability to suppress DA activity in situations of hyperdopaminergia. Similarly, in behavioural models of hyperdopaminergia, the two partial agonists blocked locomotor hyperactivity induced by psychostimulants or genetic deletion of the DAT, behaving as the full agonists, RO5256390 and RO5166017 (Revel et al., 2011, Revel et al., 2012b, Revel et al., 2013). In this regard, partial TAAR1 agonists may act to stabilize the DA system, decreasing DA when there is excessive transmission but also helping maintain DA at sustainable levels under basal or hypodopaminergic conditions. This idea of TAAR1 as a biological sentinel fits well with the results from experiment 1 showing that RO5203648 time-dependently modulated METH-stimulated locomotion, with an early attenuation followed by a late potentiation when the effects of METH began to subside. Furthermore, this state-dependent DA regulation by TAAR1 partial agonists highlights their superior therapeutic potential for treating drug

addiction over conventional medications, especially given that DA fluctuates dramatically during the addiction cycle, encompassing both “high” (intoxication and relapse) and “low” phases (withdrawal).

6.3 TAs vs. Selective TAAR1 Agonists

It is important to understand the differences between TAAR1’s endogenous ligands and selective agonists in the regulation of DA activity. The TAs, such as *β*-PEA and tyramine, inhibited the spontaneous discharge rate of DA neurons in the VTA measured by both *in vitro* and *in vivo* electrophysiology (Geracitano et al., 2004, Ishida et al., 2005, Lindemann et al., 2008) and reduced evoked striatal DA release in *in vivo* voltammetry experiments (Stamford et al., 1986). However, *in vivo* microdialysis revealed that local or systemic *β*-PEA increased extracellular DA levels in the VTA and the striatum including the NAc (Nakamura et al., 1998, Sotnikova et al., 2004, Ishida et al., 2005, Murata et al., 2009), which contradicts the apparent DA-suppressant effects of TAs observed in the electrophysiology and voltammetry studies. In fact, as previously described, *β*-PEA has been considered as an “endogenous amphetamine” due to its ability to mimic the major DA-releasing and locomotor/stereotypy-stimulating effects of amphetamines (Jackson, 1975, Tinklenberg et al., 1978, Ortmann et al., 1984, Dourish, 1985, Gianutsos and Chute, 1986, Philips, 1986, Bailey et al., 1987, Barroso and Rodriguez, 1996, Janssen et al., 1999). Evidence indicates that *β*-PEA-induced DA release is calcium ion-independent and involves stimulating the efflux of newly synthesized DA from reserpine-insensitive pools via carrier-dependent and independent mechanisms (Geracitano et al., 2004, Sotnikova et al., 2004, Ishida et al., 2005). Therefore, it has been suggested that the inhibition of midbrain DA neuron firing by *β*-PEA and tyramine occurs via indirect activation of D2 autoreceptor-mediated autoinhibition that is mediated by an increase in DA release. In support of this, the inhibitory effects of these TAs were abolished when D2 receptors were selectively antagonized or the synthesis of DA was blocked (Geracitano et al., 2004, Ishida et al., 2005). Similarly, the ability of *β*-PEA to reduce electrically evoked striatal DA release may also involve the activation of D2 autoreceptors, and may reflect the depletion of cytoplasmic DA by *β*-PEA (Sotnikova et al., 2004).

However, the mechanisms underlying TAs modulation of dopaminergic activity are complex and may involve both inhibitory and excitatory effects. Firstly, *β*-PEA acting at TAAR1 leads to inhibited uptake and increased efflux of DA through the DAT, which may partially account for its DA-releasing effects (Xie and Miller, 2008). Intriguingly, the fact that TAAR1 KO mice exhibited elevated discharge rate of VTA DA neurons suggests that, under physiological conditions, TAs may exert tonic inhibitory control over DA neurons through TAAR1, which might rely on TAAR1 interaction with the D2 autoreceptors. Moreover, *β*-PEA and tyramine have been shown to depress D2 autoreceptor-activated GIRK currents on SNr pars compacta (SNpc) DA cells in a TAAR1-independent manner (Ledonne et al., 2010). These two TAs also suppressed $GABA_B$-mediated slow inhibitory postsynaptic potential and GIRK currents on DA neurons in the VTA and SNpc (Federici et al., 2005). Thus, TAs may potentially increase the excitability of midbrain DA neurons by antagonizing D2 autoreceptor- and $GABA_B$-mediated inhibition of DA. On the other hand, *β*-PEA and tyramine were able to reduce $GABA_B$-mediated presynaptic inhibition of GABA release to SNpc DA neurons, leading to increased GABAergic input and thus enhanced DA inhibition (Berretta et al., 2005). It has been suggested that the inhibitory effects of TAs on DA neuron prevail over the excitatory effects under normal conditions, but pathological conditions may overturn the balance (Ledonne et al., 2011).

On the contrary, unlike TAs which may exert effects on targets other than TAAR1, rendering it extremely difficult to untangle their actions, the selective agonists bind exclusively to TAAR1, making it possible to decipher the functional role of TAAR1. As mentioned before, the ability of the selective full agonists to inhibit the spontaneous discharge of VTA DA neurons and to reduce electrically evoked NAc DA release is most likely mediated by a potentiated D2 autoreceptor-mediated autoinhibition as a result of structural and functional interactions between TAAR1 and D2 autoreceptors. Moreover, given the *ex vivo* observation that TAAR1 activation releases DA through altering the uptake and efflux function of the DAT, it is reasonable to suspect that the triggering of D2-mediated autoinhibition may be due in part to this increased DA outflow. However, the direct involvement of the DAT in the

action of these selective TAAR1 agonists is not fully supported by current evidence and further research should explore this possibility using techniques monitoring DA release in real time.

6.4 Non-Drug Related Effects of TAAR1 Agonists

Apart from demonstrating the remarkable therapeutic potential of TAAR1 selective agonists in modulating effects associated with psychostimulant abuse, the current work also revealed several interesting findings that are relevant to the clinical application of TAAR1-based pharmacotherapies. Experiments 4 and 5 showed that the partial TAAR1 agonists, RO5203648 and RO5263397, enhanced the motivation for food, evidenced by an increased BP for food self-administration under the PR reinforcement schedule, in direct contrast with the clear-cut inhibition these two agonists produced on cocaine and METH-maintained BP. Complicating the picture is the finding from experiment 7 which showed that, under a FR1 reinforcement schedule, RO5203648 delayed the initiation of responding for food without affecting the total number of reinforcers obtained; and the full agonist, RO5256390, not only delayed response but also reduced the total number of pellets obtained. It is important to bear in mind that these suppressant effects on food-maintained response were absent at the low dose, which was still effective at preventing cocaine relapse. Together, the immediate message arising from these food experiments is that TAAR1 activation has differential regulatory effects on behaviour mediated by drug and natural reward. However, the exact role of TAAR1 in food-related process remains to be fully elucidated. A relevant finding showed that the selective TAAR1 partial agonist, RO5263397, prevented body weight gain and fat accumulation induced by the atypical antipsychotic olanzapine which are noticeable side effects produced by this type of drugs that compromise treatment compliance (Revel et al., 2013). In addition, when administered alone, RO5263397 exhibited a propensity to decrease body weight and fat mass (Revel et al., 2013). Thus, it seems that TAAR1 selective agonists may possess anti-obesity properties, which would be consistent with the reduced food intake observed under a FR1 reinforcement schedule. This reduction is not due to a depression of the motivational circuit for food reward as indicated by the enhanced BP under PR schedule,

but instead might depend on TAAR1 activation of the peripheral nervous system. Studies have identified the expression of TAAR1 mRNA in the rat adipose tissue where TAAR1 activation facilitates fat depletion (Mitchell et al., 2008). TAAR1 is found in peripheral organs that are closely associated with the control of food absorption, energy homeostasis, and blood glucose concentration, including the stomach, intestine, and insulin-secreting β cells of the pancreas (Revel et al., 2013). Accordingly, it has been postulated that TAAR1 may be involved in the regulation of insulin secretion and body fat metabolism (Mitchell et al., 2008, Revel et al., 2013). Actually, the dysregulation of the TAs, especially PEA, has long been implicated in metabolic disorders, such as diabetes (Mosnaim et al., 1979, Mosnaim et al., 1982). Moreover, octopamine and synephrine were shown to stimulate metabolism and may potentially promote weight loss (Bour et al., 2003, Haaz et al., 2006, de Oliveira et al., 2013). Notwithstanding, the body weight regulatory effects of TAAR1 should not be considered as a limiting factor that hinders the application of TAAR1-based therapies in the treatment of addictive disorders. In our experiments, animals, which were given unlimited food or kept on a maintenance diet, did not show significant weight drop following either acute or repeated treatment with TAAR1 selective agonists. It is thus possible that the role of TAAR1 in weight modulation only becomes apparent under conditions of metabolic disturbance, which prompts further evaluation of TAAR1 agonists to treat metabolic diseases such as diabetes and obesity and to relieve side effects associated with antipsychotic treatment.

Apart from body weight regulation, other non-drug specific processes have also been suggested to involve TAAR1 activity, which encourages the versatile usage of TAAR1-based compounds in both clinical and non-clinical contexts beyond the addiction field (Pei et al., 2016a). For example, given that TAAR1 is expressed in the amygdala, which is a cardinal control centre for emotional regulation (Phelps and LeDoux, 2005), and that TA deficiency has been historically linked to affective disorders such as depression and bipolar disorder (Sandler et al., 1979, Karoum et al., 1982, Wolf and Mosnaim, 1983, Sabelli et al., 1995), it has been argued that amygdala TAAR1 may serve as an emotional stabilizer and play a role

in the actions of antidepressants and anxiolytics. Accordingly, TAAR1 selective agonists were shown to reduce immobility time in a forced-swim test and prevent stress-induced hyperthermia, supporting the anti-depressant and anxiolytic properties of TAAR1 (Revel et al., 2011, Revel et al., 2012b, Revel et al., 2013). Moreover, these compounds improved performance in tasks requiring sustained attention and response inhibition, and increased wakefulness in a manner similar to caffeine without increasing locomotion and core body temperature, which are characteristic side effects of caffeine (Revel et al., 2012b, Revel et al., 2013). Together, these findings support the broad application of TAAR1-selective agents as mood stabilisers, cognitive enhancers, and general health supplements.

Most importantly, cumulative evidence suggests the notion that pharmacologically targeting TAAR1 may provide a novel avenue for the treatment of Parkinson's disease and psychosis. Parkinson's disease is characterized by a progressive degeneration of dopaminergic cells along the nigrostriatal pathway and subsequent loss of DA in the striatum (Lotharius and Brundin, 2002). L-DOPA treatment remains the gold standard pharmacotherapy for Parkinson's disease due to its ability to partially replenish striatal DA levels. However, chronic L-DOPA produces disabling motor side effects such as dyskinesia and motor fluctuation (Lloyd et al., 1975, Dauer and Przedborski, 2003). Given the ability of TAAR1 to downregulate DA transmission, medications that suppress TAAR1 activation level may hold promise for treating this disease.

In the case of psychosis, it is shown that TAAR1 KO mice exhibited elevated brain high-affinity D2 receptors and dopaminergic supersensitivity (Wolinsky et al., 2007), which resembles the neurological modifications identified in patients with schizophrenia (Laruelle et al., 1996, Abi-Dargham et al., 1998, Seeman, 2011). These TAAR1 mutant mice also had significant deficits in sensorimotor gating, which is a known behavioural signature of schizophrenia involving both DA- and glutamate-mediated mechanisms (Wan et al., 1995, Wan and Swerdlow, 1996, Geyer et al., 2001). Moreover, all human *TAAR* genes are tightly clustered in the chromosomal region that has been reproducibly associated with

schizophrenia in linkage or association studies (Straub et al., 1995, Cao et al., 1997, Lindholm et al., 1999, Borowsky et al., 2001, Bunzow et al., 2001). Together, these findings suggest that abnormal TAAR1 function may contribute to the aetiology and neuropathology of psychosis, and TAAR1-based treatments may modulate psychotic symptoms through interacting with the DA and glutamate system.

6.5 Limitations and Future Directions

The current body of work has several limitations that should be addressed in future investigations. Firstly, in experiment 2, compound M concurrent administration with METH had no effect on METH-induced hyperlocomotion and behaviour sensitization. This finding is inconsistent with other studies showing that partial TAAR1 agonism with RO5203648 or RO5263397 blocked both the induction and expression of behavioural sensitization induced by METH or cocaine (Thorn et al., 2014b, Cotter et al., 2015); and contradicts the notion that TAAR1 activation downregulates DA activity that is potentiated by psychostimulants. Apart from a different pharmacokinetic/dynamic profile (which is not well suited for rat studies) or a therapeutic dose range beyond the tested dosage for compound M, we could not think of other explanations for the lack of effect. Thus, one direction for future research is to fully characterize the pharmacological properties of compound M (since it is being developed for human use) and establish its therapeutic window. Moreover, our data also revealed a dose-dependent effect of compound M treatment alone on the subsequent locomotor response to a METH probe, suggesting that repeated compound M stimulation at TAAR1 might produce neuroadaptive changes that render the neuronal response to METH hypersensitive or hyposensitive, depending on the concentration of compound M. This could be linked to the cross-sensitization between RO5203648 and METH reported by Cotter et al. (2015), which suggests that chronic TAAR1 partial activation may produce neuroadaptations in a way similar to METH. In addition, our data also imply the possibility that lower doses of partial agonists might produce the opposite effects. In this regard, further research should unravel the short- and long-term neurological effects produced by chronic TAAR1

activation/deactivation and its functional implications in relation to the actions of psychostimulants.

Another limitation of the project is that, although we aimed to model in rats some of the aspects of the addiction cycle that are characterized by DA fluctuations, we did not capture all the situations that human addicts may encounter. Our data strongly support the therapeutic potential of TAAR1 selective agonists in modulating behaviours related to drug intoxication and relapse to drug seeking, but did not provide information on their effects on the negative withdrawal symptoms, which are an important drive for drug craving and drug seeking. Since withdrawal from chronic drug use leads to reduced DA transmission, which may underlie anhedonia and reward hypofunction (Robertson et al., 1991, Weiss et al., 1992), selective partial agonism of TAAR1 may improve the withdrawal state through increasing DA transmission. Future studies should be directed to address this question.

Furthermore, the current study did not test the effects of TAAR1 agonists on the locomotor response induced by acute and repeated cocaine. Although both cocaine and METH produce their reinforcing and psychostimulating effects by dramatically increasing extracellular DA level, they have different mechanisms of action. METH competes with DA at the DAT for reuptake, causes transporter internalization, and reverses the transport direction of the DAT (Sandoval et al., 2001, Elliott and Beveridge, 2005). METH also depletes DA vesicular storage through interfering with vesicular monoamine transporter-2, leading to increased cytosolic DA ready for reverse transport by the DAT (Sulzer et al., 2005, Fleckenstein et al., 2009). On the contrary, cocaine binds directly to the DAT, blocking DA reuptake from the extracellular space and thus leading to DA accumulation in the synaptic cleft (Volkow et al., 1997, Beuming et al., 2008). Critically, METH itself is a potent agonist at TAAR1 but cocaine is not. METH stimulation of TAAR1 leads to a series of cellular phosphorylation cascades resulting in functional regulation of the DAT, that partially accounts for its DA-releasing effects (Xie and Miller, 2009a). Therefore, the regulation of METH's effects by TAAR1 agonists is likely to be more complex than that of cocaine, with the former also

involving direct interaction of the agonist with METH at TAAR1. In experiment 6, we used FSCV to show that partial TAAR1 agonism with RO5263397 blocked METH-induced DA overflow in the NAc, which is consistent with a recent report using cocaine and RO5203648 (Pei et al., 2014). However, as revealed in experiment 1, the TAAR1-mediated attenuation of METH-induced locomotor response was followed by a potentiation when METH's effect began to subside, which suggests a DA state-dependent modulation by TAAR1 partial agonist and potentially reflects the dynamics of TAAR1 activity in response to the competition between METH and the partial agonist. This later potentiation effect of TAAR1 could not be captured in the FSCV experiment because the drug-induced DA accumulation was still at a moderately high level at the end of the 20 min recording period (Figure 4.5e). Therefore, it is uncertain whether TAAR1 partial agonism would upregulate DA transmission after the effects of cocaine wane off. The ideal way to test this question would be to examine the effects of RO5203648 on cocaine-induced locomotor response during an extended session (3-h), allowing a straightforward comparison with the METH experiment reported here. Based on our hypothesis that TAAR1 partial agonism has the unique ability to stabilize DA transmission through bidirectional regulation of DA regardless of the drug's affinity at TAAR1, we expect a similar time-dependent modulation as the net behavioural outcome for cocaine and METH.

Finally, while the current project utilized extensive behavioural models to characterize the pharmacotherapeutic properties of TAAR1 agonists as treatment for stimulant addiction, less attention has been drawn to the neuronal basis of the observed effects. Physiological data was collected in experiment 2, where compound M was shown to modulate chronic METH-induced changes in early gene inducibility, and in experiment 6, in which we showed that RO5263397 prevented acute METH-evoked DA release in the NAc measured by FSCV. Although these findings are informative, they could not directly explain all the behavioural data, especially those involving chronic METH treatment, including the relapse and reinstatement experiments, because the response to drugs is different between naïve and drug-experienced rats. Therefore, to best understand TAAR1 modulation on

psychostimulant-induced behaviour, animals whose brains are used for the FSCV should undergo identical chronic METH treatment as those in the behavioural models. Future studies should aim at deciphering the underlying neurophysiological mechanisms of TAAR1-mediated effects, not only at neurochemical levels but also at cellular and molecular levels. Only by doing so will we fully understand the functional role of TAAR1 in regulating psychostimulant action and design more efficacious TAAR1-based therapies for the treatment of addiction.

6.6 Conclusion

To conclude, the current thesis directly tested the hypothesis that pharmacologically targeting TAAR1 may present a novel approach for treating psychostimulant addiction. This was achieved through a systematic investigation of TAAR1 regulation of key behavioural and neuronal markers of METH and cocaine abuse. Our findings provided evidence of the complex bidirectional modulation of the locomotor-stimulating effects of METH by TAAR1 partial agonists, and demonstrated the remarkable ability of full and partial TAAR1 activation to block the reinforcing and motivational properties of cocaine and METH. Furthermore, the TAAR1 selective agonists effectively prevented cocaine and METH-induced relapse of drug seeking, further strengthening the therapeutic potential of TAAR1-based compounds to treat addiction disorders. Moreover, our data indicated differential regulation by TAAR1 on food and drug reinforcement and suggested a wide therapeutic window for TAAR1-based pharmacotherapies. At the neurochemical level, we showed for the first time that TAAR1 partial agonism completely reduced METH-evoked DA overflow in the NAc, adding to the support for TAAR1's inhibitory control over DA transmission that is potentiated by stimulants. Finally, these agonists did not exhibit METH-like discriminative effects or produce cross-reinstatement with METH or cocaine, indicating low abuse liability, which is a desirable feature for a pharmacotherapy to have. Future research should fully elucidate the pharmacological properties and the therapeutic window of compound M as well as other selective TAAR1 agonists and further explore the proposed TAAR1 "DA state-dependent regulation" by examining its effects on the negative drug withdrawal symptomatology. In

addition, comparing the ability of TAAR1 partial agonist to modulate the locomotor-stimulating effects of cocaine with those of METH in an extended session would provide valuable insight into the role of TAAR1 in the actions of METH and cocaine. Lastly, more efforts should be devoted to understand the neurophysiological effects of acute and repeated TAAR1 agonism and antagonism, the neural basis of TAAR1 ability to prevent relapse after protracted withdrawal from chronic drug exposure, and the mechanisms mediating the biphasic modulation of DA transmission by TAAR1 partial agonists. However, the current study represents an important step towards understanding the pivotal role of TAAR1 in the functional regulation of DA transmission and psychostimulant action. The data presented here provide new and substantial evidence of potential therapeutic effectiveness of TAAR1 agonists in modulating abuse-related effects of METH and cocaine, and strongly support the development of TAAR1-based therapies as a new generation of medicines for the treatment of psychostimulant addiction.

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8 APPENDIX A

Pei Y, Asif-Malik A, Canales JJ (2016) Trace Amines and the Trace Amine-Associated Receptor 1: Pharmacology, Neurochemistry, and Clinical Implications. Frontiers in Neuroscience 10:148. doi:10.3389/fnins.2016.00148.

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