# THE EFFECT OF MITRAGYNINE ADDICTION IN RATS ON HISTONE REGULATIONS AND NEUROPLASTICITY

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# THE EFFECT OF MITRAGYNINE ADDICTION IN RATS ON HISTONE REGULATIONS AND NEUROPLASTICITY

**by**

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## LIST OF SYMBOLS

| | |
|---|---|
| β | Beta |
| ℃ | Degree Celsius |
| g | Gram |
| h | Hour |
| L | Litre |
| µg | Microgram |
| µL | Microliter |
| M | Molar |
| % | Percentage |

## LIST OF ABBREVIATIONS

| | |
|---|---|
| ACh | Acetylcholine |
| AChE | Acetylcholinesterase enzyme |
| AMPAR | Alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor |
| ANOVA | Analysis of variance |
| ARASC | Animal Research and Service Centre |
| BBB | Blood-brain barrier |
| BDNF | Brain-derived neurotrophic factor |
| CaMKII | Calcium/calmodulin-dependent protein kinase II |
| CDR | Centre for drug research |
| CA1 | Cornu ammonis 1 |
| CM | Centimetre |
| CREB | Cyclic AMP response element binding protein |
| DA | Dopamine |
| DORs | Delta opioid receptors |
| DG | Dentate gyrus |
| DTT | Dithiothreitol |
| GABA | Gamma aminobutyric acid |
| GLU | Glutamate |
| ELISA | Enzyme-linked immunosorbent assay |
| ERK | Extracellular signal-regulated kinase |
| FC | Frontal cortex |
| fEPSP | Field excitatory post-synaptic potentials |
| HATs | Histone acetyltransferase |
| HDACs | Histone deacetylase |
| HP | Hippocampus |
| I/O | Input-output |
| IACUC | Institutional Animal Care and Use Committee |
| ISIs | Interstimulus intervals |
| KORs | Kappa opioid receptors |
| LTP | Long-term potentiation |
| mGluRs | Metabotropic glutamate receptors |

| | |
|---|---|
| ML | Millilitre |
| MIT | Mitragynine |
| MOR | Morphine |
| NM | Nanometre |
| NMDAR | N-methyl-D-aspartic acid receptor |
| GPCRs | G protein-coupled receptors |
| PPF | Paired-pulse facilitation |
| PVDF | Polyvinylidene fluoride |
| RCF | Relative centrifugal force |
| SDS | Sodium dodecyl sulfate |
| SD | Sprague Dawley |
| SEM | Standard error of the mean |
| TBS | Theta-burst stimulation |
| TBST | Tris-buffered saline/Tween 20 |
| TrkB | Tyrosine kinase B |
| USA | United States of America |
| USM | Universiti Sains Malaysia |
| vs | Versus |
| WB | Western blotting |
| WHO | World Health Organization |

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**KESAN KETAGIHAN MITRAGININA DALAM TIKUS TERHADAP PENGAWALATURAN HISTON DAN NEUROPLASTISITI**

## ABSTRAK

Mitraginina (MIT) dilaporkan menyebabkan ketagihan dan menjejaskan fungsi kognitif. Dalam kajian akut, MIT didapati mengganggu penghantaran sinaptik *hippocampal* dan memesongkan ingatan melalui mekanisme yang tidak diketahui. Projek ini menilai kesan pendedahan MIT selama 14 hari pada modulasi histon, penghantaran sinaptik dan mekanisme potensinya serta sistem dopaminergik, GABAergik, kolinergik, dan glutamatergik dalam otak tikus. Kesan perencatan farmakologi enzim deacetylase histon pada tindakan MIT juga dinilai. Tikus *Sprague Dawley* (SD) disuntik (i.p.) setiap hari sama ada dengan kumpulan kawalan *(20% Tween 80),* morfina (MOR) 5 mg/kg, atau MIT (1, 5, 10 dan 30 mg/kg) selama 14 hari berturut-turut. Kesan ketarikan daripada rawatan terhadap ketagihan dan tingkah laku kognitif ditentukan dengan menggunakan pemarkahan ketarikan dan *passive avoidance task*. Otak tikus diperolehi untuk pengukuran tahap dopamina, GABA dan asetilkolina (ACh) dengan menggunakan *kit ELISA*. Perubahan dalam ekspresi *Rab35* dan protein histon juga ditentukan dalam *Western blot*. Dalam set 14 hari yang lain, MIT (1, 5 dan 10 mg/kg), MOR 5 mg/kg atau SAHA (20 mg/kg), MOR 5 + SAHA, MIT 10 + SAHA yang dirawat terhadap tikus dinilai kesan ke atas pemarkahan keseluruhan, kognisi dan fEPSP, PPF dan LTP direkodkan di kawasan CA1 kontralateral hipokampus tikus bius menggunakan elektrofisiologi *in vivo*. Pada akhir eksperimen, hippocampus dan otak tengah diperoleh dan kepekatan glutamat diukur menggunakan kit cerakin kolorimetri dan perubahan dalam ekspresi histon dan penanda neuroplastisiti seperti GluR-1, NMDAε2, pCaMKII, pERK, pCREB, BDNF,

synaptophysin, PSD-95, Delta fosB dan CDK-5 dalam hipokampus telah disiasat dalam penutupan barat. MIT dan MOR meningkatkan tanda-tanda penarikan ketara yang menunjukkan potensi ketagihan. MIT merosakkan ingatan dalam *passive avoidance task* manakala, MOR tidak. Kepekatan dopamina berkurangan dalam hippocampus tikus tetapi meningkat di kortek hadapan dan otak tengah selepas rawatan MIT, manakala tahap GABA dan ACh menurun. Ekspresi *Rab35* telah meningkat dalam kedua-dua tikus dengan kesan penarikan MIT dan MOR. Kesan penarikan MIT tetapi bukan MOR menghasilkan penurunan yang ketara dalam H3, H3K9, jumlah H4, H4K12 manakala HDAC2 mempunyai peningkatan berlebihan ekspresi. Rawatan bersama dengan SAHA, membalikkan MIT secara berkesan tetapi bukan pada tindakan MOR ke atas pemarkahan ketarikan, *passive avoidance task* dan epigenetik (H3K9, H4K12, HDAC2). SAHA memberi kesan negatif kepada MOR dalam *passive avoidance task*. Ketarikan MIT telah mengurangkan dengan ketara amplitud fEPSP bagi lengkung I/O, nisbah PPF dan LTP, kesan yang telah diterbalikkan sepenuhnya oleh rawatan bersama dengan SAHA. Ketarikan MOR sedikit menekan LTP, kesan yang diperkuatkan selepas rawatan bersama dengan SAHA. Aras glutamat meningkat dalam kedua-dua hipokampus dan otak tengah selepas MIT tetapi bukan ketarikan MOR. Rawatan bersama dengan SAHA membalikkan tindakan MIT pada kepekatan glutamat. Ekspresi GluR-1 kekal tidak terjejas; walaubagaimanapun, ekspresi NMDAε2 telah dikurangkan dengan ketara selepas rawatan MIT. Pengurangan ketara dalam ekspresi pCaMKII, pERK, pCREB, BDNF, synaptophysin, PSD-95, Delta fosB dan CDK-5 juga dikesan dalam tikus dengan kesan ketarikan MIT. Tidak seperti MIT, MOR didapati mengimbangi synaptophysin dan ekspresi Delta fosB. Data ini menunjukkan bahawa perubahan ekspresi histon (H3K9, H4K12 & HDAC2) serta perubahan dalam protein yang

berkaitan dengan neuroplastisiti mungkin merupakan mekanisme molekul gangguan kognitif yang disebabkan oleh MIT serta gangguan PPF dan LTP.

**THE EFFECT OF MITRAGYNINE ADDICTION IN RATS ON HISTONE REGULATIONS AND NEUROPLASTICITY**

## ABSTRACT

Mitragynine (MIT) was reported to develop addiction and impair cognitive functions. In acute studies, MIT was found to disrupt hippocampal synaptic transmission and distort memory via unknown mechanisms. This project evaluated the impact of 14 days of MIT exposure on the modulation of histone, synaptic transmission, and its potential mechanisms as well as dopaminergic, GABAergic, cholinergic, and glutamatergic systems in the rats' brains. The impact of pharmacological inhibition of histone deacetylase enzyme on MIT action was also evaluated. Sprague Dawley's (SD) rats were injected (i.p.) daily with either vehicle (20% tween 80), morphine (MOR) 5 mg/kg, or MIT (1, 5, 10, and 30 mg/kg) for 14 consecutive days. Effects of the withdrawal from the treatments on addiction and cognitive behaviors were determined using withdrawal scoring and passive avoidance task. Brains were harvested and used to quantify dopamine, GABA, and ACh levels using ELISA kits. Changes in the expressions of Rab35 and histone proteins were also determined in the western blot. In another set of 14 days of MIT (1, 5, and 10 mg/kg), MOR 5 mg/kg or SAHA (20 mg/kg), MOR 5 + SAHA, MIT 10 + SAHA treated rats, the effects on global scoring were assessed, and fEPSP of PPF, and LTP were also recorded in the contralateral CA1 region of the hippocampus of anesthetized rats using an *in-vivo* electrophysiology. At the end of the experiment, the hippocampus and midbrain were harvested. Glutamate concentration was measured using a colorimetric assay kit, and changes in the expression of histone and neuroplasticity markers such as GluR-1, NMDAε2, pCaMKII, pERK, pCREB, BDNF, synaptophysin, PSD-95,

Delta fosB and CDK-5 in the hippocampus were investigated in western blot. MIT and MOR markedly increased withdrawal signs which suggests addiction potential. MIT but not MOR impaired memory in the passive avoidance task. Dopamine concentration was decreased in the rats' hippocampus but raised in the frontal cortex and mid-brain after MIT treatments, while GABA and ACh levels were lowered. Rab35 expression was increased in both MIT and MOR withdrawn rats. MIT but not MOR withdrawal yielded a profound decrease in total H3, H3K9, total H4, and H4K12, while HDAC2 overexpressed. Co-treatment with SAHA effectively reversed MIT but not MOR action on withdrawal scoring, passive avoidance task, and epigenetics (H3K9, H4K12, HDAC2). SAHA negatively impacts MOR in the passive avoidance tasks. MIT withdrawal markedly reduced the fEPSP amplitude of the I/O curves, PPF ratio, and LTP, an effect that was wholly reversed by co-treatment with SAHA. MOR withdrawal mildly suppressed LTP, an effect that was potentiated following co-treatment with SAHA. Glutamate level was increased in both the hippocampus and midbrain after MIT but not MOR withdrawal. Co-treatment with SAHA reversed MIT action on glutamate concentration. GluR-1 expression remained unaffected; however, the expression of NMDAε2 was significantly reduced after MIT treatment. A marked reduction in pCaMKII, pERK, pCREB, BDNF, synaptophysin, PSD-95, Delta fosB and CDK-5 expression was also detected in MIT withdrawn rats. Unlike MIT, MOR was found to upregulate synaptophysin and Delta fosB expression. The data indicate that changes in histone (H3K9, H4K12 & HDAC2) expressions and alteration in neuroplasticity-associated proteins may be a molecular mechanism of MIT-induced cognitive impairments, PPF, and LTP disruption.

# CHAPTER 1

# GENERAL INTRODUCTION

## 1.1 Overview

Drug addiction is a recurring brain illness that is associated with an obsessive drug use despite its negative consequences (George & Koob, 2017). It involves functional changes in the brain circuitry areas responsible for reward (Volkow, 2018). Addiction is a complex, relapsing disorder in which illicit drugs hijack, overstimulate, and compromise reward-processing systems and associated networks (Darcq & Kieffer, 2018). Addiction-causing substances typically alter synaptic transmission in the brain's reward-related mesocorticolimbic and corticostriatal circuits (de Ceglia *et al.,* 2021).

Long-term exposure to substances with abuse potential causes neuroadaptations and neuronal degeneration, resulting in cognitive dysfunction ( Rolland *et al.,* 2019; Melugin *et al.,* 2021). Therefore, drug addiction is a disorder of cognition that may need urgent intervention (Melugin *et al.,* 2021). Addiction and cognitive impairments implicate numerous neurotransmitter systems, including dopaminergic, GABAergic, cholinergic, and glutamatergic systems. Dopamine is one of the neurotransmitters found in the brain that is mainly associated with addiction (Assar *et al.,* 2016). Activating GABAergic neurotransmission in the hippocampus provides neuroprotection and reinstates memory (Juan Li *et al.,* 2020). Moreover, the enhancement of cholinergic neurotransmission is extensively associated with improved cognition (Hampel *et al.,* 2018). Therefore, while dopamine plays a significant role in addiction, both GABAergic and cholinergic systems may be implicated in cognitive impairment. Another system that plays a role in cognition is

glutamatergic, as increased glutamate concentration causes neurotoxicity and subsequent memory loss (Lee *et al.,* 2022).

Research in the epigenetics have shown that, drug use can lead to changes in the epigenetic landscape of the brain, which may contribute to the development of addiction (Abel & Poplawski, 2014). Chronic drug use can lead to changes in the acetylation and methylation patterns of specific proteins, such as histone, which can affect the activity of those proteins and lead to drug addiction and related cases (Browne *et al.,* 2020). These epigenetic changes have been shown to regulate gene expression, affecting various aspects of cognition, such as memory, learning, and brain development. Changes in the acetylation of histones involved in neural activity may affect cognitive function (Burns & Gräff, 2021).

Mechanisms of neuronal chromatin modifications, such as histones acetylation, outlined the mechanism of learning and memory. The histone acetyltransferase activity (HAT), which is responsible for histones acetylation, was found to be increased amidst memory formation (Volmar & Wahlestedt, 2015).Another critical enzyme that modulates histone acetylation is a histone deacetylase enzyme type 2 (HDAC2), which removes the acetyl group from the histone N-terminal amino acid residue. HDAC2 overexpression in the hippocampus impairs memory and learning (Volmar & Wahlestedt, 2015; Wong *et al.*, 2020). Long-term memory impairment in animals was rescued after treatment with an HDAC2 inhibitor (Korzus *et al.,* 2004; Wong *et al.*, 2020), indicating that histone acetylation is critical in cognitive function.

The hippocampus is a crucial structure in the limbic system that plays a vital role in establishing and storing both the episodic and semantic declarative forms of

memories (Avchalumov & Mandyam, 2021). It is a crucial region in the brain responsible for long-term memory. Signals are transmitted to the hippocampus from the entorhinal cortex via dentate gyrus (DG) and passed to the pyramidal neurons of CA3 via the mossy fibers, then to the pyramidal neurons of CA1 region known as Schaffer collateral and commissural pathway (Amaral and Witter, 1989). Action potential generation via activation of the Schaffer collateral pathway (CA3-CA1) is responsible for long-term potentiation (LTP) in the hippocampus, and is crucial in the memory development and its retrieval (Whitlock *et al.,* 2016). Stimulation of the CA3 region causes calcium influx and the subsequent release of some chemical transmitters, typically glutamate, which plays a crucial role in drug-related synaptic plasticity, including the modulation of dopaminergic neurons for the development and preservation of drug-related memories (Hautrive *et al.,* 2021; Portugal *et al.,* 2014). Repeated drug use may alter synaptic plasticity in the hippocampus, and hence, may impact drug context associations (Portugal *et al.,* 2014). LTP, one of the forms of synaptic plasticity in the hippocampus, is induced via Schaffer collateral CA1 pathway stimulation and is dependent on NMDAR-mediated Ca2 influx (Alkadhi, 2021).

*Mitragyna speciosa* (Kratom/Ketum/Biak biak), belonging to the family "Rubiaceae" is an evergreen plant that has naturalized in many areas of South-Asian countries such as Thailand and Malaysia (Gong *et al.,* 2012; Hassan *et al.,* 2020) where it has been used for its sedative and stimulant mimicking actions (Suwanlert, 1975; Tanguay, 2011). Mitragynine (9-methoxy-corynantheidine) is the most studied active indole alkaloid from Kratom that constitutes more than 60 percent of the total alkaloids in Kratom (Hassan *et al.,* 2013; Obeng *et al.,* 2021). While the addiction and cognitive deficit potentials of kratom and mitragynine seem to be questionable following a study involving humans (Vicknasingam, *et al.,* 2019) and even therapeutic

use against other drug addictions becomes possible (Gutridge *et al.,* 2020; You *et al.,* 2022; Yusoff *et al.,* 2022), chronic use may cause cognitive impairments. There have been reports of learning and memory impairments after either short or prolonged exposure to kratom or mitragynine in animal studies (Apryani *et al.,* 2010; Hazim *et al.,* 2011; Suhaimi *et al.,* 2016; Yusoff *et al.,* 2016; Ismail *et al.,* 2017; Iman *et al.,* 2021), however, the molecular mechanism behind the cognitive deficit remains elusive and hence requires further investigation (Apryani *et al.,* 2010; Suhaimi *et al.,* 2016; Hassan *et al.,* 2019). Kratom preparations were previously reported to significantly reduce non-potentiated fEPSPs and block LTP in the CA1 hippocampal area of rats (Ilmie *et al.,* 2015; Senik, 2012). According to some recent studies, acute mitragynine treatment in rats reduced synaptic transmission by slightly inhibiting LTP in the hippocampus (Hassan *et al.,* 2019; Effendy *et al.,* 2022), however, an adequate knowledge of the impact of repeated exposure to mitragynine on synaptic transmission in the hippocampus and the potential mechanism underlying mitragynine-induced synaptic alterations is lacking (Hassan *et al.,* 2019). Therefore, this project evaluated the impact of 14 days of daily exposure to mitragynine on addiction, Rab35 modulation and histone expressions associated with addiction and cognition, synaptic transmission, and its potential mechanism in the rat's hippocampus. Roles of dopaminergic, GABAergic, cholinergic, and glutamatergic systems on mitragynine-induced synaptic changes were also investigated.

## 1.2 Statements of problem and study's justification

Drug use for recreational purposes leads to addiction, a severe brain disorder that affects people from all walks of life (Darcq & Kieffer, 2018). Drug addiction alters the typical structure and function of specific brain regions, leading to memory

modifications that favor continued drug use through maladaptive behaviors and preventing the development of adaptive behaviors that create an environment conducive to stopping drug use (Melugin *et al.,* 2021). As a result, drug addiction leads to cognitive decline, and has remained a global public health issue (WHO guidelines, 2019). Cognitive deficiencies are caused mainly by hippocampal-dependent memory problems, which may call for an immediate intervention (Titus *et al.,* 2015). Since mitragynine has been associated with abuse potential and significant cognitive function deterioration, in the first part of this project, it was hypothesized that changes in cognitive performance might be influenced by mitragynine repeated treatment in the epigenetic alteration of cell transcriptional signaling.

The knowledge of the cellular and molecular mechanisms governing the epigenetic in mitragynine-induced cognitive impairment may offer a practicable therapeutic strategy to prevent addiction-related types of memory decline. The second part of this project hypothesized that repeated mitragynine exposure induces long-lasting neuroplasticity alterations. Mitragynine acute administration mildly suppressed LTP in the CA1 hippocampal region of urethane anesthetized rats (Hassan *et al.,* 2019; Effendy *et al.,* 2022), but the molecular mechanisms behind the suppression of LTP after mitragynine administration remain unresolved (Hassan *et al.,* 2019). Therefore, the other part of this study targeted the effects of mitragynine repeated administration on PPF, LTP, and the potential mechanisms.

## 1.3 Research hypotheses

1. Administration of mitragynine for 14 days in rats can induce addiction.
2. Repeated mitragynine use can impair memory and causes long-lasting changes in neuroplasticity.
3. Epigenetic modifications may regulate the cognitive decline elicited by mitragynine. In mitragynine-addicted rats, histone acetylation and other chromatin modifications are altered.
4. Modulating the transcriptional capability of cells through chromatin modification by *in-vivo* administration of a histone deacetylase (HDAC) inhibitor enhances cognitive function. It augments the process of long-term memory formation in rats.
5. Changes in neuroplasticity are mediated by the alteration of protein markers in the rats' hippocampal region. Expressions of neuroplasticity-associated proteins are reduced in the mitragynine-addicted rats' brain areas.
6. Modification of cognition and synaptic transmission following mitragynine repeated exposure are influenced by the changes in dopaminergic, GABAergic, cholinergic, and glutamatergic systems. The level of neurotransmitters in these systems is modified in the mitragynine-addicted rats' brains.

## 1.4 Objectives of the study

General objective:

To understand the effects of mitragynine addiction in rat on histone regulations and neuroplasticity.

Specific objectives:

1. To develop a mitragynine-addicted rat model.
2. To determine the effects of repeated exposure to mitragynine on cognitive behaviours and the role of neuroplasticity in regulating cognitive functions.
3. To examine the role of epigenetics in regulating the cognitive functions of rats addicted to mitragynine.
4. To assess the effects of pharmacological inhibition of the negative epigenetic regulation on animals' behavioural responses to mitragynine.
5. To study the involvement of neuroplasticity biomarkers in mitragynine-induced synaptic changes.
6. To elucidate the contribution of dopaminergic, GABAergic, cholinergic, and glutamatergic neurotransmissions in the cellular mechanisms of mitragynine-induced synaptic plasticity.

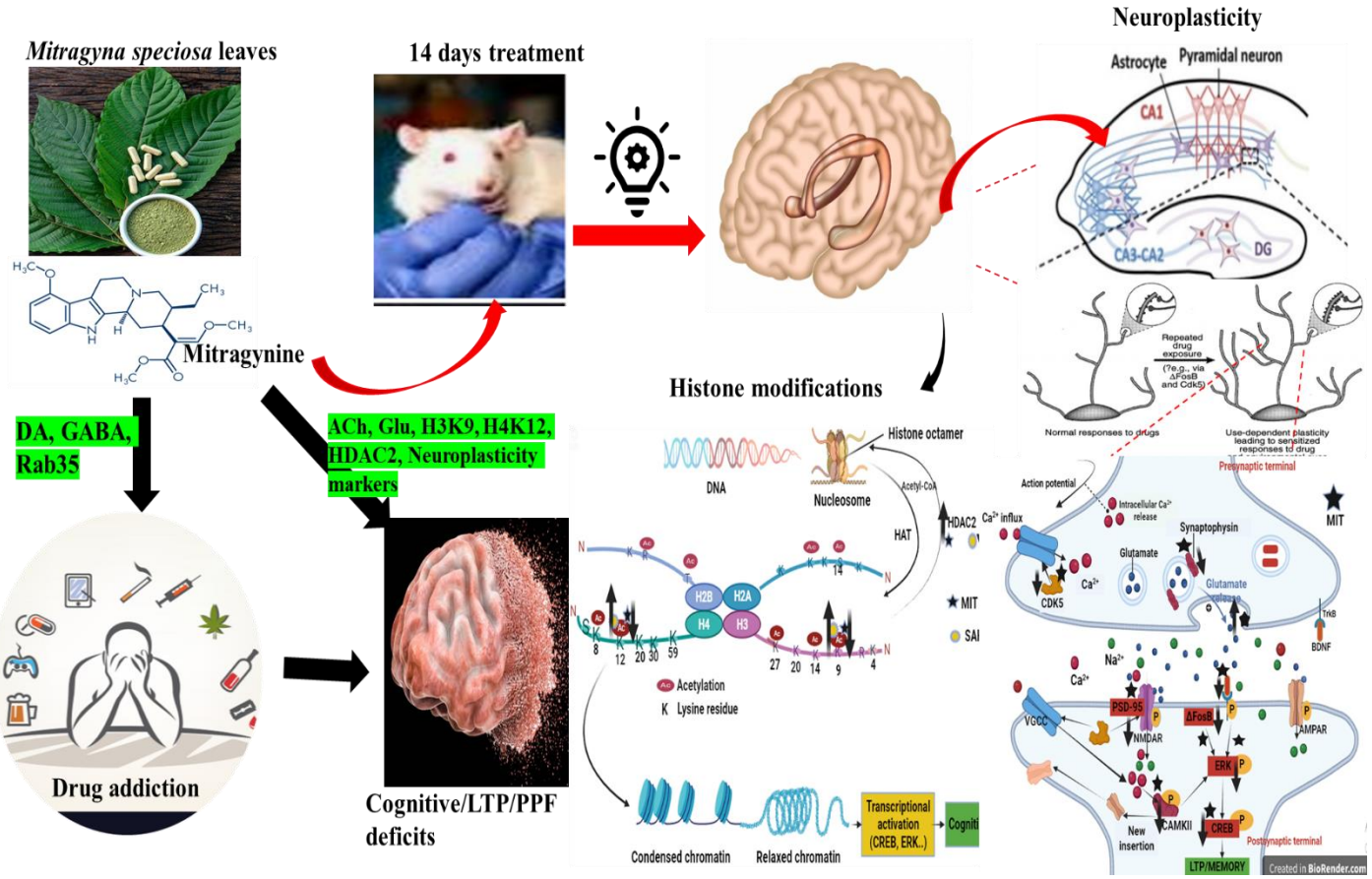


Figure 1.1 Graphical illustration of the study summary of findings.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Drug Addiction

Drug addiction is a substantial public health problem. About thirty-five million individuals worldwide suffer from disorders due to the abuse of drugs, and the incidence is increasing daily (Wei & Shah, 2020). In 2017 alone, about 271 million people were reported to participate in drug abuse, which may account for more than 5% of the global population between the age of fifteen to sixty-four years (Navarro *et al.*, 2022). Data from Africa also suggest an increased use of opioids which causes addiction and related disorders (Harker *et al.,* 2020). Drug addiction produces euphoria and lessens discomfort; these favourable transitory benefits drive the initial purposeful seeking behaviours during addiction. Most studies on the process of addiction have focused on finding and describing the various brain systems that contribute to the rewarding effects of substances with addictive potential and how these systems are altered during drugs intake (Robinson & Berridge, 2003). It has been well established that addictive drugs hijack and corrupt the brain circuitry centres that typically mediate motivation, pleasure, and cognitive processes (Darcq & Kieffer, 2018; Volkow *et al.*, 2019; Umberger & Gaddis, 2020). These brain reward and associated memory centres contain glutamate inputs from the frontal cortex, amygdala, and hippocampus; GABA from the nucleus accumbens (NAcc) to the ventral tegmental area (VTA); as well as dopamine projections from the substantia nigra and VTA to the NAcc as well as striatum (Cox & Witten, 2019; Robinson & Berridge, 2003).

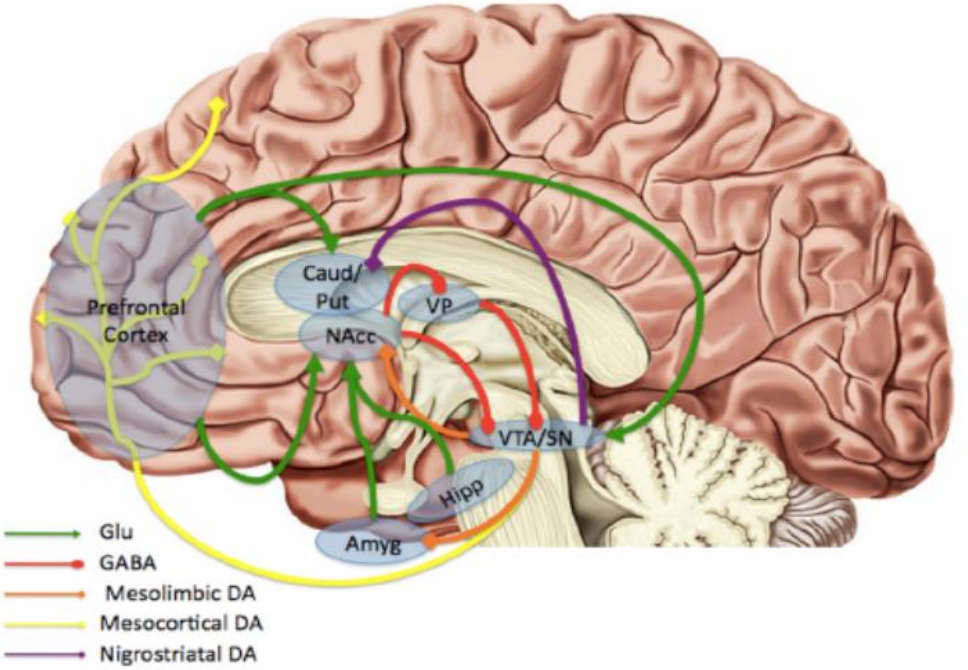


Figure 2.1 Theoretical explanation for how drugs hijack multiple learning processes to produce maladaptive and enduring addictive behaviors. Adopted from Volkow et al. (2019). VTA= ventral tegmental area, NAc = Nucleus accumbens, Amy = Amygdala, Hipp = Hippocampus

### 2.1.1 Addiction cycle

Impulsivity (uncontrolled responses to internal and external stimuli without consideration for the outcome) and compulsivity (the persistent repetition of inappropriate behaviours) are two components of drug addiction, which result in a recurring cycle of addiction that commence with binge/intoxication, followed by withdrawal, and then finally craving/ preoccupation leading to neuroplastic changes in both rewards and executive functions-controlled areas (Koob & Volkow, 2016). The stage of intoxication is when a person consumes an intoxicant and feels it's gratifying, euphoric, and pleasurable effects. This stage is preceded by the withdrawal phase, which is the stage at which a person suffers a negative emotional state in the absence of substance and finally enters the preoccupation stage, which is characterized by a period

at which a person is forced to start retaking the substance due to the unfavourable circumstances experienced during withdrawal stage (US Department of Health and Human Services (HHS) & Office of the Surgeon General, 2016). Fig 2.2 below illustrates the progression through the three stages of the addiction cycle. The release of dopamine and opioid peptides by neurons in the basal ganglia during the intoxication stage contributes to the rewarding effects of the addictive substance. The withdrawal stage causes the corticotropin-releasing factor, norepinephrine, and dynorphin stress neurotransmitters to be activated, which contributes to the general loss of reward sensitivity. During the preoccupation stage, neurons from the prefrontal cortex stimulate the habit systems of the dorsal striatum through connections that use glutamate, leading to executive function impairment (US Department of Health and Human Services (HHS) & Office of the Surgeon General, 2016).

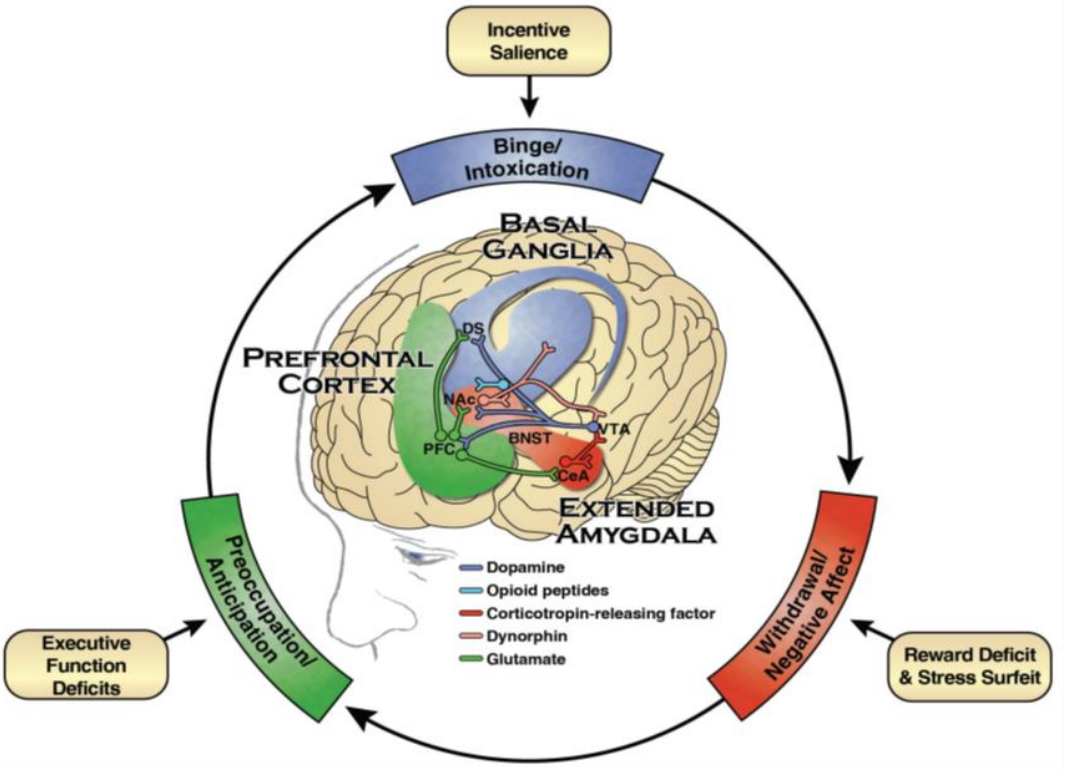

Figure 2.2 Stages of the addiction cycle (Adopted from the US Department of Health and Human Services (HHS) & Office of the Surgeon General (2016) reports.

## 2.2 Drug dependence, tolerance, and withdrawal syndrome

### 2.2.1 Definition of drug dependence

Drug dependence occurs when an individual is compelled to take a drug regularly to avoid physical discomfort or to achieve a pleasurable state (Pantazis *et al.*, 2021). It is characterized by the appearance of a withdrawal syndrome following the cessation of drug use (Koob, 2021). Dependence can either be physical or psychological. Physical dependence is a severe physical disturbance when drug use is discontinued. In contrast, psychological dependence is defined as a condition in which a drug produces a feeling of satisfaction and a psychic drive that necessitates continuous drug administration in order to have a pleasure or to avoid discomfort (Koob, 2021). Physical withdrawal symptoms are reflected by physical signs and symptoms that are usually opposite to the acute effects of the drug itself. This project focuses on physical dependence.

### 2.2.2 Definition of tolerance

Tolerance to a drug is defined as a state in which the previously rewarding drug must be used in higher doses and more frequently to maintain euthymia (Koob, 2021). During tolerance, an increased drug dose is required to produce the same effect as the previously used lower dose. Regular drug intake typically causes a decline in drug response, leading to relative tolerance to the drug's effects (Katzung *et al.*, 2012).

Tolerance to a drug develops mainly due to increased drug metabolism or decreased interaction between the drug and its receptor (Ehsani *et al.,* 2021).

### 2.2.3 Definition of withdrawal syndrome

Withdrawal syndromes are devastating physical and mental reactions that appear following cessation of recreational drug intake (Connor et al, 2022). The maximal effects are observed within 24 to 48 hours after the stoppage of drug consumption (Robinson & Berridge, 2003; Hassan *et al.,* 2020) The occurrence of withdrawal syndrome depends on the type of drug administered and may include changes in appetite, restlessness, insomnia, tremor, anxiety, etc (Connor et al, 2022). The manifestation of a physical withdrawal syndrome after 24 h is the primary focus of this project.

## 2.3 Brain's reward and memory circuitry: Interactions between the neural networks

Various neural structures critically affect information processing related to diverse types of memory and learning (Yang & Chang, 2019).The crucial interplay between these structures determines the duration of task acquisition and the magnitude, content, and durability of memories. When a specific brain's region is damaged, the participation of the affected neural system in-memory processing decreases from its regular proportionate contribution (du Toit *et al.,* 2020).

The hippocampus is a fundamental brain structure involved in forming and consolidating memories. However, it does not work in isolation as it interplays with

other brain regions, including midbrain structures that are involved in the regulation of motivation and reward, such as the VTA and the substantia nigra (Aberg *et al.*, 2020). These structures are part of the mesolimbic dopamine system, critical in processing rewarding stimuli and regulating motivated behaviours (Lowes & Harris, 2022). The hippocampus receives input from the VTA and projects back to the same region (VTA), indicating a bidirectional communication between these structures. This interaction is critical for consolidating memories associated with reward and motivation (James *et al.*, 2021). Furthermore, the hippocampus has been shown to regulate the function of dopaminergic neurons in the VTA and substantia nigra, indicating that it can influence dopamine release in response to rewarding stimuli (Tsetsenis *et al.*, 2021). Dysfunction of this interaction has been implicated in several psychiatric disorders, including addiction and dementia.

The frontal cortex, which is implicated in higher-order cognitive functions, such as working memory and decision-making, also interacts with the hippocampus during memory formation as the frontal cortex provides feedback to the hippocampus on the relevance of the information being processed, which helps to prioritize which memories should be consolidated for long-term storage (Yavas *et al.*, 2019). The hippocampal formation and frontal cortex are closely linked in structure and function owing to their reciprocal connections with other areas of the cerebral cortex, such as the temporal and parietal association areas (Jobson *et al.*, 2021). Recent research has documented the existence of unmediated projections originating from the subiculum within the hippocampal formation to the medial frontal cortex, along with mutual connections between the caudal section of the pre-subiculum and the dorsolateral prefrontal cortex (Nelson *et al.*, 2020). Additionally, the medial and orbital prefrontal cortex has been demonstrated to receive direct inputs from the hippocampal CA1 field (Dolleman-Van

Der Weel *et al.,* 2019). This project has explored the involvement of these brain regions vis-a-vis the hippocampus, midbrain, and frontal cortex in mitragynine-treated rats.

## 2.4 The neurotransmitter systems involved in the addiction and cognitive deficit pathways

The neurological effects of many drugs have been the subject of extensive research as the basis of addiction, cognitive and behavioural processes, suggesting a plausible therapeutic target for managing several neurological disorders (Antons *et al.,* 2020). This complex mechanism implicates multiple systems in the body, such as dopaminergic, GABAergic, cholinergic, and glutamatergic (Juan Li *et al.,* 2020).

### 2.4.1 Dopaminergic system

Extensive research has established the crucial role of the dopaminergic system in modulating addiction, learning, and memory processes across several addictions and memory-associated brain regions, such as the hippocampus, frontal cortex, amygdala, VTA, and nucleus accumbens (De Sa Nogueira *et al.*, 2022). The dopaminergic system is a complex network of neurons in the brain responsible for producing and releasing dopamine, a neurotransmitter associated with reward, motivation, and pleasure. This system involves many aspects of behaviours, including motor control, cognition, reward processing, emotion, and synaptic plasticity (Speranza *et al.*, 2021). Drug abuse increases dopamine levels in the brain, leading to feelings of pleasure and reward that eventually manifest in compulsive drug use and subsequent addiction development (Dresp-Langley & Hutt, 2022). Long-term drug use can cause changes in the dopaminergic system that can cause tolerance and withdrawal symptoms during drug abstinence (Potla *et al.,* 2023). Dysfunction of the dopaminergic system has been linked

to various neurological disorders, such as Parkinson's disease and associated cognitive impairments (Aarsland *et al.*, 2021). Research suggests that, drug addiction can hijack reward centres such as VTA, amygdala, and hippocampus. These structures also participate in cognitive function; therefore, their malfunction can predispose to cognitive impairment (Guzmán-Ramos *et al.*, 2022).

Dopamine receptors at the postsynaptic nerve terminal are part of a more prominent family of G protein-coupled receptors (GPCRs) (Teng *et al.*, 2022). There are five different subtypes of dopamine receptors, denoted as D1, D2, D3, D4, and D5. Each subtype has a slightly different structure and function, and they are distributed in different areas of the brain and other organs, such as the kidneys and the heart. When dopamine binds to a receptor, it can activate or inhibit downstream signaling pathways in the cell, leading to various physiological and behavioural effects (Cai *et al.*, 2021). D1 and D5 are D1-like receptors, while D2, D3, and D4 are D2-like dopamine receptors (Ouchi *et al.*, 2022). D1-like receptors promote the conversion of ATP to cAMP, which then triggers the activation of cAMP-dependent protein kinase. This activation changes membrane permeability to ions, resulting in excitation from sodium influx or inhibition due to potassium influx (Klein *et al.*, 2018).

Conversely, the activation of D2-like receptors can impede adenylate cyclase activity, ultimately causing cAMP levels to decrease (Xin *et al.*, 2019). Recent research has also indicated that D2 receptors can impact voltage-dependent calcium channels, resulting in neurotransmitter release and synthesis changes, ultimately modulating neuronal excitability, which is implicated in almost all neural mechanisms, including cognitive function (Xin *et al.*, 2019). Abnormalities in dopamine receptor function have been implicated in several brain disorders such as addiction and Parkinson's disease, which are associated with memory loss (Cai *et al.*, 2021).

Rab35 is a small GTPase protein that regulates endocytosis, a process by which cells take up molecules from their environment (Haley & Zhou, 2021). It has been shown that Rab35 may play a role in addiction by regulating the endocytosis of dopamine receptors (Lejia Xu *et al.,* 2021), and could potentially lead to changes in dopamine signalling and reward-seeking behaviour. Proteomic analysis reveals that mitragynine, known to increase the dopamine level in the reward circuitry centres of the brain, was able to upregulate Rab35 expression (Hassan *et al.,* 2021).

### 2.4.2 GABAergic system

The GABAergic system utilizes gamma (γ)-aminobutyric acid (GABA) as the neurotransmitter. GABA remains the major inhibitory neurotransmitter in the brain (Isler *et al.,* 2022) and activation of this system has been reported to improve cognition (Isler *et al.,* 2022). When released to the synaptic cleft, GABA interacts with its receptors, such as GABA-A, GABA-B, GABA-C, and GABA-D, to elicit the inhibitory action (Luo, 2022). GABA-A, GABA-B, and GABA-D are predominant in the CNS, while GABA-C is densely found in the retina (Ali *et al.,* 2023). The GABA-A and GABA-C receptors are ion channels activated by ligands at pre- and post-synaptic neurons. GABA-A receptor is primarily gated by GABA and secondarily modulated by other agents, including benzodiazepines. When GABA or a GABA-A receptor agonist binds to the receptor, the chloride channel opens, leading to influx of chloride ions, resulting in the hyperpolarization of neurons (Bryson *et al.,* 2023). This process reduces the synaptic transmission of other neurotransmitters, including dopamine and acetylcholine, and modulates addiction, cognition (Bryson *et al.,* 2023) and other brain functions. The GABA-B and GABA-D receptors are associated with G-proteins and a second-messenger system that facilitates the activation of calcium or potassium channels. The latter leads to the production of slow inhibitory post-synaptic potentials

in various regions of the CNS (Bassetti, 2022). Intraperitoneal administration of GABA, A receptor agonist to Wistar rats improved memory in robust behavioural test models such as the Morris water maze, object recognition test and social novelty discrimination test (Santrač *et al.,* 2022).

### 2.4.3 Cholinergic system

Numerous studies have revealed the cholinergic system's participation in modifying learning and memory processes, encouraging the interest of many researchers in this area (Hampel *et al.,* 2018; Lysenkov *et al.,* 2023). The cholinergic system releases acetylcholine (ACh), a neurotransmitter essential to many bodily functions (Kopańska *et al.,* 2022). ACh is produced in the nerve endings through the choline acetylation with acetyl-CoA by the action of the choline acetyltransferase (ChAT) enzyme. Once synthesized, ACh is stored in the synaptic vesicles, where proton-pumping ATPase facilitates it's accumulation (Kopańska *et al.,* 2022). The release of ACh into the synaptic cleft is quantal and is triggered by $Ca^{2+}$ flux induced depolarization (Deschenes *et al.*, 2022). Once released, ACh can bind to pre- and post-synaptic muscarinic (mAChR) and nicotinic (nAChR) receptors. The mAChR family consists of M1, M2, M3, M4, and M5, which are G-protein coupled receptors extensively found in the central nervous system, especially in the hippocampus and amygdala (Dwomoh *et al.,* 2022). On the other hand, nAChRs are ligand-gated ion channel receptors widely distributed in the thalamus, hippocampus and amygdala (Lee & Hung, 2022). Activation of these receptors results in swift changes in cellular permeability, causing depolarization and excitation by allowing Na+ and $Ca^{2}$+ influx (Maltan *et al.*, 2021). Studies have shown that cholinergic agonists or antagonists

administration into the hippocampus affects learning and memory performance (Upright & Baxter, 2021). Enhanced cholinergic neurotransmission plays a vital role in several bodily processes, including muscle contraction, memory formation, learning, and other cognitive functions. Defects in this system can result in neurobiological disorders such as dementia (Lysenkov *et al.*, 2023).

### 2.4.4 Glutamatergic system

The glutamatergic system plays a crucial role in the excitatory neurotransmission of the brain. Glutamate, the primary neurotransmitter of this system, is involved in various cognitive functions such as learning and memory (Santrač *et al.*, 2022). Stimulation of the presynaptic nerve terminal causes the influx of calcium ions or their intracellular release, which then mediate the release of glutamate from the vesicles and subsequent release to the synaptic cleft (Lin *et al.*, 2022). Upon release, glutamate interacts with its receptors, such as NMDA, AMPA and kainate, which are widely expressed throughout the brain (Moroz *et al.*, 2021). Glutamate receptors are responsible for mediating the effects of the excitatory neurotransmitter glutamate. NMDA, AMPA and kainate receptors are ionotropic glutamate receptors, while metabotropic glutamate receptors are G protein-coupled receptors. NMDA receptors are crucial in synaptic plasticity and learning, while AMPA and kainate receptors are essential in regulating synaptic transmission and excitability (Moroz *et al.*, 2021). Metabotropic glutamate receptors modulate synaptic transmission and plasticity by regulating intracellular signalling pathways. Dysfunction in glutamate receptor signalling has been implicated in various neurological and psychiatric disorders, including Alzheimer's Disease, epilepsy and schizophrenia (Su *et al.*, 2022).

The memory process begins with activating relevant synapses, which release glutamate from the pre-synaptic neuron (Lin *et al.,* 2022). This glutamate then binds to the AMPA receptor, leading to the influx of Na+ into the post-synaptic neuron and causing depolarization (Zanetti *et al.,* 2021). Enhanced depolarization triggers the activation of NMDA receptors, which removes Mg+ that blocks the NMDA receptor channel. This, in turn, leads to a massive influx of both Na+ and $Ca^{2}$+ into the post-synaptic neuron, which activates various protein kinases, including CaMKII (Endo *et al.*, 2019). Adenylyl cyclase can be activated by multiple modulatory inputs, such as the influx of $Ca^{2+}$ through G-protein-coupled receptors, leading to the elevation of intracellular cyclic adenosine monophosphate (cAMP) levels (Sanchez-Collado *et al.,* 2019). This increase in cAMP levels eventually leads to the activation of protein kinase A (PKA), which regulates the availability of new proteins by activating CREB in the nucleus (Gao *et al.*, 2022), which plays a crucial role in memory. In vitro activation of NMDA receptors in the CA1 hippocampal area increased histone H3 acetylation (Levenson *et al.,* 2004), a phenomena that is known to reinstate memory in cognitively impaired rodents (Wong *et al.,* 2020).

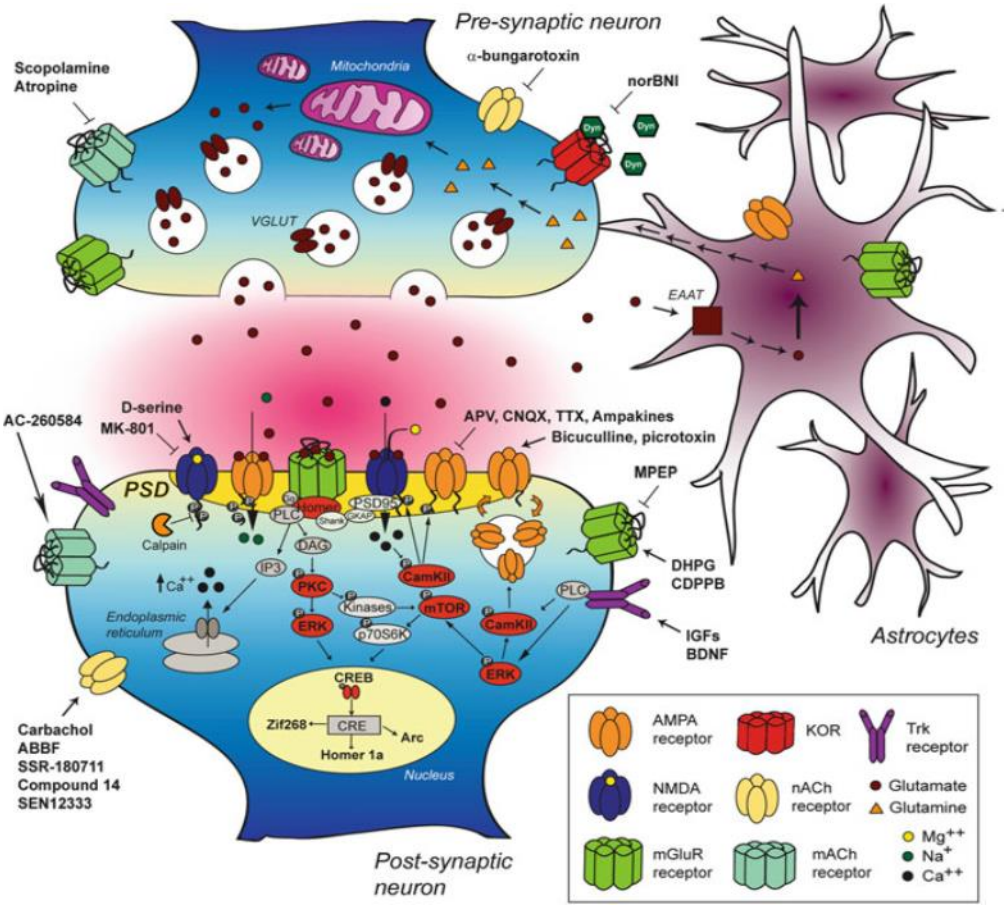


Figure 2.3 The roles of glutamate signalling in memory. When released, glutamate activates postsynaptic AMPA, NMDA, and mGluR receptors. This activation leads to phosphorylating downstream signalling effectors, notably PKC, CaMKII, ERK, mTOR, and CREB. Additionally, it triggers the expression of immediate early genes such as Arc, Homer 1a, and Zif268. Other neurotransmitters like ACh, BDNF, and growth factors (IGF) can influence glutamatergic neurotransmission through Trk receptors. Neuromodulators like dynorphins (Dyn) can modulate glutamate release by inhibiting presynaptic κ-opioid receptors (KOR). Astrocytes actively participate in the activity of the glutamatergic system. They recapture glutamate through excitatory amino-acid transporters (EAATs) and convert it into glutamine, which is then reconverted into glutamate within neuron mitochondria. Adopted from Ménard et al. (2015).

Glutamate excitotoxicity is a pathological process that occurs when excessive amounts of glutamate accumulate in the synaptic cleft and overstimulate receptors on the postsynaptic neuron, which causes excessive activation of the glutamate receptors (Shen *et al.*, 2022). The over-activation of these receptors can lead to a cascade of intracellular events that result in neuronal damage and death, including excessive

calcium influx, mitochondrial dysfunction (Shen *et al.,* 2022), and the production of reactive oxygen species and ultimately, this process can lead to a loss of brain function that contribute to the progression of neurodegenerative diseases (Liu *et al.,* 2022). This project has assessed the changes in the glutamate level and its associated receptors in relation to cognitive deficits induced by mitragynine.

## 2.5 Epigenetics in addiction and cognitive deficit

Epigenetics refers to the mechanisms that impact gene expression, resulting in phenotypic changes without altering the underlying DNA sequence (Elvir *et al.,* 2019). Epigenetic regulation control gene expression and establishes a multi-dimensional, collaborative, and cross-regulating mechanism that is sometimes reversible. The primary epigenetic control mechanisms are histone regulation, DNA methylation and RNA-based mechanisms (Zhang *et al.,* 2016). The chronic intake of psychoactive substances can induce persistent modifications in an individual's behaviour by disrupting the cellular transcriptional potential through genomics and interfering with the neuronal information transmission pathways within the mesocorticolimbic and corticostriatal outflows (Meccariello *et al.,* 2020). This phenomenon can lead to long-lasting neuroadaptations characterized by complex alterations in synaptic connectivity and neural plasticity (Meccariello *et al.,* 2020). Memory formation in specific brain regions that regulate cognition, such as the hippocampus, requires gene transcription at the epigenetic level (Poplawski & Abel, 2012).

## 2.6 Histone proteins

Histones are primary and positively charged proteins that give chromosomes structural support (Sato *et al.,* 2022). Each chromosome has a long DNA molecule that needs to fit into the cell nucleus, and this is accomplished by the DNA wrapping around

assemblies of histone proteins, which results in a more compact form for the chromosome (Simpson *et al.,* 2022). DNA is packaged in highly organized structures known as nucleosomes which serve as chromatin's fundamental unit (Mariño-Ramírez *et al.,* 2005). In nucleosomes, double-stranded DNA is wrapped around an octamer of histone proteins containing two copies of core histones: H2A, H2B, H3, and H4 (McGinty & Tan, 2015; Sato *et al.,* 2022). Two H2A-H2B dimers and two H3-H4 dimers make up the four-helix bundle that makes up the octamer structure (McGinty & Tan, 2015). H1, another histone protein not part of the core histone octamer, but contributes to nucleosome stability by acting as a linker protein (Kalashnikova *et al.,* 2016). An additional factor that gives stability to the nucleosome is that histones contain a high level of lysine and arginine amino acids (Smith & Denu, 2009) which makes them positively charged, and this favours their interaction with negatively charged DNA (Dueva *et al.,* 2019) and hence providing stability to the nucleosome. Fig 2.5 illustrate (a) Nucleosome core particle structure, depicting histone proteins octamer wrapped by DNA base pairs. (b) Histone-fold heterodimer H3-H4. (c) Histone-fold heterodimer H2A-H2B.

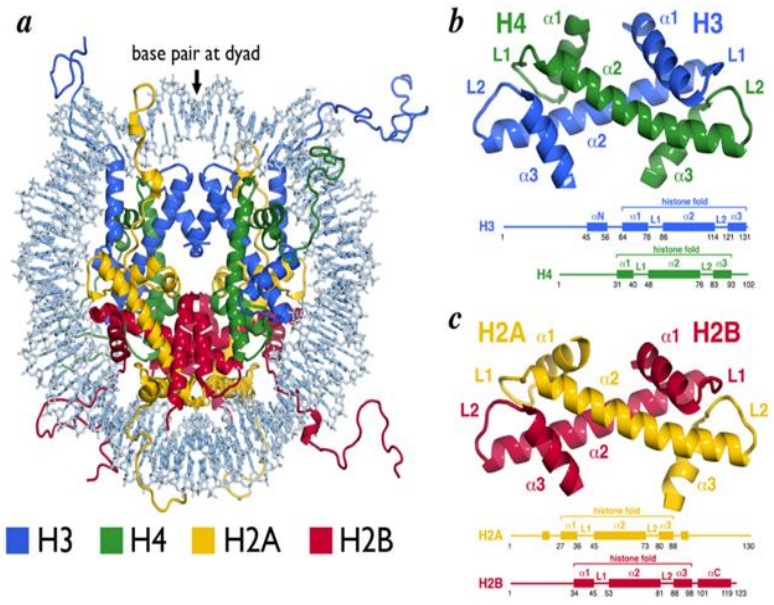


Figure 2.4 Structure of nucleosome core particles and histone-fold heterodimers adopted from McGinty & Tan (2015).

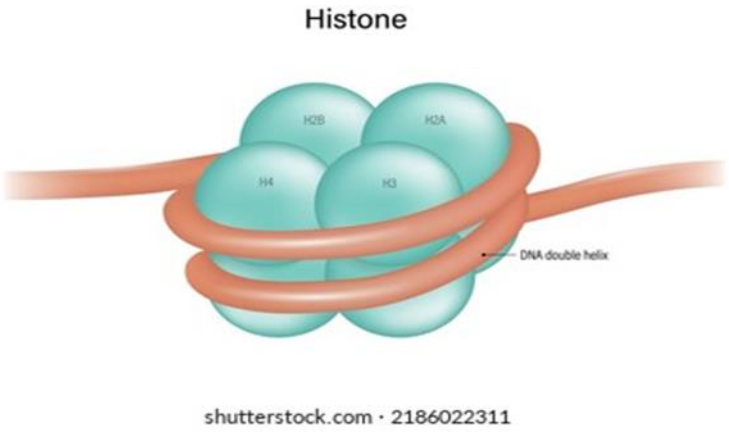


Figure 2.5 Histone proteins assembled in the nucleosome.

### 2.6.1 Histone modification

Histone modifications, a collection of post-translational alterations to histone proteins' amino acid end terminal tails, are one of the most studied epigenetic mechanisms (Ramazi *et al.*, 2020). Histone modifications are critical cellular mechanisms that tightly regulate the chromatin structure and expression of genes which