THE ROLE OF MESOLIMBIC SYSTEM AND LATERAL HABENULAR MOLECULAR TARGETS (CB_1, $GluA_1$ AND NK_1 RECEPTORS) IN *Mitragyna speciosa* Korth (KETUM) ADDICTION IN THE MITRAGYNINE-SENSITISED SWISS ALBINO MICE

NURUL IMAN BINTI WAN ISMAIL

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by

NURUL IMAN BINTI WAN ISMAIL

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF SYMBOLS

%	percentage
°C	degree Celcius
μ	mu
Δ	Delta
δ	delta
κ	kappa
γ	gamma
β	beta

LIST OF ABBREVIATIONS

2-AG	2-arachidonylglycerol
7-HMG	7-hydroxymitragynine
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate acid
ARASC	Animal Research and Service Centre
BSA	Bovine Serum Albumin
CA1	Cornu Ammonis area 1 (a region in the hippocampus)
CA3	Cornu Ammonis area 3 (a region in the hippocampus)
cAMP	cyclic adenosine monophosphate
CB_1	cannabinoid type-1
CB_2	cannabinoid type-2
cDNA	complementary deoxyribonucleic acid
CNS	Central nervous system
CPA	Conditioned place aversion
CPP	conditioned place preference
ECB	endocannabinoid
GluA	Glutamate AMPA
$GluA_1$	Glutamate AMPA subunit-1
$GluA_2$	Glutamate AMPA subunit-2
$GluA_3$	Glutamate AMPA subunit-3
$GluA_4$	Glutamate AMPA subunit-4
GR	Glucocorticoid receptor
Hb	Habenula
ID	identification
IPS	Institut Pengajian Siswazah
LHb	Lateral habenula
LTD	long-term depression
LTP	long-term potentiation
MHb	Medial habenula
mPFC	medial prefrontal cortex
NAcc	Nucleus accumbens
NADA	Malaysian National Anti-Drugs Agency

NBF	Neutral Buffered Formalin
NK	Neurokinin
NK_1	Neurokinin type-1
NK_2	Neurokinin type-2
NK_3	Neurokinin type-3
OD	Optical density
PEMADAM	Malaysian Drug Prevention Association
qPCR	quantitative real-time PCR
RFID	Radio-Frequency Identification
RNA	ribonucleic acid
RNases	ribonucleases
SUD	substance use disorder
TBS	Tris Buffer Saline
TBST	Tris Buffer Saline + Tween 20
THC	Δ-9-tetrahydrocannabinol
US FDA	US Food and Drug Administration
USM	Universiti Sains Malaysia
VTA	Ventral tegmental area

PERANAN SASARAN MOLEKULAR DALAM SISTEM MESOLIMBIK DAN LATERAL HABENULA (RESEPTOR CB_1, $GluA_1$ DAN NK_1) UNTUK KETAGIHAN *Mitragyna speciosa* KORTH (KETUM) MELALUI PEMEKAAN MITRAGININA TERHADAP MENCIT ALBINO SWISS

ABSTRAK

Trend penggunaan daun *Mitragyna speciosa* Korth. (pokok ketum), iaitu sejenis tumbuhan ubatan tradisi di Malaysia, sebagai dadah rekreasi dan perubatan alternatif kepada opioid semakin meningkat dan berisiko menyebabkan masalah ketagihan. Mitraginina (alkaloid utama ketum) merupakan agonis reseptor opioid unik yang menghasilkan kesan dwi-dos sebagai perangsang (psikostimulan) dan analgesik (seperti morfin), namun mekanisme yang terlibat masih tidak jelas. Penyelidikan terkini menunjukkan kewujudan pelbagai pertindihan dan interaksi di litar ganjaran otak, termasuk sistem opioid-kanabinoid-glutamat AMPA-neurokinin-dopaminergik. Kajian ini dijalankan bertujuan untuk mengkaji penglibatan reseptor kanabinoid (CB_1), glutamat ($GluA_1$) dan neurokinin (NK_1) di bahagian hipokampus, *ventral tegmental area* (VTA) dan lateral habenula (LHb) sebagai asas dalam penyalahgunaan ketum, melalui interaksi dengan mitraginina. Seratus dua puluh ekor (n=120) mencit albino Swiss jantan dipeka selama 28 hari (kronik) dengan kumpulan kawalan (tidak dirawat dan vehikel Tween 20), morfin sulfat, THC atau mitraginina, sama ada dengan/tanpa kombinasi dengan NIDA-41020 (antagonis reseptor CB_1), PhTx-74 (antagonis reseptor $GluA_1$) atau RP-67580 (antagonis reseptor NK_1). Sistem IntelliCage® telah digunakan sebagai model pemekaan untuk menilai perubahan kognitif dan tingkahlaku ketagihan selepas pendedahan dadah secara kronik. Hasil kajian menunjukkan pendedahan mitraginina kronik (dos peningkatan dari 5 hingga

25 mg/kg) menyebabkan hiperlokomotor ($p < 0.05$), meningkatkan kecenderungan dan kegigihan pengambilan ganjaran semulajadi (10% sukrosa) ($p < 0.01$), ketahanan terhadap rangsangan aversif ($p < 0.05$) dan menjejaskan pembelajaran dan memori spatial ($p < 0.05$), setanding dengan kumpulan tikus yang dipeka oleh morfin dan THC ($p > 0.05$). Gangguan proses pembelajaran dan memori yang dihasilkan oleh pendedahan kronik mitraginina, morfin dan THC tersebut didapati mampu dikurangkan oleh NIDA-41020 ($p < 0.05$), PhTx-74 ($p < 0.05$) dan RP-67580 ($p < 0.05$), seterusnya mencadangkan peranan putatif reseptor-reseptor CB_1, $GluA_1$ dan NK_1 dalam mekanisme tindakan bahan-bahan tersebut. Manakala proses neuroadaptasi dalam sistem mesolimbik otak mencit, yang melibatkan reseptor CB_1, $GluA_1$ and NK_1, telah dikaji melalui kaedah immunohistokimia, *Western Blot* dan *quantitative real-time PCR* (qPCR). Kumpulan mitraginina menunjukkan peningkatan ekspresi reseptor dan gen CB_1 di kawasan CA1 hipokampus ($p < 0.001$) dan VTA ($p < 0.001$). Ekspresi reseptor dan gen $GluA_1$ turut meningkat di kawasan CA1 hipokampus ($p < 0.001$), manakala ekspresi NK_1 meningkat di LHb ($p < 0.05$). Peningkatan ekspresi reseptor dan gen tersebut adalah menyerupai seperti yang dihasilkan oleh pendedahan kronik morfin ($p > 0.05$). Perubahan-perubahan neuron tersebut didapati tidak berlaku dalam kumpulan mitraginina dan morfin yang dipasangkan bersama reseptor-reseptor antagonis NIDA-41020, PhTx-74 dan RP-67580, seterusnya memberikan petunjuk afirmatif kepada dapatan awal dari perubahan tingkahlaku mencit. Secara kesimpulan, hasil kajian kami menunjukkan kemungkinan penglibatan bersama reseptor CB_1, $GluA_1$ dan NK_1 dalam proses ketagihan ketum/mitraginina, seterusnya menghasilkan perubahan tingkahlaku dan proses neuroadaptasi di litar ganjaran otak. Namun begitu, sejauh mana tahap interaksi reseptor-reseptor ini dalam penyalahgunaan ketum masih tidak diketahui. Hasil kajian

ini juga memberikan bukti awal yang melibatkan sasaran molekular yang tidak diketahui sebelumnya (iaitu sistem kanabinoid-glutamat AMPA-neurokinin) dalam pemahaman konsep dan potensi penyalahgunaan ketum/mitraginina secara kronik. Selain itu, hasil kajian juga dapat memberikan maklumat baru mengenai potensi fitoperubatan yang berkaitan dengan pokok ketum.

THE ROLE OF MESOLIMBIC SYSTEM AND LATERAL HABENULAR MOLECULAR TARGETS (CB_1, $GluA_1$ AND NK_1 RECEPTORS) IN *Mitragyna speciosa* KORTH (KETUM) ADDICTION IN THE MITRAGYNINE-SENSITISED SWISS ALBINO MICE

ABSTRACT

There is a growing trend to use the leaves of Malaysian folklore medicinal plant *Mitragyna speciosa* Korth. (or ketum) for recreational high and as a self-medication alternative to traditional opiates, hence subjecting it to addictive liabilities. Mitragynine (ketum major alkaloid) is an atypical opioid agonist exhibiting complex psychostimulant and morphine-like analgesic effects, although the exact mechanisms remain unclear. In recent years, studies demonstrated a wide array of overlapping and integrated neuronal circuits in addiction, including the opioid-cannabinoid-glutamate AMPA-neurokinin-dopaminergic systems. This study aimed to demonstrate the involvement of the cannabinoid (CB_1), glutamate ($GluA_1$) and neurokinin (NK_1) receptors in the hippocampus, ventral tegmental area (VTA) and lateral habenula (LHb) brain regions as the neurobiological bases of ketum abuse potential through its interaction with mitragynine. One hundred and twenty (n=120) male Swiss albino mice were subjected to 28-days (chronic) regimen with untreated and Tween-20 vehicle control, morphine sulphate, THC or mitragynine, either with/without co-administration with CB_1, $GluA_1$ or NK_1 receptor antagonists (i.e. NIDA-41020, PhTx-74 or RP-67580, respectively). The IntelliCage® system was used as the behavioural sensitisation setting to assess mice cognitive performances and addiction-like behaviours following chronic drug treatment. Findings revealed that chronic mitragynine exposure (incremental doses of 5 to 25 mg/kg) resulted in

hyperlocomotion ($p < 0.05$), potentiated preference and persistence for natural reward (i.e. 10% sucrose) ($p < 0.01$), resistance to punishment ($p < 0.05$), and spatial learning memory deficit ($p < 0.05$), comparable to those observed in morphine- and THC-sensitised mice ($p > 0.05$). The mitragynine-, morphine- and THC-induced spatial learning and memory impairments were attenuated by NIDA-41020 ($p < 0.05$), PhTx-74 ($p < 0.05$) and RP-67580 ($p < 0.05$), suggesting the CB_1, $GluA_1$ and NK_1 receptors putative role in the drugs' mechanism of actions. The underlying adaptations in mice key brain mesolimbic areas, with regards to CB_1, $GluA_1$ and NK_1 receptors, were investigated using immunohistochemistry, Western Blot and quantitative real-time PCR (qPCR) studies. Mitragynine-sensitised mice demonstrated enhanced CB_1 receptor proteins and genes expression at hippocampus CA1 ($p < 0.001$) and VTA regions ($p < 0.001$). $GluA_1$ receptor proteins and genes were also up-regulated at hippocampus CA1 regions ($p < 0.001$), whereas NK_1 were up-regulated at the LHb ($p < 0.05$). These mitragynine-induced receptor up-regulations resembled those observed with chronic morphine ($p > 0.05$). Neuronal changes as seen in mitragynine- and morphine-sensitised mice appeared to be absent in drug paired with respective receptor antagonist groups, thus providing affirmative clues to the behavioural changes observed. Taken together, these findings demonstrate the seeming integrated role of brain CB_1, $GluA_1$ and NK_1 receptors in mitragynine/ketum addictive liabilities, leading to behavioural and probable adaptive changes in the brain mesolimbic reward pathway. However, the extent and nature of these receptor interactions in ketum misuse remain unclear. The study findings lend the first correlative relationship that implicates drug molecular targets not previously known (i.e. cannabinoid-glutamate AMPA-neurokinin systems) in relation to chronic mitragynine misuse. This may also

provide new insight to inform the phytomedicinal potentials that are linked to this plant.

CHAPTER 1

INTRODUCTION

1.1 Background

Substance abuse and its consequent addiction pose global major public health concerns to that of epidemic proportions. Malaysia is one of the many countries in the world not spared of the addiction menace. In an attempt to tackle the prevalent substance-related mental health issue in the society, Malaysia has declared substance abuse as the 'Nation's No. 1 Enemy' since 1983 – an effort which has attained a varying degree of success with continuing challenges as it remains an enemy to present day.

The Malaysian National Anti-Drugs Agency (NADA) identified a total of 149,322 substance users/addicts in Malaysia from the year 2014 to 2018 alone; this equals to proximately 404 users for every 100,000 individuals. Of this figure, a mere 12.4% (18,534 users) received treatment and rehabilitation through NADA in 2018. Illegal substance use in Malaysia reached a peak in the year 2016, with a total of 30,844 reported new users, a substantial increase of 13.5% compared to 2015 (26,668 users) (NADA, 2018). Aimed at reducing the extent of illegal substance use in this country, NADA had initiated endless transformational efforts, including educational awareness programs, Cure & Care Clinics and Rehabilitation Centres, legal intervention, and community-based supervision (NADA, 2018).

Consequently, illegal substance use among Malaysians had declined from the 2016 peak by 25,922 users in 2017, a modest 5.3% decrement. Moreover, illicit substance use continues to present a fairly stable annual reduction from the year 2017 to 2019. The latest figures available indicate only a 2.5% decline of substance users in 2018 (25,267 users) (NADA, 2018). Despite these accomplishments, at this point, Malaysia's vision of becoming a 'Drug-free Nation' remains a tough challenge to achieve.

In recent years, emerging new psychoactive and recreational substances further impose public health implications in Malaysia. NADA (2018) has declared a sudden rise in the raid and seizures of new synthetic substances, such as ecstasy and psychotropic pills, as well as a local psychoactive plant, namely ketum. According to the drug report, nearly 233,600 litres of ketum drinks and 87,600 kg of ketum leaves were seized by the local law enforcement agents in 2018 alone. Currently, raw ketum leaves are being sold at RM30 to 40 per kg in local market and RM80 to 100 per kg in Thailand market, while processed ketum drinks are being sold at RM15 per litre (The Star, 11 June 2019; NADA, 2018). Oftentimes, ketum drink concoctions (locally known as 'cakoroi' or 'koroi') are adulterated with insect repellent, isotonic drink and cough medicine, for more mind-altering and luring effects.

Ketum has been reported to produce a unique, yet contradictory, combination of psychostimulant (at low doses) and opioid-like depressant effects (at high doses), which possibly render its abuse liability and addictive potentials (Suwanlert, 1975; Ahmad and Aziz, 2012; Saingam *et al.*, 2013; Iman *et al.*, 2017). Mitragynine is the main active alkaloid isolated from ketum leaves and accounts for the plant's opioid-like properties (including antinociceptive, anaesthetic, antitussive, analgesic and narcotic effects)

which are primarily mediated by the opioid receptors (Takayama, 2004; Matsumoto *et al.*, 2006; Hassan *et al.*, 2013). Nonetheless, the exact molecular and cellular targets in the brain for ketum/mitragynine addiction remain unknown, and may partly relate to its opioid cross-dependence and agonistic properties.

To date, the trend of recreational use of ketum suggests the probable stimulation of brain reward circuitry, comparable to that of the cannabinoids and opioids. In this context, the dopaminergic mesolimbic system [including hippocampus, ventral tegmental area (VTA), amygdala, and nucleus accumbens] has been implicated as the crucial molecular and cellular targets for substance-induced synaptic plasticity, that may contribute to the transition from a pattern of recreational substance-taking to the development of an addictive pattern of behaviour (Koob and Volkow, 2010; Koob and Volkow, 2016; Uhl *et al.*, 2019). Recently, the lateral habenula (LHb), a small structure located within brain reward circuit, has emerged as a novel target for addiction via its glutamatergic and GABAergic neuronal projections to the VTA to modulate reward-related processes of morphine and cocaine (Wang *et al.*, 2017; Zapata *et al.*, 2017). The synergistic involvement of the classical mesolimbic system and novel LHb in reward and addiction, motivation, learning and pain processing, makes both structures as the ideal target for understanding the effects of substances of abuse on the brain, as observed with the use of ketum.

Recent evidence has attested the reciprocal interaction between opioid and endocannabinoid (ECB) systems in reward-processing and addiction, and the underlying biochemical and molecular mechanisms in brain reward circuitry (Justinova *et al.*, 2009; Scavone *et al.*, 2013). Data pertaining to the ECB system role in modulating brain reward function of various addictive substances, and the ensuing aberrant synaptic

plasticity, are supported by studies with CB_1 receptor antagonists and gene deletion, thus implicating wider potential roles of this receptor in addiction disorders (Parsons and Hurd, 2015; Manzanares *et al.*, 2018). Recent studies from our laboratory indicated the first evidence to the mitragynine-CB_1 receptor interaction in the modulation of ketum abuse potential from mice sensitisation model (Nanthini *et al.*, 2015; Iman *et al.*, 2017), however, further investigation is necessary to determine the extent of its involvement, and/or the synergistic involvement of other targets that may exist within the brain reward circuitry.

The mesocorticolimbic dopaminergic system is recognised to intricately interact with the glutamatergic AMPA (GluA) system (Chartoff and Connery, 2014; Huijstee and Mansvelder, 2014). GluA has a prominent role in modulating dopaminergic release and long-lasting neuroadaptations in the mesocorticostriatal circuitry that represents the putative neural substrate of enduring vulnerability to relapse through the interaction of $GluA_1$ receptors involving various classes of abused substances (Xia *et al.*, 2011; White *et al.*, 2016). In addition, the Neurokinin-1 (NK_1) receptor system represents a novel class of signalling molecules that contribute to short- and long-term synaptic plasticity throughout the brain as evident in studies involving opiates and nicotine (Ripley *et al.*, 2002; Dao *et al.*, 2014; Bowman *et al.*, 2015). Both $GluA_1$ and NK_1 receptors are widely expressed in the brain reward circuits and LHb (Mantyh, 2002; Huijstee and Mansvelder, 2014), thereby suggesting their putative synergistic interaction with opioid and ECB systems in the modulation of the brain reward function. However, to date, data on the potential interaction between mitragynine with $GluA_1$ and NK_1 receptor systems within the central nervous system is currently nonexistent.

1.2 Problem statement

The relevance of identifying and understanding the potential threats from emerging new psychoactive substances is widely recognised worldwide owing to the substantial public health and policy implications. Ketum, once a region-confined herbal product native of South East Asia, has now widely marketed as a psychotropic dietary supplement worldwide, primarily on users' interests on its opioid-like anti-nociceptive properties. In Malaysia, the growing concerns over ketum use and misuse have prompted the selling and possession of ketum leaves to be classified under the Poisons Act of 1952, where those found guilty will be imposed with a maximum imprisonment of four years, or RM10,000 fine, or both. Albeit this restriction, recreational use and misuse of ketum remain widespread, particularly in the Northern states of Malaysia, as well as illegal smuggles across borders of Thailand, Singapore and Indonesia.

Recently, the Malaysian government, with advise from the NADA and Malaysian Drug Prevention Association (PEMADAM), has proposed a bill in the parliament to reschedule ketum leaves under Dangerous Drugs Act of 1952 with more severe sentences, and to initiate an Islamic Ruling (Fatwa) against ketum use. At present, Kedah, Perlis and Kuala Lumpur are the only states in Malaysia, and likely the first among Muslim nations to have proclaimed an Islamic Ruling against ketum leaves (Majlis Jawatankuasa Fatwa Negeri Kedah, 2005; Jabatan Mufti Wilayah Persekutuan, 2016). Much of these developments owed to the growing concerns of ketum misuse and abuse in the country in specific, and substance addiction in general. Nonetheless, the bill is currently placed on hold by the parliament as a group of policymakers remained unconvinced on hard scientific evidence that ketum poses enough threat which merits its inclusion in the Dangerous Drugs Act, 1952.

In addition, ketum is currently banned in Thailand, Myanmar, Bhutan and Australia, as well as several European Union states including Denmark, Finland, Romania and Sweden. Despite cautioning alerts imposed by the US Food and Drug Administration (FDA), ketum is still largely considered as a "safe legal-high" and remain unregulated in the USA and UK (Hassan *et al.*, 2013; Cinosi *et al.*, 2015; Singh *et al.*, 2017). Collectively, at present, ketum and mitragynine still lingers outside the control of the international controlled substance, and is widely accessible on the internet, reflecting its sustained and growing demand. This apparent variable regulatory stance is underpinned by the current lack of understanding of the exact molecular and cellular candidate targets in the brain for ketum addiction. Hence, further advances in our understanding of ketum abuse potential and addiction biology are critical if they are to be adopted to guide law and policy-makers.

1.3 Hypothesis and rationale of the study

Increasing evidences have elucidated the potent opioid agonistic properties of mitragynine, as well as the existence of anatomical and functional overlap between opioid, endocannabinoid, glutamate AMPA and neurokinin system within the brain reward circuitry. In general, the central hypothesis guiding this study is that ketum/mitragynine addiction may involve CB_1, $GluA_1$ and NK_1 receptors in the brain mesolimbic dopaminergic pathway and LHb. If this hypothesis is substantiated, it raises plausible new link on ECB, GluA and NK systems in ketum misuse, and could offer extended targets for future development of fundamentally novel treatments to tackle ketum addiction. At present, the discovery of the opioid-ECB-GluA-NK-dopaminergic systems synergistic interaction holds the promise of providing potentially important novel insights into the underlying mechanism of ketum addiction, thereby serve to

inform towards a uniform global stance to the current contentious debates on ketum use and misuse liability among human users worldwide.

1.4 Objective

1.4.1 General objective

This rodent-based study was designed to demonstrate the involvement of CB_1, $GluA_1$ and NK_1 receptor of the mesolimbic system and lateral habenula as the bases of ketum abuse potential through its interaction with ketum dominant alkaloid, mitragynine.

1.4.2 Specific objectives

Objective 1. To establish behavioural and locomotor alterations in the IntelliCage® system using mitragynine sensitisation model in Swiss albino mice.

Objective 2. To determine the immunohistochemical distribution and Western Blot detection of CB_1 receptors within the different regions of the mesolimbic system of mitragynine-sensitised Swiss albino mice with and without the co-administration of CB_1 receptor antagonist.

Objective 3. To determine the immunohistochemical distribution and Western Blot detection of $GluA_1$ receptors within the hippocampus of mitragynine-sensitised Swiss albino mice with and without the co-administration of $GluA_1$ receptor antagonist.

Objective 4. To determine the immunohistochemical distribution and Western Blot detection of NK_1 receptors within the lateral habenula of mitragynine-sensitised Swiss albino mice with and without the co-administration of NK_1 receptor antagonist.

Objective 5. To establish the levels of CB_1 receptor gene expressions using quantitative real-time polymerase chain reaction (qPCR) within the mesolimbic system of mitragynine-sensitised Swiss albino mice with and without the co-administration of CB_1 receptor antagonist.

Objective 6. To establish the levels of $GluA_1$ receptor gene expressions using quantitative real-time polymerase chain reaction (qPCR) within the hippocampus of mitragynine-sensitised Swiss albino mice with and without the co-administration of $GluA_1$ receptor antagonist.

Objective 7. To establish the levels of NK_1 receptor gene expressions using quantitative real-time polymerase chain reaction (qPCR) within the lateral habenula of mitragynine-sensitised Swiss albino mice with and without the co-administration of NK_1 receptor antagonist.

CHAPTER 2

LITERATURE REVIEW

This chapter attempts to review the relevant literature related to opioid, ECB, GluA and NK systems co-interactions within brain reward circuitry as the neurological bases of ketum/mitragynine abuse potential. The chapter begins with defining some common terminologies in addiction, followed by the classical addiction neurocircuitry and plasticity theories. This chapter also discuss on the animal behavioural model of addiction including the novel IntelliCage® automated learning system. Next, this chapter discusses the ECB, GluA and NK system in the brain mesolimbic system pertaining to addiction. This is followed by details on ketum plant use and misuse, and the neuropsychopharmacology of mitragynine. Finally, a brief overview is provided on the hypothetical co-interactions between brain CB_1, $GluA_1$ and NK_1 receptor systems in relation to ketum/mitragynine abuse liability.

2.1 The neurobiology of addiction

2.1.1 Introduction to addiction

Drug addiction, or recently termed as substance use disorder (SUD), is a behavioural pattern of substance use, characterised by compulsive and at times, uncontrollable substance-seeking and use, which persists even in the face of negative consequences (Koob and Volkow, 2016; Uhl *et al.*, 2019). Different classes of addictive substances share common neurobiological underpinnings, despite their diverse pharmacological profiles and resultant side-effects. Advances in current understanding of the neurobiological mechanism of addiction have been derived from extensive studies in human and animal models of addiction on widely popular substances of

abuse, such as opioids, cannabinoids, stimulants, nicotine, and alcohol (Goldberg and Mitchell, 2018; Uhl *et al.*, 2019).

Despite their harmful reality, these substances are initially sought for their ability to produce rewarding and pleasurable effects (i.e. positive reinforcement), or to temporarily relieve negative feelings (i.e. negative reinforcement), which increase the probability of pursuing substance intake. Repetitive substance-taking will induce changes in the central nervous system (CNS), thus leading to tolerance (i.e. diminished reinforcing effects of substances with repeated use) and dependence (i.e. adaptive changes which develops from constant substance exposure). Further increment in substance dosage and frequency of substance-taking, for its reinforcing effects, will exacerbate neurophysiological alteration causing long-term synaptic plasticity (Koob and Volkow, 2016; Ramirez and Arbuckle, 2016; Uhl *et al.*, 2019). As an individual falls deeper into addiction, distressing withdrawal syndromes will occur with any attempt to abstain from the abused substance. As a mean to avoid withdrawal symptoms, addicts will maintain to use the substance. The inability to reduce and control substance use increases the incidence of relapse even after years of abstinence (Koob and Volkow, 2010; Goldberg and Mitchell, 2018; Uhl *et al.*, 2019). Increasing evidences suggest the disruption of neuronal networks in the brain reward pathway accounts for the development and persistence of addictive behaviours (Koob and Volkow, 2016; Ramirez and Arbuckle, 2016; Uhl *et al.*, 2019).

2.1.2 Brain reward pathway and mesolimbic system

The brain reward pathway plays a major role in the motivational system regulating responses to biologically essential natural rewards, such as food, music, sex, maternal/paternal behaviours and social interactions. This circuitry primarily involved the mesocorticolimbic dopaminergic system (see Figure 2.1), which governs cognitive and emotional brain functions. The neurotransmitter dopamine controls brain's reward and pleasure centre, thus critical in the rewarding effects of biologically essential activities and substance rewards, as well as the consequent development of addiction (Koob and Volkow, 2010; Uhl *et al.*, 2019). The mesolimbic pathway runs from the ventral tegmental area (VTA) of the midbrain through the mesolimbic system of the temporal lobe, which are the hippocampus, amygdala, nucleus accumbens (NAcc) and lateral habenula (LHb), as depicted in Figure 2.2 (Russo and Nestler, 2013; Uhl *et al.*, 2019). In response to reward-related stimuli, VTA releases dopamine to NAcc, as well as to associated cortical and limbic structures. Activation of this pathway motivates individuals to continuously engaged in these rewarding activities (enabling habit formation) by linking it with the pleasurable effects associated with the natural (or substance) rewards, thereby serving for species survival with natural rewards (Sjoerds *et al.*, 2014; Torregrossa and Taylor, 2016), or for addiction with substances of abuse (Koob and Volkow, 2016; Uhl *et al.*, 2019).

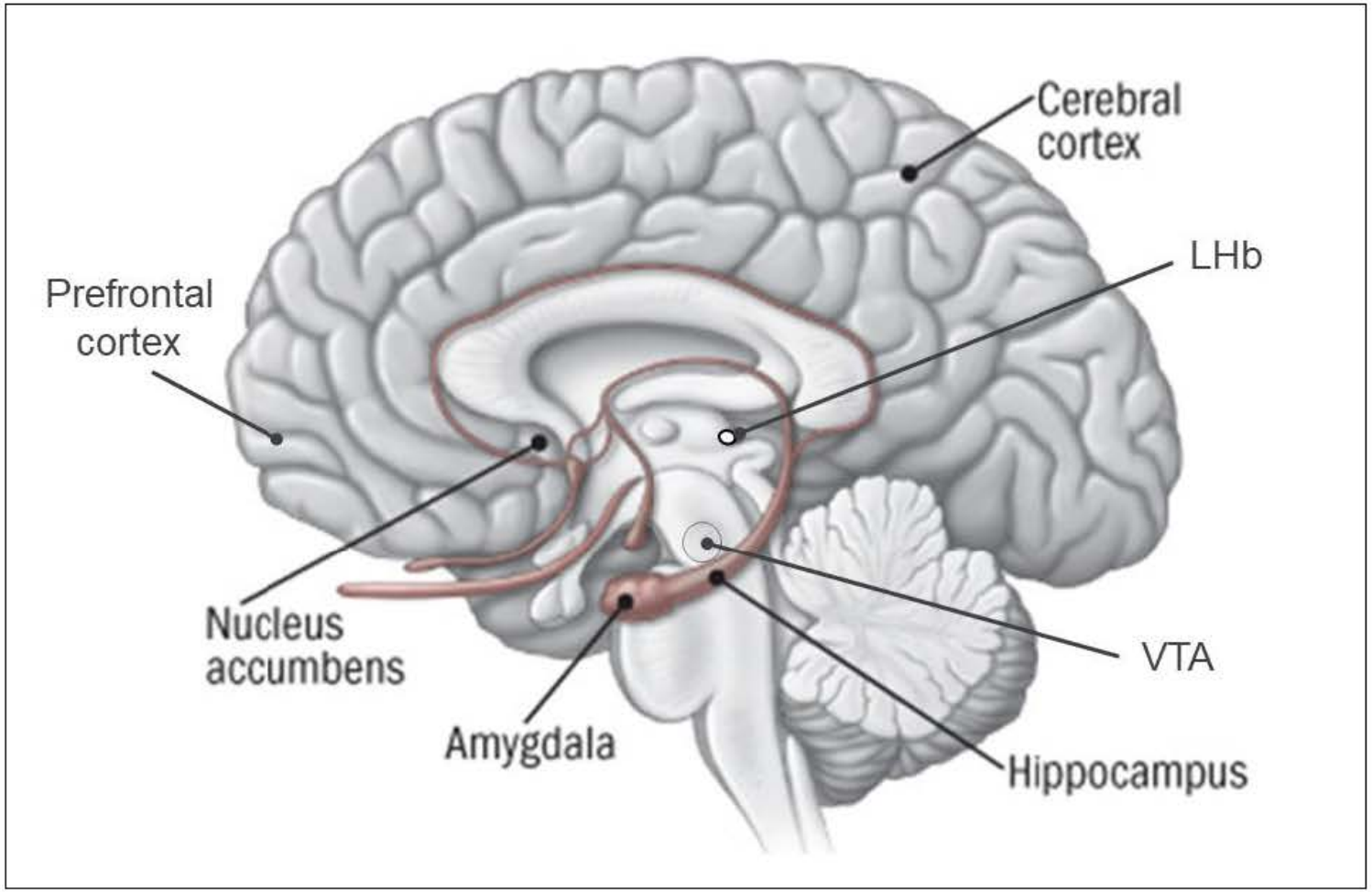

Figure 2.1 The key regions in the human brain mesocorticolimbic system involved in reward circuitry (adapted from Recovery Research Institute, 2019). VTA=Ventral tegmental area; LHb=lateral habenula

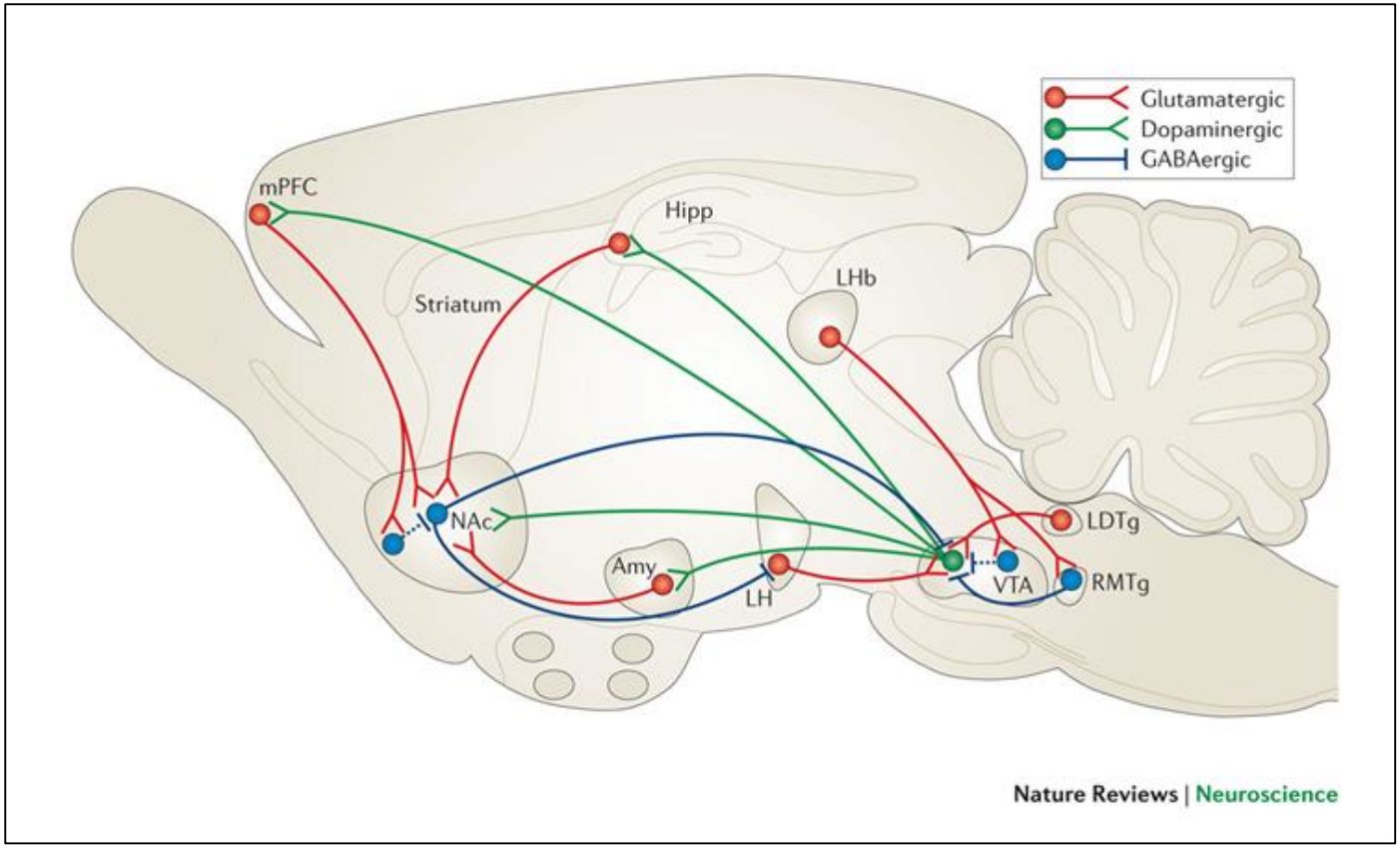

Figure 2.2 Illustration shows the major dopaminergic, GABAergic and glutamatergic connections in the mouse brain reward circuitry. The primary reward circuit includes dopaminergic projections from the ventral tegmental area to the nucleus accumbens and other mesocorticolimbic structures, upon stimulation by reward-related stimuli and addictive substances (from Russo and Nestler, 2013)
mPFC=medial prefrontal cortex; Hipp=hippocampus; NAcc=nucleus accumbens; Amy=amygdala; LH=lateral hypothalamus; VTA=ventral tegmental area; LHb=lateral habenula; LDTg=laterodorsal tegmental area

There are substantial evidences suggesting that all substances of abuse, directly or indirectly, target the brain's reward system by exaggerating release of dopamine in the circuit (Koob and Volkow, 2010; Uhl *et al.*, 2019). The amplified firing of dopaminergic cells projecting from VTA to NAcc and other associated structures, encode the substance's primary reinforcing effects, thus facilitates conditioned learning and memory mechanisms associated with the substance, involving the hippocampus, amygdala, and medial prefrontal cortex (mPFC). Human brain imaging and animal studies reported the reinstatement of substance-seeking behaviours were elicited by dopamine agonist and reversed through blockage by dopamine antagonist (Koob and Volkow, 2010; Uhl *et al.*, 2019). Concurrent phasic release of other neurotransmitters, such as opioid peptides, γ-aminobutyric acid (GABA) and endocannabinoids upon primary substance-taking, further aggravates NAcc stimulation (Koob and Volkow, 2010; Uhl *et al.*, 2019).

However, dopaminergic neurons merely fire in response to novel rewards. With continuous procurement of addictive substances, the discharge of dopamine declines, which explains the need for dosage and frequency increment in substance-taking to regain pleasure (Koob and Volkow, 2010; Sjoerds *et al.*, 2014; Uhl *et al.*, 2019). Therefore, while the requirements for increased dopamine levels in the reward pathway may describe the acute reinforcing effects of various substances of abuse, it does not fully account for the long-lasting craving and relapse, which are the other hallmarks of SUD. In this context, it is crucial to relate our current understanding on the role of synaptic plasticity mechanisms in addiction.

2.1.3 Synaptic plasticity

It has been a general consensus that the storage of information in the brain results from synaptic plasticity, which is the alterations in synaptic connections between neurons. In 1973, the discovery of long-term potentiation (LTP) at the excitatory synapses in the hippocampus, which is the key brain region for declarative memory formation, initiated further exploration on the molecular and behavioural basis of neuroplasticity (Bliss and Gardner-Medwin, 1973). The transition from substance-taking to substance-craving is widely regarded as a pathological form of learning and memory. Different classes of addictive substances, with distinct molecular targets and behavioural traits, have been shown to hijack, thus fine-tunes neural circuits in the brain reward pathway (Mameli and Luscher, 2011; Ramirez and Arbuckle, 2016; Oliver and Perrone-Bizzozero, 2017).

Studies demonstrated that the blockade of N-methyl-D-aspartate (NMDA) receptor, which is predominant for neuroplasticity and memory formation, within VTA, thwarted rodents' behavioural sensitisation and conditioned place preference (CPP) of addictive substances (Tomazi *et al.*, 2017; Galaj *et al.*, 2018). The synapses between dentate granule cells and the CA3 pyramidal region of the hippocampus have shown a distinct form of LTP upon acute drugs exposure (Nicoll and Schmitz, 2005). Furthermore, the application of cocaine, nicotine, amphetamine, morphine and ethanol had been shown to cause elevation of excitatory postsynaptic currents in VTA brain slices recording. These effects were significantly reversed when the VTA slices were pre-treated with NMDA receptor antagonist (Mameli and Luscher, 2011; Oliver and Perrone-Bizzozero, 2017). Furthermore, results from *in vivo* studies have shown that the administration of addictive substances to brain slices elicit LTP, as well as blocked

long-term depression (LTD) at the same sites, thus aggravating its potentiation effects (Oliver and Perrone-Bizzozero, 2017).

Another study reported that the exposure to cocaine on mutant mice, with a deficiency in any form of synaptic plasticity, did not increase excitatory synaptic currents in VTA. The mutant mice also failed to show CPP to cocaine when compared to the wild-type mice (Oliver and Perrone-Bizzozero, 2017). Collectively, these results provide strong evidence that synaptic plasticity at the brain reward pathway is vital to associate learning and memory formation with substance-taking experiences.

2.1.4 Substance-craving and relapse

Relapse to substance use, even after prolonged periods of abstinence, is the core limitation in the treatment of addiction. Substance-craving and relapse are often triggered by acute substance re-exposure, substance-associated cues, or stress (Koob and Volkow, 2010; Uhl *et al.*, 2019). Extensive evidence implicates amygdala in the acquisition, storage and emotional memories. With repeated substance use, memories of reward experiences, as well as associated substance cues (such as people, place, mood and drug paraphernalia) are embedded in brain reward circuitry, particularly in the amygdala and dorsal striatum (Dong *et al.*, 2017; Uhl *et al.*, 2019). As a result, exposure to substance-associated cues and stimuli may initiate spontaneous dopamine phasic firing, thus serve as triggers to substance-craving, compulsive substance use, and relapse (Ramirez and Arbuckle, 2016; Dong *et al.*, 2017; Uhl *et al.*, 2019).

Studies have shown that animals pre-trained to associate a particular surrounding with drug administration, shows high tendency and preference to return to that surrounding to secure drug. Removal and/or lesions in amygdala results in the rodents' failure to show preference for drug-associated cues and contexts (Marchant *et al.*, 2013; Dong *et al.*, 2017). Additionally, synaptic plasticity and functional decrease of central amygdala system had been shown to result in heightened corticotropin-releasing factor (CRF) dependent signalling, thus causing anxiety and stress response (Ramirez and Arbuckle, 2016; Uhl *et al.*, 2019). Animals studies show significant and persistence degrees of drug reinstatement following foot-shock, which acts as a stressor. This occurrence is reversed by the administration of CRF-antagonist (Ramirez and Arbuckle, 2016; Uhl *et al.*, 2019).

2.1.5 Behavioural sensitisation model

Animal studies using laboratory rodents are widely acknowledged to be useful for defining current understanding of the neurobiology of SUD, notably the dopaminergic mesocorticolimbic circuitry, and the neuropharmacological aspects of substances of abuse (Justinova *et al.*, 2009; Venniro *et al.*, 2016; Müller, 2018). Whilst animal models may not fully reflect and reproduce the complex human experience, they nevertheless provide means for researchers to conduct SUD research under highly-controlled conditions that may not be possible or ethical to replicate in humans. Earlier animal models of SUD emphasised on the mechanisms of acute reward, but current research has shifted to include consequent neuroadaptations in long-term or chronic substance use paradigms.

The behavioural sensitisation model involves a progressive increase in the motor stimulatory effects of a drug following repeated intermittent administration. The development of behavioural sensitisation has been hypothesised to represent a transition from drug 'liking' to 'wanting' that underlies compulsive substance use as demonstrated with chronic treatment of morphine, cocaine, amphetamine, ethanol, nicotine and cannabinoids (Venniro *et al.*, 2016; Müller, 2018). Literature indicates that Swiss albino mice have been widely used to model addiction study. Behavioural sensitisation challenge with numerous psychoactive substances including morphine (Cheaha *et al.*, 2017), alcohol (Mitra and Nagaraja, 2020), amphetamine (Ridzwan *et al.*, 2017), cannabinoids, mitragynine (Iman *et al.*, 2017), and ecstasy (Ebrahimian *et al.*, 2017) in Swiss albino mice demonstrated the hyper-activation of brain reward circuitry, thus resulting in distinctive behavioural and neurological alterations that are highly relevant and reproducible for addiction studies.

To date, the CPP box is one of the most widely-used testing protocol for rewarding behaviours in rodents. It generally consists of two or three standard experimental chambers for screening the reinforcing properties of psychoactive substances (or natural stimuli), as well as for investigating the neurobiological systems implicated in reward and addiction. A more specialised CPP box (i.e. the Shuttlebox system) is also equipped with foot-shock floor as an aversive stimulus, thus enabling active/passive avoidance and fear-conditioning memory studies in substance-dependent rodents. Assessment of general exploration and motor coordination can be performed with open-field and rotarod platforms, respectively. Additionally, the effects of substance dependence on rodent's cognition and learning and memory performances are commonly tested using the Morris water maze, T maze, radial maze, or novel object/location recognition tasks (Spanagel, 2017; Müller, 2018; Kuhn *et al.*, 2019).

2.1.5(a) IntelliCage® as a novel system for cognitive assessment of group-housed mice

In the field of cognitive neuroscience, there is a growing need for robust testing protocols to evaluate the cognitive and behavioural performance of model organisms, including rodents. Substantial evidence has documented and recognised the practicality and effectiveness of IntelliCage®, a fully-automated novel system for short-term and/or long-term cognitive assessment of group-housed mice. To date, many new parameters to analyse mice social behaviours and cognitive abilities have been tested and designed in the IntelliCage® system. One of the early works by Galsworthy *et al.* (2005) explored simple exploratory behaviours, circadian patterns and learning paradigms between two sympatric wild-caught rodents (i.e. wood mice and bank voles). This study acknowledged the IntelliCage® system as a novel and valuable behavioural testing module for both wild and laboratory rodents, as well as for inter-species comparison. In 2006, Knapska *et al.* tested the system for place preference (by the acquisition of sweetened water at a specific corner) and avoidance (by avoiding corner associated with air-puff) tasks (Knapska *et al.*, 2006). A study by Onishchenko *et al.* (2007) demonstrated that IntelliCage® system is more sensitive in the detection of behavioural alterations and learning paradigms in comparison to Morris water maze, rotarod test and forced swimming test. It was assumed that the absence of mice social deprivation and minimal human interference may be the contributing factor to the sensitivity of behavioural assessment.

In the last decade, increasingly more sophisticated and specialised IntelliCage® protocols were employed to characterise mouse models for Huntington's disease (Rudenko *et al.*, 2009; Balci *et al.*, 2013), Alzheimer disease (Codita *et al.*, 2010; Ryan *et al.*, 2013), Down syndrome (Faizi *et al.*, 2011), and autism (Puścian *et al.*, 2014;

Mitjans *et al.*, 2017). In addition, a modified and adjusted prototype of IntelliCage® for rats has recently been tested and validated with transgenic Huntington's disease rats (Urbach *et al.*, 2014).

2.1.5(b) IntelliCage® for animal model of addiction

Thus far, a growing body of literature has attested the practical importance of IntelliCage® system in addiction-related mouse model, primarily in alcoholism research. Each IntelliCage® corner chamber permits voluntary oral consumption of liquid reward (via nosepoke) which is useful for self-administration paradigms, as well as the application of operant and Pavlovian conditioning tasks for studying the rewarding properties of various substances of abuse.

In 2012, Radwanska and Kaczmarek designed the first longitudinal study of animal models of addiction using extensive IntelliCage® parameters in BALB/cJ and C57BL/6 male mice. The study successfully elucidated the behavioural traits associated with alcohol addiction, such as novelty-seeking, impulsivity, anxiety, motivation and persistence for natural reward, withdrawal and relapse in mice for a span of 128 days (Radwanska and Kaczmarek, 2012). Parkitna *et al.* (2013) later adapted their alcohol abuse model for the assessment of ethanol self-administration, abstinence and circadian pattern on chronic ethanol consumption. Ensuing published studies utilised the IntelliCage® paradigms to develop alcohol addiction models using intermittent-access schedule (Smutek *et al.*, 2014; Koskela *et al.*, 2018), delay-discounting impulsivity (Szumiec and Parkitna, 2016), alcohol-seeking (Stefaniuk *et al.*, 2017), alcohol deprivation-induced effect (Thomsen *et al.*, 2017) and cue-induced conditioning procedures (Koskela *et al.*, 2018).

Additionally, IntelliCage® has been used for oral morphine self-administration (0.1 – 0.5 mg/ml) in a progressive ratio nosepokes, with co-administration of dexamethasone [a selective glucocorticoid receptor (GR) agonist], and conditioned place preference paradigms to evaluate GR effects on the rewarding properties of morphine in mice (Marut *et al.*, 2017). Overall, considering the flexible task design and longitudinal monitoring in social cage environment, the IntelliCage® system indicates invaluable and promising abilities to be a novel model for short-term and long-term addiction study for other substances of abuse. Therefore, based on this knowledge, our laboratory had previously successfully designed a new model, which was an adaptation from Radwanska and Kaczmarek (2012), for the study of extended behavioural and cognitive effects of socially-interacting Swiss albino mice chronically exposed to the widely-abused substances, i.e. morphine, THC and mitragynine (Iman *et al.*, 2017).

2.2 Endocannabinoid (ECB) system and addiction

2.2.1 Introduction to the ECB system

The endocannabinoid (ECB) system was first discovered in the early 1990s in an attempt to study the mechanism of action of Δ-9-tetrahydrocannabinol (THC), the principal psychoactive constituent of *Cannabis sativa* or marijuana. The ECB system constitutes the cannabinoid (CB) receptors, their endogenous ligands (known as endocannabinoids), and associated enzymes and proteins. Two CB receptors, the CB_1 and CB_2 receptors have been isolated and pharmacologically characterised to date (Katona, 2009; Di Marzo, 2018). CB_1 receptors are primarily located in the CNS and responsible for mediating the psychoactive effects of THC. Lower densities of CB_1 receptors are also found in the testes, ovary, heart, lung and immune cells. In contrast,

the CB_2 receptors are abundantly present on immune tissues. Interestingly, despite early claims of the nonexistence of CB_2 receptors in CNS, recent work has indicated their presence in the cerebellum, hippocampus and spinal cord (Baek *et al.*, 2013). However, this current study only focuses on the CB_1 receptors in the brain mesolimbic system. Meanwhile, the two endocannabinoids that have been isolated so far are anandamide and 2-arachidonylglycerol (2-AG) (Katona, 2009; Di Marzo, 2018).

The CB_1 receptor, which is a G-protein-coupled receptor, strikingly accumulates on the plasma membrane of axon terminals, indicating its pivotal role in synaptic neurotransmission. Upon activation by endocannabinoids or natural rewards stimuli, CB_1 receptor activates G-protein, producing interconnected neural signalling cascades through different neurotransmitter systems which subsequently inhibits the release of GABA, glutamate and acetylcholine from presynaptic terminals. The reciprocal down-regulation of inhibitory (primarily GABA) and excitatory (primarily glutamate) inputs in synapse produce homeostatic excitatory and inhibitory neurotransmission across associated cortical dopaminergic projections (Katona, 2009; Parsons and Hurd, 2015; Di Marzo, 2018). However, acute and chronic drug stimuli can hijack and modify CB_1 receptor signalling, which results in the down-regulation of GABA and/or up-regulation of glutamate inputs, hence disturbing the GABAergic/glutamatergic equilibrium. This homeostatic imbalance eventually disrupts dopaminergic system regulations, thereby contributes to the greater vulnerability of developing substance dependence, withdrawal symptoms and propensity for relapse in substance users (Katona, 2009; Parsons and Hurd, 2015; Koob and Volkow, 2016).

2.2.2 ECB system in brain reward circuitry

The CB_1 receptor shows a ubiquitous distribution throughout the CNS, but with characteristic cell-specific differences in expression levels. The highest density of CB_1 receptor is found in the cerebellum, especially at the molecular layer (Tsou *et al.*, 1998; Katona, 2009). Its regional distribution overlaps with brain reward circuitry, with high to moderate localisation on both GABAergic and glutamatergic axon terminals of hippocampus, amygdala, VTA, NAcc, mPFC and neocortex (Tsou *et al.*, 1998; Katona, 2009; Parsons and Hurd, 2015).

The ECB system has been shown to modulate the positive reinforcement of both cannabinoid and non-cannabinoid drugs. Acute and chronic exposure to addictive substances results in the increment of anandamide and 2-AG levels in midbrain and striatum (Justinova *et al.*, 2009; Di Marzo, 2018). These findings are consistent with the concept that up-regulation of endocannabinoids further enhance the drug's reinforcing effects by inhibiting GABA and glutamate releases, thus causing persistent short-term and long-term plasticity at both excitatory and inhibitory synapses in the limbic system. Furthermore, the central injection of CB_1 receptor antagonist into rodent's VTA was shown to dramatically reduced nicotine-taking and seeking, partly due to the diminished dopamine release in NAcc (Parsons and Hurd, 2015; Manzanares *et al.*, 2018). CB_1 receptor knockout mice also reported a reduction in ethanol self-administration, as well as cocaine and ethanol CPP (Parsons and Hurd, 2015; Manzanares *et al.*, 2018).

2.2.3 ECB system in substance-seeking and relapse

Continuous procurement of THC has been reported to cause functional down-regulation of CB_1 receptors in the striatum, cortex, limbic system and cerebellum (Justinova *et al.*, 2009). Neural adaptation in some brain areas resulted from the hijacking of cAMP pathway following chronic THC exposure, which subsequently heightened dopamine release into the synapse (Justinova *et al.*, 2009; Parsons and Hurd, 2015; Manzanares *et al.*, 2018). De Vries *et al.* (2001) pioneers the findings of ECB involvement in the reinstatement of substance-seeking behaviours. The study reported that the use of synthetic CB_1 receptor agonist reinstated substance-seeking behaviour in rodents. This effect was reversed upon introduction of rimonabant, a CB_1 receptor antagonist (De Vries *et al.*, 2001).

Rimonabant also blocks reinstatement of cocaine, heroin, methamphetamine and ethanol, as well as attenuates cue-induced reinstatement of cocaine, nicotine, methamphetamine, heroin and alcohol (Justinova *et al.*, 2009; Manzanares *et al.*, 2018). As depicted by early clinical trials, rimonabant shows promising results as a putative treatment to smoking problem, obesity and metabolic syndrome (Justinova *et al.*, 2009). However, rimonabant had to be withdrawn from the market since it was found to induce depression and suicidal ideation in patients (Di Marzo, 2018). More recently developed CB_1 receptor antagonists and rimonabant analogues (i.e. SR147778, AM4113, and NIDA-41020) have been reported to cause blockade of THC, ethanol and nicotine self-administration and reinstatement of substance-seeking behaviours in rodents (de Bruin *et al.*, 2011; Schindler *et al.*, 2016; Manzanares *et al.*, 2018). These findings serve as a ground basis to possible manipulations of the ECB system, primarily the CB_1 receptor system, as potential therapeutic targets in the treatment of SUD.