THE EVALUATION OF THE POTENTIAL OF TETRAHYDRO-β-CARBOLINE DERIVATIVES FOR THE TREATMENT OF ADDICTION USING *IN-VIVO* MODEL WITH ZEBRAFISH AND USING LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY FOR THE NEUROTRANSMITTER ANALYSIS

SITI MAZLEENA BINTI MOHAMED

UNIVERSITI SAINS MALAYSIA

2022

THE EVALUATION OF THE POTENTIAL OF TETRAHYDRO-β-CARBOLINE DERIVATIVES FOR THE TREATMENT OF ADDICTION USING *IN-VIVO* MODEL WITH ZEBRAFISH AND USING LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY FOR THE NEUROTRANSMITTER ANALYSIS

by

SITI MAZLEENA BINTI MOHAMED

Thesis submitted in fulfilment of the requirements
for the degree of
Doctor of Philosophy

October 2022

ACKNOWLEDGEMENT

In the name of Allah, the Most Gracious and the Most Merciful.

First of all, praise be to Allah Almighty for his consent and blessing to complete the project. I wish to express my heartfelt gratitude to my main supervisor, Professor Dr. Mohd Nizam Mordi, for devoting his utmost time and generous efforts, and patience to his supervision. I want to extend my deepest thanks and appreciation to my co-supervisor, Professor Dr. Shariff Mahsufi Mansor, for his assistance, patience, encouragement, and dedication in accomplishing this project. Also, I would like to express my gratitude to my co-supervisor, Assoc. Prof. Dr. Mohd Nazri Ismail and his staff for providing me with guidance and research facilities. I want to thank my co-supervisor, Professor Dr. Chong Shu Chien @ Alexander, and his students, who gave me new experimental skills in the animal study.

I want to extend my appreciation to the Centre for Drug Research (CDR) staff especially Mr. Abdul Rahim Ali Musa, Mrs. Nur Aziah Hanapi, Mrs. Siti Najmi Syuhadaa Bakar, Mr. Mohd Khairil Ismail, Mr. Mohd Hilman Sulaiman, and others who were always helpful and assisted in my Ph.D. journey. Special thank goes to Dr. Mohammed Oday Ezzat, Ahmad Saifuddin Mohamad Arshad, and all my friends for their valuable suggestions and support over the last few years.

This work was supported by the Research University (RUI) Grant Scheme (1001/CDADAH/8011705), Universiti Sains Malaysia, and the Ministry of Higher Education of Malaysia under the HICoE Programme (311/CDADAH/4401009). Last but not least, thanks to the Ministry of Higher Education of Malaysia for awarding me the Skim Latihan Akademik Bumiputera (SLAB) during my Ph.D. journey.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF SYMBOLS

µ	Mu
µM	Micro Molar
mm	Millimeter
mg	Milligram
m/z	Mass To Charge Ratio
eV	Electronvolt
min	Minute
mL	Milliliter
^{o}C	Degree Celcius
U	Unit
µL	Micro Liter
nm	Nanometer
rpm	Revolutions Per Minute
L	Liter
S/N	Signal To Noise Ratio
µS	Microsiemens
hrs	Hours

LIST OF ABBREVIATIONS

AA	Arachidonic Acid
AC	Adenyl Cyclase
AADK	National Anti-Drug Agency
AANTs	Amino Acid Neurotransmitters
Ach	Acetylcholine
ACN	Acetonitrile
cAMP	Cyclic Adenosine Monophosphate
CNS	Central Nervous System
COX	Cyclooxygenases
COMT	Catechol-O-Methyltransferase
CPP	Conditioned Place Preference
DA	Dopamine
DOPAC	3,4-Dihydroxyphenylacetic Acid
DST	Drug Substitution Therapy
EMEM	Eagle's Minimum Essential Medium
ECD	Electrochemical Detection
GCMS	Gas Chromatography-Mass Spectrometer
GABA	ɤ-Aminobutyric Acid
Glu	Glutamate
GPCR	G-Protein-Coupled-Receptor
HPLC	High-Performance Liquid Chromatography
HVA	Homovanillic Acid
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
MAO	Monoamine Oxidase
MHPG	3-Methoxy-4-Hydroxy Phenylglycol
MOP	μ-Opioid Receptor
MTT	3-(4,5-Dimethyl-2-Thizolyl)-2,5-Diphenyl-2H-Tetrazolium Bromide
MeOH	Methanol
Nac	Nucleus Accumbens
NE	Norepinephrine

NT	Neurotransmitters
NG108-15	Neuroblastoma X Glioma Hybrid Cells
NIH	National Institutes Of Health
NOP	Nociception Opioid Receptor
ORL1	Opioid Receptor-Like-1
PBS	Phosphate Buffer Saline
PKA	Protein Kinase A
RA	Retinoic Acid
SH-SY-5Y	Human Neuroblastoma Cell Line
SK-N-SH	Human Neuroblastoma Cell Line
THβC	Tetrahydro-β-Carbolines
VMA	Vanillylmandelic Acid

PENILAIAN POTENSI TERBITAN TETRAHIDRO-β-KARBOLINA UNTUK RAWATAN KETAGIHAN MENGGUNAKAN MODEL *IN-VIVO* DENGAN IKAN ZEBRA DAN MENGGUNAKAN KROMATOGRAFI CECAIR JISIM -TANDEM SPEKTROMETRI UNTUK ANALISIS NEUROTRANSMITTER

ABSTRAK

Rawatan ketagihan dadah merangkumi terapi tingkah laku, ubatan atau gabungan. Ubatan yang digunakan dalam rawatan adalah terhad dan diketahui kesan sampingannya. Kajian ini menilai sifat ketagihan sebatian tetrahidro-β-karbolina yang disintesis kerana kajian sebelumnya menunjukkan bahawa sebatian tersebut memberi kesan positif terhadap aktiviti analgesik. Sebanyak empat puluh sembilan sebatian telah disaring untuk sifat fizikokimia sebelum dipilih untuk kajian *in vitro* dan *in vivo* yang lebih lanjut. Daripada empat puluh sembilan sebatian, empat belas sebatian dipilih untuk penyaringan *in-vitro*. Empat sebatian menunjukkan ketoksikan terhadap sel SK-N-SH, dua sebatian tidak larut di dalam media sel. Dengan itu lapan sebatian diteruskan untuk aktiviti fungsi reseptor melalui penghasilan cAMP. (6-methoxy-1,3,4,9-tetrahydro-2H-β-carbolin-2-yl)(phenyl)ethanone (OD1b) and 6-methoxy-2,3,4,9-tetrahydro-1H-β-carbolin-1-one (OD2a) menunjukkan rangsangan cAMP yang tertinggi. Radar bioavailibiliti keserupaan drug menunjukkan OD1b and OD2a terletak di dalam julat optimum yang mencadangkan sebatian tersebut mepunyai sifat menyamai dadah. Kesan ganjaran OD1b dan OD2a dinilai melalui teknik tingkah laku kecenderungan tempat berkondisi (CPP) menggunakan ikan zebra sebagai model haiwan dan neurotransmiternya di dalam otak dan badan diukur. Ikan zebra didedahkan kepada tiga kepekatan iaitu 0.75, 1.5, and 3.0 mg/L melalui

rendaman terus ke dalam air. CPP telah mencadangkan bahawa kedua-dua OD1b dan OD2a sebatian menunjukkan perubahan kecenderungan paling ketara pada kepekatan 1.5 mg/L. Satu kaedah HPLC-MS/MS yang cepat, peka dan memilih telah dibangunkan untuk mengukur pelbagai neurotransmiter, dan metabolitnya serta prostaglandin E2 di dalam sampel ikan zebra dalam masa 5 minit. Had pengkuatitian untuk dopamina (DA), serotonin (SE), norepinefrina (NE) dan metabolitnya iaitu asid 3,4-dihidrosifenilasetik (DOPAC), asid homovanilik (HVA), dan asid 5-hidroksiindola-3-asetik (5-HIAA) adalah 2.0, 1.5. 5.0, 30, 600 and 5.0 ng/mL. Kaedah ini juga dapat mengukur OD1b, OD2a, and prostaglandin E_2 (PGE_2) dengan had pengkuatitian 0.3, 2.0, and 2.0 ng/mL. Selepas pemberian tunggal 1.5 mg OD1b atau OD2a ke dalam satu liter air sistem, tiada perubahan pada aras dopamina dalam sampel ikan zebra tetapi peningkatan pada aras SE and 5-HIAA serta pengurangan aras NE. Secara keseluruhannya, kajian CPP menunjukkan bahawa OD2a dan OD1b mempunyai profil ganjaran yang lebih rendah daripada morfin.Bagi SE dan 5HIAA, perolehan SE yang lebih rendah (nisbah 5HIAA/SE) untuk OD2a dan OD1b mencadangkan kemungkinan kesan sampingan yang berkaitan dengan sebatian ini. Sebatian OD1b dan OD2a memerlukan pemeriksaan lanjut untuk laluan β-arrestin. Kajian lanjut diperlukan mengenai kesan ganjaran untuk memahami keselamatan dan mekanisme tepat interaksi OD2a dan OD1b dengan reseptor sebagai calon untuk rawatan ketagihan dadah.

THE EVALUATION OF THE POTENTIAL OF TETRAHYDRO-β-CARBOLINE DERIVATIVES FOR THE TREATMENT OF ADDICTION USING *IN-VIVO* MODEL WITH ZEBRAFISH AND USING LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY FOR THE NEUROTRANSMITTER ANALYSIS

ABSTRACT

Drug addiction treatment can include behavioural therapies, medications, or their combination. The available drug used in medication treatment is limited and has known side effects. The present study evaluates the addictive property of synthesized tetrahydro-β-carboline derivatives compounds since the previous study showed that the compounds positively impact analgesic activity. Forty-nine compounds have been screened for physicochemical properties before selecting further for *in-vitro* and *in-vivo* studies. Out of forty-nine compounds, fourteen compounds were chosen for *in-vitro* screening. Four compounds showed toxicity toward SK-N-SH cells, and two compounds were insoluble in the cell medium. Thus, only eight compounds proceeded for receptor functional activity through cAMP production. (6-methoxy-1,3,4,9-tetrahydro-2H-β-carbolin-2-yl)(phenyl)ethanone (OD1b) and 6-methoxy-2,3,4,9-tetrahydro-1H-β-carbolin-1-one (OD2a) showed the highest cAMP stimulation. Bioavailability radar for drug-likeness showed OD1b and OD2a located inside the optimal range suggesting good drug-like properties of the compounds. The rewarding effects of OD1b and OD2a were evaluated using the behavioural technique of conditioned place preference (CPP) using zebrafish as an animal model, and their neurotransmitters in the brain and body were measured. The zebrafish were exposed to three concentrations of 0.75, 1.5, and 3.0 mg/L by immersion in water.

The CPP has suggested both OD1b and OD2a showed the most significant change in preference at 1.5 mg/L of concentration. A quick, sensitive, and selective HPLC-MS/MS method has been developed to simultaneously quantify various neurotransmitters and their metabolite and prostaglandin (PGE_2) in zebrafish samples with a run time of 5 min per sample. The limit of quantification (LOQ) of dopamine (DA), serotonin (SE), norepinephrine (NE), and their respective metabolites of 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 5-hydroxyindole-3-acetic acid (5-HIAA) were 2.0, 1.5, 5.0, 30, 600 and 5.0 ng/mL respectively. The method can also quantify OD1b, OD2a, and prostaglandin E2 (PGE_2) with LOQ levels of 0.3, 2.0, and 2.0 ng/mL, respectively. After a single administration of 1.5 mg OD1b or OD2a into per liter system water, there were no changes in the level of dopamine in zebrafish samples but an increase in the level of SE and 5HIAA while NE level was reduced. Overall, the CPP study demonstrates that OD2a and OD1b have a rewarding profile of lower than morphine. As for SE and 5HIAA, a lower SE turnover (ratio of 5HIAA/SE) for OD2a and OD1b suggests a possible side effect associated with these compounds. The OD1b and OD2a compound warrants further screening for the β-arrestin pathway. Further investigations are needed regarding rewarding effects to understand the safety and precise mechanism of OD2a and OD1b interaction with the receptors as a candidate for drug addiction treatment.

CHAPTER 1

INTRODUCTION

1.1 Background

Addiction is a condition that occurs when an organism engages in compulsive behaviour despite the possibility of negative consequences. This is a rewarding reinforcement or behaviour (NIDA, 2007). Drug addiction can be defined as a need to seek and consume the drug, a lack of control in limiting consumption, and the development of harmful emotional symptoms such as dysphoria, anxiety, and irritability when access to a drug is restricted (Koob et al., 2009).

Opioid addiction and abuse of opioids (heroin or morphine) are the major problems that cause severe social and economic issues worldwide. According to the U.S. National Survey on Drug Use and Health, 165.3 million people, 12 or older, used a substance such as tobacco (21.1%), alcohol (50.8%), kratom (0.3%), or an illicit drug (13%) in 2019. In Malaysia, 128325 drug and substance abusers and addicts were registered with the National Anti-Drug Agency (AADK) in 2020, with 95.5% men and 4.5% women, and 65.0% contributed by youths aged 19-39 years old. The statistics showed that 30.8% of them had taken opiates 9 (https://www.adk.gov.my/en/).

Drug addiction treatment may include medications, behavioural therapies, or a combination. Medication treatments are critical for many patients, especially when combined with counseling and behavioural therapies. Buprenorphine and methadone have been approved as safe and effective treatments for opioid addiction. However, because of the limited choice of drugs for medications, besides the side effects of the available drugs, researchers are attracted to the drug discovery field for an alternative to the available medicines with lesser side effects.

In 1841, the first β-carboline alkaloid known as harmalin was isolated from *Peganum harmala* or Zygophillaceae, Syrian Rue. β-carbolines are likely to occur naturally due to their simple biogenesis from tryptamine or tryptophan (Laine *et al*.,2014). β-carbolines have been reported to appear in mammalian fluids and tissues and the human brain (Herraiz & Galisteo, 2003) and are also rich in marine invertebrates (Shen et al., 2011).

Tetrahydro-β-carbolines (THβCs) (1,2,3,4-tetrahydro-9H-pyrido[3,4-b]indole) are naturally occurring indole alkaloids formed by Pictet-Spengler condensation of indoleamines, aldehydes, and/or alpha-keto acids. A pyridine ring is integrated into an indole skeleton to form a three-ring structure in THβCs. Many synthetic pharmaceuticals and naturally occurring indole alkaloids contain the THβC ring system, with various structural types and biological activities (Shi et al., 2013, Bondzic and Eilbracht, 2008). THβC exhibits a wide range of biological, psychopharmacological, and toxicological activities. The THβC was found to possess of anti-convulsant (Barbero et al., 2010), anti-tumor (Shi et al., 2013), antimalarial (Gupta et al., 2008), antiviral and fungicidal (Song et al., 2014),

antiparasite (Walton et al., 2009), PDE5 inhibitors (Mohamed et al., 2011), antimicrobial, anti-inflammatory, antioxidant, neuroactive, vasorelaxant, and psychoactive or neurotoxic actions (Herraiz & Galisteo, 2014).

The effects of β-carbolines on the central nervous system (CNS) have been documented in previous research. β-carboline skeletons are widely used in pharmacology research due to their high affinity for serotonin receptors in the CNS. THβC is also known to inhibit monoamine oxidase and monoamine uptake. Besides, THβC also binds to the benzodiazepine receptor. Researchers have hypothesized the potential involvement of these alkaloids in the central nervous system, where they could act as mild neuromodulators (Herraiz & Galisteo, 2003). Various β-carboline derivatives are involved in the treatment of alcoholism (Kotha et al., 2011).

1.2 Problem Statement

Addiction and abuse of opioids have become a significant health crisis worldwide. Morphine is a popular and potent analgesic. Morphine relieves pain by acting directly on the central nervous system (CNS). At the same time, morphine leads to many side effects, including respiratory depression, addiction, and tolerance. The morphinan skeleton is a chemical structure of morphine. Morphinan is a broad chemical class that includes opiate analgesics and other psychotropic drugs. It has been chemically modified to develop selective molecules for different opioid receptors and has agonistic or antagonistic activity. The skeleton of 6-methoxy THβC (6MTHβC) is not a morphinan but a simplified mitragynine structure, an alkaloid isolated from *Mitragyna speciosa* through a ring reduction (Al-Azzawi,

2018). Agonist opioid receptor molecules different from morphine scaffold may induce other conformational changes in the opioid receptor and activate their signaling pathways. Thus, the 6MTHβC compounds might present significant therapeutic advantages in treating addiction and enhanced analgesic effects without addiction development. A previous study showed that most 6MTHβC derivatives synthesized in the laboratory possess analgesic activity (Al-Azzawi, 2018). Hence, the present study aims to evaluate the addictive properties of 6MTHβC derivatives with various substitutions at the C1 and N2 positions of the scaffold synthesized by Al-Azzawi 2018. The objectives of this study are listed as follows:

1. To evaluate the purity, ~~s~~olubility, and toxicity of 6MTHβC compounds.
2. To measure the cAMP concentration in human neuroblastoma (SK-N-SH) cell line treated with selected 6MTHβC compounds
3. To predict the physicochemical and absorption, distribution, metabolism, and excretion (ADME) properties of the selected 6MTHβC compounds using *in silico* technique.
4. To evaluate addictive behaviour in terms of rewarding property following 6MTHβC compound exposure to zebrafish animal model using the Conditioned Place Preference (CPP) paradigm.
5. To develop and validate a sensitive and selective analytical method using the LCMS/MS technique for measuring simultaneously the neurotransmitters of dopamine (DA), serotonin (SE), and norepinephrine (NE) and their respective metabolites of 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 5-hydroxyindoleacetic acid (5-HIAA),

together with prostaglandin E_2 (PGE_2) and 6MTHβC compounds in the zebrafish brain and biological matrix

6. To determine the concentration of neurotransmitters (DA, SE, and NE) and their metabolites (DOPAC, HVA, and 5-HIAA), selected 6MTHβC compounds and PGE_2 in the brain and body of zebrafish following the CPP experiment.

1.3 Research flowchart

Figure 1.1 shows the summary of the research flowchart of this study.

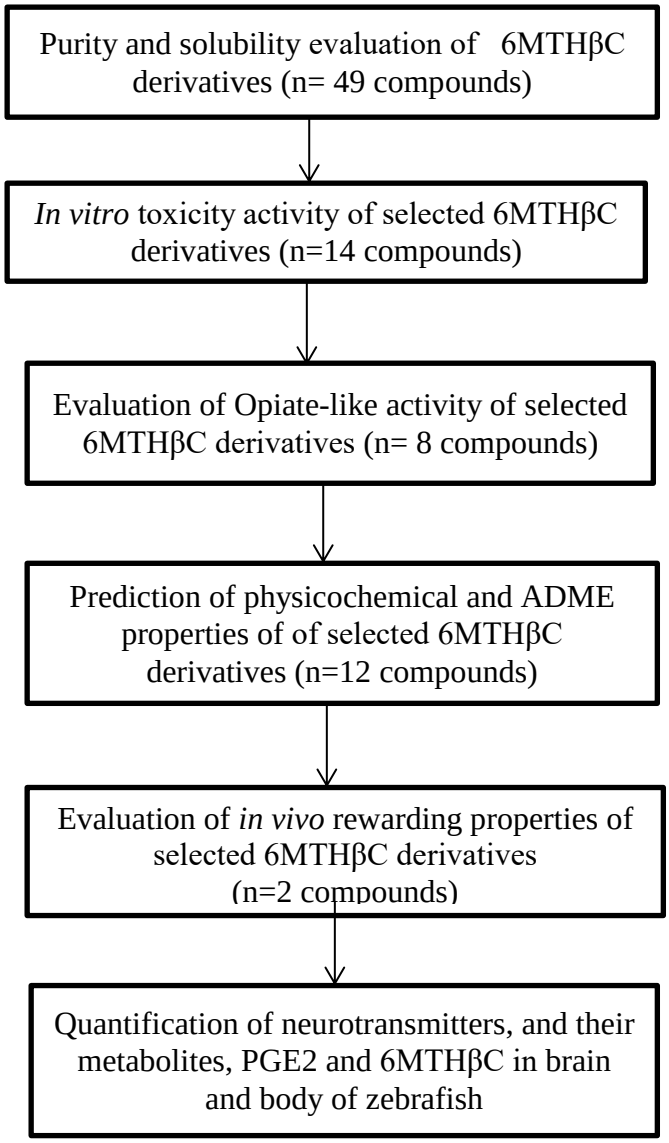

Figure 1. 1 Research flowchart of the present study
*Note: n= number of compounds

CHAPTER 2

LITERATURE REVIEW

2.1 Drug addiction and its treatment

Drug addiction is a chronic disease characterized by compulsive or uncontrollable drug seeking and use despite harmful consequences and changes in the brain. The changes in the brain may lead to unhealthy behaviours (NIDA, 2019). The goal of drug addiction treatment is to assist addicts in ending their obsessive drug seeking and usage. The therapy can take various approaches, handle many forms, and last for different time lengths. Drug addiction treatment can include behavioural therapies, medications, or a combination. Many patients require medications, especially counseling and other behavioural therapies. A patient may require various services and treatment components during treatment and recovery. Individuals addicted to heroin or opioids are prescribed medication to stabilize their lives and reduce illicit drug use (NIDA 2018). Methadone, buprenorphine, and naltrexone are an example of drugs used in medication treatment. The government decided to start medication treatment in Malaysia after realizing that the institutional and community treatment and rehabilitation program was ineffective (Vicknasingam & Mazlan, 2008). The government introduced Drug Substitution Therapy (DST) in early 2005 to manage nicotine and opioid dependence (Mohamed & Kasa, 2005). DST is also known as agonist pharmacotherapy, agonist replacement therapy, or agonist-assisted therapy. DST is a medically supervised administration of a psychoactive substance that is pharmacologically dependent on

the substance delivers the desired therapy outcome (Mohamed & Kasa, 2005). The therapy was initially started with antagonist medication (naltrexone) and moved towards agonist medication (buprenorphine and methadone) to treat drug users (Vicknasingam & Mazlan, 2008). Table 2.1 lists several medicines used for the treatment of drug addiction.

Table 2. 1 Medication used for the treatment of drug addiction (Koob et al., 2009)

Name	**Addiction**	**Year of FDA approval**
Disulfiram	Alcohol	1954
Methadone	Opiate	1972
Naltrexone	Alcohol	1994 and 2005 (extended-release formulation)
Bupropion (Wellbutrin/Zyban; GlaxoSmithKline)	Nicotine	1997
Buprenorphine (Subutex; Schering–Plough)	Opiate	2002
Acamprosate (Campral/Aotal; Merck–Serono/Forest Laboratories)	Alcohol	2004
Varenicline (Chantix/Champix; Pfizer)	Nicotine	2006
Nicotine replacement therapy	Nicotine	-

*Note: FDA, US Food and Drug Administration; NMDA, N-methyl-d-aspartate.

Methadone maintenance therapy (MMT) is one of the most effective methods of addiction pharmacotherapy. Methadone is an agonist to the opioid receptor with similar pharmacological properties to morphine. It exists as d and l forms, and 1 isomer has the best analgesic activity (Verthein et al., 2005). The majority of methadone used in clinics is a racemic mixture. Methadone is an excellent maintenance agent due to its unique properties. It is orally active and long-acting (a single dose suppresses opioid withdrawal symptoms) for 24-36 hours without euphoria, sedation, or analgesia (Mohamed & Kasa, 2005). Methadone is well absorbed, and levels are detectable at 30 minutes, with the highest concentrations

occurring four hours later, and it is 90% bound to plasma protein. Methadone effects allow patients to function normally while still experiencing normal pain and emotional responses. Methadone also has the added benefit of suppressing cravings (Leri et al., 2007). Pyrrolidines and pyrroline are methadone metabolites excreted in urine and bile (Shurman et al., 2010). Methadone's elimination half-life ($t_{1/2}$) is approximately 22 hours, but there is significant interindividual variability, ranging from 5 to 130 hours (Eap et al., 2002). According to research, methadone improves overall health and well-being, decreases criminal activity, boosts mortality, decreases blood-transmitted diseases, improves psychosocial functioning, and is a reasonably inexpensive treatment method (Mohamed & Kasa, 2005).

Buprenorphine is a synthetic opioid that is 25 to 40 times more potent as an analgesic than morphine following parenteral administration (Huang et al., 2001). It is a methadone substitute with agonist/antagonist properties, has prolonged action duration, and is given on alternate days (Mohamed & Kasa, 2005). Buprenorphine, like methadone, has several modes of action and is a partial agonist activity at the μ-opioid receptor (MOP) and nociception opioid peptide receptors (NOP). Its analgesic activity *in vivo* at low and intermediate doses has a bell-shaped response curve (Mcdonald, 2005). Buprenorphine has a high affinity for μ-opioid receptors, and high lipophilicity may cause it to act longer at higher doses. Buprenorphine, unlike methadone, is poorly absorbed orally and must be administered sublingually. Buprenorphine also has a relatively good safety profile. It can block the exogenous opioid euphoria and prevent opioid withdrawal, and doses many times higher than usual therapeutic doses appear to result in clinically minor respiratory depression. This property makes buprenorphine an appealing alternative to methadone as it has lower abuse potential than full opioid agonists, which have higher efficacy.

Buprenorphine has significant advantages over methadone in terms of pharmacology. Aside from the lower risk of overdose, it suppresses gonadotropin-releasing hormones less than methadone, which has a less impact on libido (Blanco-Gandía & Rodríguez-Arias, 2018).

Alternative drug substitutions are required because methadone does not work for everyone (Mohamed & Kasa, 2005). Thus, this present study targeted to screen the properties of tetrahydro-β-carbolines (THβCs) compounds, which might be the alternative to methadone and buprenorphine.

2.2 Drug addiction theory

Addiction is a chronic relapsing condition marked by a lack of control over drug intake, a solid urge to receive the drug, and a continuous desire to obtain the drug (Yager et al., 2015). Individuals addicted to alcohol, tobacco, or other drugs continue to use them in ways that injure themselves or others physically, psychologically, or socially. The following features describe drug addiction (Carter & Medzihradsky, 1992):

1. An decreased ability to control drug use
2. Drug use causes significant physical and psychological harm that worsens over time.
3. A long period of heavy drug use leads to tolerance to drug effects and withdrawal symptoms. Even if there is a high risk of relapse to drug use, individuals should quit if the service is abruptly terminated.

Drugs that are addictive have different molecular targets and, as a result, physiological impacts. By definition, addictive drugs have the same propensity to generate a state in which a person (human or model organism) seeks the drug compulsively despite harmful consequences and the presence of other typically enjoyable stimuli (Liat, 2016). Different drugs develop varied addiction patterns depending on the dose, length of use, and cultural circumstances. A compulsive intravenous or smoked drug-taking habit develops with opioids, characterized by extreme intoxication, tolerance development, escalation in intake, profound dysphoria, physical discomfort, and physical and emotional withdrawal indicators during abstinence (Spanagel, 2003). Several biological pathways linked to associative learning can be triggered by addictive drugs, including stimulation of dopamine D1 receptors, activation of the cAMP/PKA/CREB signal transduction pathway, and a temporary burst of altered gene expression synaptic rearrangements (Berke & Hyman, 2000).

2.2.1 Mechanism of drug addiction

A reward is a property that is shared by many addictive drugs. Addictive drugs may act upon the brain's central "reward pathway" to motivate some people to use them repeatedly, often despite the harm, they cause to themselves and others (Carter & Medzihradsky, 1992). It is enjoyable to receive rewards. Addiction, on the other hand, causes harm. A reward is a pleasurable and arousing experience that responds to discrete stimuli. Addictions are chronic, compulsive, uncontrollable maladaptive, and destructive behaviors. The researchers hypothesized that rewards and addiction have similar neurobiology (Adinoff, 2004).

Several brain parts are involved in the reward pathway (Figure 2.1). The ventral tegmental area (VTA), the nucleus accumbens, and the prefrontal cortex were all involved. Information travels from the VTA to the nucleus accumbens and then to the prefrontal cortex when activated by a rewarding stimulus (NIDA, 2020). Stimulating receptors result in the release of various neurotransmitters in the brain. Dopamine (DA) is one neurotransmitter that gives a pleasurable experience and is necessary for drug reinforcement. In a drug-induced reward, dopaminergic neurons in the VTA of the midbrain and the shell of the nucleus accumbens are required, as both regions are involved in pleasure and reward perception (Benowitz, 2010).

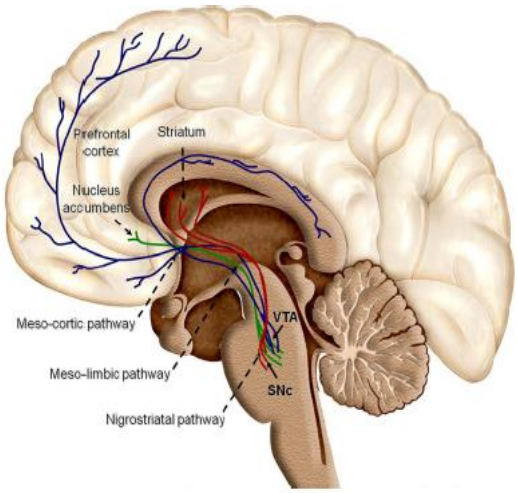

Figure 2. 1 Reward pathways in the human brain. The reward's brain structures were the ventral tegmental area (VTA), the nucleus accumbens, and the prefrontal cortex. The midbrain structures substantia nigra (SNc), and the VTA comprises the dopaminergic neurons (Arias-Carrián et al., 2010)

2.2.2 Opioid system in drug addiction

Opiates, such as morphine and endogenous opioid peptides, are classified as having pharmacological and physiological effects on target tissues by binding to opioid receptors on the cell surface. Opiates are categorized as agonists, partial

agonists, or antagonists based on their impact on opioid receptors. Agonists interact with the receptor to elicit the most excellent possible response from that receptor. Antagonists, on the other hand, bind to receptors but have no functional response. At the same time, it prevent an agonist from binding to that receptor. While partial agonists bind to receptors, they only elicit partial active reactions regardless of the amount of drug administered (Pathan & Williams, 2012). Opioid receptors are members of the large superfamily of G-protein-coupled-receptor (GPCR). The receptor subtypes have been identified pharmacologically and genetically by researchers. Hydrophobicity analyses of the cloned opioid receptors' deduced amino acid sequences revealed that these receptors have seven putative transmembrane helices, typical of the G-protein coupled receptor family (Satoh & Minami, 1995). The original receptor subtypes are Mu (μ), kappa (κ), and delta (δ), with opioid receptor-like-1 (ORL1) being the least studied (Al-Hasani & Bruchas, 2011). Numerous studies have linked all four opioid receptors to behavioural effects such as analgesia, reward, depression, anxiety, and addiction. Opioid receptors are found throughout the central and peripheral nervous system and play a minor role in various physiological processes.

The Mu (μ) opioid receptors (MOR) were the last to be cloned. MOR receptors have more than 15 serine, threonine, and tyrosine residues that can be phosphorylated by the protein kinases (Al-Hasani & Bruchas, 2011). It is found throughout the central nervous system in sensory and motor function areas and regions concerned with integrating and perceiving these senses, such as the cerebral cortex of the amygdala (Mcdonald, 2005). Morphine has a high binding affinity to μ-opioid receptors; thus, it is of particular interest and is linked to drug addiction and

pain perception modulation (Kaufman et al., 1995). μ-opioid receptor agonists, such as morphine, are extensively used to treat severe pain, with severe side effects, including respiratory depression, tolerance, withdrawal symptoms, decreased gastric motility, and emesis (Zhang et al., 2004). Most clinical opioid analgesics and anesthetics have significant agonist activity at the μ-opioid receptors (Volpe et al., 2011).

MOR receptors are the primary molecular target for morphine *in vivo* and mediate beneficial and harmful effects in most opiate users. The μ-opioid receptor also mediates rewarding properties of non-opioid abuse drugs, such as cannabinoids, alcohol, and nicotine, as well as natural reinforcers such as social interactions. As a result, the μ-opioid receptor is a critical molecular trigger for reward and is thought to play a role in initiating addictive behaviours (Contet et al., 2004). The intracellular signaling molecules of the second messenger change when a ligand binds to its receptor. The second messenger relays other signals to the ultimate target, which can cause a cellular response. Cyclic AMP, cyclic GMP, 1,2-diacylglycerol, inositol 1,4,5-triphosphate, and Ca^{2+} are the most important secondary messengers produced in cells (Newton et al., 2016).

Kappa opioid receptors (KORs) are found throughout the central and peripheral nervous systems, regulating various physiological systems (Jacobson et al., 2020). KOR signaling is a critical component of the brain reward function, with the hypothalamus being the most abundant. The KOR was the second opioid receptor family member to be cloned. MOR and KOR receptor shares some regulation characteristics as they are quickly phosphorylated, desensitized, and internalized with

MOR regulation (Al-Hasani & Bruchas, 2011). KORs are found presynaptically on dopaminergic, glutamatergic, and serotonergic terminals and inhibit the release of neurotransmitters (Clark & Abi-Dargham, 2019; Pirino et al., 2020). The KOR system, in particular, is significantly associated with stress and addiction because of its role in aversive, dysphoric, and anxiety-related behaviours (Przybysz et al., 2021). In humans and animals, stimulation of KOR receptors, the endogenous receptor for the dynorphin-like peptides, inhibits dopamine release in the striatum and causes a negative mood state. KOR antagonists also promise to treat addiction and depression (Lewicky et al., 2020). KOR receptor agonists have an advantage over other opioid ligands because they do not induce respiratory depression (Mcdonald, 2005).

The delta-opioid receptors (DORs) are found throughout the central nervous system, including the hippocampus, hypothalamus, basal ganglia, and amygdala. The most abundant opioid receptor protein in the striatum is DOR. Unlike MOR and KOR, the DOR receptor was thought to exist more than 90% at intracellular sites (Al-Hasani & Bruchas, 2011). The stimulation of the DOR initiates intracellular signaling via G protein-dependent or independent pathways for most GPCRs (Pradhan et al., 2011). In contrast to MOR, the density of DOR receptors in nociceptive circuits of the midbrain and brainstem is extremely low. DORs are promising targets for treating pain and mental disorders because of their low potential for addiction (Zhou et al., 2021). DORs are widely dispersed in nociceptive pathways in the peripheral and central nervous systems; hence DOP agonists were hypothesized to be analgesics for chronic pain (Nagase & Saitoh, 2020). Previous research has suggested that DOPs play a role in primary pain processing and emotional and cognitive aspects of pain (Wawrzczak-Bargieła et al., 2020).

According to pharmacological and genetic studies, the DOR activity modifies the rewarding properties of various abused substances. It influences several aspects of addictive behaviour, such as drug-seeking, emotional responses, and learning processes.

2.2.3 Cyclic adenosine monophosphate (cAMP)

In recent years, researchers have discovered that opioids had additional excitatory effects on the same cellular effectors. Adenylyl cyclase (AC) synthesized cyclic adenosine 3',5'-monophosphate (cAMP) from adenosine triphosphate (ATP). AC is regarded as an essential mediator of opioid-induced analgesia and reward. The cAMP pathway is a critical mediator of opioid action (Zhou et al., 2021). The cAMP was the first recognized second messenger. It is found in almost all organisms, from prokaryotes to eukaryotes (Antoni, 2000). It is essential for cellular responses to many hormones and neurotransmitters (Sassone-Corsi, 2012). Hormones and neurotransmitters increase Ca^{2+} current, Ca^{2+} uptake, and intracellular calcium level by stimulating adenylyl cyclase activity and cyclic AMP formation (Sarne et al., 1998). The cAMP is a tightly controlled molecule influenced by G-protein-coupled-receptor (GPCR) activation and other cellular processes. Cellular cAMP levels indicate receptor activation and other cellular processes (Chiulli et al., 2000). Hormones and neurotransmitters act through receptors that activate GTP-binding proteins (G proteins), which causes adenylyl cyclase to synthesize cAMP at the cell membrane (Avidor-Reiss et al., 1997). The interplay of AC and cyclic nucleotide phosphodiesterase (PDE) enzymes regulate intracellular cAMP levels.

Cellular cAMP production begins with a stimulatory ligand binding to GPCR on the cell surface. The G_s protein is activated in the molecular process, increasing the AC activity and converting ATP to cAMP and inorganic pyrophosphate. A single ligand-binding event synthesizes hundreds of cAMP molecules, dramatically increasing the cAMP level. Ligands can inhibit AC activity, stimulating GPCRs linked to Gi and cAMP degraded by phosphodiesterases (PDEs). The combination of these mechanisms allows for continuous cAMP concentration control. The mechanism of cAMP production as illustrated in Figure 2.2. GPCRs activate most ACs through interactions with the G_s protein subunit (α). Agonist ligands activate AC when they bind to GPCRs. Heterotrimeric αβγ G-protein complexes emit α_s. The βγ subunits can also stimulate some AC isoforms. As a result of AC activation, cAMP is produced, activating cAMP-dependent protein kinase (PKA). Acute activation of inhibitory G_i receptors inhibits adenylyl cyclase inhibition, whereas chronic activation of such receptors increases cAMP accumulation (Avidor-reiss et al., 1997).

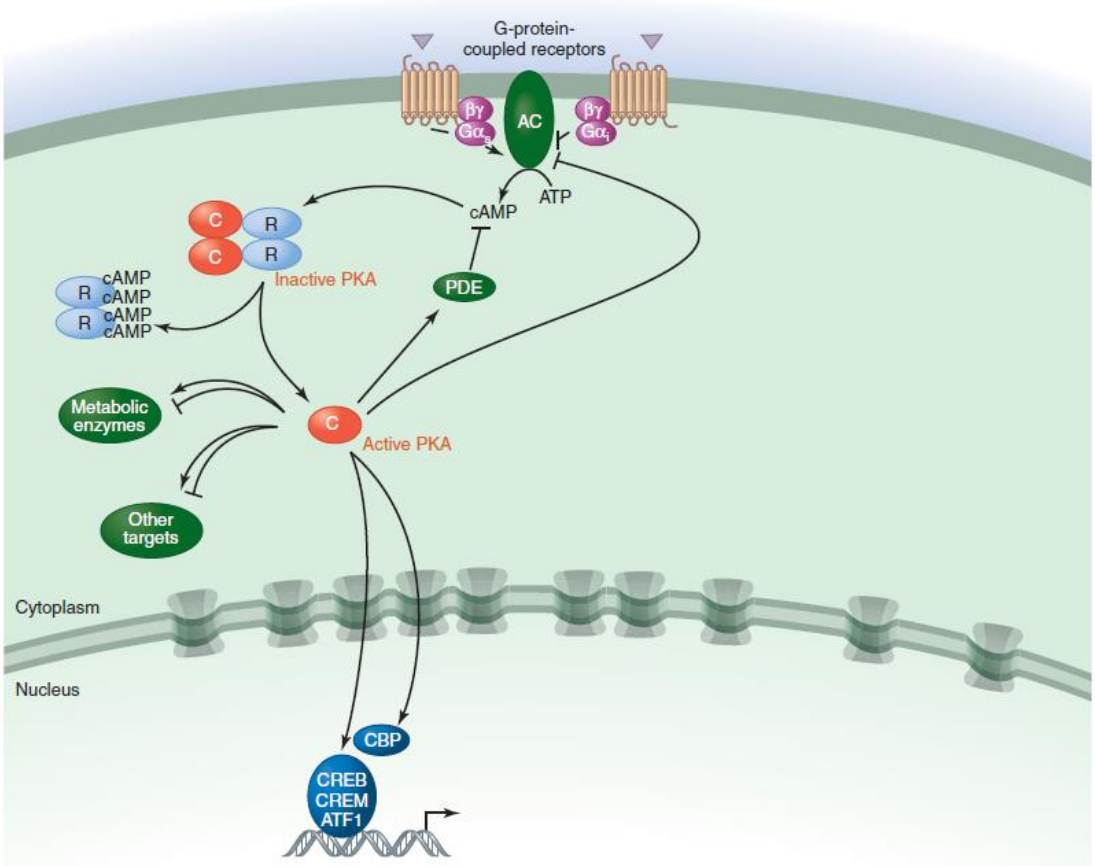

Figure 2. 2 The cAMP mechanism. The symmetrical complex of two regulatory (R) subunits and two catalytic (C) subunits cAMP-response element-binding protein (CREB), cAMP- responsive modulator (CREM), transcriptional coactivators CREB- binding protein (CBP), Activating Transcription Factor 1(ATF1) (Sassone-Corsi, 2012)

2.2.3(a) Human Neuroblastoma Cell line (SK-N-SH) As Cellular Model for Measurement of cAMP Production

Human neuroblastoma cells are valuable models of μ-receptor systems because the functional effects of opioids are primarily mediated through the μ-receptors in this cell line (Yu et al., 1988). Another human neuroblastoma cell line model, SK-N-SH and SHSY-5Y, was recently established. The SHSY-5Y was estimated to express 50,000 μ and 10,000 δ sites/cells, respectively (Yu et al., 1986).

Therefore, the μ-receptor dominates the opioid effects rather than the δ-receptor (Yu et al., 1990).

Marshall Nirenberg's group at the National Institutes of Health (NIH) was the first to study morphine addiction using a cellular model system of Neuroblastoma x Glioma hybrid cells (NG108-15) (Xia et al., 2011). Among the several cell lines tested, the researchers discovered that Neuroblastoma x Glioma hybrid cells (NG108-15) have an abundance of opiate receptors. Table 2.2 summarizes the data on using a human neuroblastoma cell line to determine cell activation via cAMP accumulation.

Table 2. 2 Human Neuroblastoma Cell line in the cAMP determination

Cell Type	Cell treatment	Reference
SH-SY5Y & SK-N-SH	PGE2	(Yu et al., 1988)
SH-SY5Y	Morphine & Naloxone	(Yu et al., 1990)
Neuro 2A	Morphine & Naloxone	(Chakrabarti et al., 1995)
SK-N-SH	Morphine	(Ratka and Simpkins, 1997)
NG108-15	Mitragynine	(Tohda et al., 1997)
SK-N-SH	DAMGO & Naloxone	(Fields & Sarne, 1997)
SK-N-SH	DAMGO & DPDPE	(Sarne et al., 1998)
NG108-15	Methadone	(Liu et al., 1999)
SK-N-SH	Morphine-3-glucuronide, Morphine-6-glucuronide, Naloxone	(Baker et al., 2000)
SK-N-SH	Morphine, endomorphin	(Yu et al., 2003)
SH-SY5Y	Cumene Hydroperoxide	(Juan et al., 2011)
NG108-15	Morphine & Naloxone	(Xia et al., 2011)
SK-N-SH	Mitragynine & Morphine	(Jamil et al., 2013)

2.2.4 Neurotransmitters

Most psychoactive drugs act as agonists or antagonists at the receptors of the central nervous system's endogenous chemical messengers (Wise, 1998). The human brain contains approximately 100 billion nerve cells and neurotransmitters for over

99% of the communication between neurons. Neurotransmitters (NTs) and their metabolites are abundant in mammals' central nervous systems and peripheral body fluids. They play an essential role in the nervous systems of many organisms (Maximino et al., 2010). Endogenous metabolites known as neurotransmitters serve as messengers in intracellular signaling across the central nervous system (Tufi et al., 2016). Investigating the NT's metabolic profile requires trace level studies in biological material. The changes of NTs and their metabolites in neurophysiology, behavioural effects, pathology, and disease diagnosis are associated with Alzheimer's disease, Parkinson's disease, Down's syndrome, schizophrenia, depression, cocaine addiction, and epilepsy (Akyuz et al., 2021; Carlsson et al., 1999; Factor et al., 2017; Moret & Briley, 2011; Sangubotla & Kim, 2018; Tomkins & Sellers, 2001; Walker et al., 2011).

Neurotransmitters are classified according to their chemical structure: catecholamines, amino acids, indoleamines, neuropeptides, and acetylcholine (Ach). For amino acid neurotransmitters (AANTs), there is glutamate (Glu) and its metabolite ɤ-aminobutyric acid (GABA). Meanwhile, the monoamine neurotransmitters (MANTs) are norepinephrine (NE), serotonin (5-hydroxytryptamine, 5-HT or SE), and dopamine (DA). The acidic metabolites, 5-vanillylmandelic acid (VMA), hydroxyindole-3-acetic acid (5-HIAA), 3-methoxy-4-hydroxy phenylglycol (MHPG), and homovanillic acid (HVA) 3,4-dihydroxyphenylacetic acid (DOPAC) also the MANTs. Figure 2.3 shows the molecular structures of studied neurotransmitters. SE is synthesized from the amino acid tryptophan, whereas DA is synthesized from tyrosine. Monoamine oxidase

(MAO) metabolized both DA and SE to metabolite DOPAC and 5-HIAA. DOPAC is further metabolized to HVA by catechol-O-methyltransferase (COMT) (Figure 2.4).

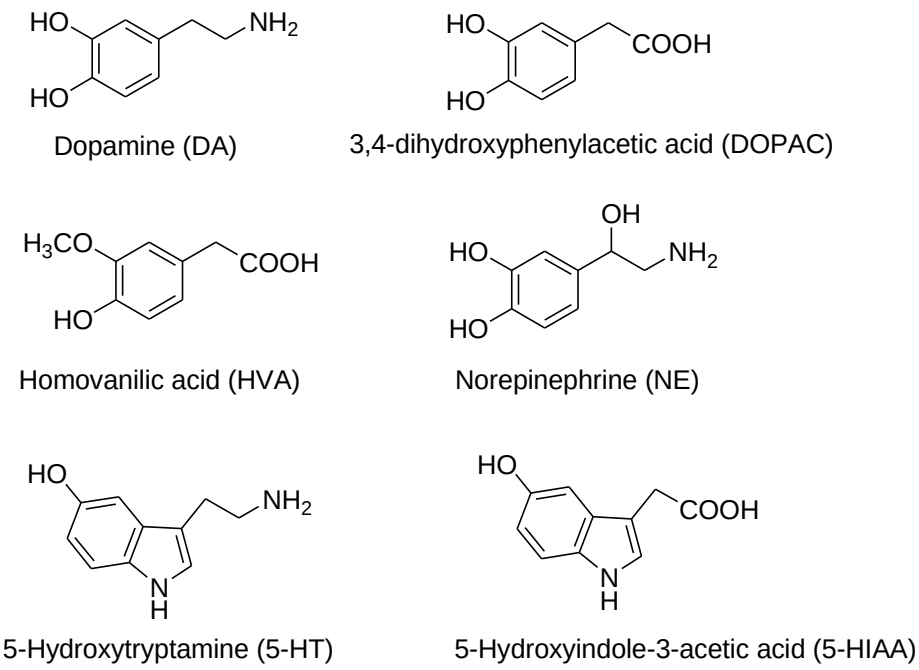

Figure 2. 3 Structure of the neurotransmitters examined in brain and body of zebrafish samples

Dopamine was first implicated in medial forebrain bundle electrical stimulation (Wise, 1998). DA is a neurotransmitter produced in the substantial nigra, ventral tegmental area, and brain's hypothalamus. DA and SE were reported to be the two primary monoamine neurotransmitters in the brain. DA is the most widely implicated in the drug addiction mechanism, not just as a substrate of psychostimulant reward but also as a substrate of drug-related learning and neuroadaptation (Di Chiara, 2000).

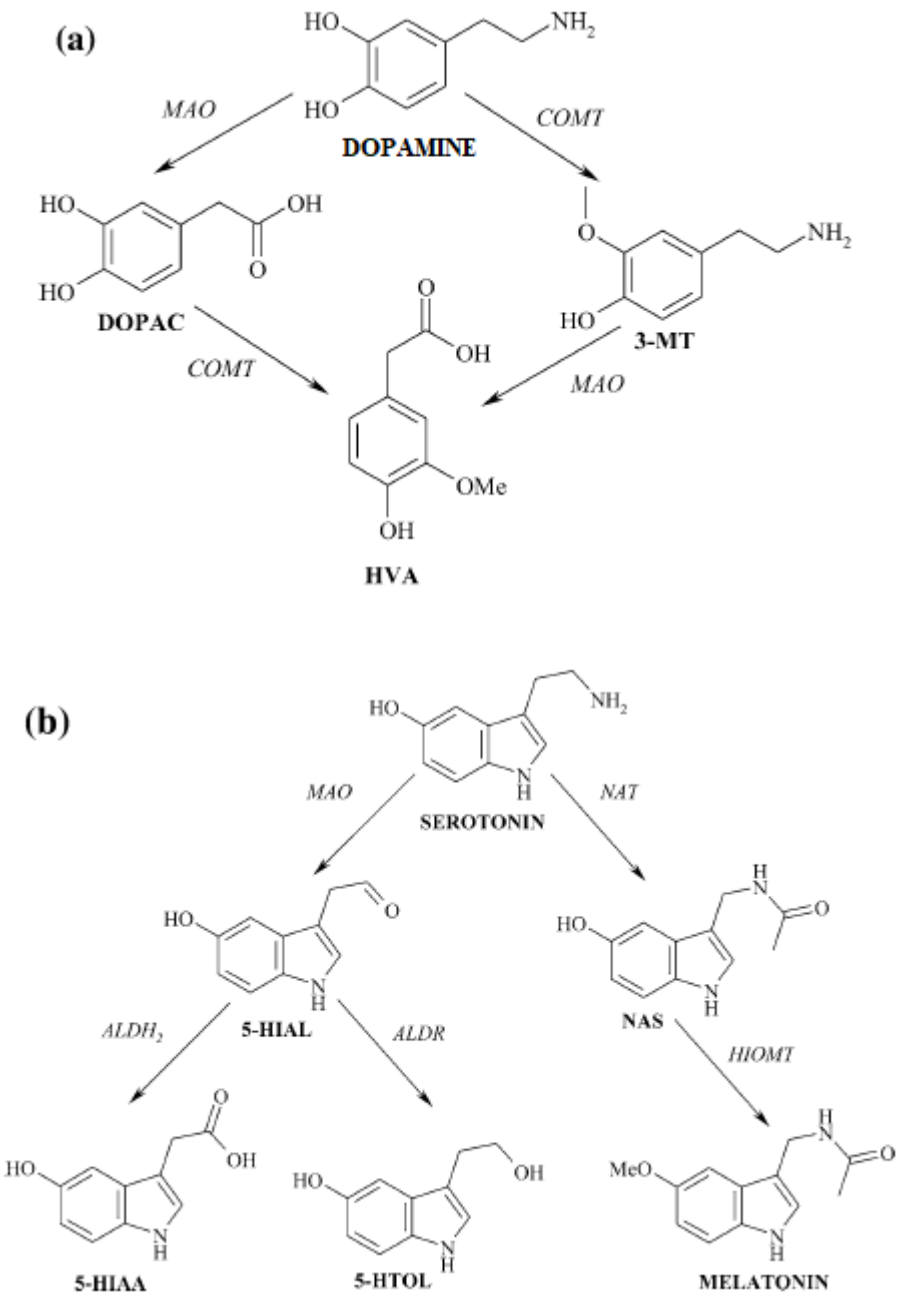

Figure 2. 4 Enzyme-catalyzed metabolic pathways of (a) DA and (b) SE (Najmanová et al., 2011)

The DA and SE mediate several essential brain functions, where DA regulates locomotion, cognition, emotion, and reward (Rico et al., 2011). Abnormalities in their levels are associated with different human central nervous system (CNS) diseases. G–protein-coupled receptors mediate the effects promoted

by DA. DA plays an essential role in the abuse mechanism (Charbogne et al., 2014). One of the essential systems involved in the reward system is the dopaminergic system. This system includes the midbrain dopaminergic system in vertebrates (Simpson & Kelly, 2011). Dopamine projections to the striatum and frontal cortex have been shown to play an essential role in mediating behaviour and learning in behavioural studies (Schultz, 2002). DA neurons in the mesolimbic reward circuit respond to rewarding stimuli, such as food, sexual interaction, drug abuse, and music. Changes in dopamine activity caused by drugs are critical in developing addictive behaviours. Human abused substances, such as alcohol, amphetamine, caffeine, cocaine, marijuana, nicotine, and opiates, increased the extracellular DA concentrations.

SE functions as a neurotransmitter in the body's central and peripheral nervous systems (Moriarty et al., 2011). SE is an important modulator of brain physiology and behaviour and plays a role in vertebrate CNS development and plasticity. Studies have shown that brain SE neurons' cell bodies are located primarily in the medial and the dorsal raphe nuclei (Ciccocioppo, 1999). SE is responsible for perception, aggression, anxiety, sexual behaviour, appetite, vascular function, and pain. Serotonergic neuron dysfunction during development or adulthood has been linked to various psychiatric disorders, including depression, drug addiction, and schizophrenia (Rico et al., 2011). Gerlai et al. (2009) found an increase in DA and SE levels in adult zebrafish brains due to alcohol addiction. In zebrafish, the role of serotonin in locomotor behaviour has been studied in both larvae and adults. While in larvae, SE does not change the frequency of spontaneous

swimming. The opposite is seen in adult zebrafish exposed to a bath application of SE, reducing locomotor rhythms frequency (Maximino et al., 2010).

The noradrenergic system is made up of both central and peripheral sympathetic pathways. Norepinephrine (NE) is its primary chemical messenger (Fisch et al., 1983, Sofuoglu & Sewell, 2009). NE serves multiple brain functions, including arousal, attention, mood, learning, memory, and stress response. NE is a catecholamine actively transported from the blood and formed from the amino acid L-tyrosine (Figure 2.5). The metabolic pathway's rate-limiting enzyme regulates converting L-Tyrosine to L-dihydroxyphenylacetic acid (L-dopa) via tyrosine hydroxylase. Dopa-decarboxylase converts L-dihydroxyphenylacetic acid into DA, the immediate precursor of NE, and transports it into storage vesicles. DA-β-hydroxylase (DBH), containing storage vesicles found in NE-specific neurons, convert the stored DA to NE via β-hydroxylation. DBH-free storage vesicles are found in dopamine-producing neurons. A loss or change in any neurotransmitter is likely to affect other neurotransmitters and potentially lead to behavioural deviations (Fisch et al., 1983). In preclinical addiction models, NE plays an important role in mediating stimulant effects such as sensitization, drug discrimination, and drug-seeking reinstatement. Even though the role of NE in reward mediation has been recognized for over four decades, NE has received little attention as a potential treatment target for stimulant addiction (Sofuoglu & Sewell, 2009).