MITRAGYNINE-INDUCED CONDITIONED PLACE PREFERENCE (CPP) AND THE PROFILES OF DOPAMINE AND ITS METABOLITES IN RATS

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by

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Nurul Hasnida Mohammad Yusoff

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LIST OF ABBREVIATIONS

ALT	alanine transaminase
ANOVA	analysis of variance
AP	anteroposterior
AST	aspartate aminotransferase
C_{max}	maximum serum concentration
C_{mea}	measured concentration
C_{nom}	nominal concentration
Ca^{2+}	calcium
cAMP	cyclic adenosine 3',5'-monophosphate
Cl^-	chloride
Cl/*F*	clearance rate
CNS	central nervous system
COMT	catecholamine-*O*-methyltransferase
CPA	conditioned place aversion
CPP	conditioned place preference
CPu	caudate putamen
CRE	cAMP response element
CREB	cAMP response element binding protein
CRF	corticotrophin-releasing factor
CV	coefficient of variation
DA	dopamine
DAT	dopamine transporter
DOPAC	3,4-dihydroxyphenylacetic acid
DRRF	dopamine receptor-regulating factor
DV	dorsoventral
ECF	extracellular fluid
eg.	example
et al.	and others
fEPSP	field excitatory post-synaptic potentials
FST	forced swimming test

GABA	gamma-aminobutyric acid
GIRK	Kir3/G protein-coupled inwardly rectifying K^+
7-HMG	7-hydroxymitragynine
HPA	hypothalamic-pituitary-adrenal
HPLC-ECD	high performance liquid chromatography coupled with electrochemical detector
HVA	homovanillic acid
ICSS	intracranial electrical self-stimulation
i.d.	internal diameter
i.p.	intraperitoneal
K^+	potassium
L-DOPA	L-dihydroxyphenylalanine
LLOQ	lower limit of quantification
LTP	long term potentiation
M	Molar
MAO	monoamine oxidase
MGM-9	ethylene glycol-bridged and C10-fluorinated derivative of mitragynine
ML	mediolateral
3-MT	3-methoxytyramine
n	number of animals/samples
NAc	nucleus accumbens
NPY	neuropeptide Y
PFC	prefrontal cortex
QC	quality control
RE	relative error
s.c	subcutaneous
SEM	standard error of means
SN	substantia nigra
$t_{1/2}$	elimination half life
T_{max}	maximum time
TH	tyrosine hydroxylase
TST	tail suspension test

vs.	versus
VTA	ventral tegmental area
v/v	volume over volume

LIST OF SYMBOLS

°	degree
°C	degree celcius
%	percent
-	minus
±	plus minus
X	multiply
=	equals to
/	per (for each)
µ	micro
µ	mu
<	less than
>	more than
Δ	delta
Κ	kappa
Υ	gamma
λ_z	elimination rate constant

KECENDERUNGAN TEMPAT BERKONDISI (CPP) TERARUH MITRAGININA DAN PROFIL DOPAMINA SERTA METABOLIT-METABOLITNYA DI DALAM TIKUS

ABSTRAK

Mitragyna speciosa (*M. speciosa*) Korth ialah tumbuhan herba tradisional berasal dari Asia Tenggara. Penggunaan tetap tumbuhan ini oleh manusia boleh menyebabkan ketagihan dengan simptom-simptom penarikan yang ketara. Dihipotesiskan bahawa penyalahgunaan penyediaan-penyediaan *M. speciosa* mungkin disebabkan oleh mitraginina, bahan psikoaktif utama yang dipencilkan dari daunnya. Oleh itu, kajian secara komprehensif ke atas sifat-sifat ganjaran mitraginina, beserta mekanisma-mekanisma yang terlibat diperlukan. Dalam bahagian pertama kajian, sifat-sifat ganjaran mitraginina (1, 5, 10 dan 30 mg/kg) dan kesan-kesan lokomotor dikaji di dalam tikus Sprague-Dawley, menggunakan paradigma kecenderungan tempat berkondisi (CPP). Sebagai keputusannya, tikus yang dikondisi dengan mitraginina menghasilkan CPP pada dos 10 dan 30 mg/kg ($P<0.05$) seperti yang ditunjukkan oleh kecenderungan tikus terhadap persekitaran berkondisi mitraginina, tanpa perubahan aktiviti lokomotor pada semua dos yang diuji ($P>0.05$). Tambahan lagi, adalah menarik juga untuk mengkaji sama ada CPP teraruh mitraginina kekal semasa ketiadaan mitraginina, dan muncul semula berikutan suntikan mitraginina. Dari pemerhatian, CPP teraruh mitraginina (10 mg/kg) kekal sekurang-kurangnya 7 hari ($P<0.001$) selepas ujian CPP pertama, lenyap selepas 8 latihan pemadaman ($P>0.05$) dan muncul semula oleh suntikan mitraginina ($P<0.05$). Untuk memahami mekanisma-mekanisma yang menyebabkan

CPP teraruh mitraginina, penglibatan sistem reseptor opioid dan asid gamma-aminobutirik $_B$ ($GABA_B$) dalam fasa pemerolehan dan pengekspresan dikaji. Eksperimen-eksperimen dijalankan menggunakan antagonis reseptor opioid, nalokson (0.1, 0.3 dan 1.0 mg/kg) dan agonis reseptor $GABA_B$, baclofen (1.25, 2.5 dan 5.0 mg/kg). Sebagai keputusannya, dalam kajian nalokson, pra-rawatan dengan nalokson (0.1-1.0 mg/kg) sebelum kondisi mitraginina menghalang CPP teraruh mitraginina ($P>0.05$) sementara nalokson (0.1-1.0 mg/kg) yang diberi sebelum ujian CPP tidak menghalang pengekspresan CPP teraruh mitraginina ($P<0.05$). Dalam kajian baclofen, CPP teraruh mitraginina diperhatikan selepas 1.25 mg/kg baclofen tetapi tidak lagi kelihatan selepas 2.5 atau 5 mg/kg baclofen, sama ada diberi sebelum kondisi mitraginina atau ujian CPP ($P>0.05$). Keputusan-keputusan ini menunjukkan bahawa CPP teraruh mitraginina dipengaruhi oleh sistem opioid pada fasa pemerolehan tetapi tidak pada fasa pengekspresan, sementara sistem reseptor $GABA_B$ mempengaruhi CPP teraruh mitraginina pada kedua-dua fasa pemerolehan dan pengekspresan. Akhir sekali, adalah penting untuk memeriksa sama ada mitraginina meningkatkan pengeluaran dopamina (DA) pada kawasan-kawasan ganjaran otak seperti yang diperhatikan pada dadah-dadah lain yang disalahgunakan. Untuk tujuan ini, pengeluaran DA otak ekstraselular serta metabolit-metabolitnya, asid 3,4-dihidroksifenilasetik (DOPAC) dan asid homovanilik (HVA), diperiksa dalam korteks prefrontal (PFC), nukleus akumbens (NAc) dan kaudat putamen (CPu) berikutan pendedahan mitraginina (10 mg/kg) akut. Daripada penemuan hasil, mitraginina tidak mengubah paras DA ekstraselular basal dalam semua bahagian otak yang diperiksa ($P>0.05$), tetapi meningkatkan paras DOPAC dalam CPu (183%; $P<0.05$) dan HVA ($P<0.05$) dalam PFC (168%), NAc (144%) dan CPu (157%) berikutan pemberiannya. Secara keseluruhannya, kajian ini merungkai sifat-sifat

ganjaran mitraginina, dengan terhasilnya CPP teraruh mitraginina. CPP teraruh mitraginina boleh dikekalkan dan muncul semula, menunjukkan kehadiran perlakuan mendapatkan dadah dan relaps dadah. Penemuan-penemuan ini menyokong peranan mitraginina terhadap potensi ketagihan *M. speciosa* yang dilaporkan dalam manusia. Sebagai tambahan, sistem reseptor opioid dan $GABA_B$ bertindak sebagai sebahagian dari mekanisma-mekanisma melibatkan CPP teraruh mitraginina. Sistem DA mungkin memainkan peranan penting dalam mempengaruhi CPP teraruh mitraginina, kerana pendedahan mitraginina akut menghasilkan kesan penggalak akut terhadap sintesis DA.

MITRAGYNINE-INDUCED CONDITIONED PLACE PREFERENCE (CPP) AND THE PROFILES OF DOPAMINE AND ITS METABOLITES IN RATS

ABSTRACT

Mitragyna speciosa (*M. speciosa*) Korth is a traditional herbal plant indigenous to Southeast Asia. Regular use of this plant by human may lead to addiction with profound withdrawal symptoms. It is hypothesized that the abuse of *M. speciosa* preparations could be attributed to mitragynine, the main psychoactive compound isolated from its leaves. Therefore, comprehensive studies on the rewarding properties of mitragynine, as well as the mechanisms invoved, are needed. In the first part of the study, the rewarding properties of mitragynine (1, 5, 10 and 30 mg/kg) and its locomotor effects were investigated in Sprague-Dawley rats, using a conditioned place preference (CPP) paradigm. As a result, rats conditioned with mitragynine possesses CPP at dose of 10 and 30 mg/kg ($P<0.05$) as shown by the rats' preference for mitragynine-conditoned environment, with no changes in locomotor activity at all doses tested ($P>0.05$). Furthermore, it is of interest to investigate whether the mitragynine-induced CPP can be maintained in the absence of mitragynine, and reinstated following mitragynine administration. From the observations, the mitragynine (10 mg/kg)-induced CPP was maintained for at least 7 days ($P<0.001$) after the initial CPP test, extinguished after 8 extinction trainings ($P>0.05$) and reinstated by priming mitragynine injection ($P<0.05$). In order to understand the mechanisms underlying the mitragynine-induced CPP, the involvement of opioid and gamma-aminobutyric acid $_B$ ($GABA_B$) receptor system in the acquisition and expression phase was studied. The experiments were performed

using an opioid receptor antagonist, naloxone (0.1, 0.3 and 1.0 mg/kg) and a $GABA_B$ receptor agonist, baclofen (1.25, 2.5 and 5.0 mg/kg). As a result, in naloxone study, pre-treatment with naloxone (0.1-1.0 mg/kg) before mitragynine conditioning blocked the mitragynine-induced CPP ($P>0.05$) while naloxone (0.1-1.0 mg/kg) given before the CPP test did not prevent the expression of mitragynine-induced CPP ($P<0.05$). In baclofen study, the mitragynine-induced CPP was observed after 1.25 mg/kg baclofen but no longer observed after 2.5 or 5 mg/kg baclofen, either given before mitragynine conditioning or CPP test ($P>0.05$). These results showed that the mitragynine-induced CPP were mediated by opioid system at the acquisition but not expression phase, while $GABA_B$ receptor system affects the mitragynine-induced CPP at both the acquisition and expression phases. Finally, it is of great importance to examine whether mitragynine increases the dopamine (DA) release at brain reward areas as seen with other drugs of abuse. For this reason, the release of extracellular brain DA and its metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), were monitored in the prefrontal cortex (PFC), nucleus accumbens (NAc) and caudate putamen (CPu) following acute mitragynine exposure (10 mg/kg). From the findings, mitragynine did not alter the extracellular basal DA level in all brain regions examined ($P>0.05$), but increased DOPAC in the CPu (183%; $P<0.05$) as well as HVA ($P<0.05$) in the PFC (168%), NAc (144%) and CPu (157%) following its administration. Taken together, the present study reveals the rewarding properties of mitragynine, by the establishment of mitragynine-induced CPP. The mitragynine-induced CPP could be maintained and reinstated, indicating the presence of drug-seeking behaviour and drug relapse. These findings support the role of mitragynine for the abuse potential of *M. speciosa* reported in humans. Additionally, the opioid and $GABA_B$ receptor systems serve as parts of the

mechanisms underlying the mitragynine-induced CPP. The DA system may play an essential role in mediating the mitragynine-induced CPP, since acute mitragynine exposure produced acute stimulating effect on DA synthesis.

CHAPTER 1

OVERVIEW

1.1 General introduction

Mitragyna speciosa (*M. speciosa*) Korth is a plant native to Southeast Asia. It is known as ketum in Malaysia and kratom in Thailand (Hassan et al., 2013; Suhaimi et al., 2016). It is traditionally used for its medicinal values and stimulatory actions (Singh et al., 2018; Suhaimi et al., 2016). Increasing reports on *M. speciosa* abuse and its addictive potential has led to the extensive study of mitragynine, the major active alkaloid extracted from *M. speciosa* leaves (Harun et al., 2015; Sufka et al., 2014). Mitragynine is believed to be responsible for various pharmacological effects exerted by *M. speciosa*, mainly through opioid receptors (Matsumoto et al., 2005a; Taufik Hidayat et al., 2010; Watanabe et al., 1997). Though the *M. speciosa* extracts and its compounds may be potentially used in the management of pain, opioid withdrawal symptoms and other illnesses (Boyer et al., 2008; Kumarnsit et al., 2007a), it is pertinent to clarify in depth the abuse and addiction potential of these substances at its therapeutic doses as well as its mechanism of actions before any recommendation for its application in the patient treatments can be made.

The rewarding properties of a particular substance may lead to drug dependence and addiction (Esch & Stefano, 2004; Koob & Le Moal, 2001). The rewarding properties can be assessed preclinically in animal model using CPP paradigm, a widely known screening tool for addictive potential of drug (Bardo & Bevins, 2000; Tzschentke, 1998). A subject is said to develop CPP when it spent more time in a drug-paired environment, following several drug and vehicle pairings with neutral environment.

The drug-induced CPP occurs as a result of the acquisition (learning) and expression (retrieval of learning) phases (Carboni & Vacca, 2009). In the absence of drug, the established CPP can be maintained for a certain period of time, promoting drug-seeking behaviour. In certain circumstances, this drug-induced CPP can be extinguished and reinstated, which could be of beneficial value to study drug relapse phenomenon in human (Mueller et al., 2002; Mueller & Stewart, 2000; Sakoori & Murphy, 2005).

Different classes of drugs of abuse share a final common neuronal pathway for its rewarding effects, by activating the mesolimbic dopaminergic activity (Pierce & Kumaresan, 2006; Spanagel & Weiss, 1999; Wise, 1998). Their actions could be either by exerting direct influence on the dopamine (DA) neurones, and/or by altering other neurotransmitter systems that modulate the mesolimbic dopaminergic pathway (Pierce & Kumaresan, 2006; Tomkins & Sellers, 2001). Other than DA, the role of various neurotransmitter systems in the rewarding properties of drugs of abuse have been implicated, including opioidergic, γ-aminobutyric acid (GABA), serotonergic, cholinergic, glutamatergic, noradrenergic and endocannabinoid system (Mathon et al., 2003; Ross & Peselow, 2009).

1.2 Problem statements

The available human reports on *M. speciosa* abuse and addiction (Ahmad & Aziz, 2012; Saingam et al., 2013; Singh et al., 2014; Vicknasingam et al., 2010) have highlighted the need for comprehensive studies on the addictive properties of mitragynine in animal model of addiction. The elucidation of mitragynine's rewarding properties in laboratory animals is required to reveal whether or not it contributes to *M. speciosa* dependence and abuse. Since mitragynine is the major constituent of the total alkaloid contents of *M. speciosa* plants cultivated in Malaysia (Chan et al., 2005), therefore, it may have a major role in the addictive effects exerted by this plant. Most of the drugs of abuse by human, such as morphine, posses rewarding properties, as indicated by the establishment of CPP in laboratory animals (Tzschentke, 1998), which is believed to be responsible for its abuse. Therefore, the present study was performed to examine the rewarding properties of mitragynine using CPP paradigm. It is hypothesized that mitragynine would exhibits CPP in the experimental animals based on the addictive features reported in human with regular *M. speciosa* consumption.

In the absence of drug, the maintenance of the drug-induced CPP can promote drug-seeking behaviour in laboratory animals (Mueller et al., 2002; Sakoori & Murphy, 2005). Moreover, in human addicts, exposure to environment associated with drug increased the urge to use drug (Ludwig & Stark, 1974; O'Brien et al., 1977). Therefore, in the case of mitragynine, it is of interest to know whether the established mitragynine-induced CPP is maintained for extended periods of time and whether the environment associated with mitragynine is able to promote drug-seeking behaviour. It is hypothesized that mitragynine's rewarding properties may persist over

considerable time and this would make the subjects attracted to the environment which has been previously associated with mitragynine.

In laboratory animals, the drug-induced CPP could be extinguished when the subject is no longer exposed to the drug for a certain period of time (Mueller et al., 2002; Sakoori & Murphy, 2005) and this would lessen the desire to seek for the drug. Nevertheless, the drug-induced CPP could be triggered when the subject is reintroduced to the drug (Do Couto et al., 2003; Itzhak & Martin, 2002; Parker & McDonald, 2000). With regard to mitragynine, this has not yet been studied. Therefore, it is necessary to elucidate whether the mitragynine-induced CPP disappear following several extinction trainings, and whether the preference to mitragynine could be reinstated with priming mitragynine administration, after the extinction period. If mitragynine acts in a similar way as other drugs of abuse, it is hypothesized that mitragynine-induced CPP can be extinguished, and triggered by priming mitragynine administration following the extinction period.

The brain reward mechanisms involve several interactions among multiple neurotransmitter systems (Ross & Peselow, 2009; Tomkins & Sellers, 2001). Previous observations revealed that the opioidergic, adrenergic, serotonergic and/or dopaminergic receptors play a role in several mitragynine's activities (Matsumoto et al., 1996a,b; Thongpradichote et al., 1998), thus open a question for the role of these receptor systems in mitragynine reward, or CPP. Owing to the opioid characteristics showed by mitragynine (Matsumoto et al., 2005a; Taufik Hidayat et al., 2010; Watanabe et al., 1997), the current study tested the hypothesis that the opioid

receptor system would mediate the acquisition and expression of mitragynine-induced CPP.

The importance of $GABA_B$ receptor system on reward mechanism has been implicated in many studies (Le Foll et al., 2008; Li et al., 2001). For certain drugs of abuse, inhibition of $GABA_B$ activity partly mediates its rewarding effect, by releasing tonic inhibition exerted on DA neurones (Chen et al., 2005). Activation of opioid system has been demonstrated to inhibit the activity of $GABA_B$ on DA neurones (Spanagel & Weiss, 1999). Based on these facts, indirect involvement of $GABA_B$ receptor system in the acquisition and expression of mitragynine-induced CPP is sought to be an important issue that warrants further investigation. It is hypothesized that the $GABA_B$ receptor system involves in the pharmacological mechanisms underlying the opioid-mediated mitragynine-induced CPP.

Drugs of abuse are well known to activate the mesolimbic dopaminergic activity as shown by the increased DA levels in the nucleus accumbens (NAc) (Di Chiara, 1998; Tomkins & Sellers, 2001). In addition, the increased NAc DA has been suggested to mediate the drug conditioned rewarding effects, or CPP (Shoaib et al., 1995). To date, there is no study performed to examine the activity of the dopaminergic system in brain reward areas following mitragynine administration. This posing a question whether acute mitragynine administration affects the release of extracellular DA levels within the brain areas that received dopaminergic projections. Therefore, investigation on the neurochemical events in the prefrontal cortex (PFC), NAc and caudate putamen (CPu) of the rat brain with regard to DA system following acute mitragynine administration was addressed in the present study. It is hypothesized that

mitragynine will increase the extracellular DA release, thus supports the effect of mitragynine on CPP paradigm.

1.3 Objectives

In the present study, four specific objectives have been constructed to address the abovementioned problem statements:

1. To investigate the locomotor effects of mitragynine as well as the establishment, maintenance, extinction and reinstatement of mitragynine-induced CPP.
2. To reveal the role of opioid receptor system on acquisition and expression of mitragynine-induced CPP using non-selective opioid receptor antagonist, naloxone.
3. To elucidate the role of $GABA_B$ receptor system on acquisition and expression of mitragynine-induced CPP using $GABA_B$ receptor agonist, baclofen.
4. To discover the effects of acute mitragynine administration on the extracellular release of DA and its metabolites, 3,4-dihydroxyphenylacetic acid and homovanillic acid, in the PFC, NAc and CPu regions using *in vivo* brain microdialysis coupled with high performance liquid chromatography-electrochemical detector.

1.4 Summary of the research design

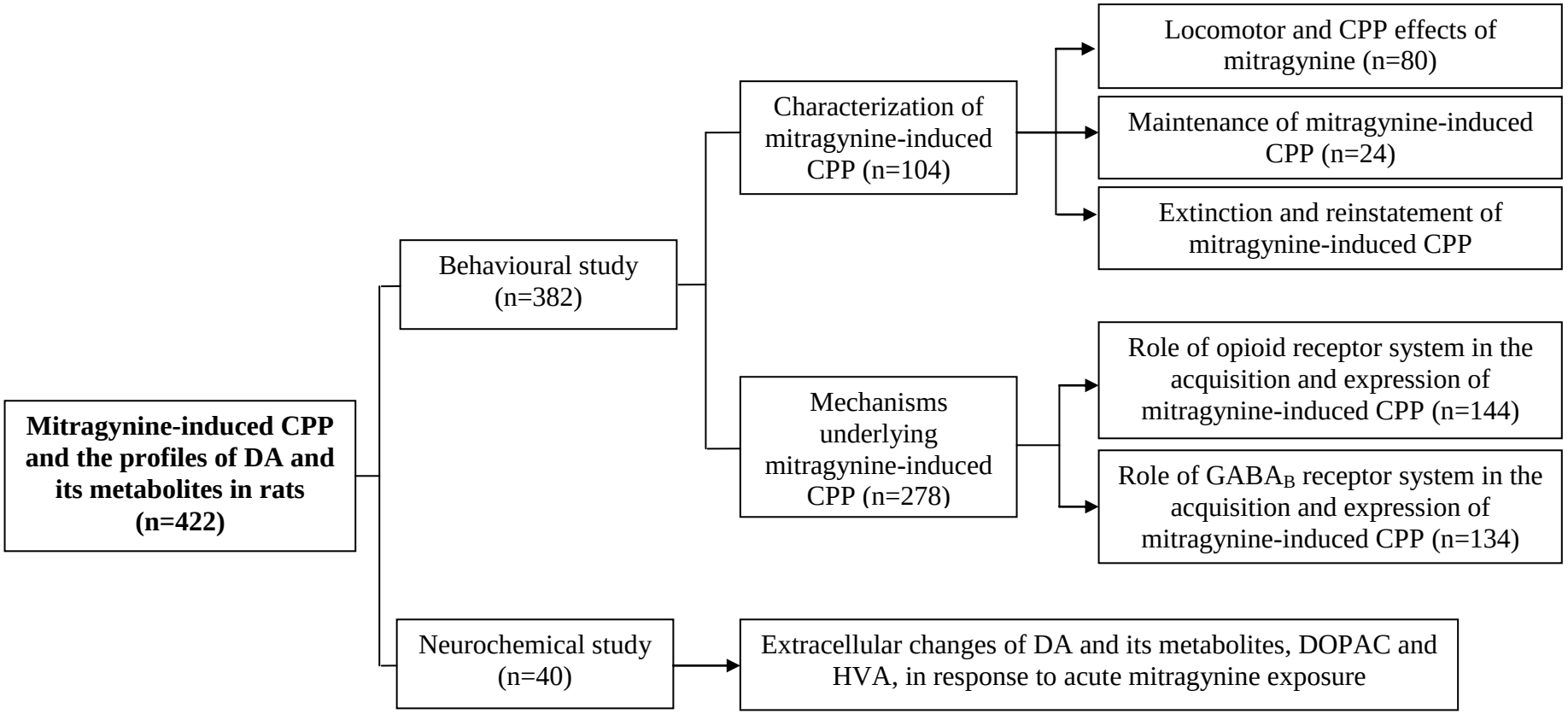

Figure 1.1 Research design and the number of animals used in the study.

CHAPTER 2

LITERATURE REVIEW

2.1 Drug addiction

Drug addiction can be classified as a chronic disease of the central nervous system (CNS), characterized by a loss of control over impulsive behaviour that leads to compulsive drug-seeking and drug-taking behaviour, and to relapse even after a long period of abstinence (Feltenstein & See, 2008). The clinical diagnosis of addiction includes the use of a psychoactive substance for a longer period or at higher doses than initially prescribed, drug cravings or failure to cease the drug use, compulsive drug taking despite negative consequences, increasing tolerance, withdrawal syndromes and desire to take the drug to mitigate these syndromes (Filip & Frankowska, 2008).

2.1.1 Theories of addiction

Early theories of addiction postulated that the ability of the substance to produce a pleasurable effect (reward) is the one that initiates drug consumption, and drug dependence occurs as a consequence of repeated drive for reward (Wise, 1980). The theory of positive reinforcement, which refers to the ability of the reinforcer (e.g.; addictive drug) to promote behavioural responses responsible for the pleasurable effects, has been accepted as a main factor that contributes to drug dependence. However, with repeated use, many drugs exhibit tolerance effect, a condition at which the reinforcing properties of the drug is attenuated, and therefore the drug dosage needs to be continually increased to maintain the desired effects. Meanwhile, some drugs produce sensitization on specific behaviours, as oppose to tolerance

effects. Based on these facts, the positive reinforcement theory alone is insufficiently strong to serve as the only causative factor for drug addiction. A negative reinforcement theory, which refers to continued drug use as a means to prevent the aversive consequences of drug withdrawal (aversive psychological and physiological effects), could account for the maintenance of compulsive drug use. Nevertheless, both positive and negative reinforcement theories are inadequate to comprehensively describe all aspects of addiction cycle, and several researchers have proposed some addiction theories which had a substantial influence on our understanding about addiction (Feltenstein & See, 2008).

Koob & Le Moal (1997) have proposed that drug addiction is accompanied by the development of allostasis, a state where the brain reward regulatory system deviates chronically from its homeostatic level to new reward set points. The dysregulations of reward system with repeated drug intake result in an allostatic state that drives further drug intake, and ultimately lead to compulsive drug use, and in turn exaggerates the allostatic state. The process of maintaining the reward function stability is hypothesized to involve alterations in reward neurotransmission (mesolimbic dopamine and opioid peptide system) as well as brain (corticotrophin-releasing factor; CRF, neuropeptide Y; NPY and norepinephrine system) and hormonal stress system (hypothalamic-pituitary-adrenal; HPA axis).

Alternatively, Robinson & Kent (1993) have suggested an incentive sensitization theory of addiction, whereby recurrent use of addictive drugs can persistently alter the neural circuits that normally involves in motivated behaviour, including incentive motivation and reward for natural appetitive reinforcers. These neuroadaptations

cause the brain circuits hypersensitive to the drug and drug-associated stimuli, thus results in a transition from drug 'liking' to drug 'wanting' behaviour. This pathological incentive motivation for drugs last for years resulted from the persistent incentive sensitization.

Several research groups had made an assumption that a shift to addiction stems from aberrant learning caused by the drug itself. The implicit stimulus-response learning promotes the progression from initially conscious behaviour to more automatically (unconscious) habit behaviour (Everitt & Robbins, 2005; Robinson & Berridge, 2003; Wise, 2002). In another point of view, Jentsch & Taylor (1999) have postulated that alterations in the cortical and limbic circuits causing deficits in behavioural control, impaired decision-making processes as well as augmented incentive motivational qualities of the drug and drug-associated stimuli.

2.1.2 Neurobiology of addiction

Activation of dopaminergic mesocorticolimbic system that arises from the ventral tegmental area (VTA) of the midbrain accounts for the final common neuronal pathway by which various drugs of abuse mediate their acute reinforcing effects (Pierce & Kumaresan, 2006; Spanagel & Weiss, 1999; Wise, 1998). This dopaminergic mesocorticolimbic system plays pivotal roles in reward, motivational control and modulation of cognitive functions. The mesolimbic circuit includes projections from cell bodies of the VTA to limbic structures such as nucleus accumbens (NAc), amygdala and hippocampus (Figure 2.1). Meanwhile, the mesocortical circuit includes projections from the VTA to the PFC, orbitofrontal cortex, and anterior cingulate. The activation of VTA DA neurones lead to an

increase in dopaminergic neuronal firing rate as well as DA synthesis and release. The increased DA concentration in the NAc appears to be responsible for the rewarding effects or feeling pleasure (Koob & Le Moal, 1997).

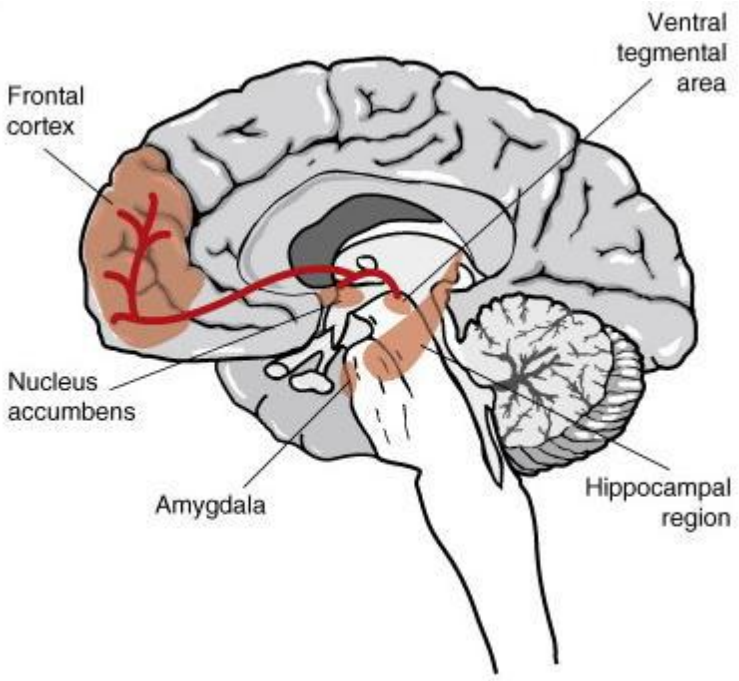

Figure 2.1 Brain reward circuit (adapted from Kalsi et al., 2009).

Other than the addictive drugs, natural rewards such as food, drink and sex also stimulate the release of DA into the NAc, producing euphoric effect. As for the natural rewards, novelty contributes to the initial response, and habituation occurs after a few experiences. In the case of addictive drugs, the concentration of NAc DA increases as the drug dosage increases. Overstimulation of DA will then results in dysregulation of the natural reward pathways as well as learning process in the brain (Cami & Farre, 2003; Di Chiara, 1998).

DA is a catecholamine, a subclass of the monoamine neurotransmitters synthesized from an amino acid known as tyrosine (Yang & Beal, 2011). The synthesis of DA and its metabolism are depicted in Figure 2.2. In the dopaminergic neurones, tyrosine is converted to 3,4-dihydroxyphenylalanine (L-DOPA) by the cytosolic enzyme tyrosine hydroxylase (TH). Aromatic amino acid decarboxylase (AADC) converts cytosolic L-DOPA to DA. From the cytoplasm, DA is stored in specialized storage vesicles until the arrival of an action potential, whereby DA is being released into the synapse by a process of exocytosis. In normal conditions, the reuptake mechanisms by presynaptic DA transporter (DAT) are responsible for transporting extracellular DA back into the cytoplasmic nerve terminal. In the cytoplasm, DA is either repacked into storage vesicles or broken down by the enzyme monoamine oxidase (MAO) to DOPAC. The extracellular DA left in the synaptic gap is methylated to 3-methoxytyramine (3-MT) by the enzyme catecholamine-*O*-methyltransferase (COMT), which can be taken back into the cytoplasm and further oxidized to HVA by MAO. The cytoplasmic DOPAC may leak out to the extracellular space and be methylated to HVA by COMT. The determination of DA and its metabolites DOPAC and HVA in biological samples provides an important key to understand the neurobiology of addiction (Yang & Beal, 2011). The molecular structure of DA, DOPAC and HVA are shown in Figure 2.3.

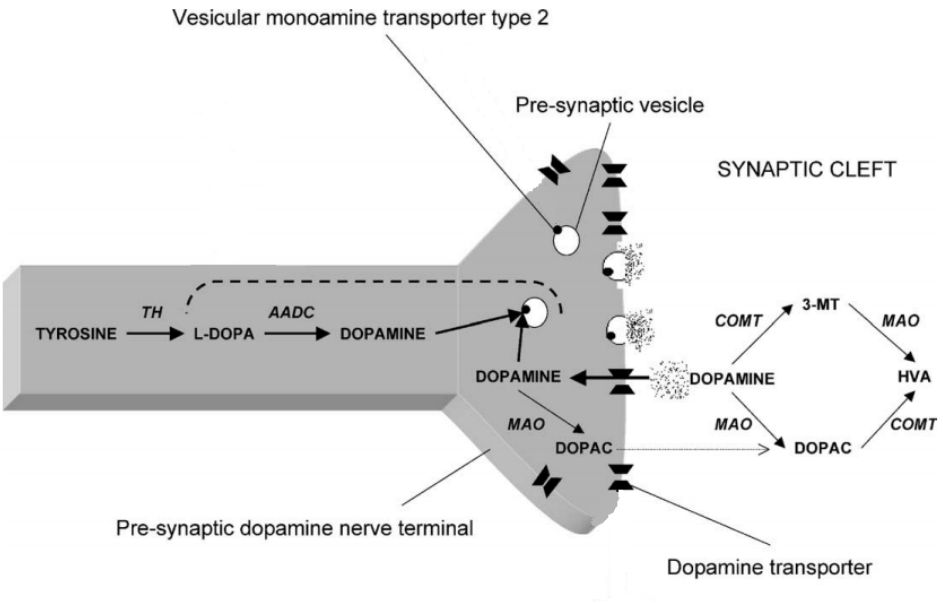

Figure 2.2 Schematic representation of dopamine synthesis and metabolism (AADC: aromatic amino acid decarboxylase; COMT: catechol-*O*-methyltransferase; DOPAC: 3,4-dihydroxyphenylacetic acid; HVA: homovanillic acid; L-DOPA: 3,4-dihydroxyphenylalanine; MAO: monoamine oxidase; 3-MT: 3-methoxytyramine; TH: tyrosine hydroxylase) (adapted from Mazzio et al., 2011).

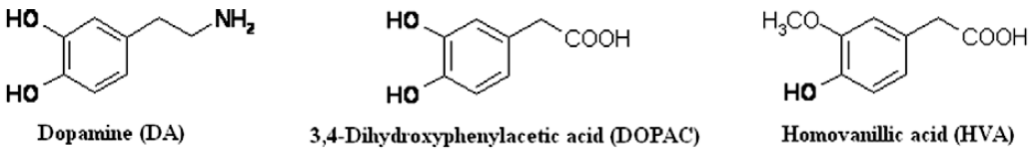

Figure 2.3 Structure of DA and its metabolites (adapted from Cai et al., 2010).

The physiological actions of DA are mediated by five distinct G protein-coupled receptor subtypes (Bressan & Crippa, 2005). Two D_1-like receptor subtypes (D_1 and D_5) coupled to G protein causes activation of adenylyl cyclase and stimulate cyclic adenosine 3',5'-monophosphate (cAMP) formation whereas the D_2-like receptor

subtypes (D_2, D_3 and D_4) coupled to G protein causes inhibition of adenylyl cyclase and activate potassium (K^+) channels. The expression of both D_1 and D_2 receptors differs in distinct neuronal populations that project to different brain regions (Bressan & Crippa, 2005).

Despite dopaminergic system, converging evidence have implicated the role of various neurotransmitter systems in acute reinforcing properties of drugs of abuse, including opioidergic, γ-aminobutyric acid (GABA), serotonergic, cholinergic, glutamatergic, noradrenergic and endocannabinoid system (Mathon et al., 2003). Each of these systems acts either in parallel with dopaminergic system or via independent pathways of reinforcement (Ross & Peselow, 2009). Among these, the involvement of opioid and GABA systems in mediating the reward processes are chosen as subject of interest in the present study.

GABA is the main inhibitory neurotransmitters in the mammalian CNS (Chalifoux & Carter, 2011). Immunocytochemical studies revealed that GABA is the most abundant non-dopaminergic cells in the VTA. These GABAergic neurones project to the NAc, as well as other brain regions (Steffensen et al., 1998; Van Bockstaele & Pickel, 1995). Several lines of evidence suggested that GABAergic interneurones provide tonic inhibitory control over dopaminergic neuronal activity in the VTA (Chen et al., 2005; Xi & Stein, 2002). GABA causes hyperpolarisation either by acting on ionotropic $GABA_{A/C}$ or metabotropic $GABA_B$ receptors. $GABA_A$ receptors are ligand-gated chloride (Cl^-) channel that mediate the fast synaptic inhibition, while $GABA_B$ receptors are linked to K^+ channel through G protein and mediate slow onset of actions (Mathon et al., 2003; Zarrindast et al., 2006) . The GABAergic system is

crucial in modulating the rewarding effects of addictive drugs, particularly through $GABA_B$ receptors (Spano et al., 2007) since majority of $GABA_A$ receptors in the VTA are not located on DA neurones (Churchill et al., 1992). The $GABA_B$ receptor exists as a heterodimer which composed of $GABA_{B(1)}$ and $GABA_{B(2)}$ subunits. The agonist binding site resides on the $GABA_{B(1)}$ subunit whereas the $GABA_{B(2)}$ subunit links to G proteins (Chen et al., 2005). Previous studies have indicated that stimulation of $GABA_B$ receptors reduced the dopaminergic cell firing and bursting activity in the VTA (Erhardt et al., 2002). Moreover, $GABA_B$ receptor agonists are effective in the treatment of substance abuse disorders (Filip & Frankowska, 2008). Baclofen, a lipophilic $GABA_B$ receptor agonist, has been reported to cause reduction in the reinforcing effects of several addictive drugs, such as opiates, cocaine and nicotine (Fadda et al., 2003). In addition, $GABA_B$ receptor activation results in attenuation of methamphetamine, cocaine, heroin, alcohol and nicotine self-administration in rodents (Brebner et al., 2002). Enhancement of GABAergic transmission also inhibit conditioned place preference effects induced by morphine (Kaplan et al., 2003; Zarrindast et al., 2006), methamphetamine (Li et al., 2001) and nicotine (Le Foll et al., 2008) in rats.

Besides GABA, extensive studies have shown the modulatory role of opioidergic system in motivational processes and rewarding behaviours (Narita et al., 2001; Self & Stein, 1992; Van Ree et al., 1999). The opioid receptors are mainly classified into mu (μ), delta (δ) and kappa (κ) subtypes (Kieffer & Evans, 2009). The μ-opioid receptors primarily mediate the rewarding effects of both opioid and non-opioid drugs, although δ-opioid receptor system has also been implicated in the rewarding processes. In contrast, stimulation of κ-opioid receptor produces aversive events

(Liang et al., 2006). Activation of μ-opioid receptors enhance DA neurotransmission by inhibiting GABAergic interneurones, thereby disinhibiting mesolimbic DA neurones and increasing both somatodendritic and axonal DA release (Spanagel & Weiss, 1999). Administration of μ-opioid agonists such as morphine and endogenous opioid peptide, endomorphin-1 produced conditioned place preference effect, a behavioural measure of drug-induced reward, as a result of increased DA release in the mesolimbic regions (Terashvili et al., 2008). In addition, selective agonists acting at μ-opioid receptors supported self-administration behaviour induced by morphine. Conversely, application of opioid antagonists such as naloxone effectively blocked both morphine-induced conditioned place preference and morphine self-administration, therefore strengthening the roles of opioid receptors in mediating the reward mechanisms (Van Ree et al., 1999).

2.1.3 Molecular and cellular adaptations in addiction

Repeated exposure to the addictive drugs may gradually and progressively develop several addictive behaviours that can sustain after a long period of abstinence. Alterations of gene expression in specific brain regions have been suggested to mediate the persistence of the addictive behaviours in drug addicts through changes in transcription factors.Administration of drug of abuse causes changes in various intracellular signalling pathways, eventually signals to the cell nucleus leading to alteration of transcription factors. These transcription factors bind to the regulatory region of the DNA hence controlling the rate of gene transcription. To date, drugs of abuse have been demonstrated to cause alterations in numerous types of transcription factors, including cAMP response element binding protein (CREB) (Nestler, 2004). CREB is one of the transcription factors that mediate the effects of cAMP second

messenger pathway on gene expression. Up-regulation of the cAMP pathway is the best established molecular adaptation to chronic drug exposure. This occurs through the phosphorylation of CREB on a single serine residue, namely Ser133, by protein kinase A, which is activated by cAMP. After phosphorylation process, CREB dimers bind to specific cAMP response element (CRE) sites and regulate the transcription of target genes via interaction with basal transcriptional complex. Several lines of evidence have implicated drug-induced activation of CREB underlying up-regulation of cAMP pathway in NAc, VTA, amygdala, hippocampus, lateral hypothalamus, frontal cortex and locus ceruleus (Nestler, 2004).

2.1.4 Drugs of abuse

The drugs of abuse can generally be classified into different categories, including psychostimulants (cocaine, amphetamine), cannabinoids (marijuana), depressants (ethanol), hallucinogens (ecstacy), inhalants (toluene) and opiates (morphine, heroin) (Feltenstein & See, 2008). Virtually all abused drugs have a common ability to produce rewarding effects and/or relieve negative emotional states by enhancement of dopaminergic activity (Feltenstein & See, 2008). These drugs exert their actions either by directly influencing the principal DA neurones (either by opening or closing specific ion channels or through second messenger systems) or by acting specifically on secondary neurones that modulates the dopaminergic neurones (Mathon et al., 2003).

Psychostimulants exert its euphoric effects through interactions with DA transporters. While cocaine blocks the activity of DA transporters which reduce the rate of DA reuptake, most amphetamine derivatives increased DA concentration by

reversing the action of transporters at the plasma membrane (Luscher & Ungless, 2006; Pierce & Kumaresan, 2006).

Nicotine is the main psychoactive compound founds in tobacco (Tomkins & Sellers, 2001). Increased DA release in the NAc following nicotine administration mainly results from direct stimulation of nicotinic acetylcholine receptors. Nicotine binds to the nicotinic acetylcholine receptors in the brain, become cation-permeable and depolarise the cell leading to an increase in DA concentration (Luscher & Ungless, 2006). These ionotropic receptors are expressed on GABA and dopaminergic neurones, as well as glutamatergic inputs to DA neurones (Pierce & Kumaresan, 2006).

Δ^9-tetrahydrocannabinol (THC), the active ingredient in cannabis, mainly binds to the type 1 cannabinoid receptors (CB1Rs) (Feltenstein & See, 2008; Luscher & Ungless, 2006). These receptors are highly expressed in several brain regions including the VTA, NAc, cortex, hippocampus and striatum. In the VTA, these receptors are expressed on the GABA neurones and on terminals of glutamatergic synapses on DA neurones (Feltenstein & See, 2008; Luscher & Ungless, 2006). It has been demonstrated that application of THC in acute midbrain slices causes a net disinhibition by decreasing GABA release (Szabo et al., 2002).

Since ethanol has complex pharmacological effects within the brain, a wide variety of neurotransmitter systems have been linked to its reinforcing effects, such as GABA ($GABA_A$ receptors), opioid peptides (δ receptors), glutamate (NMDA receptors), acetylcholine (nicotinic receptors) and serotonin ($5\text{-}HT_3$ receptors)

(Feltenstein & See, 2008; Koob & Le Moal, 1997; Luscher & Ungless, 2006). Ethanol increased the NAc DA release either by direct or indirect modulation of the mesolimbic DA activity (Tomkins & Sellers, 2001).

Morphine exerts its rewarding action primarily by binding to μ-opioid receptors. The direct action of morphine is inhibitory, therefore, the excitatory effects of morphine on VTA dopaminergic neurotransmission occurs via indirect mechanisms (Tsuji et al., 1996). Previous report has indicated that the VTA DA neurones are under tonic inhibition of GABAergic interneurones (Johnson & North, 1992). Anatomical evidence has suggested that μ-opioid receptors are predominantly expressed on GABAergic interneurones of the VTA (Mansour et al., 1988). μ-opioid receptors have dual action, either acting post-synaptically or pre-synaptically (Luscher & Ungless, 2006). μ-opioid receptors expressed on the post-synaptic terminals hyperpolarise GABA neurones mediated by Kir3/G protein-coupled inwardly rectifying K^+ (GIRK) channels coupled to μ-opioid receptors on the soma and the dendrites, which in turn reduces GABA release. Meanwhile, μ-opioid receptors expressed on the presynaptic terminals decreased GABA release by inhibiting calcium (Ca^{2+}) channels or activating voltage-gated K^+ channels. These dual actions of μ-opioid receptors lead to a strong inhibition of GABA neurones which contributes to the disinhibition of DA neurones, resulting in augmented DA release in the NAc (Luscher & Ungless, 2006).

2.1.5 Drug dependence and relapse (reinstatement)

Contradictory to the significant increase in neurotransmitter activity (DA) following acute drug exposure, chronic administration of addictive drugs involves complex

alterations in the NAc activity (Feltenstein & See, 2008). The persistent alterations in stress hormone system, receptor and/or neurotransmitter activity act as compensatory mechanisms for restoring homeostatic functions in response to the drug. These changes could account for the negative emotional states manifested after drug withdrawal, as well as enhanced sensitivity to stressful stimuli, which both could lead to greater vulnerability to relapse (Koob & Le Moal, 1997; Weiss, 2005).

Relapse to drug use after prolonged periods of abstinence is a core feature of drug addiction and becomes the foremost challenge to the drug addiction treatment (Mueller & Stewart, 2000). With the use of reinstatement model in laboratory animals, relapse can be measured when the response to the drug is reinitiated, following the establishment and subsequent extinction of particular behaviour response. Reinstatement can be defined as recovery of a learned response when a subject is exposed to an unconditioned stimulus after extinction. The major precipitating factors for reinstatement include interactions with stimuli previously associated with the drug, exposure to the drug itself, and/or stressors (Aguilar et al., 2009). To date, the development and application of reinstatement model of relapse have contributed towards neurocircuitry mapping essential for maintaining the relapse-like behavior. From previous findings, it appears that the neurocircuits involved in cue-, drug- and stress-induced reinstatement of drug-seeking behavior are distinct, yet overlapping. Evidence has shown the interactions of dorsomedial prefrontal cortex (dmPFC), amygdala and VTA with the NAc core via glutamatergic and dopaminergic pathways in the reinstatement of cue-induced drug-seeking behavior. In another studies, dmPFC glutamatergic projections to the NAc core and dopaminergic innervations of the dmPFC and NAc shell play important roles in

mediating relapse behavior following exposure to the previously administered drug. Increased drug craving and relapse following exposure to stressful stimuli, such as acute foot shock and administration of noradrenergic α_2 receptor antagonist, yohimbine, a drug that exert anxiety-like states in both humans and animals, are suggested to be attributed by CRF and noradrenergic inputs to the central nucleus of amygdala and the lateral bed nucleus of the stria terminalis that serially project to the dmPFC and NAc core. In addition, other brain structures such as dorsal striatum, dorsal hippocampus and NAc shell, may play critical roles in producing drug-seeking behavior, depending upon the nature of the withdrawal history or contextual environment that triggers relapse (Feltenstein & See, 2008).

Functional brain imaging in humans as well as brain lesion and site-specific pharmacological manipulations in animals have acknowledged an interconnected set of cortical and limbic brain regions in associative learning mediating craving and relapse. Among components of this circuitry include the orbitofrontal cortex, anterior cingulate, prelimbic cortex, basolateral amygdala, hippocampus, NAc, and dorsal striatum (Weiss, 2005).

2.1.6 Animal models of addiction

The use of animal models has provided an invaluable means for understanding the neurobiology of addiction and mechanisms of action of abused drugs. Examples of such models include intracranial electrical self-stimulation, drug self-administration paradigms and conditioned place preference.

(a) Intracranial electrical self-stimulation

Intracranial electrical self-stimulation (ICSS) is useful for localizing brain regions that mediate drug reward, as well as elucidating the brain reward mechanisms. In the ICSS procedure, animal is equipped with electrodes implanted into specific brain regions. Specific behavioural response (e.g.; pressing a lever) will be followed by a short-pulse train of electrical current via the electrode. Animal will initiate and maintain lever-pressing for obtaining the stimulus if the electrodes were implanted in brain areas associated with reward pathways (Van Ree et al., 1999).

(b) Drug self-administration

Drug self-administration model has been extensively used in basic and preclinical drug abuse research. It is based on the concept of operant conditioning, in which animals are trained to perform an operant behaviour (e.g.; nose poke or lever press) for a certain stimulus (e.g.; administration of a drug). The drug is said to be self-administered, and serves as a positive reinforcer, when the frequency of the behavioural response is increased. In a controlled laboratory setting, animals self-administer drugs that consumed by human for recreational purposes, including opiates, cannabinoids, alcohol, nicotine, amphetamine and cocaine. Most of the intravenous self-administration studies employ rats and monkeys to assess the reinforcing effects of drugs. However, other species such as dogs, cats, mice and pigeons, have been used. For this method, several routes of drug administration were developed, including intragastric, oral, inhalation, intracerebroventricular, and intracerebral (Van Ree et al., 1999).

(c) Conditioned place preference (CPP)

CPP paradigm, which is a standard preclinical behavioural test, has been widely used to assess the motivational, including rewarding and aversive effects of drugs (Tzschentke, 1998). Generally, addiction can be characterized by a loss of behavioural control over the desired drug due to its unconditioned or rewarding properties. In the absence of drug, the drug-associated stimuli can acquire the abilities to control the behaviours. CPP takes place when a subject prefers an environment (conditioned stimulus) over the other because the preferred environment has been previously associated with pleasurable or rewarding events (unconditioned stimulus). The drug-associated conditioned stimuli are presumed to be responsible for drug craving and relapse. Most drugs of abuse, such as morphine, amphetamine, cocaine, ethanol and nicotine, elicit a CPP in laboratory animals (Tzschentke, 1998). CPP has been demonstrated in various species, including zebrafish, flies, mice, rats, primates and humans (Huston et al, 2013; Mathur et al., 2011). Despite addictive drugs, natural stimuli such as food and sexual activity also produce CPP. In addition, conditioned place aversion (CPA) of a particular drug stimulus can be detected using place conditioning paradigm. CPA refers to the avoidance behaviour of a subject for one place over the other because of the previously associated aversive events (Tzschentke, 1998).

There are numerous methodological approaches reported in performing the CPP based on the route of drug administration (e.g.; intraperitoneal, subcutaneous), the choice (e.g.; mice, rat) and age (e.g.; adolescent, adult) of the model organism, as well as design of the conditioning chamber (e.g.; two chambers, three chambers) and experiments (e.g.; biased, unbiased) (Bardo et al., 1995).