The effects of a street cocktail drug (nyaope) on the opioid signalling system in the rat brain and benefits of *Moringa oleifera* extracts

Matome Michael Sekhotha

790890

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Declaration

I, **Matome Michael Sekhotha**, declare that this dissertation 'Characterizing the effects of a street cocktail drug (nyaope) on the opioid signalling system in the rat brain' is my unassisted work. It is being submitted for the Degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

(Signature of candidate) 17 June 2022 in Polokwane

Abstract

The use of adulterated drugs is a worldwide public health problem. Since 2010, South African black communities have been administering an adulterated drug called nyaope. The method of administration is mainly through smoking and intravenous injection on an interval basis. There is evidence that the active ingredient of nyaope is heroin. Heroin has devastating health outcomes. Several drug rehabilitation centres have used phytomedicine to mitigate the curb substance use disorder. The study aimed to assess the effect of nyaope on the behavioural pattern of rats and the ability of moringa to reverse the negative outcome. Firstly, the nyaope samples were analysed using gas chromatography-mass spectrometry. It was subsequently found that nyaope consists mainly of a high grade of heroin. For the animal studies, forty Wistar rats (20 males and 20 females) were subjected to the open field test, elevated plus maze, novel object recognition test, and Y-maze before and after being treated with nyaope. Subsequently, animals were sacrificed, and the brain and liver were harvested for further analysis using ELISA and IDEXX respectively. The active ingredient of nyaope was heroin mixed with other human health toxic compounds. Animals that were administered with nyaope displayed slow locomotor activity and high levels of anxiety-like behaviour in the open field test and the elevated plus-maze. To our knowledge, this is the first study to determine the effects of nyaope on the liver and brain. In healthy individuals, locomotor activity is mainly centred and controlled by the brain. When a person experiences SUD, BDNF or other neurotrophic factors are often prescribed to impact the functioning of the nigrostriatal dopaminergic pathway, thereby influencing locomotor behaviour. Administration of nyaope led to significant abnormalities in locomotor activity as reflected by a marked decrease in total distance travelled in the open field test, and this observation was more pronounced in female animals. In addition, female animals also displayed a greater likelihood of anxiety-like behaviour by spending significantly more time against the wall of the open field in comparison to saline controls as well as male animals. The histology results showed that some organelles in the liver were damaged as a result. There were modified liver enzyme concentration levels in the nyaope-treated animals suggesting nyaope-induced hepatic damage. Nyaope-treated animals that were also treated with *Moringa oleifera* showed some improvement. The plant extract improved total distance travelled and therefore locomotor activity as well as reduced the amount of time spent against the wall of the open field, reflecting decreased levels of anxiety-like behaviour. The levels of the liver enzymes of nyaope-treated animals were also similar to that of saline controls. The present study showed that administration of nyaope has some negative health impacts on

the body, and that phytotherapeutics can reverse some of the deleterious effects of the drug. Our findings indicate that nyaope may have deleterious effects on specific organs that may contribute to its malfunctioning. There is a need to do further research to investigate the health impacts of nyaope on the hippocampus and compare the effectiveness of herbal extracts and opioid antagonists. The use of *Moringa olifeira* showed some promising results. But further research is needed to look at phytomedicine and substance abuse disorders.

Acknowledgements

I am grateful to be allowed to express my profound thanks and appreciation to Professor W Daniels, the head of the School of Physiology in the Faculty of Health Science. He was my supervisor during my PhD. I would like to recognise his efforts as supervisor, his continuing guidance, support, enthusiasm, advice, constructive criticism and the great latitude he provided me to conduct this study and complete this thesis. I never can repay him for all his efforts. I also like to thank Dr Dee Muller for your support. I would also like to extend my gratitude and thanks to Sharlot Malatshwana, Khethani Mathikhi and Monica Gomes for your kind support and friendship. Mr Vincent "Jones" Njoni, you are a star with your technical abilities and support. Wits Central Animal Services staff members, thank you for your kind support. Wits Physiology staff members, you treated me like one of your staff members. A special thanks go to Mr Eliot M Sibuyi, Ms L Tladi and Ms K Mpherwane for their wise words. Thank you to the University of Limpopo for funding my studies and to the NRF for providing grants through my supervisor.

My late Daddy (Alpheus M Sekhotha) and late mother (Johannah N Sekhotha) will be proud of my achievement. Thanks and appreciation to my family (Lesedi Odirile Thabang, Naled iOmelemo Boitumelo, Kholofelo Angela) and extended family (Rose-Mary, Omphile and Onkgopetso) who were always beside me with their prayers, patience, unconditional love, inspiration and support whenever I needed it. Finally, my thanks go out to anyone who has helped in one way or another during this degree. Thank God for giving me the patience, guidance, wisdom and strength to complete my study.

Vincent "Jones" Njoni

My journey towards obtaining a PhD

It all started in 2016 when I approached Prof W Daniels as Dean and Head of the University of Kwa-Zulu Natal's School of Laboratory Medicine and Medical Sciences, the Disciplines of Clinical Anatomy and Forensic Medicine during the Physiological Society of South Africa (PSSA) conference. He was then appointed as the head of school at the University of the Witwatersrand in 2017. I approached him to pursue a Doctor of Philosophy in physiology. I got registered in the second semester of 2017 with the help of the University of Limpopo staff support grants. Our main focus was to investigate the effect of nyaope using Wistar rats. This topic was selected to provide adequate scientific evidence of the effect of nyaope on the human brain. Currently, there is not adequate scientific literature to provide clear evidence of the effect of nyaope on the brain or any other body system in South Africa.

In order to obtain an adequate nyaope sample, we had to request permission for a licence to be in possession of such a chemical compound. The application form was sent to the South African Health Product Regulatory Authority (SAHPRA). The licence was issued and was valid for 12 months. The next step was to obtain the nyaope samples without jeopardising the study, researcher and the institutions. I approached the South African police service forensic laboratory official to provide us with the 500g nyaope sample. It took us almost 18 months to obtain such a quantity with the help of General Nghokha. Prior to getting hold of the samples, there were several meetings held between myself and the General, who later introduced me to the laboratory manager. The SAPS procedure was very extensive. On one occasion, I had to locate the General while he was attending a strategic meeting. This was to show the seriousness of my attitude in order to obtain the nyaope sample. My credentials and criminal record were checked before the release of the sample.

Obtaining nyaope illegally through the black market would have been very expensive and dangerous. One could get arrested or killed by the nyaope dealers. Another nyaope sample was obtained from the Mankweng Police station with the help of station commander Colonel Mathabatha. The local police station does not have a consistent protocol for disposing of the nyaope samples. The Mankweng sample was sent to the Witwatersrand chemical pathology laboratory for analysis. The samples were given to the Master Science student to analyse as part of her dissertation in order to attain a qualification. The M.Sc. student failed to deliver the protocol and later deregistered.

In 2019, we recruited a Master's student under the supervision of Prof Daniels Williams to investigate the effect of nyaope on neuroblastoma cells (Ms Sharlot Malatswana). Necessary connections were made with the local supply and we later purchased two vials of neuroblastoma cells, only to discover that they were contaminated. One of the biggest challenges with neuroblastoma development was contamination and the use of the appropriate culture medium. The cell culture team struggled to grow the neuroblastoma, but it ended up being contaminated. The journey to attain a PhD was travelled with my supervisor. We then initiate a consolation period at least once or twice a month. This was of great help to understand the addiction mechanism that might lead to the subsequent consequences of the administration of nyaope over time. The biggest obstacle was that the topic was very new to my line of learning. As a result, I had to read a lot to familiarise myself with the terminology in the neurosciences. The topic changed several times. Once, we were looking into the lateral habenula, and later into the immunology. None of those topics made it into the final protocol that was submitted to the research committee for approval.

Finally, we decided to look for the effect of nyaope on certain sections of the brain. I compiled a protocol and submitted it to my supervisor. My supervisor was a perfectionist especially when it came to writing. I had to rewrite my protocol several times before it made sense to him. Thanks to Prof for such support. Later, the research committee invited, Prof Daniels and Dr Neville to defend the protocol. One needs to applaud the Witwatersrand research committee for their prompt response, which was later approved. A special thanks must go to Prof Kennedy for providing unlimited support throughout the stages of this project. All of the abovementioned activities were conducted by myself, travelling between the University of Limpopo and the University of the Witwatersrand. In April 2019, I submitted my first manuscript to my supervisor and never received any feedback. I had hoped that I would receive feedback within a year, but it never happened. Yet, with this set back one had to work very hard to achieve a qualification.

This never lowered my commitment and dedication to strive for more and work harder to complete my qualification within a stipulated duration. This never stopped my monthly meetings with my supervisor to pave the way forward. In 2019, I was offered a sabbatical leave from 01 July 2019 to 15 January 2020. This time was dedicated solely to data collection in the Witwatersrand Central Animal Services (CAS). The old section of the CAS was under intended renovation initiated almost a year ago and never happened until I finished my data collection. In June, we met with CAS official Ms Amelia to outline the plan for my project.

List of abbreviation

ACC - Anterior cingulate

CG - anterior cingulate gyrus

CBHSQ - Center for Behavioral Health Statistics and Quality

CICAD - Inter-American Drug Abuse Control Commission

CNS – Central nervous system

COVID-19 – Coronavirus disease

CRF - Corticotrophin-releasing factor

DA - Dopamine

DAT - dopamine transporter

dPFC - dorsal prefrontal cortex

DSM - Diagnostic and Statistical Manual of Mental disorders

FC - Frontal cortex

GABA - Gamma-aminobutyric acid

GC - Gas chromatography-

GDP - Guanosine diphosphate

GIRK - G-protein activated inward-rectifying potassium

GPCR - G-protein-coupled receptor

GTP - guanosine-5′-triphosphate

MDMA - Methylenedioxymethamphetamine

MRC - Medical Research Council

MS - Mass spectrometry

NAcc - nucleus accumbens

NR - Negative reinforcement

OFDA - Office of U.S. Foreign Disaster Assistance

PTSD - Post-traumatic stress disorder

SA- -South Africa

SACENDU - South African Community Epidemiology Network on Drug Use

S-R - Stimulus-response

SUD - Substance use disorder

THC - Tetrahydrocannabinol

UNODC - United Nations Office of Drugs and Crime

USA - United States of America

VTA - Ventral tegmental area

WDR - World Drug Report

WHO - World Health Organisation

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CHAPTER 1: Introduction

1.1 Background of the study

According to the United Nations Office of Drugs and Crime (UNODC), the use of adulterated illicit street drugs is a worldwide public health problem (UNODC, 2004). These commonly consumed adulterated illicit street drugs are often mixed with other unknown substances, generally only known to the producers and dealers of these substances (Coomber, 1997). World Health Organisation (WHO) and Inter-American Drug Abuse Control Commission (CICAD) stated that the addition of other materials to the core compound is a common practice worldwide meant to increase the efficacy of the drug (WHO, 2007; CICAD, 2019). The resulting drug mixture usually does not have any scientific nomenclature but is given a local name (WHO, 2017). For example, in the USA cocaine is often referred to as crack, coke or snow; the street name for heroin is Big H, hell dust or smack methamphetamine is called speed, meth or ice; and MDMA (3,4 methylenedioxymethamphetamine) is called ecstasy, eve or love drug. Other street drugs are krokodil (desomorphine which is an opioid derivative), butane hash (pure tetrahydrocannabinol, THC) extracted from cannabis (Lazarjani *et al*., 2021), khat (naturally occurring cathinone, an amphetamine-like substance), flakka (alpha-pyrrilodinopentiophenone, a cathinone-like substance), and mandrax (methaqualone) (Rock & Moore, 1976). In South Africa, similar drugs of abuse are known by different names for instance heroin-based drugs are known as “whoonga” and “nyaope”, while “tik” is a methamphetamine-based street drug (Shukla, 2016). Nyaope has become a substantial community health challenge amongst both black and white community members in South Africa (Nkosi, 2017).

Thus, the purity of street drugs is extremely adjustable and can be subject to the region of production or synthesis. Evidence shows that there is a broad spectrum of ingredients in adulterated street drugs (UNODC, 2008). As a result, several of these ingredients used during the preparation are typically commonly available substances that are easily accessible. Ingredients include household chemicals, e.g., VIM dish detergents, rodenticides that can be purchased over the counter or prescribed medication (e.g., Codeine, fentanyl, hydromorphone, morphine, oxycodone, oxymorphone and tramadol (Coomber, 1997; Fernandes & Mokwena, 2016; Mokwena, 2015). The variability in the components of street drugs makes it essential

to establish the exact chemical composition of the drug of choice before it is used in scientific experiments. The drug of interest in the current study was nyaope. Ingredients added during the synthesis of nyaope are only known to the primary dealer. Notwithstanding, the variety of additives used during the preparation of nyaope may add another level of complexity to the effect of these drugs on the brain. Repetitive consumption of nyaope is associated with undesirable changes to lifestyle that may involve aggressive sensation-seeking behaviour (Morgan *et al*., 2018). If this sensation-seeking behaviour is not properly controlled by health care professionals and family members, it tends to develop into a full-blown substance use disorder (SUD) that over time may lead to death (Fernandes and Mokwena, 2015). SUD is defined as a mental sickness or illness that negatively affects a person's thinking, feeling, mood, and behaviour in the brain leading to a person's inability to control the administration of substances of abuse such as legal or illegal drugs, and alcohol, or medications over time.

Despite this tragic possibility, to sustain their adulterated illicit drug lifestyle, the user is often forced to develop unlawful strategies to enable them to purchase these drugs. Consequently, these users adopt various skills to acquire the necessary funds to sustain their lifestyles, including criminal activities such as theft, robbery, and assault, (Coomber, 1997), and menial jobs such as car guards at parking lots, garden services, and recycling of refuse (Morgan *et al*., 2018). The World Drug Report (WDR), stated that the prevalence of adulterated illegitimate drug usage in South Africa is considerably lower compared to Europe, Asia and America (WDR, 2010). Nyaope, being a relatively new prohibited recreational depressant adulterated illicit drug, is mainly consumed by South African youths residing in townships (Peltzer *et al*., 2010). Sadly, the Office of U.S. Foreign Disaster Assistance (OFDA) reported that the use of adulterated illicit drugs has a significant negative effect on the socio-economic status of families, communities and countries at large (OFDA, 1997). Despite this rather alarming societal situation, limited scientific studies are investigating the pathophysiology of nyaope in South Africa. In the last two decades, a few scattered reports focused on nyaope and its consumption by school learners and other youths in socio-economically deprived settings (Fernandes and Mokwena, 2016; Mokwena, 2006). Due to the scarcity in the scientific literature on nyaope, the present study relied on data from heroin literature to extract the appropriate information for its experiments. This study was conducted using previous research that suggested that the main active ingredient of nyaope was heroin (Mokwena, 2015; Mokwena & Huma, 2014; Khine *et al*., 2015; Mthembi *et al*., 2019). Considering the above,

the foremost aim of the current study was to characterise the toxic effects of nyaope on the body, with a focus on brain and liver function, using animal models. The following questions were addressed:

1. What is the chemical composition of nyaope?
2. How does nyaope intake affect behaviour such as locomotor activity, anxiety-like behaviour, and learning and memory?
3. To assess whether the changes in behaviour are associated with alterations in brain chemistry due to the administration of nyaope.
4. Can these changes in behaviour be reversed by *Moringa olifeira* treatment?
5. Does nyaope intake damage the liver?

I hypothesised that nyaope, with its high heroin content, will alter behavioural patterns, underpinned by changes in neurochemistry in the frontal cortex. I further proposed that nyaope is toxic to the liver. In addition, I hypothesised that these deleterious effects can be reversed by treatment.

1.2 Outline of the thesis

Herewith is a framework of this thesis

Chapter 1 introduces the context in which the study was conducted.

Chapter 2 gives an overview of the important topics related to the study, together with background to some of the pertinent methodological approaches.

Chapter 3 describes the methodology adopted to collect data.

Chapter 4 presents all the results generated by the study.

Chapter 5 interprets and discusses the results using the current literature**.**

Chapter 6 reports on the potential of *Moringa oleifera* to reverse nyaope-induced deleterious effects

Chapter 7 contains our conclusions of the study, reflections on its limitations and recommendations for further experimentation.

The references used in the study are listed at the end

CHAPTER 2: Literature Review

2.1 The scope of substance abuse

Substance abuse is a worldwide problem (Ali *et al*., 2011). There is a continuous increase of people that administer substance abuse. According to several studies, in 2014, a total of 21.5 million people aged 12 or older, had SUDs during their developmental years ranging from early childhood to adolescence. Of these, 20.2 million were adults aged 18 years or older and represented 94.2 per cent of people who had experienced a SUD early in their childhood years (Raninen *et al*., 2016; Lipari & Van Horn, 2017; Laslett *et al*., 2012). This statistic suggests that SUD may have its origins early in life and manifests strongly during young adulthood. The latest World Drug Report of the United Nations Office of Drugs and Crime (UNODC) estimated that 35 million individuals suffer from SUDs (WDR, 2019). Sadly, only one in six of these individuals are receiving some form of treatment.

Illicit drugs have been transported into sub-Saharan Africa through a variety of world economic systems. This has led to exacerbating the use of drugs problem. The distribution of these drugs is orchestrated by transnational organized crime and drug syndicates of various sizes and individual traffickers (Affinnih, 2002; OFDA, 1997). Of the many known illicit drugs, the global production, transportation and consumption of opioids, especially heroin, have been particularly high and worrisome in many countries (WHO, 2019). The surge in substance use has an enormous impact on the economy. A dated but relevant study by Mark *et al*., (2001) estimated the economic burden of heroin usage in large parts of the United States of America (USA) in 1996 to be in the region of $21.9 billion per year. The estimate was quite comprehensive as it included costs related to treatment, forfeiture of throughput, delinquency and social welfare. In a separate study, also conducted in the USA, illicit drug use increased significantly amongst low-income individuals during times of economic difficulty (Carpenter *et al*., 2017). In South Africa, it has been found that unemployment was high among men who have been associated with drug use (Mokwena, 2014). The increase in economically inactive individuals has placed an additional burden on society (Peltzer *et al*., 2010). According to WHO reports (2004 & 2008), more than 80% of people living with a mental disorder, reside in low-middle income countries (LMICs). Although South Africa is considered an upper-middle-income country, a large portion of the

South African population experiences a low-income standard of living, and hence the prevalence of mental disorders is similar to LMICs.

Together, these statistics reflect a dire situation and are particularly alarming for developing countries where a high prevalence of low socio-economic conditions exists (WDR, 2010). Additionally, there are usually limited treatment options and rehabilitation facilities for drug users in these low-income countries (Patel *et al.,* 2007; Morgan *et al*., 2018). The enormity of the substance use problem necessitates the search for alternative solutions in addition to current management practices. As such, other forms of harm reduction interventions, abstinence programmes and replacement therapies have to be explored. Numerous African states experience a deficiency of proper monitoring systems for drug use behavioural and lifestyle patterns (EMCDDA, 2015). Therefore, the data on the prevalence of drug consumption in Africa is not available. In South Africa, though, the South African Community Epidemiology Network on Drug Use (SACENDU) systematically monitors abuse (WHO, 2017). SACENDU is a substance abuse monitoring system, driven by the Medical Research Council (MRC) of South Africa, based on individuals seeking treatment at rehabilitation centres throughout the country (UNCCP, 1999). A shortcoming of the monitoring approach is that neither the success of prevention campaigns nor the need for treatment and rehabilitation of drug abusers is included (WHO, 2007). Some drug users required accurate assessments to implement better mitigation strategies for their condition. Most national estimates of the prevalence of substance abuse are based only on rapid assessments of substance abuse among specific groups within the drug-abusing population and a limited number of school surveys. The cross-country comparability of national substance abuse estimates is therefore severely limited in Africa (UNODC, 2017; NlDA, 2021).

As a result, the gap between substance dependence and treatment services is significant and growing wider as the rate of SUD increases in different countries (Adelekan & Morakinyo, 2000). Some African countries, including Tanzania, Kenya, Mauritius, Nigeria and South Africa, have resorted to religious and traditional healing methods to identify and treat drug misuse (UNODC, 2017). Interestingly, all these countries report an increase in injectable drug use over time (Degenhardt *et al*., 2017; WHO, 2018) suggesting that these alternative

methods are also struggling to hold back the substance abuse tide. While approaches such as opioid substitution therapy have proven to reduce opiate dependence and frequency of consumption (Gowing *et al*., 2000), the need for more complementary therapies remains high, especially in areas where access to traditional treatment is limited.

2.2 Substance use disorder (SUD)

According to the fifth edition of the Diagnostic and Statistical Manual of Mental disorders (American Psychiatric Association, 2013, 5th ed.), addictive behaviours emanate from progressive adaptations occurring in the brain in response to repeated exposure to substances that trigger the reward system. It is worth noting that addictive behaviours are not limited to the abuse of substances only, but also include gambling, internet use, gender, gaming, smartphone use and shopping (Banz *et al*., 2016; Jorgenson *et al*., 2016; Derevensky *et al*., 2010; Parke & Griffiths, 2004). However, since the current study was interested in the effects of a specific street drug (nyaope) consumed by a local population, the literature review of this thesis will mainly focus on SUD and its consequences.

2.2.1 The development of substance use disorder (SUD)

SUD is a complex brain abnormality that typically develops from an initiation phase where the early recreational intake of the drug produces pleasurable effects. According to the Inter-American Drug Abuse Control Commission (CICAD), withdrawal from drug intake then leads to unpleasant side effects precipitating the repetitive use of the drug to alleviate negative symptoms (CICAD, 2019). As the drug is consumed repeatedly, often in large quantities (binge consumption), neuroadaptations take place at molecular, synaptic and pathway levels, leading to a loss of control of drug intake, eventually resulting in compulsive drug-seeking behaviour (Figure 2.1).

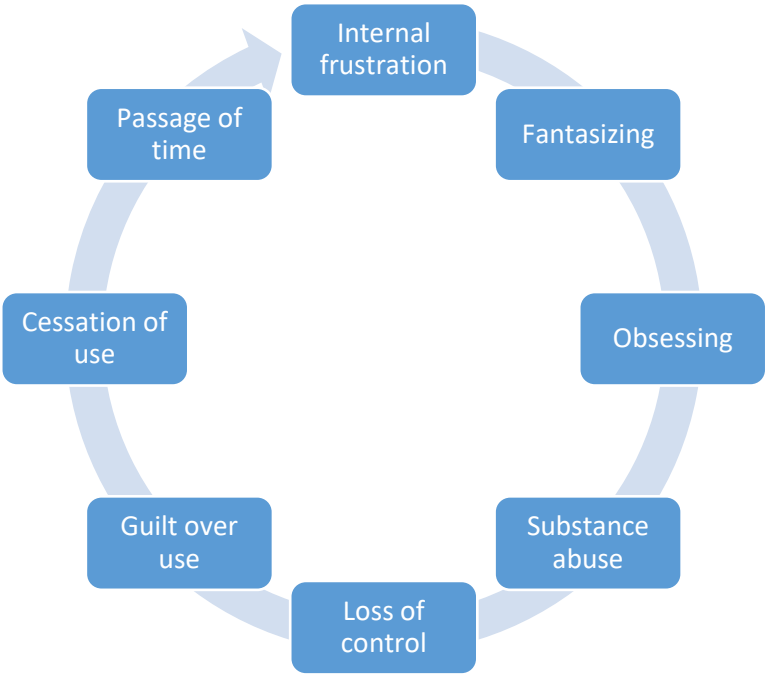


Figure 2.1: The cycle of addiction adapted from Alksne *et al*., (1967) (Appendix 3)

Substance use disorder (SUD) is a chronic, relapsing condition categorized by the obsessive use of a substance that is characterised by three distinct phases according to Koob & Le Moal (1997). The first phase is the binge–intoxication phase, whereby the substances of abuse are utilized and the principal motivation is the rewarding experience (Appendix 1). During this phase, downregulation of positive reward pathways occurs, such that increasing levels of substance intake are required to obtain similar positively rewarding experiences. The second phase is the withdrawal phase during which the drug user experiences negative consequences (i.e. unpleasant withdrawal symptoms) when consumption of the substance is discontinued. Consequently, the intake of a substance may commence primarily to avoid the negative experiences (i.e. by negative reinforcement). The third and last phase entails a preoccupation–anticipation stage, which is categorized by an inflated inspiration for the administration of a substance (i.e., craving) (Appendix 1) (Figure 2.2).

Figure 2.2 depicts a broad representation that illustrates the amalgamation of neuroadaptations in the circuitry of certain brain areas that underlie the three phases of the addiction cycle (Appendix 3), the activation of the dopaminergic (DAergic) connections linking the ventral tegmental area (VTA), and the ventral striatum/nucleus accumbens (NAcc) in the binge intoxication phase (O'Donnell, 2003). During the withdrawal negative-effect phase, the DA systems are compromised and activate the brain stress systems such as corticotropin-releasing factor (CRF) (Venton *et al*., 2004). These brain stress systems reset after detecting the presence of drugs and drug-related stimuli in the perspective of causing strong dislike when a person experiences intense feelings of depression and discontent. During the preoccupation–anticipation phase, contextual cues via the hippocampus and stimuli cues via the basolateral amygdala converge with frontal cortex (FC) activity to drive drug-seeking. Other components in the FC are also compromised, producing deficits in executive function.\

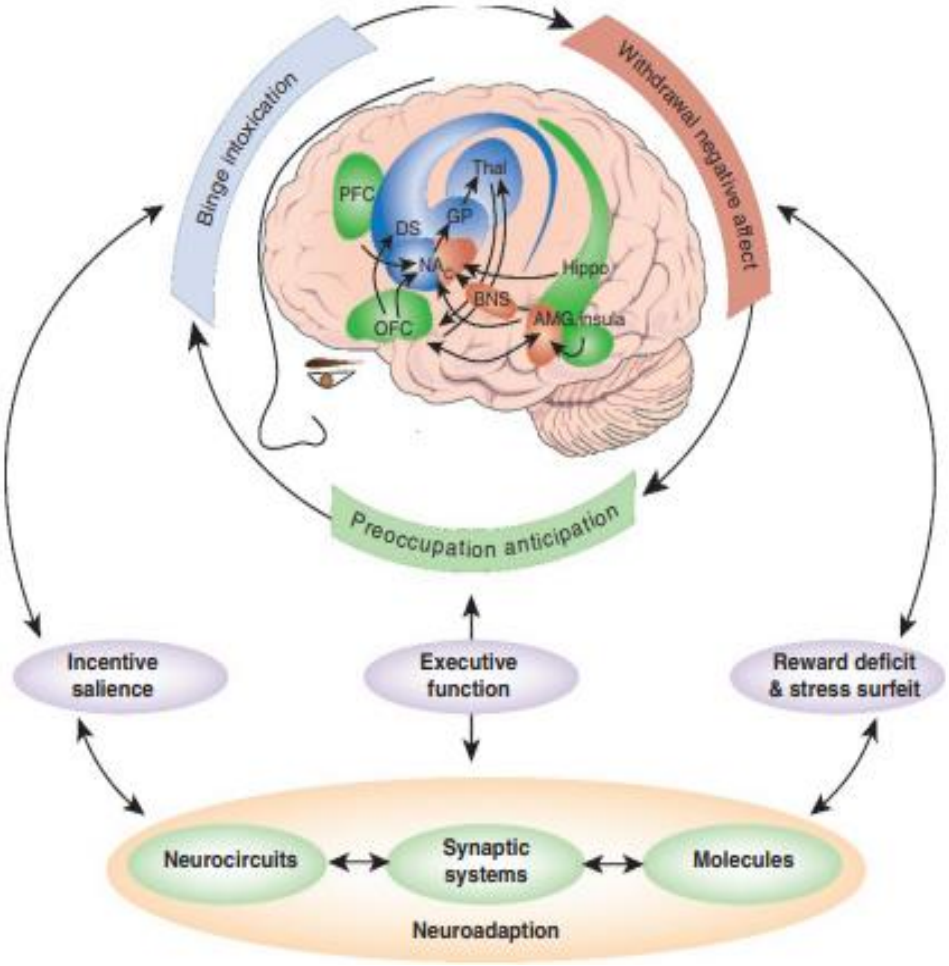


Figure 2.2: The processes that might lead to substance use disorder (Wise & Koob, 2014)

Thus, SUD manifests as a cluster of cognitive, behavioural and physiological symptoms. The main features of this disorder can be grouped as impaired control, social impairments, risky use and pharmacological criteria ((American Psychiatric Association, 2013, 5th ed.). Interestingly, substance abuse was first characterised by the hazardous use of a substance, in conjunction with social and interpersonal problems related to the use of the substance and consequent legal challenges arising from the use of the substance. Drug dependence, on the other hand, was originally defined by features such as withdrawal, tolerance, increasing consumption quantities, repeated attempts to quit, and spending much time to obtain and use the substance. Lately, both the characteristics of substance abuse and the features of drug dependence are combined, together with cravings for the substance, to make up the criteria for SUD (Hasin *et al*. 2015). Moreover, SUD often occurs as a comorbidity with other disorders. It has been noted that individuals suffering from either attention deficit or hyperactivity disorder (Van der Burg *et al*., 2019); schizophrenia (Arranz *et al*., 2018), eating disorders (Bahji *et al*., 2019), depression and anxiety-like behaviour disorders (Zhou *et al*., 2020), post-traumatic stress disorder (PTSD), may also display symptoms of SUD. Other primary mental conditions may therefore precipitate SUD (Petrakis *et al*., 2012), increasing the complexity of the manifestation, treatment and management of individuals using illicit substances.

2.3 Theories explaining the development of SUD

Below are brief descriptions of some pertinent theories that have been put forward by several scientists to explain the development of SUD.

2.3.1 Negative reinforcement (NR)

The negative reinforcement theory is based on the continuous need to consume substances of abuse driven by external factors to the consumer. These factors most often relate to economic, social, or health factors. The main aim of drug consumption in this situation is to alleviate the stress, pressure and perception of external factors, minimising the negative impact of the external factors on the mental wellbeing of the consumer. Negative reinforcement, therefore, sustains a behavioural pattern of drug-seeking and consumption over a long period, culminating in perpetual substance abuse (Wise & Bozarth, 1987). Often, the purpose of maintaining the habit is to escape the existing surroundings to avoid the noxious and unpleasant symptoms associated with drug withdrawal. In this way, the cycle

of drug-seeking, drug consumption and minimising withdrawal symptoms, characterises addictive behaviour (Siegel & Ramos, 2002; Siegel. 1988).

Interestingly, negative reinforcement can also stem from self-medication by the individual to relieve pre-existing symptoms such as pain, anxiety-like behaviour or depression. The danger of this approach to drug use is that it easily evolves into a consumption pattern underlined by a strong motivational process, resulting in regular drug use. At this point, addictive behaviour can develop without the individual realising the problem (Baker *et al*., 2004). The individual's attention is mainly focused on reducing perceived threats and negative effects and not on the damaging impact the drug may have on his/her body. An individual's response to avoid or escape negative thinking induced by external factors, therefore, stimulates the body to continuously desire more drugs. Under these circumstances, attitudes and expectations influence decision-making, leading to regular drug consumption and, eventually, to addictive behaviour (Koob, 2013).

2.3.2 Positive reinforcement (pleasure-seeking behaviour)

The positive reinforcement theory of SUD postulates that drug self-administration is maintained because of the mental state drugs induce, and not because the consumer wishes to alleviate an unpleasant condition (Stewart *et al*., 1984; Wise, 1988). In this case, drug consumption is triggered by a desire to seek the rewarding, euphoric effects of the drug. The positive reinforcement of drugs is therefore primarily ascribed to their ability to produce a pleasurable emotional state. These subjective pleasurable experiences following drug consumption are adequate to evoke compulsive drug-seeking behaviour over time (Wise & Koob, 2014). Strong support for this theory came from preclinical studies where rats were placed in chambers enabling them to self-administer drugs by pressing a lever to dispense either cocaine or heroin via intravenous or intracerebral catheters placed into the body (Mendez *et al*., 2010). These authors found that the rats would repeatedly press the lever for them to experience the positive, rewarding sensation. When these positive feelings outweigh the negative consequences, then drug use becomes perpetual and drug-seeking behaviour is positively reinforced.

2.3.3 Incentive motivational salience

Incentive motivational salience is a cognitive process that motivates an individual's behaviour towards or away from a particular object, perceived event, or outcome (Puglisi-Allegra & Ventura, 2012). It also regulates the intensity of behaviours to attain a particular goal and the amount of time and energy that an individual is willing to expend to attain a particular goal. Furthermore, it assesses the level of risk that the individual is willing to accept while working to attain a particular goal (Puglisi-Allegra & Ventura, 2012). Motivational salience consists of two components namely incentive and aversive salience (Puglisi-Allegra & Ventura, 2012). Whereby, the motivation to acquire a rewarding stimulus is an incentive salience (Berridge, 2012). A person that experiences an incentive salience treats the administration of the drug as the reward as an attractive property of a stimulus that induces approaches, or consummatory behaviour (Schultz, 2015). It is also important to distinguish between "liking" which is the immediate pleasure gained from a stimulus (Berridge, 2012; Berridge & Kringelbach, 2015), and "wanting" which is the quality of a rewarding stimulus that demands attention, induces approach, and occupies a lot of thought. The incentive motivational theory of addictive behaviour is based on the premise that an individual suffering from substance abuse has lost the ability to behave appropriately. It is mainly due to the toxic effect of the drug on neurons and circuits that normally regulate the incentive salience stimuli, particularly the DAergic pathway and limbic system in the brain (Robinson & Berridge, 2008). The neuro-adaptive changes and modifications that occur due to continuous drug administration, result in a pathological level of incentive salience such that the desire to acquire the drug replaces the initial enjoyment of using the drug. Drugs, therefore, are no longer pursued because of their euphoric effects ("liking"), but because of their incentive motivational properties ("wanting") (Berridge & Robinson, 2003). It is postulated that drug-induced neuroadaptations may persist for an extended period after discontinuation of drug use. In this way, drug-abstinent individuals become prone to relapse when exposed to "wanting" stimuli that usually present as drug-associated cues (Berridge, 2012). This explains why so many individuals relapse long after their withdrawal symptoms have disappeared: drug cues remain desired and highly salient even after physical needs to use the drugs have been extinguished (Berridge & Robinson, 2003).

2.3.4 Stimulus-response (S-R) or habit theory

The stimulus-response theory is a psychological concept based on the belief that behaviour manifests because of the interplay between stimulus and response (Spence, 1950) An individual will respond to a stimulus and develop a particular behaviour over time. In the case of addictive behaviour, the initial goal-directed drug-seeking behaviour of a drug abuser will over time change into a stimulus-response habit (habit theory). Drug-associated stimuli learnt through Pavlovian mechanisms during previous bouts of drug-taking, will trigger a response (drug seeking and intake) by the abuser without any consideration for the consequences of drug consumption (Hogarth, 2018; 2020; Everitt, 2014). The implication is that a substance abuser takes drugs in response to a drug-related cue with no consideration of the negative health effects of drug intake such as euphoric effects or pain, or excessive stress. In S-R theory, therefore, addictive behaviour is conceptualized as the reflexive response to drug-associated environmental stimuli, not driven by the value of the outcome of drug intake but rather resulting from the habitual actions of the abuser (Dickson *et al*., 1983).

2.3.5 Inhibitory control dysfunction

The inhibition of impulsive, irrational voluntary responses to stimuli is a cognitive process that directs self-control. Inhibition control is therefore an executive function that permits an individual to inhibit their impulses and natural, habitual, or dominant behavioural responses to stimuli (Malenka *et al*., 2009). Self-control is therefore important to orchestrate an appropriate behaviour to achieve specific goals emanating from the initial stimulus (Diamond, 2013). It is well-known that the chronic use of drugs leads to cognitive abnormalities including impaired self-control (Fillmore & Rush, 2002). Inhibitory control dysfunction in addictive behaviour implies that the abuser cannot stop taking a substance and consequently maintains drug dependence behaviour (Goldstein & Volkow, 2002).

In summary, SUD can be characterised by impairments in physical and mental behaviours. The drug-induced neuroadaptations in certain brain areas render the brain hypersensitive in a way that inappropriate levels of incentive salience are attributed to a drug or its associated cues. The improper accredited value to a drug or its cues, in combination with defects in cognitive decision-making ability and loss of inhibitory control, seem to underpin the motivational drive to obtain and consume a drug irrespective of its disastrous consequences.

2.4. Neurobiology underpinning substance use disorder (SUD)

SUD is defined as a recurrently degenerating condition characterised by an obligation to pursue and administer a drug (Koob & Le Moal, 1997; Koob, 2009). The reluctance of individuals who suffer from SUD to make use of available professional help and to present themselves for rehabilitation is a great concern (Lander *et al*., 2013). Understanding the neurobiological underpinnings of drug abuse is fundamental in unravelling the intricacies of addictive behaviour (Koob, 2013 & 2004). Investigations into the neurobiology of SUD that concentrate on the function of various brain areas and their involvement in different phases of the addiction cycle (Figure 2.3) is an ongoing process. Additional knowledge about the biochemical and pharmacological mechanisms of action of substances of abuse is equally important for understanding the aetiology of SUD (Table 2.1).

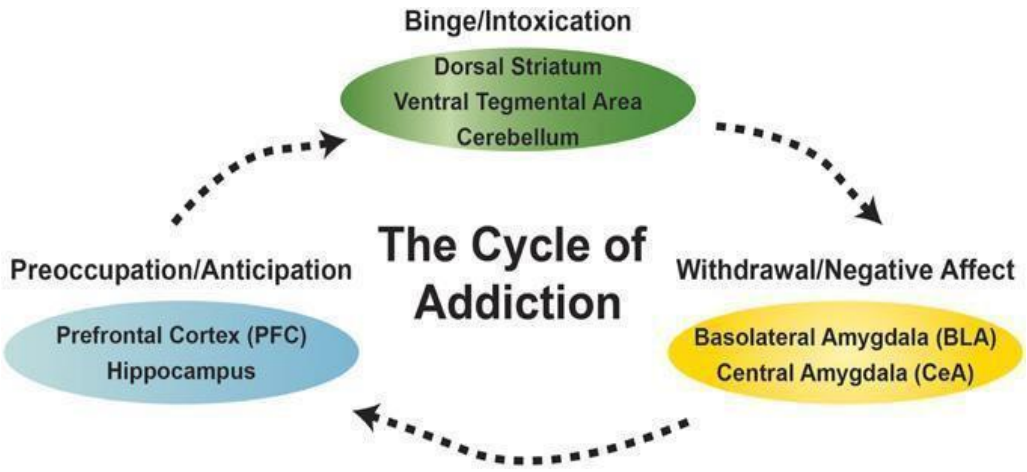


Figure 2.3: The relationship between binges, negative affect and anticipation in the cycle of addiction (Herman & Roberto, 2015).

Table 2.1: Summary of the mechanisms of action of drugs that have common addictive potential (McCracken *et al*., 2014; Nestler, 2005).

Drug	**Class of Drug**	**Mechanism of action**
Codeine; Fentanyl; Hydrocodone; Oxycodone Oxymorphone; Morphine, heroin	Opioids	Opioids produce analgesia by acting at several levels of the nervous system, particularly by inhibiting neurotransmitter release from the primary afferent terminals in the spinal cord and activating descending inhibitory controls in the midbrain
Alcohol	Hypnotic sedative	A positive allosteric modulator of GABAA receptors inhibits NMDA receptors.
Delta-9-tetrahydrocannibinol (THC)	Cannabinoids	CB1 receptor agonist
Indoleamines (lysergic acid dimethylamide – (LSD); psilocybin; N,N-dimethyltryptamine (DMT) Phenethylamines (methylenedioxymethamphetamine – MDMA, or ecstasy; phencyclidine – PCP) Serotonin re-uptake transporter (SERT), Norepinephrine transporter (NET) and dopamine transporter (DAT)	Hallucinogens	Binds to postsynaptic 5-HT_2 receptors, antagonistically binds to NMDA receptors and to D_2 receptors
Volatile alkyl nitrite: Amyl Nitrite and cyclohexyl nitriteNitrous oxides Solvents, fuels and anaesthetic	Inhalants	Affects certain ligand-gated, and ion channel receptors, including those for $GABA_A$, glutamate, and acetylcholine
Tobacco	Nicotine	Acts as an agonist at nicotinic acetylcholine receptors. These are ionotropic receptors composed of up to five homomeric or heteromeric subunits. In the brain, nicotine binds to nicotinic acetylcholine receptors on dopaminergic neurons in the cortico-limbic pathways.

2.4.1. The mesocorticolimbic dopaminergic reward system

The mesocorticolimbic DAergic reward system plays an integral role in the addiction to certain drugs (Table 2.1). The mesocorticolimbic DA is illustrated as the key component that plays a crucial role in the reward system of the brain (Figure 2.4). It connects the VTA, one of the principal DA-producing areas in the brain, with the nucleus accumbens (Gonon, 1997). The NAcc is found in the ventral striatum and is closely related to the motivation and reward system (Figure 2.4). The projections from the NAcc innervate cortico-limbic areas such as the prefrontal cortex (PFC), hippocampus and amygdala. These brain areas are responsible for the cognitive aspects of addictive behaviour (McCusker, 2001). An upsurge in the activity of the mesocorticolimbic pathway initiates the brain's reward system that is characterised by enhanced DAergic neurotransmission and more specifically an escalation of the intrasynaptic concentration of DA levels in the NAcc (Arias-Carrión *et al*., 634 2010).

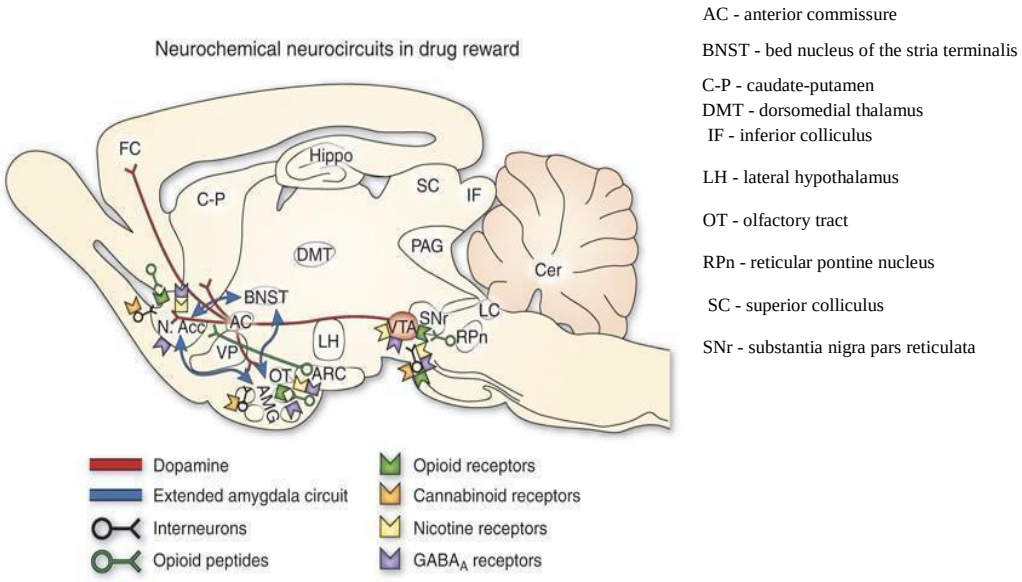


Figure 2.4: The pathways and receptor systems implicated in the acute reinforcing actions of drugs of abuse using a sagittal section of a rodent brain (Koob, 2004).

Figure 2.4 depicts the interplay between the opioid, cannabinoid, nicotinergic and GABAergic systems. Opioids affect DA transmission at both the VTA and NAcc levels. Opioids will activate opioid receptors located on neurons in the VTA, directly increasing the firing rate of these neurons. Opioids can promote DA release indirectly by binding to opioid receptors located on interneurons in the VTA. Similarly, opioids can stimulate the release of

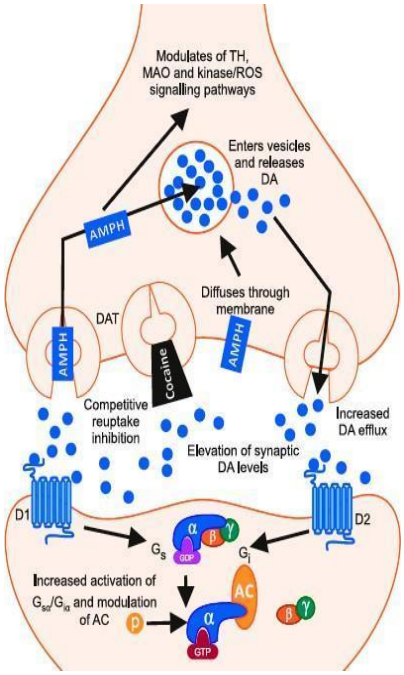
DA in the NAcc through direct actions on DA terminals, or indirectly, via interneurons in the ventral striatum. The administration of nicotine and cannabinoids will trigger the nicotinic acetylcholine and cannabinoids CB1 receptor respectively. Activation of these receptors follows a similar process to that stimulated by opioids (De Biasi & Dani, 2011; Richardson *et al*., 1998). Stimulation of neurons located in the VTA leads to the release of DA in the NAcc shell rather than in the core part of this brain area. From here, DA can follow a few possible routes (Figure 2.5).

DA is released from the presynaptic terminal into the synapse where it can bind to DA receptors located both pre-and postsynaptically. DA receptors mediate a wide variety of cellular functions, including altering the production of second messengers such as cyclic adenosine monophosphate (cAMP), mobilizing internal calcium stores, modulating ion channel function, altering neurotransmitter release, and influencing gene expression (Figure 2.5). Despite the large number of crucial functions it performs, the DA chemical messenger is found in a relatively small number of brain cells. While there are a total of 10 billion cells in the cerebral cortex alone there are only one million DAergic in the entire human brain (Mamelak, 2018).

Dopamine is removed from the synapse by the presynaptic monoamine transporter or dopamine transporter (DAT). Interestingly, stimulants such as cocaine act upon DATs, blocking DA re-uptake to increase and maintain high levels of synaptic DA to mediate their rewarding effects (Dela Peña *et al*., 2015; Wang *et al*., 2007; Volkow *et al*., 2004). Amphetamine, on the other hand, acts on intra-presynaptic vesicles by interfering with the functioning of the vesicular monoamine transporter (VMAT). This may lead to a reversal of VMAT direction, impede the filling of synaptic vesicles, and promote the non-vesicular release of DA, increasing synaptic DA concentrations (Fleckenstein *et al*., 2007).

Although DA neurons are relatively scarce in the brain, their importance is highlighted by the number of normal brain processes (e.g. learning, memory, motor behaviour and reward) and disorders that are modulated by DA receptor signalling (Gonon, 1997). For instance, having too much, or increased concentrations of DA in some parts of the brain and not

enough in other parts is linked to being more competitive, aggressive and having poor impulse control. DA imbalances can lead to conditions such as ADHD, binge eating, addiction and gambling. (Rogers *et al.* 2010; Volkow *et al*., 1997). Central to these pathologies is the dysregulation of DA receptors and production. Therefore, studies continue to focus on elucidating the mechanisms through which DA reuptake suppression may lead to mental disorders such as SUD (Volkow *et al*., 2010; Everitt, 678 2014).



GTP - guanosine-5′-triphosphate

AC - Adenylate cyclase

G – Protein guanine nucleotide-binding proteins

α - Alpha

β – Beta

γ – Gamma

Figure 2.5: The fate of synaptic dopamine (DA) at the terminal of dopaminergic neurons (Tritsch & Sabatini, 2012)

DA receptors are mainly found in the central nervous system (notably in the striatum hippocampus, hypothalamus, midbrain and fronto-cortical areas), but may also occur in the periphery (cardiovascular system and kidneys). These receptors are members of the G-protein-coupled receptor (GPCR) superfamily and are pharmacologically divided into two broad categories namely, D1-like (D1 and D5) and D2-like (D2S, D2L, D3 and D4)

receptors, based on their ligand recognition properties and their effects on cAMP production (Garau *et al*., 1978; Kebabian *et al*., 1972). G- Protein activation is the first step in the signal transduction cascade of GPCR. It is worth noting that GPCRs are intimately involved in cell recognition and communication processes, and have emerged as a prominent superfamily for drug targets. In the resting state, GDP is bound to the Gα subunit of the heterotrimeric (αβγ) G-protein (Sprang, 2016). When the receptor is activated, it promotes the dissociation of guanosine diphosphate **(**GDP) and the association of guanosine-5′-triphosphate (GTP) to the G-protein. This induces the active state of the G-protein, leading to the heterotrimer dissociating into Gα-GTP and Gβγ. The GTPase activity of the Gα subunit hydrolyses the GTP to GDP, causing the inactivation of the G-protein. Gα-GDP and Gβγ then re-associate, returning to the resting state and completing the cycle (Groves *et al*., 1978).

In addition to G_{α}-mediated effects, the $G_{\beta\gamma}$ subunits liberated by GPCR activation can influence neuronal physiology via direct interactions with voltage-gated calcium channels and the G-protein-activated inward-rectifying potassium (GIRK) channels, promoting vesicular neurotransmitter release. DAergic GPCRs are critical mediators of the neurochemical and behavioural effects of drugs of abuse. Repeated exposure to drugs may produce long-lasting changes in the ability of some presynaptic GPCRs to modulate the release of neurotransmitters, particularly glutamate, in addiction-relevant circuits (Sesack *et al*., 2003). More recently, it has been realized that glutamate also plays a central role in processes underlying the development and maintenance of addiction (Tzschentke & Schmidt, 2003). These processes include reinforcement, sensitization, habit learning and reinforcement learning, context conditioning, craving and relapse. Such drug-induced neuroadaptations likely play key roles in the transition to problematic drug-related behaviours including escalation of drug-taking and relapse following abstinence (Belin-Rauscent *et al*., 2016; Scofield *et al*., 2016). Pharmacological manipulation of presynaptic GPCRs has been shown to reduce drug-seeking and self-administration of drugs in both rodent and non-human primate models, suggesting that targeting these receptors could be a viable therapeutic approach for treating addictive behaviour (Johnson & Lovinger, 2016).

The mechanism by which opiates stimulate DA transmission is different to that of cocaine and amphetamine. While cocaine prevents the synaptic reuptake of DA by blocking DAT and amphetamine increases vesicular DA release, opioids exert their effects by binding directly to opioid receptors on NAcc neurons to inhibit DA re-uptake. Additionally, opioids may employ an indirect action by inhibiting GABAergic interneurons in the VTA, while simultaneously stimulating the firing rate of VTA DAergic neurons to cause enhanced DA release in the NAcc (Al-Hasani, Gowrishankar, & Schmitz, 2021).

2.4.2 The role of the frontal cortex (FC) in substance use disorder (SUD)

Several pieces of evidence from both clinical and animal models of drug addiction have revealed various roles for the FC in the processing of addiction-related learning, memory, and drug-seeking behaviour (Perry *et al*., 2011; Volkow *et al*., 2019; Ndlovu *et al*., 2021). The FC is organized hierarchically and can be divided into functional regions: the orbital frontal cortex (OFC) and anterior cingulate (ACC) (Fuster, 1989; 2000; 2001). These functional regions are generally involved in reward, emotion and motivation. The dorsal prefrontal cortex (dPFC) specifically, is involved in higher cognitive processes or executive functions, while the premotor and motor areas are involved in the planning and the execution of voluntary movements (Smith & Jonides, 1999).

The mammalian PFC serves as a critical functional interface between several subcortical neural regions involved in the processing of motivationally salient, reward-related information. The PFC keeps the reward noted by the NAcc in check by inhibiting the decision to use drugs when there are obvious negative consequences. Therefore, continual administration of substances of abuse, despite negative health consequences, is a definitive characteristic of addiction and a strong indication of PFC damage (Goldstein & Volkow, 2011; Volkow *et al*., 2016). Damage to, and loss of physiological function of the medial PFC compromises the ability to regulate the reward system. As a result, the substance of abuse will eventually take free reign over the brain, prioritizing the experience of pleasure while losing the ability to ‘just say no’.

Impulsivity is considered to occur in the absence of a decision-making process. It is therefore logical that damage to the FC, where critical thinking, decision making and planning take place, will also increase a substance abuser's impulsivity. Researchers have found that PFC development is halted when substances are abused during early development. Certainly, chronic exposure to drugs alters the prefrontal cortex, which governs motivation, inhibitory control and choice. But it also alters an area of the brain called the basolateral amygdala, which is associated with the link between a stimulus and an emotion (Winters, 2011). It is found that for young people to avoid substance abuse because drugs and alcohol dramatically impede the brain's development and can cause long-term damage. In some cases, the effects are irreversible (Natalia & MacQueen, 2015)

This outcome supports the notion that prefrontal-cortical aberrations are responsible for some of the behaviours of substance abusers (Winters & Arria, 2011; Renard *et al*., 2017; Dhein, 2020). In summary, the mammalian PFC serves as a functional nexus point controlling subcortical neural reward and motivational pathways. In patients suffering from SUD, the aberrant amplification of reward signals is associated with drugs of abuse and powerful memories are formed that are associated with the drug-taking experience. Glutamate and gamma-aminobutyric acid (GABA) are the major neurotransmitters in the brain. Inhibitory GABA and excitatory glutamate work together to control many processes, including the brain's overall level of excitation due to the administration of drugs (Rage *et al*., 1992).

2.4.3. The role of the hippocampus in substance use disorder (SUD)

The hippocampus is central to cognitive processes such as learning and memory. Anatomically it is an extension of the cerebral cortex embedded in the temporal lobe and forms part of the limbic region in the brain (Anand & Dhikav, 2012). Since the hippocampus is closely linked with cognitive functions, this region has been implicated in many neurological disorders such as Alzheimer's disease (AD) and epilepsy (Frisoni *et al*., 2010; Dhikav & Anand, 2007(b)).

Several imaging studies reported neurochemical and functional alterations in this brain region in drug-dependent individuals, highlighting the significant role of the hippocampus in SUD (Volkow *et al*., 1997; Wightman & Robinson 2002). The hippocampus is well known for its role in learning and memory, where it synthesizes complex representations of various sources of information. In the case of substance abuse, the hippocampus will facilitate associations between drug intakes and the surrounding environment or circumstances and embed these associations as memory traces (Kutlu & Gould, 2016). The hippocampus possesses two neurophysiological properties that enable this brain structure to perform its learning and memory functions. This role is underpinned by direct glutamatergic projections between the NAcc and the hippocampus (Floresco *et al*., 2001). Firstly, hippocampal neurons are highly neuroplastic (Kolb & Gibb, 2011). The neurotransmitter system involved in the neurocircuitry of addiction stages and functional may a change as in Appendix 1. Secondly, neurogenesis has been observed within the hippocampus (Silva & Brandao, 2000). These processes allow the hippocampus, together with other brain regions such as the amygdala and dorsal striatum, to encode drug-related events into consolidated memories (Goodman & Packard, 2016).

There is adequate evidence that shows that drugs of abuse may induce long-lasting changes in the brain (van Huijstee & Mansvelder, 2015; Gould, 2010; Kosten & George, 2002). This modification mainly occurs at the level of the physiological functioning of neurotransmitters. Drugs of abuse can activate neurons since their chemical structure often mimics that of the natural neurotransmitter in the body. This allows the drugs to bind to neurotransmitter receptors, thereby initiating neurotransmission. Prolonged high-frequency activation of such receptors leads long term potentiation and the physical modification of the synapse cytoarchitecture and structure. Addictive drugs, therefore, remodel the brain's reward circuitry, e.g. the mesocorticolimbic dopamine (DA) system, by inducing widespread adaptations of glutamatergic synapses.

In summary, SUD involves multiple brain circuits. According to Lovinger & Gremel, (2021) it is commonly understood that a network of four major circuits is involved in substance abuse: (a) the reward circuit, located in the NAcc and ventral pallidum; (b) the

motivation/drive circuit, located in the OFC and the subcallosal cortex; (c) the memory and learning circuit, located in the hippocampus and amygdala; and (d) the control circuit, located in the PFC and the anterior cingulate gyrus (CG). These four circuits receive direct DAergic innervations but are also connected through direct or indirect projections (mostly glutamatergic). Although specific brain regions and pathways are described in this thesis, other brain regions are also involved in the development of SUD (e.g. the thalamus, insula and habenula). Since chronic morphine administration may lead to a reduction in acetylcholinesterase (AChE) activity in the MHb (Neugebauer, *et al*., 2013). During morphine withdrawal, AChE activity in MHb returns to baseline, suggesting a homeostatic balance. However, a second study using higher doses of morphine administered for a longer period (15 days), showed increased AChE activity in the habenula (Mohanakumar & Sood, 1983)

Furthermore, one region may participate in more than one circuit and function (e.g. the CG is actively involved in both control and motivation/drive circuits), and other brain regions (e.g. the attention and emotion circuits in the cerebellum) are also likely affected by repetitive drug consumption. While current hypotheses tend to focus mainly on DA, it is evident from preclinical studies that modifications in glutamatergic projections may mediate many of the adaptations observed in SUD (Cornish & Kalivas, 2001). According to Volkow *et al*. (2003), SUD is a mental state initiated by the qualitatively different and larger reward value attributed to the drug, which triggers a series of adaptations in the reward, motivation/drive, memory, and control circuits of the brain. These changes result in an enhanced and permanent saliency value for the drug, and in the loss of inhibitory control, favouring the emergence of compulsive drug-seeking. Based on neurobiological changes, the behaviour of an individual suffering from SUD may show alteration of locomotor activity, poor self-control, inappropriate cognitive behaviour, and aberrant learning and memory functioning (Greengard, 2001).

2.5 Nyaope

Nyaope, a street drug commonly found in South Africa, is a mixture of heroin, cannabis products, antiretroviral drugs, antidepressants and other materials added as cutting agents (Khine *et al*., 2015; Mthembi *et al*., 2019). This highly physiologically addictive substance is often smoked by youths from poorer socioeconomic backgrounds, with a history or high risk of homelessness and incarceration (Mokwena & Huma, 2014; Masombuka, 2013; Degenhardt *et al*., 2017). While substance misuse is most prevalent among males, recent trends suggest a roughly 80/20% male/female split across the country (TEDS, 2016). Men are more likely than women to use almost all types of illicit substances (CBHSQ, 2017). The factors that might lead to such discrepancy of high usage of illicit drugs between females and males may include access to treatment facilities, cultural practices, or social pressures on males eg fulfilling their duties as the primary breadwinner of the family (Morgan *et al*., 2019). The Attempts to clamp down on illicit drug usage by legislation have escalated the introduction of man-made cocktail drugs with different street names and different combinations of ingredients (Coomber, 1997). Nyaope is also known as "whoonga", "whunga", "sugar", or "ungah" in parts of South Africa. The low cost of nyaope makes it easily accessible. While the literature on the effects of nyaope is limited, studies have reported on its psychoactive properties, its potentially damaging effects on the mucosa lining of the respiratory passages, and infective endocarditis associated with intravenous use (Mokwena, 2015; Meel & Essop, 2018). Misdiagnosis of endocarditis from intravenous use has led to an increased mortality rate amongst nyaope users.

2.6. Heroin toxicity

Since heroin is said to be the major constituent of nyaope, the present study focused on literature about this drug. Heroin, also known as diacetylmorphine, is a very efficient prodrug, more potent than morphine. After administration, heroin is rapidly metabolized through sequential deacetylation to 6- monoacetylmorphine (6-MAM) and morphine by different tissues in the body, including the brain and blood (Figure 2.6). Since morphine has been thought to be the major active metabolite involved following heroin exposure, many of the studies published have used this drug to mimic the effect of heroin. However, 6-MAM and morphine-6-glucuronide are also pharmacologically active (Umans &

Inturrisi, 1982; Hubner & Kornetsky, 1992; Handal *et al*., 2002) and 6-MAM may be more effective than morphine in activating μ-opioid receptors (Selley *et al*., 2001).

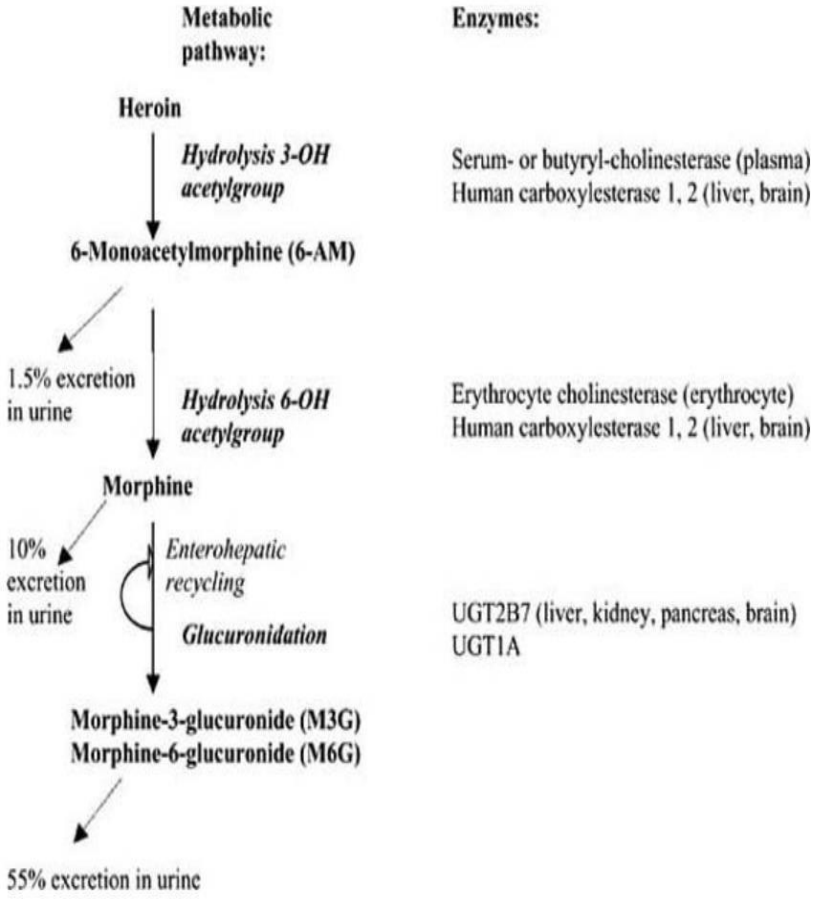


Figure 2.6: Heroin metabolism and related enzymes (Rook *et al*., 2006(a)(b)).

Recent publications have renewed concerns regarding the rise in opioid-related deaths (Lyden & Binswanger, 2019; Clark *et al*., 2014; Martins *et al*., 2015). It is therefore not surprising that there is an increased interest in heroin-induced toxicity. Heroin is a strong agonist of opioid receptors. The most recent classification scheme identifies four major classes of opioid receptors, with several minor classes. The three most clinically relevant opioid receptors are the mu (μ), kappa (κ) – and delta (δ) - receptors. The fourth receptor is called the nociceptor/orphanin FQ receptor (Waldhoer *et al*., 2004). In general, activation of these receptors leads to a reduction in excitability and neurotransmission. Stimulation of central μ-receptors causes respiratory depression, analgesia (supraspinal and peripheral), and euphoria. κ- and δ-opioid receptors also have potent analgesic effects, with the κ-receptors being known to cause disassociation, hallucinations, and dysphoria δ- receptors also

modulate the activity of μ-receptors and are thought to influence mood. Nociceptor/orphanin FQ receptors generally have a very low binding affinity for endogenous opioids but seem to have a greater affinity for dynorphin-related compounds and opioid antagonists (naltrexone and naloxone) (Toll *et al*., 2016). These receptors bind to nociception/orphanin FQ, which is said to be an opioid-like peptide found in the brain, immune cells and vascular endothelial cells (Uetrecht, 2009). They supposedly regulate several biological functions, including locomotor 846 activity, memory, emotional states, food intake, drug abuse, pain transmission, micturition, cough reflexes, and cardiovascular, respiratory, and immune functions (Toll *et al*., 2021) (Ndlovu, et al., 2021).

The toxicity of heroin can be assessed at the whole body, organ system or cellular level. The behavioural effects of heroin highlight the impact of heroin on the function of the central nervous system. However, heroin as an opioid may also affect other organ systems including the respiratory, and gastrointestinal system. Of relevance to the current study is the effect of heroin on the liver (Jezequel *et al.,* 1976). Liver toxicity due to adulterated drugs has been investigated in several studies. For instance, by adopting chromatographic techniques, Al-Asmari (2020) was able to demonstrate the presence of heroin metabolites (6-monoacetyl-morphine, morphine, 6-acetylcodeine, and codeine) in post-mortem liver tissues of heroin-related fatalities. An interesting study, (Qian *et al., 2020*) showed that cannabis products could inhibit the hydrolysis of heroin in the liver. Decreased hydroxylation of both cannabis and heroin in the liver is of particular importance in the South African context where nyaope (heroin) is commonly used in conjunction with cannabis and may explain the persistence of high levels of the drug in the body of nyaope users (Zhu & Peltekian, 2019). While heroin has not yet been implicated in clinically relevant hepatic injury, the prevalence 862 of infectious diseases, such as hepatitis C, in substance users has been documented (Zeremski *et al*., 2013).

Nevertheless, acute liver injury has been reported in cases of a heroin overdose with marked increases in serum aminotransferases (Hoofnagle *et al*., 2013). Serum biomarkers such as aspartate and alanine aminotransferase are useful parameters to indicate liver damage and fibrosis. Liver enzymes reside within hepatocytes (liver cells) so that when these cells are damaged the enzymes leak into the systemic circulation (Zeremski & Martinez, 2017). In a

preclinical study, David & Hamilton, (2010) were able to show heroin-induced liver damage by increasing the production of reactive oxygen species, activating the mitogen-activated protein kinase pathway, and increasing the expression of tumour necrosis factor-alpha (TNF α) and interleukin-1 beta (IL-1β). A clinical study was conducted by Farooqi *et al*., (2016) to determine the degree of liver damage in participants who were abusers of heroin using the blood plasma as compared to serum. The difference between using plasma (unclotted blood) or serum (clotted blood) is often assay dependent. However, a study by Nwosu *et al*., 2009, showed that there is no significant difference in whether liver enzymes are measured in serum or plasma, but rather that storage conditions are more important. The sample size was comparable to my local nyaope studied cohort with 25 patients suffering from SUD using heroin between ages 25 and 45 years old. They investigated the activity of aspartate aminotransferase, alkaline phosphatase and alanine aminotransferase. Approximately 52% of the study population which suffered from SUD showed higher than normal liver enzyme levels, while 4% of the participants displayed below normal plasma enzyme levels (Morgan *et al*., 2018), however, almost third of the participants showed increased serum enzyme levels. Some participants that suffer from SUD also tested positive for hepatitis B or C, raising the possibility that prior infection could have contributed to the observed liver damage. The study concluded that elevations in liver enzyme serum concentrations were a consequence of liver injury induced by heroin. The authors further proposed that intravenous administration of heroin predisposes the liver to morphological alterations which may result in liver steatosis, vesicular changes, chronic hepatitis, and fibrosis, culminating in liver cirrhosis (Farooqi *et al.* 2016) and hepatitis (Berson *et al*., 2001). The literature on the direct effects of heroin on the liver remains scarce. Henceforth any data on this topic will improve my current understanding of the effect of heroin on liver structure and function.

2.7 Therapeutic approaches to managing substance use disorder (SUD)

2.7.1 Conventional approaches

Individuals suffering from SUD face several challenges as they battle to overcome changes in the physiological and chemical functioning of their brains and body. In many instances, the conventional approach to managing the disorder includes immediate detoxification followed by cognitive behavioural therapy and social support over an extended period. However, sudden discontinuation of substances of abuse typically results in a tremendously unpleasant withdrawal syndrome distinguished by severe headaches, vomiting and diarrhoea (Hodding *et al*., 1980). These symptoms are said to underlie the craving period for the substance of abuse that may persist for some time after the last usage, and may eventually precipitate relapse. Failures within this treatment strategy have called for a need to shift the addiction treatment field from an acute care model to a chronic disease management paradigm (Scott *et al*., 2011). Therefore, treating SUD as a chronic disorder has yielded better long-term results compared to acute interventions (McLellan *et al*., 2000).

Following the need for medication-based approaches, the use of opioid replacement therapy programs has been effective in the United States of America. Methadone was part of a maintenance management treatment strategy developed in the 1960s (EMCDD, 2015) and was initially only administered to individuals who were dependent on heroin and resistant to other treatments. Methadone doses ranging from 80-to 150 mg/kg were initially used to block the euphoric effects of heroin. Treatment was successful, not only in reducing heroin cravings and illicit drug use but also in helping the individuals to become self-supporting and well-functioning.

Methadone is a synthetic opioid agonist that eliminates withdrawal symptoms. It relieves drug cravings by acting on opioid receptors in the brain with the same receptors that are activated by other opioids such as heroin, morphine, and opioid pain medications. Buprenorphine, a partial μ-receptor antagonist, was originally developed as an analgesic but has lately been used in the management of opioid dependence (Kuehn, 2005). Reported advantages of using buprenorphine include (i) a lower risk of toxicity at higher doses

compared to naloxone (another opioid receptor antagonist) (ii)greater effectiveness at dosages lower than that recommended for daily analgesic use, (iii) less severe withdrawal symptoms after discontinuation, (iv) lower abuse potential, and (v) greater accessibility for office-based treatment programs (Dole & Nyswander, 1965; Jansinki *et al*., 1978). However, methadone and buprenorphine administration have their disadvantages. Methadone side effects include drowsiness, bigheadedness, weakness, dry mouth, urinary retention, constipation and slow or troubled breathing (Giacomuzzi *et al*., 2001). Buprenorphine on the other hand may cause respiratory depression (Jansinki *et al*., 1978).

Naloxone is a non-selective, short-acting opioid receptor antagonist that has been used to diminish craving in individuals suffering from SUD (Carroll & Schottenfeld, 1997). Naloxone is often used in the treatment of opioid overdose-induced respiratory depression, in rapid detoxification or, in combination with buprenorphine, for maintenance therapy. However, drugs that induce or inhibit the cytochrome CYP450 3A4 enzyme can interact with opioids, resulting in potentially fatal respiratory depression (Dahan *et al*., 2013). Thus, risks related to naloxone use in opioid-dependent patients are the induction of an acute withdrawal syndrome with the occurrence of vomiting and aspiration which are potentially life-threatening (Wermeling, 2015). In patients treated for severe pain with opioids, high-dose, or rapidly infused naloxone may cause catecholamine release and consequently pulmonary oedema and cardiac arrhythmias (Pugsley, *et al*., 2015). These risks warrant the cautious use of naloxone and careful monitoring of the cardiorespiratory status of patients after naloxone administration.

2.7.2 Alternative therapies

The side effects of conventional treatments have promoted a growing interest in the use of natural plant products for medicinal purposes. Although abstinence is the most desired method of changing opioid usage behaviour, this has proven impractical and difficult for those afflicted by illicit drug consumption. In certain communities, herbal medicine has been used extensively to treat a variety of pathological conditions (Ullah *et al*., 2014). The rise in the use of plant extracts has primarily been driven by its availability, affordability and advocacy by traditional healers for its disease-healing benefits. Additionally,

preference for natural remedies has been encouraged by the inefficiency or undesirable side effects of conventional drugs, and hence scientific investigation into the pharmaceutical potential of plant extracts has increased over the past decades. Moreover, the attitude changes of the general population to prefer naturally derived substances and extracts as treatments has boosted the growing interest in alternative phytochemical measures (Ji *et al*., 2009).

Opioid maintenance treatment has reduced harm in patients suffering from SUD (Cheung & Chien, 1999; Cohen, 2004). Unfortunately, a maintenance therapeutic approach is not commonly available in resource-stricken countries where the abuse of substances is equally as rife as in more affluent states. In disadvantaged populations, traditional medicine has received wide attention in the treatment of drug misuse. Traditional Chinese medicine, including therapies like acupuncture, has already been approved for the treatment of opiate addiction by the Chinese State Food and Drug Administration (SFDA) (Shi *et al*., 2006; Su & Zheng, 1998). In comparison to modern medicine, traditional Chinese medicine claims to have fewer adverse effects, be safer to use and be more effective in treating refractory chronic diseases. These natural treatment approaches have subsequently been indicated for a variety of conditions including pain relief, local anaesthesia, stabilization of blood sugar, improvement of protein metabolism, protection of the liver, blood pressure control, fatigue, stress, convulsions and the modulation of immune function (Shi *et al*., 2006). The wide scope of medicinal benefits of traditional Chinese medicine has also encouraged its use in the treatment of substance abuse (Doosti *et al*., 2013).

Some cases show the detrimental effect of the use of ginseng extracts with stimulant drugs that might cause cardiovascular diseases (Ratan *et al*., 2021). Studies have shown that extracts of ginseng suppress place preference behaviour in cocaine-treated mice (Kim *et al*., 1999), and attenuate the deleterious effects of morphine, methamphetamine, cocaine and alcohol in both preclinical and clinical cohorts of addiction (Takahashi & Tokuyam, 1998). The advantages of traditional Chinese medicine are its multi-system, multi-target mechanism of action and reduced side effects. This approach could therefore aid the rehabilitation program and enhance the recovery process. Therefore, traditional Chinese medicines could be promoted as an alternative 980 or supplement to conventional medicine.

The medicinal benefits of certain botanical species have led to an extensive search for a plant-based remedy for SUD (Jilek, 1994; Lu *et al*., 2009; Sarkar & Varshney, 2017). As a result, several studies have shown the potential of herbal derivatives to restore substance-induced behavioural changes (Winkelman, 2014; De Veen *et al*., 2017; Brown *et al*., 2010; Nunes *et al*., 2016; Hurtado-Gumucio, 2000). The reported benefits of aqueous and ethanolic extracts from various plant species are described in Table 2.2. We focus on the two extracts (ibogaine and ayahuasca) that have been subjected to extensive clinical and preclinical investigations and provide a summary of the latest findings regarding their use in SUD.

2.7.2.1 Tabernanthe iboga

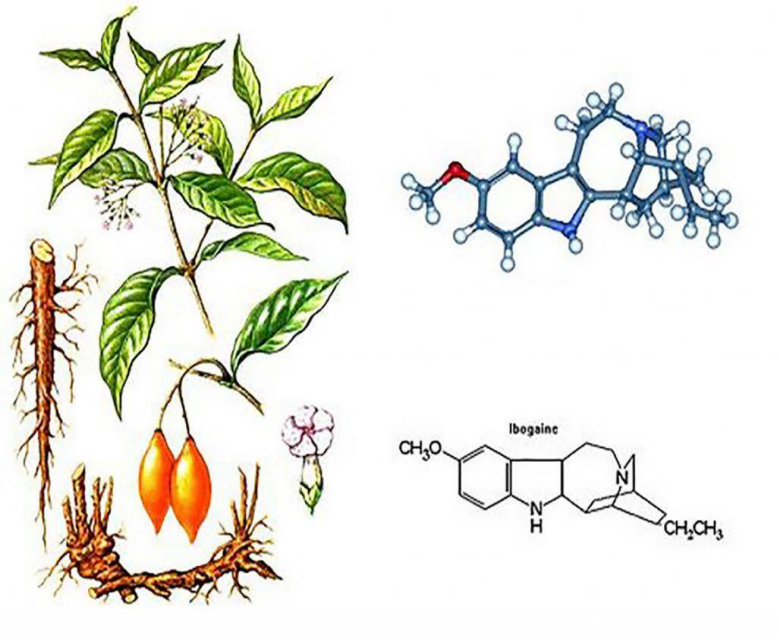


Figure 2.7: The *Tabernanthe iboga* shrub tree

Tabernanthe iboga is a well-known shrub mainly used by African traditional healers for a variety of health conditions (Figure 2.7). The compound ibogaine is extracted from the root bark of the iboga plant. Ibogaine is an alkaloid that is reported to effectively reduce drug cravings and withdrawal symptoms in opioid users (Brown & Alper, 2018; Yu *et al*., 1999; Szumlinski *et al*., 2000). However, the extract also has psychedelic effects (Alper *et al*., 2012; Cameron *et al*., 2021). A single dose of ibogaine is adequate to relieve withdrawal symptoms (Sheppard, 1994; Maisonneuve *et al*., 1997), although booster doses are needed to obtain optimal outcomes (Brown & Alper, 2018). The mechanism of action of ibogaine is not well understood. While the compound can bind to opioid receptors, binding does not activate G-proteins. Some studies have proposed ibogaine to be an NMDA receptor antagonist as it reduces withdrawal symptoms in a similar way to memantine, a well-known NMDA receptor antagonist (Popik *et al*., 1995, Koenig & Hilber, 2015).

Others have attributed the beneficial effects of ibogaine to its impact on neural circuitry by modulating trophic factors such as glial-derived neurotrophic factor (GDNF) (Brown and Alper, 2018). While the recognition of ibogaine as a scheduled, regulated pharmaceutical is still being debated, its use in treating SUD is gaining momentum as more studies are being undertaken. Ibogaine derivatives showed an increase in safety and efficacy as compared to other western medications (Sershen, 1994). Two synthetic iboga alkaloid congers, i.e. 18-methoxycoronaridine (Glick *et al*., 1996), as well as an engineered ibogaine analogue called tabernanthalog (TBG) (Cameron *et al*., 2021), reduce heroin-seeking behaviour without its hallucinogenic effects, demonstrating potent anti-addiction properties with greater safety

Table 2.2: Health benefits of some plants used for substance use disorder treatment**.**

Herbs	**Effects**	**Citations**
Flos *Lonicerae japonica,* Herba Taraxaci, *Gossampinus malabarica* (mumian), pumpkin, Radix Glycyrrhiza, pine leaves, small flower milkwort herbs with roots (Jinniucao) and *Hedyotic diffusa* (baihua sheshecao)	Reduces unpleasant experiences during detoxification	Gao *et al.* (2001)
Pinelliae rhizome, Semen Ziziphi Spinosae, Radix Polygalae	Induces sedation and tranquilization	
Radix Ginseng	Prevents morphine tolerances Relieves withdrawal symptoms	
Derivatives of *Daturae albae,* flos (Yang Jinhua)	Diminishes withdrawal symptoms to prevent SUD establishment and brings pain relief. Activates the respiratory centre and promotes the metabolism of morphine.	Su & Zheng (1998)
Radix Aconiti (Fuzi)	Relieves swaying (reeling) and shaking of the head and extremities (ramble) in rats during opiate withdrawal	Xu *et al.,* (2001)
*Radix Ginseng, Radix Astragali, Radix Panacis Quingueflii, Radix Aconiti lateralis praeparata, Radix Angelicae Sinesis a*nd *Cordyceps*	Healthy Qi[1] reinforcement	
Yanhusao rhizome	Controls drug-associated vomiting	

Qi[1] - the vital energy that flows through the body to maintain a person's health

2.7.2.2 The use of ayahuasca extracts

Figure 2.8: Ayahuasca leave and roots

Ayahuasca, also known as the tea, the vine, and la purge, is a brew made from the leaves of the shrub (Figure 2.8). *Psychotria Viridis* and the stalks of the *Banisteriopsis caapi* vine. It is commonly used in communities in South America (Amazon basin), where it is mainly consumed during religious rituals and is often referred to as a sacred medicine (Fábregas *et al*., 2010; Frecska *et al*., 2016). Since its users display hallucinogenic behaviour, Fabregas *et al*. (2010) investigated the presence of SUD in three communities that have been identified as frequent users of ayahuasca. Surprisingly, these authors found no symptoms of addictive behaviour and concluded that the consumption of ayahuasca did not lead to the deleterious psychosocial effects associated with other drugs of abuse. Additionally, the concoction has a little reinforcing effect and few addictive properties (Don *et al.*, 1998; Liester & Prickett, 2012). Indeed, subsequent studies have suggested that ayahuasca might have therapeutic potential for a variety of diseases including substance dependence (Frecska *et al*., 2016). A study by Rodrigues *et al*., (2022) reported that among healthy

ritual ayahuasca users and patients with SUD, reductions in drug use, anxiety, and depression, and increases in quality of life and well-being were observed.

The active ingredients of ayahuasca responsible for its effects are the beta-carboline harmala alkaloids, obtained from the stem bark of *Banisteriopsis caapi*, and the N, N-dimethyltryptamine (DMT) derivatives present in *Psychotria Viridis* (Gable, 2007). An earlier study by Callaway *et al*. (1999) has shown that the harmala alkaloids, harmine and harmaline, are monoamine oxidase inhibitors (MAOIs). The observed psychedelic effects of ayahuasca are probably mediated by its action on the monoamine oxidase enzyme, increasing the levels of monoamines, particularly serotonin, in the brain. Activation of the serotonergic 5-HT_{2A} receptors has been proposed to be the mechanism by which DMT mediates its psychoactive effects (Hamill *et al*., 2019). In summary, ayahuasca shows no addictive properties, does not activate the DAergic reward system, does not induce withdrawal symptoms, and may be considered a therapy for SUD (Nichols, 2004).

2.7.2.3 The use of *Moringa oleifera*

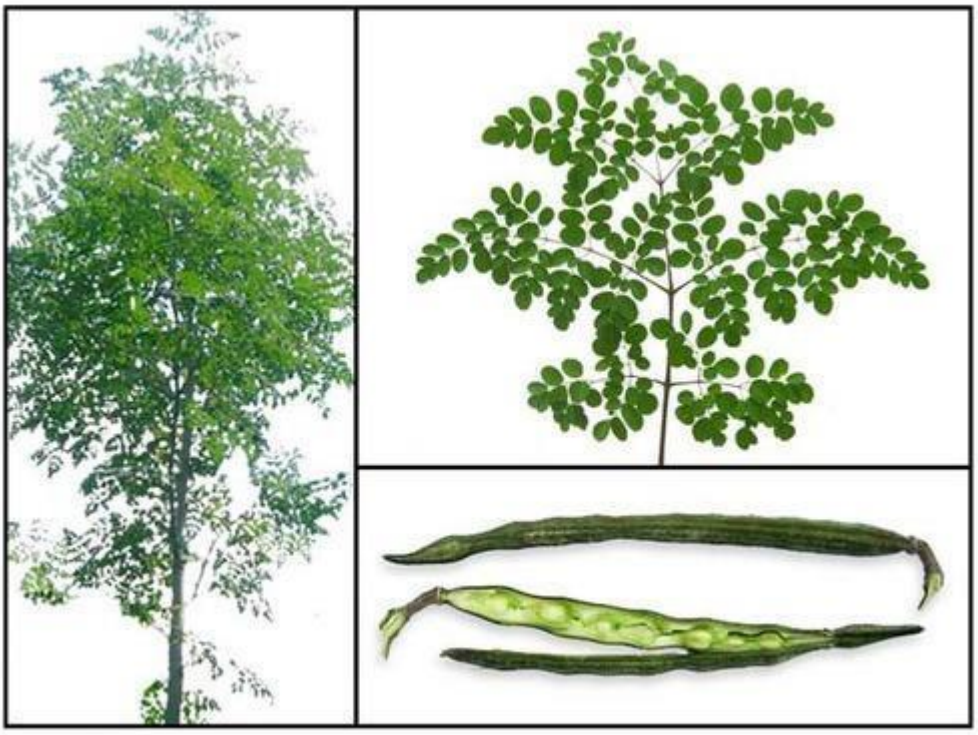


Figure 2.9: The components of *Moringa oleifera*

Moringa oleifera, or the horseradish tree, is a pantropical species that are known by regional names such as benzolive, drumstick tree, kelor, matango, mlonge, mulagay, nèbèday, saijhan and Sajna (Figure 2.9). It belongs to the Moringaceae family and is one of 13 species that can be found in several countries (Anwar *et al*., 2007; Olson & Fahey, 2011). While it is native to sub-Himalayan India, Pakistan, Bangladesh and Afghanistan, the species is now widely cultivated in Ethiopia, the Philippines, Sudan, West, East and South Africa, tropical Asia, Latin America, the Caribbean, Florida State of The United States of America and the Pacific Islands (Fahey, 2005). It is a perennial softwood tree with low-quality timber, but has, for centuries, been advocated for traditional medicinal, agricultural and other domestic uses (Table 2.3). All parts of *Moringa* trees are edible and consequently, they also hold significant nutritional value in many countries (Table 2.4).

Table 2.3: The beneficial uses of different parts of the *Moringa oleifera* tree (Paikra, *et al*., 2017)

Usage	Part of the tree
Foliar nutrient	Juice extracted from leaves
Fencing	Living trees
Fertilizer	Living trees
Alley cropping	Biomass production
Animal forage and Biogas	Leaves and treated seed cakes
Blue dye	Wood
Domestic cleaning agents	Crushed leaves
Green manure	Leaves
Gum	Trunk
Honey	Flower nectar
Honey and sugar honey clarifier	Powdered seed
Medicine	All plant parts
Ornamental plant and biopesticide	Incorporating leaves into soil and preventing damping of wood in seedlings
Pulp	Stem
Tannin or tanning hides	Barks and gums
Water purification	Powdered seed

Table 2.4: The medicinal uses of *Moringa* on various physiological systems in the body. *(Gopalakrishnan, Doriya, & Kumar, 2016)*

System	**Condition**
Integumentary	Skin infection, blackheads
Blood	Anaemia, blood impurities
CNS	Anxiety
Respiratory	Asthma, respiratory problems, chest congestion
Digestive	Cholera

The nutritional and medicinal properties of *M. oleifera* stem from the vitamins, minerals, amino acids, beta carotene, antioxidants and omega-3 and -4 fatty acids it contains (Fahey, 2005; Posmonteir, 2011). Its leaves have substantial amounts of phenols and flavonoids that provide a wide range of antimicrobial properties (Luqman *et al*., 2012).

In a study by Sutalangka *et al., (*2013) an extract of fresh leave of *Moringa oleifera* was administered via oral route to Wistar rats weighing 180 to 220 g for 7 days. The spatial memory was evaluated using the Morris water maze. Rats were sacrificed and the hippocampus harvested to measure oxidative stress markers and alterations in AChE activity. These results showed that *Moringa oleifera* possessed neuroprotective and memory-enhancing effects. This finding was endorsed by clinical investigations suggesting that extracts of *Moringa* may indeed be useful to treat neurodegenerative diseases (Adikuru *et al*., 2011; Craggs & Kalaria, 2011). The most common neurodegenerative disease amongst heroin users is tau hyperphosphorylation which develops later in life. Hyperphosphorylation of tau is responsible for its loss of normal physiological function, the gain of toxicity and its aggregation to form neurofibrillary tangles (NFTs). Hence neurofibrillary tangles (NFTs) made up of hyperphosphorylated tau are a histopathological hallmark of Alzheimer's disease (AD) and related tauopathies (Hanseeuw *et al*.,2019).

It is therefore clear that *M. oleifera* extracts affect central nervous system functioning, even though there is currently no literature showing that the administration of an aqueous extract of *M. oleifera* to nyaope-treated animals can reverse nyaope-induced effects. The high protein content in moringa promotes healthy production of neurotransmitters, which in turn affects your mood, emotions, sleep and learning abilities. Moringa can enhance your memory by altering the activity of certain enzymes in the hippocampus, the brain region that's responsible for learning and memory (Mohammed *et al*., 2018). The alkaloids, glycosides, phenols, saponins and tannins present in *M. oleifera* are most likely responsible for its analgesic effects (Kumbhare & Sivakumar, 2011; Gupta *et al*., 2002). The underlying molecular mechanism is probably due to the inhibition of cyclooxygenase-2 (Cox-2) activity, preventing prostaglandin synthesis (Anwar *et al*., 2007). One needs to be careful because the COX-2 inhibitors and other NSAIDs may increase the risk of heart attacks, stroke, and related conditions, which can be fatal (Taraneh, 2014). As a result, *Moringa* can also be used by smokers because *Moringa* tea may reduce the oxidative stress associated with nicotine consumption and limit the extent of structural damage in the brain such as the frontal cortex of Wistar rats (Gbadamosi, et al., 2016). However, Omotoso *et al*. (2018) showed that concomitant administration of *M. oleifera* with nicotine prevented severe chromatolysis and apoptosis in the cerebral cortex and inhibited the locomotive deficits observed in rats treated with nicotine only. These effects were ascribed to the antioxidative properties of *M. oleifera* extract, including the presence of phytoconstituents with free radical scavenger abilities and the activation of endogenous antioxidative enzymes (Gupta *et al*., 2003). These results were mainly obtained from the frontal cortices of the right and left lobes of each animal during the experiment. Another study demonstrated that *M. oleifera* seed extract could alleviate scopolamine-induced learning and memory impairment in mice (Zeng *et al*., 2019). Since nyaope may affect learning and memory, administration of *M. oleifera* extracts may have beneficial effects in reducing the nyaope-induced impairments.

A study by Bhairi *et al*. (2015) observed that the extracts possess both peripheral and central antinociceptive activities with the involvement of opioid receptors. The effect was significantly reversed by the opioid receptor antagonist, naloxone, indicating the role of both central and peripheral opioid receptors in alleviating pain. This result suggested that *M. oleifera* might be useful to treat opioid-related addictive disorders. Despite this connection,

we could not find any literature investigating the potential benefits of *Moringa* extracts on SUD. My study attempted to address this shortcoming.

2.8 Animal models to study human behaviour

Over the years, animal models have been used to test hypotheses related to several human pathologies. Specifically, studies using animal models have filled gaps in my understanding of the aetiology of many diseases. Gaps were created by my inability to conduct certain studies on humans. The findings of these studies can address questions related to the functioning of the central nervous system. One of the great benefits of animal models is that they can be used to conduct studies where social, environmental, behavioural and genetic factors can be controlled or standardized, allowing the deduction of clear conclusions (Beilharz & Cox, 1967). Animal models, therefore, enable the manipulation of single variables or groups of variables in a highly controlled context. Animal models also allow for the invasive examination of organ, tissue, and region-specific mechanisms at the physiological, cellular, and molecular levels (Reza Khorramizadeh & Saadat, 2020). In the subsequent sections, the animal models applied in the present study are briefly described. Specifically, animal models related to locomotor behaviour, anxiety-like behaviour and cognitive behaviour, are discussed.

2.8.1 Animal models to assess anxiety-like behaviour

Anxiety is a defence mechanism exhibited by an organism in response to novelty. It is generally characterized by negative emotions and feelings of apprehension (Nuss, 2015). People with substance use challenges often present with other mental health problems, including anxiety disorders**.** Understanding their pathophysiology will assist the development of effective prevention and treatment programs. Human anxiety disorders are approximately congregated according to their symptomology and response to pharmacological and emotional management (Nutt, 1990; Weiss, 2007). Indiscriminate anxiety-like behaviour and panic disorder are the two principal groupings of excessively mode behaviour developed in individuals with SUD (Kelly & Daley, 2013). Several researchers have employed rodent models to study pathological anxiety-like behaviour in humans (Cryan & Holmes, 2005; Ohl, 2005; Rodgers *et al*., 1997). In principle, these models evoke a fearful reaction through an aversive occasion or anticipated aversive event. Other mixed tactics to circumvent the

conflicting premeditated circumstances to prevent an ongoing behaviour reveal distinguishing features in the behaviour of the animal. These distinguishing features may include a conflicting tendency of animal models to engage in exploratory activity or social investigation against the aversive properties of an open, brightly lit, or elevated space. Numerous experiments have characterised the behavioural patterns of animal models and subsequently translated them to humans under different circumstances, including assessing the efficacy of drugs for the treatment of SUD (Wahlsten, 2001; Wahlsten *et al*., 2003; Würbel, 2001).

2.8.1.1. The open field test (OFT)

The open-field test (OFT) was developed to assess emotionality in rodents (Beilharz & Cox, 1967). It is commonly used to assess the levels of anxiety-like behaviour, exploratory behaviour and locomotor activity in rodents (Christmas & Maxwell, 1970; Rodger, 1997). Additionally, recurring exposure or extended session lengths can evaluate habituation to a progressively familiar chamber environment. It has been suggested that two factors influence the anxiety-like behaviour of rodents in the open field. When placed within a novel environment, the rodent has an internal dilemma of wanting to explore the novel environment against the fear of being exposed to brightly lit, unprotected, wide-open spaces. The amount of time spent exploring versus hiding is indicative of the emotional state of the animal (File, 1980; Prut & Belzung, 2003). These factors make the OFT useful for measuring the locomotor activity of the animals, such that the effects of interventions on anxiety-like behaviour and locomotion can be simultaneously determined over time (Reith *et al*., 1985).

This is an important factor to be considered when utilizing the OFT. If the locomotor ability is compromised, then measuring activities that rely on the ability of the subject to move will be confounded.

2.8.1.2. The elevated plus maze (EPM)

The elevated plus maze (EPM) is a well-established paradigm with a long and successful history in assessing anxiety-like behaviour in animal models (Pellow *et al*., 1985; Crawley, 2007; Rodgers & Dalvi, 1997). Similar to the OFT, this assessment also benefits from the innate propensity of rodents to explore novel environments. In this test the animal is provided with the choice of spending time in open, unprotected maze arms or enclosed, protected arms, all elevated approximately 1 m above the floor. The elevation of the maze is necessary to create the effect of a wide, open area. Once again the animal is conflicted between their wish to explore the novel environment, and their preference to avoid bright, open areas, and favour darker, enclosed spaces. Time spent in the respective arms reflects this approach-avoidance conflict and indicates the emotionality of the animal (Holmes *et al*., 2003). The EPM can successfully evaluate the anxiolytic potential of drugs (Himanshu *et al*., 2020).

2.8.2. Learning and memory

The consumption of psychoactive substances over an extended period can damage various cerebral structures, which become apparent through deficits in cognitive and affective mental functioning (Lacković, 2007). Several studies have indicated that the cognitive functions most affected by the toxic influence of opiates are attention, concentration, memory and perceptual-motor speed/coordination (Ahmad *et al.*, 1989; Davis *et al.*, 2002; Mitrović *et al.*, 2011). The overarching view is that during the initial or acute use of drugs, maladaptive memories of drug effects and environmental stimuli are formed and these associated memories direct drug-seeking behaviour and relapse. After the continued use of drugs, learning deficits emerge along with cognitive inflexibility.

These learning deficits and cognitive inflexibility combined with previously formed maladaptive drug-cue associations contribute to the maintenance of addictive behaviour. The hippocampus is associated with learning and memory. In addition to being involved in long-

term declarative memory formation, the hippocampus demonstrates high-level synaptic plasticity, often assessed by changes in long-term potentiation (LTP) (Teyler & DiScenna, 1987; Kauer & Malenka, 2007). The high degree of plasticity in the hippocampus and the ability of this region to support contextual and declarative memories may facilitate drug-induced changes in hippocampal function that have profound effects on behaviour (Dhikav & Anand, 2007(a); Dhikav, 2006). The physiological effects of drugs of abuse can become associated with contextual information, contributing to future drug-seeking behaviour (Kutlu *et al*., 2015; Tropea *et al*., 2008) and the maintenance of the addictive state (Smith & Mizumori, 2006). In support, acute administration of substances such as heroin, cocaine, nicotine, amphetamine, and alcohol can enhance hippocampus-dependent learning and memory (Kutlu & Gould, 2016).

2.7.2.1. The novel object recognition (NOR) test

The relationship between novelty and behaviour has received a lot of attention from researchers. Novelty is an alteration of the expected likelihood of an event based on both previous information and internal estimates of conditional probabilities (Antunes & Biala, 2012). Importantly, animals can be affected by a novel stimulus. According to Bevins *et al*. (2002), novel stimuli can change animals' behaviour, provoke stress responses, elicit approach behaviour, and cause an increase in corticosterone plasma levels (a major index of stress). These parameters suggest that confinement to a novel environment may be stressful to animals. However, repeated exposure to the same environment decreases this stress response and, over time, the animal becomes more comfortable exploring its surrounding. The novel object recognition (NOR) test assesses an animal's behaviour when it is exposed to a novel and a familiar object. The NOR is premised on the innate exploratory nature of rodents placed in a novel environment. This test has been used to study short-term, intermediate-term, and long-term memory (Antunes & Biala, 2012; Lueptow, 2017). In essence, the test calculates a retention index using the number of times an animal retains the memory of sample objects presented during the familiarization phase, and the amount of time the animal spends with a novel object that replaced one of the familiar objects during the test phase (Taglialatela *et al*., 2009). There is no reward in the NOR test and animals explore the novel object as part of their natural propensity to novelty (Baxter, 2010). The ability of the NOR test to assess learning and memory function has been validated by studies that demonstrated the influence of both hippocampal and cortical lesions on NOR test outcomes (Buckmaster *et al*., 2004; Clark *et*

al., 2000). The NOR task is used to evaluate cognition, particularly recognition memory, in rodent models of CNS disorders, and therefore is used to assess material-specific recognition memory deficits (O'Bryant *et al.*, 2003). Other brain areas related to learning and memory include the perirhinal cortex, said to play an important role in object recognition memory (Aggleton *et al*., 2010), and the medial temporal lobe, a structure that enables the recognition of a previously encountered item as familiar (Hammond *et al*., 2004). These brain structures play important roles in recognition memory formation, and when damage occurs, performance during recognition memory tasks is impaired (Albasser *et al*., 2009).

2.8.2.2. The Y-maze

The Y-maze, as the name implies, has three arms positioned in a Y-shape and is used to assess short-term memory in rodents. The test is driven by the innate curiosity of rodents to explore new environments. Rodents typically prefer to investigate a new arm of the maze rather than returning to one that was previously visited. This spontaneous alternation behaviour is considered a measure of spatial working memory. A rodent with an intact working memory, i.e. intact prefrontal cortical functions, will remember the arms previously visited and prefer to enter a less recently visited arm. Spatial reference memory, which is underlined by the hippocampus, can also be tested by placing the test animal into the Y-maze with one arm closed off during training (Kraeuter *et al*., 2019). It has been reported that performance in the maze relies on working memory to recall a list of previous spatial orientations concerning external surroundings (Strijkstra & Bolhuis, 1987). The enhancement of maze performance by naltrexone implies that endogenous opioid systems have a functional role to play in this aspect of cognition. While morphine does not appear to impair spatial working memory, it might impair the acquisition of reference memory (Olton, 1987; Lupien *et al*., 2009). Since chronic opiate treatment can reduce long-term hippocampal potentiation (LTP) (Pu *et al*., 2002), investigations into the impact of this class of drugs on cognitive function seem appropriate.

CHAPTER 3: Methodology

3.1 Introduction

Nyaope is an adulterated drug used mainly by youth in South Africa for recreational purposes (Mokwena, 2015). To characterize the contents of nyaope, samples were collected from the South African Police Forensic Services Laboratory (Pretoria, South Africa; SAPS 2019/05). The project obtained permission to have nyaope for experimental purposes from the National Department of Health (NDoH), South Africa (POS 140/2018/2019; POS 385/2019/2020) (Appendix 4). Samples were documented and stored under strict access control. Wits ethics committee awarded me the permission to conduct this study (Appendix 5)

3.2 Chemical analysis of nyaope

This section of the study was conducted in conjunction with the Department of Chemical Pathology in the Faculty of Health Sciences at the University of the Witwatersrand. Street samples were ground into a fine powder using a mortar and pestle. Separate aliquots of the homogenised street sample ranging from 10 mg to 16 mg were weighed into a 20 mL vial and mixed with 1 mL of each of the tertiary butyl alcohol, dichloromethane and isopropanol internal standard solution. This was done to see whether the composition of nyaope was solvent-dependent. Tertiary butyl alcohol has previously been shown to be the solvent of choice for presenting nyaope extracts to the GC–MS (Mthembi *et al*., 2018). Gas chromatography-mass spectrometry was used to analyse the samples as this is the method of choice to separate (gas chromatography) and identify (mass spectrometry) small amounts of volatile compounds. The National Institute of Standards and Technology (NIST) database was used to identify compounds according to their CAS registry number. The CAS registry number is a unique numerical identifier assigned by the Chemical Abstracts Service to every chemical substance described in the open scientific literature. The obtained identifications were verified against another database known as the Mass Spectral Library of Drugs, Poisons, Pesticides, Pollutants developed by Maurer *et al*. (2016).

3.3 Animal husbandry

A total of 72 male and female Wistar rats were used in this project. There is adequate evidence that showed that different genders react differently to the reaction of administered adulterated drugs (Whitley & Lindsey, 2009). The rats were obtained from and housed in the Wits Research Animal Facility at the University of the Witwatersrand. The six to eight-week-old rats were housed in groups of three in standard Perspex cages with wood chip bedding and *ad libitum* access to water and food mainly the pellets (90% of the diet),. The temperature in the housing area was monitored using a HOBO data logger with U10 software (Wantit All (Pty) Ltd, Linbro Park, Frankenwald Gauteng, South Africa) and maintained at 22.5ºC. The light intensity of the housing room and behavioural test area was similarly kept at about 325 lux. Humidity ranged from 60-70% and the lights were connected to a timer set at a 12-hour cycle, with lights on between 06h00 – 18h00. All animals were weighed daily to accustom them to being handled and to monitor their overall well-being. Ethical approval was obtained from the university's Animal Ethics Screening Committee (AESC) (Clearance certificate 2018/09/40/C) (Appendix 5). All procedures followed the standards approved by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Witwatersrand University Animal Care and Facilities Committee.

3.4 Determining the dose of nyaope

As there are no *in vivo* studies on nyaope in the literature, we had to determine a dose that would be suitable for my experiments. A total of 24 rats (12 male and 12 female) ages ranging from six to eight-week-old were treated with nyaope at a concentration of 10 mg/ml (approximately after conversion 30 mg/kg dose) based on literature investigating heroin toxicity in rats. This was extracted from the study by Strandberg *et al*., (2006) that groups of rats of similar age to the rats in this project were injected intravenously (i.v.) with heroin, 21.5 mg/kg, or morphine, 223 mg/kg, causing a 60–80% mortality among drug-naïve rats. Additional groups of rats were pre-treated with morphine for 14 days, with or without 1 week of subsequent abstinence. The nyoape solution was prepared by mixing the powder with saline and administered intraperitoneally with the assistance of staff members of the Wits Research Animal Facility. Animals were subsequently monitored by visual observation for two hours for any changes in locomotor behaviour, rate of breathing, piloerection and overall wellbeing. Animals that showed signs of stress were euthanized under the supervision of animal care officials. The administration of 30 mg/kg of nyaope solution led to animal deaths so the dose

was adjusted to 1 mg/kg. Animals weighing about 300 g were subsequently injected with 0.3 ml of a 1mg/ml solution (Appendix 2). The selection of 1mg/kg was selected based on the existing literature (Picetti *et al*., 2012).

3.5 Assessing the effects of nyaope on behaviour

3.5.1 Animal treatment

After the loss of the rats due to overdose administration of nyaope I was left with 40 rats i.e.20 males and 20 females from different litters. Rats were grouped into two genders of the age ranging from 8 to 10 weeks old. In subsequent experiments, rats were treated with either saline (1 ml, intraperitoneal, for five consecutive days) to serve as controls, or nyaope (1 mg/kg, intraperitoneal, for five consecutive days as indicated by the pilot study). In addition to the saline or nyaope injections, an aqueous extract of *Moringa oleifera* was administered to some animals. 10 ml of distilled water was added to 1 g of crushed leaves of *Moringa oleifera*, obtained from commercial resources (Dis-Chem Pharmacy, South Africa), and heated for 10 minutes. Studies by Ifeanyi *et al*. (2020) have shown that heating does not alter the composition of Moringa meaningfully. The aqueous extract was then allowed to cool to room temperature. After filtration, the animals received an intraperitoneal injection of 5 ml of the extract for a further five consecutive days, i.e., after the end of the saline/nyaope injections. The 5 ml extract administration approximated a concentration of 500 mg/kg (a dose of 1500 mg/kg). The dose was in alignment with that of a *Moringa* aqueous extract (2000 mg/kg) given orally to rats for 21 consecutive days and yielded no significant negative effects (Adedapo *et al*., 2009). The animals were weighed daily to monitor the effect of nyaope and *Moringa oleifera* on the body mass of the animals and to adjust the volumes of solutions to ensure correct dosing.

3.6 Experimental design and protocol

A separate batch of rats was divided into four different groups. Groups one and two had 12 rats, and groups three and four had eight rats each (Figure 3.1). The experimental procedure lasted 15 days. The first five days were reserved for pre-treatment behavioural assessments. During the next five days, (days six to ten) the animals were given either nyaope or saline, while rats in groups three and four received *Moringa oleifera* aqueous extract only and an i.p. injection of nyaope followed by an i.p. injection of *Moringa* solution respectively (Figure 3.2). Thereafter, post-treatment behavioural assessments were conducted on days 11 to 15. After

the last injection (on day ten), the animals were left alone until the commencement of post-treatment behavioural assessment (day 11). All animals were decapitated the day after the end of the experimental protocol (day 16). While treatments and assessments were staggered, the schedule was designed in such a manner that representatives of each group were decapitated on one day. Following decapitation, trunk blood was collected and centrifuged and the plasma was kept for biochemical analysis at a later stage. The entire frontal cortex (FC) was sampled by dissecting the rostral part of the brain just after the ends of the olfactory bulbs. The tissue was subsequently frozen in liquid nitrogen for subsequent analysis. Liver tissue was harvested into saline-buffered 10% formalin for histological investigation.

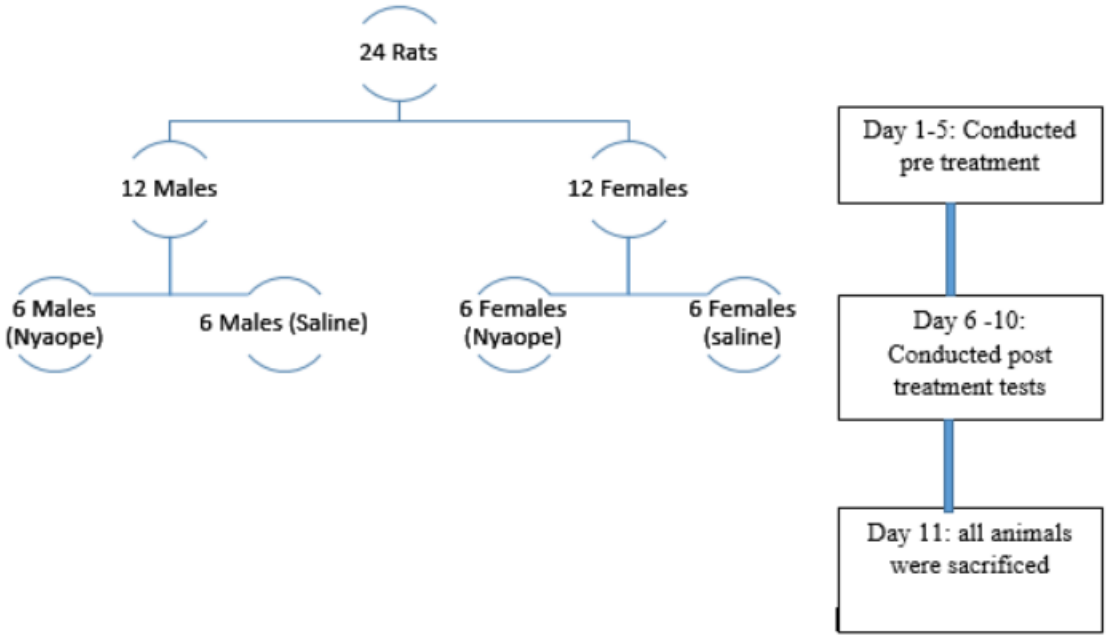


Figure 3.1: The study design for behavioural assessment following the administration of saline, nyaope, nyaope with *Moringa oleifera*, and saline with *Moringa oleifera.*

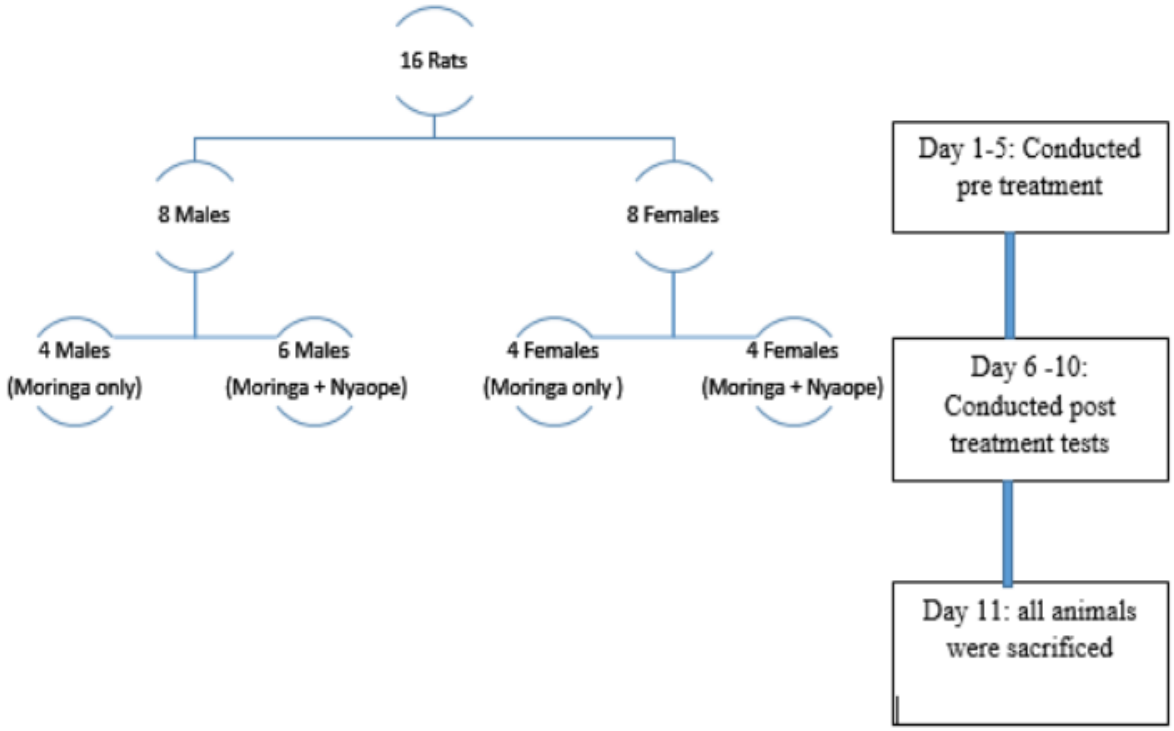


Figure 3.2: The study design for behavioural assessment following the administration of saline, nyaope, nyaope with *Moringa oleifera*, and saline with *Moringa oleifera***.**

3.7 Behavioural assessments

Behavioural assessments were performed in a dedicated room adjacent to the animal housing area. The room was blackened with dark curtaining to minimize visual distractions and white noise. Three behavioural domains were assessed in all animals: (i) locomotor activity, (ii) anxiety-like behaviour, and (iii) cognitive behaviour. The physical activity of the animals was tracked via a camera mounted directly above the apparatus. The camera was connected to a computer where all data were stored. The movements of the animals were analysed using ANY-maze software (Stoelting Co., Dublin Europe).

3.7.1 Anxiety-like behaviour and locomotor activities

3.7.1.1 The open field test (OFT)

The OFT is a common approach to measuring exploratory behaviour and both the quality and quantity of activity in mice and rats (Gould & Einat, 2007; Hasler *et al*., 2006). The open field was made of black Perspex with sides of 1.20 m and walls of 50 cm in height (Figure 3.3). The floor of the apparatus was delineated into an inner (centre) area and an outer (wall) area.

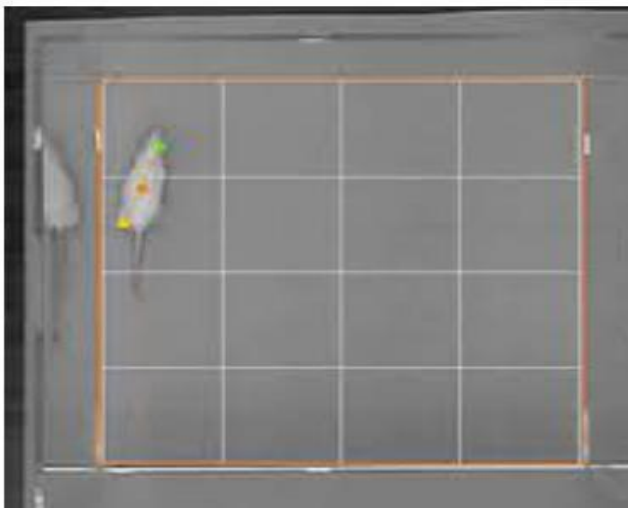


Figure 3.3**:** The open field test apparatus with demarcations created by ANY-maze software.

At the start of the experiment, the surface was cleaned with F10SC veterinary disinfectant (Health and Hygiene, Florida Hills, South Africa) all the time. The rats were placed in one corner of the apparatus facing the wall and allowed to explore the arena for five minutes. The following parameters were measured: distance travelled, time spent in the centre and wall areas, and average speed in the respective areas. After completion of the test, rats were returned to their home cages and the apparatus was cleaned with F10SC veterinary disinfectant (Health and Hygiene, Florida Hills, South Africa) to minimise factors that may influence the behaviour of the animals in the apparatus.

3.7.1.2 The elevated plus maze (EPM)

The EPM can reliably assess anxiety-like behaviour in rodents (Pellow *et al*., 1985). The apparatus was made of black Plexiglas and consisted of two open (50 cm long X 10 cm wide) and two enclosed (50 cm long X 10 cm wide X 40 cm walls) arms (Figure 3.4). The arms

were in the form of a plus sign with similar arms arranged opposite each other. The maze was elevated 65 cm above the floor to create the effect of wide-open space for the animals when they were in open arms.

Figure 3.4**:** The elevated plus maze apparatus.

Rats were placed in the central zone facing an open arm and their behaviours were subsequently recorded with the use of a camera attached to the computer hosting the ANY-maze software. The behaviour of the animals was recorded for three minutes with tracking focused on head movements. While the animals were observed for five minutes in the open field test, the animals remained in the closed arms of the EPM. Hence only the first three minutes were analysed. The behavioural parameters scored included the head distance travelled, the overall mean speed of the head, the number of times the head entered into the various arms, and the number of whole-body entries into open and closed arms (a whole-body entry was defined as when the rat had all four paws in the considered arm), and the duration of head time spent in the open and closed arms. Both apparatuses (open field and EPM) were cleaned with a damp soapy cloth after every animal exposure to minimise any scent of the previous animal influencing the behaviour of the subsequent experimental animal.

3.7.2 Learning memory

3.7.2.1 The novel object recognition test (NOR)

The NOR test was conducted according to the original procedure as described by Ennaceur & Delacour (1988). The rats were subjected to a habituation phase followed by a testing phase. For the habituation phase, the rats were allowed to freely explore an arena (a cage similar to its home cage) in which two identical objects were placed. For the test phase, one of these objects was replaced with an object of a different colour and texture but a similar size to the original objects (Figure 3.5). The time that the rat spent exploring this novel object indicated its ability to identify the new object as novel and remember that the other object was there the previous day. The novel object recognition test, therefore, provides insight into the animal's memory ability. The experiment was conducted over two days.

A. A familiar object exposure B. A novel object exposure

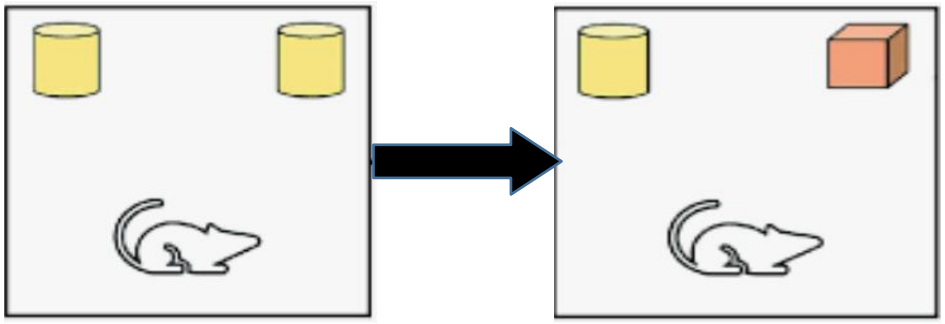


Figure 3.5: The novel object recognition test apparatus.

On the first day (habituation phase), the rat was released facing the wall opposite the objects to prevent coercion to explore the objects. On the second day, the testing phase was initiated by replacing one of the familiar objects with a novel object. The animals were once again released with their backs to the objects and allowed to explore the cage. On each occasion, the movement of the rats was tracked for three minutes using the ANY-maze software. The primary parameter of interest was the time spent interacting with the novel object. It was assumed that the animal would spend more time interacting with the novel object than with the familiar object (Figure 3.5). Object interaction was defined as an entrance into a pre-determined object-containing zone resulting in direct or nearly direct object contact with the nose or whiskers. A discrimination index was calculated using the following formula: Index

(I) = (T_n-T_f) / Total, where time was spent with a novel object (T_n), the time spent with the familiar object (T_f) and total time allowed for the exploration (Sivakumaran, 2018). Positive values indicate more time spent with the novel object and negative values indicate less time spent with the novel object, compared to the time spent with the familiar object. Distance travelled and average speed were additional parameters considered in this experiment. After which rats were returned to their home cages after the respective habituation and test trials. Due to the nature of the task and based upon the original reports of Ennaceur *et al*. (1998), practice effects were not anticipated and indeed retesting did not produce any statistically significant effects on total amounts of object interaction or novel object interaction during the test trial. The same procedure was performed before and after the various treatments.

3.7.2.2 The Y-maze

The Y-maze consists of three non-reflective plastic arms, orientated in the shape of a "Y', at an angle of 120^o to each other (Figure 3.6). Each arm is 50 cm long, 10 cm wide and has a wall of 20 cm high. The apparatus was designed to assess spatial working and reference memory in rodents (Kraeuter *et al*., 2019). The basis of these tests rests upon the willingness of rodents to explore new environments, hence the animals were not habituated to the Y-maze. In the case of the Y-maze, the natural tendency of rats is to enter a new arm rather than return to an arm previously visited. A sequential visiting of different arms is referred to as a spontaneous alternation behaviour and the successful completion of one such sequence is referred to as a triad. Structures of the brain involved in spontaneous alternation behaviour include the hippocampus and FC, and therefore the test can assess cognitive function in rats (Cleal *et al*., 2021).

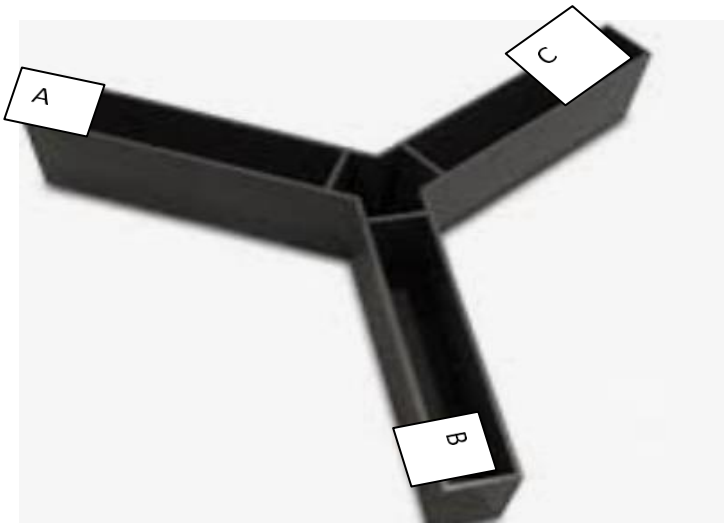


Figure 3.6: The Y-maze apparatus.

When conducting the Y-maze test, animals were placed in an arm facing the centre of the maze and allowed to freely explore the maze for five minutes. The movements of the animals were tracked by the ANY-maze software. An animal needed to have all four paws in an arm before it was counted as an arm entry. A correct alternation (triad) occurred when the animal moved to the other two arms without retracing its steps (i.e. from A to B to C). For example, movements such as C–B–C were considered incorrect. Measurements were continuous, so the reading frame of alternations constantly shifted and a percentage of correct alternations was calculated based on the animal's exploration patterns.

Using the ANY-maze programme we defined each arm and the middle area as separate zones. Data were obtained for overall movement in the maze as well as by each zone separately. By following this approach, head distance travelled, no of entries per arm, average speed per arm, and the time spent per arm were also recorded. According to standard practice, the data of an animal would be excluded when the immobilisation time between two arms exceeded two minutes.

3.8 Blood and tissue sampling

Twenty-four hours after the last behavioural test was performed, the animals were anaesthetized (ten seconds of exposure to halothane-saturated air in an anaesthetic chamber), before being decapitated. Trunk blood was collected and centrifuged at 1500 rpm for 10 minutes and the plasma was aliquot into Eppendorf tubes for subsequent biochemical analyses and stored in a -20^{o}C refrigerator. The brains of the animals were removed from the skull and the FC dissected, immediately frozen in liquid nitrogen and stored at -20^{o}C until further neurochemical analyses. Liver tissue was sampled and placed in a 10% phosphate-buffered formalin solution for subsequent histological analysis.

3.8.1 Assessment of the effect of nyaope on opioid signalling in the brain

The effect of nyaope on opioid signalling was assessed by measuring the levels of the extracellular signal-regulated kinase (Erk) 1/2, members of the mitogen-activated protein (MAP) kinase pathway, in the fronto-cortical tissues, using enzyme-linked immunosorbent assays (ELISAs). The Abcam ELISA kits were obtained from Biocom Africa (Centurion, South Africa) and are suitable for the semi-quantitative measurement of signalling proteins. The frontal cortices were minced and thoroughly rinsed in phosphate-buffered saline (PBS) to remove any blood present in the sample. The tissue was subsequently homogenized using a glass Dounce homogenizer, in 1 mL of chilled 1X Cell Extraction Buffer PTR. The mixture was incubated on ice for 20 minutes and centrifuged at 18,000 x g for 20 minutes at 4°C. The supernatants were transferred into clean tubes and pellets were discarded. The assay samples were immediately aliquoted into Eppendorf tubes and stored at -80°C. On the day of the experiment, one aliquot was to determine the protein concentration. This was done using a Bradford Protein Assay with absorbance read at 280 nm. Samples (10 µL) were subsequently diluted to the desired concentration in 1X Cell Extraction Buffer PTR. 96-well plate strips were provided together with the ELISA kits (Figure 3.7). For each assay performed, a minimum of two wells were used as the zero control.

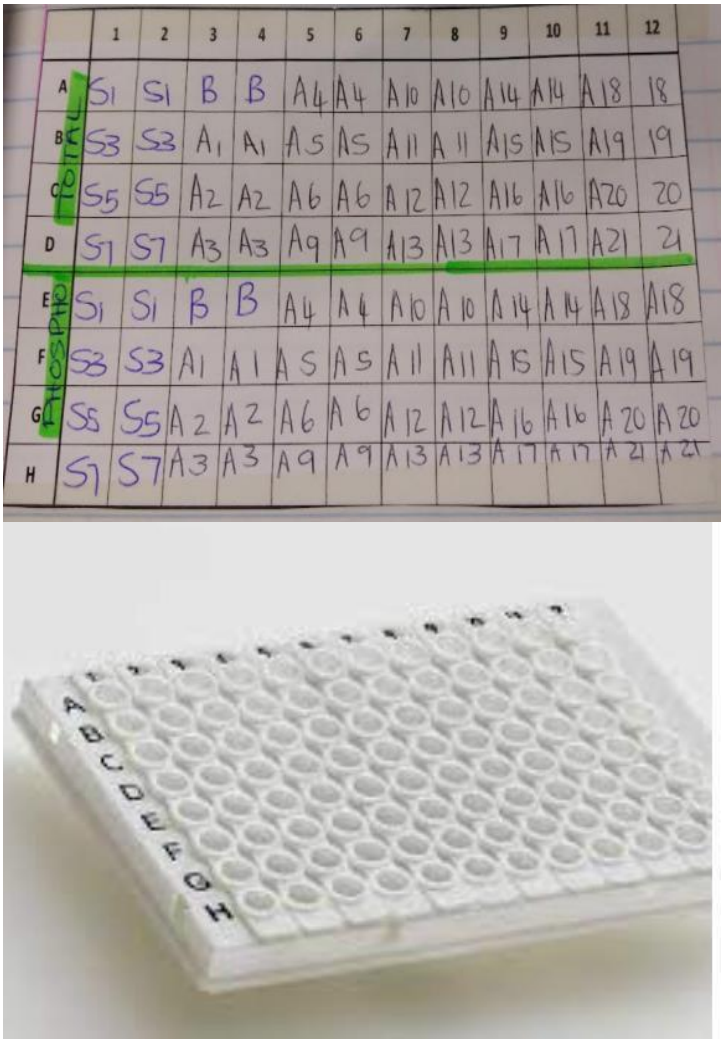


Figure 3.7: Preparation of plate (A) plan with sample labels and (B) clean wells. C = controls, S = standard, B= blank and A for the various samples 1-21.

All materials and reagents were brought to room temperature before use. All standards, controls and samples were assayed in duplicate. The reagents, working standards, and samples were prepared according to the manufacturer's instructions. A volume of 50 µL of all samples and standards was added to appropriate wells. Also, 50 µL of the Antibody Cocktail was added to each well. The antibody cocktail consisted of both a capture antibody as well as a detector antibody for c-Jun N- terminal kinase (JNK) and ERK1/2. For JNK the antibodies were specific to detect phosphorylated JNK1/2 (pT183/Y185) and total JNK. For ERK1/2 the antibodies captured and detected phosphorylated ERK1 (pT202/pY204), phosphorylated ERK2 (pT185/pY187) and total ERK1/2

The plates were sealed and incubated for one hour at room temperature on a plate shaker (MaxQ 4450 Mini Benchtop Shaker, Thermo Fischer Scientific, RHODESFIELD South Africa) set to 400 rpm. The incubation buffer was aspirated and the wells were washed three times by adding and decanting 350 µL Wash Buffer PT each time. After the last wash, the plate was inverted and blotted against clean paper towels to remove excess liquid. A volume of 100 µL of TMB Substrate was added to each well and incubated for 15 minutes in the dark on a plate shaker set to 400 rpm. After the incubation period, 100 µL of stop solution was added to each well. The plate was placed on the shaking plate for one minute after which the optical density was read at 450 nm.

3.9.1 Assessment of the effect of nyaope on the liver

3.9.1.1 Tissue Processing (Embedding)

The 10% formalin-fixed liver tissue samples were cut and mounted into a cassette for paraffin embedding. The cassette was inserted into a tissue processing machine (ATP 300 Close Tissue Processor, LABOTEC, SA) for approximately 24 hours where the liver tissue was dehydrated and infiltrated with wax, forming a wax block housing the tissue sample. After placing the wax block on ice for 20 minutes, it was positioned in a microtome (Leica RM2125 RTS, LABOTEC, South Africa) for sectioning at 5 µm thickness. The sections were initially placed in a water bath set at 25^{o}C for five minutes before being mounted onto a glass slide. Three sections per animal were placed on one slide. Slides were dried in an incubator at 30^{o}C for four hours before being stained.

3.9.1.2 Staining procedure

Two staining techniques were used. Some slides were subjected to Mayer's hematoxylin and eosin staining to gain insights into the overall cellular structure and nuclei integrity of the tissues, while other slides were stained with Masson's trichrome stain to evaluate the supporting structures within the tissues for examples of connective tissues and collagen fibres. The procedure entailed deparaffinized and dewaxing the sections with xylene for ten minutes followed by a series of immersions in 100%, 95% and 70% ethanol for rehydration. The sections were then washed with distilled water for one minute.

For Mayer's hematoxylin staining, the slides were exposed to hematoxylin solution for ten minutes and washed under slow-running tap water for five minutes. The sections were subsequently differentiated by dipping the slides twice in 1% acid alcohol. The sections were checked under the microscope to verify the appropriateness of the stain. The slide was washed under slow running tap water for five minutes, stained with Scott's tap water (alkaline medium) for five minutes and washed with running tap water for another five minutes. Sections were then counterstained with a 1% eosin solution for ten minutes.

3.9.1.3 Dehydration and Mounting

After the sections were washed under tap water for another five minutes, the slides were dehydrated by immersing them in 70%, 95% and 100% ethanol solutions sequentially. The sections were then cleared by immersing them twice in xylene for ten minutes. The sections were finally mounted in Entillin for evaluation under light microscopy (Olympus CX23 Microscope, Olympus, Auckland Park South Africa). Masson's trichrome staining was done according to the method by Suvarna *et al*. (2019). After the sections were dewaxed, rehydrated and washed under tap water, they were exposed to a celestine blue solution for five minutes. The slides were rinsed in distilled water and stained with alum hematoxylin for five minutes and washed with tap water for about ten minutes until a noticeable blue colour was obtained.

3.10 The effect of nyaope on liver enzymes

The plasma levels of several enzymes were determined, using veterinary rapid in-clinic assays, which serve as indicators of liver function. These enzymes were alanine transferase (ALT), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH). Additionally, the plasma concentration of albumin was also measured. Ten microliters of plasma were pipetted into a cassette preloaded with reagents for the specific tests and placed in a biochemical analyser (Catalyst Dx Chemistry Analyzer, IDEXX Laboratories, Midrand, South Africa). The results of this automated technology were subsequently captured on a computer connected to the equipment.

3.11 Statistical analysis

All statistical analyses were done using GraphPad Prism (Version 9.02) software. Descriptive statistics are provided that include minimum and maximum values, median, 25

and 75% percentiles, arithmetic mean, standard deviation, standard error, and lower and upper 95% confidence intervals, where appropriate. Normality of tests was performed using the Shapiro-Wilk test. For parametric data, analyses of variance (ANOVAs) or repeated ANOVAs were used, followed by t-tests. For non-parametric data, Holm-Sidak tests or Kruskal-Wallis tests were used depending on whether the data were repeated or not. This was followed by Dunn's multiple comparison test. Where group differences (saline vs nyaope; pre and post-treatment) were obtained were shown to be significantly different, and additional analyses were performed to identify sex differences. If no significant differences were found, no further analyses were conducted.

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CHAPTER 4: Results

4.1 Chemical analysis of nyaope

Nyaope samples of the same batch, obtained from the South African Police Services, were extracted using three different solvents (dichloromethane, ethanol and methanol) and subsequently subjected to gas chromatography-mass spectrometry (GC/MS) analysis (Mthembi *et al*., 2018;2018). When nyaope samples were extracted using dichloromethane, significant peaks were identified for caffeine, codeine, heroin and morphine (Figure 4.1, Table 4.1). A similar pattern was observed when nyaope samples were extracted with ethanol (Figure 4.2, Table 4.2). Extraction of nyaope using methanol yielded a more comprehensive list of compounds (Figure 4.3, Table 4.3). In the current study, significant peaks were identified as 1(3H)-isobenzofuranone, 1H-purine-2, 6-dione 3, 7dihydro-1,3,7-trimethyl-acetamide,acetylcodein, 6-MAM and diacetylmorphine (Table 4.3). Despite slight differences, all three solvents showed high concentrations of heroin and heroin-related products.

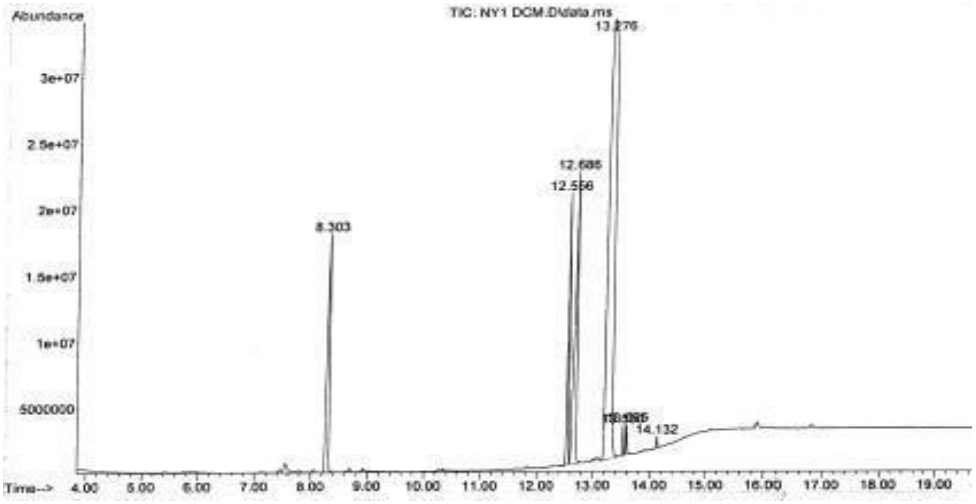

Figure 4.1: Chromatograph of nyaope samples extracted with dichloromethane.

Table 4.1: Compounds identified in nyaope samples following dichloromethane extraction.

Retention time (min)	Library / ID	Reference no	CAS #	Quality (%)
8.303	Caffeine	191	000058-08-2	95
12.556	Codeine	224	006703-27-1	93
12.685	Heroin-M (6-acetyl-morphine)	525	059833-14-6	95
13.276	Morphine 2AC	225	000561-27-3	96

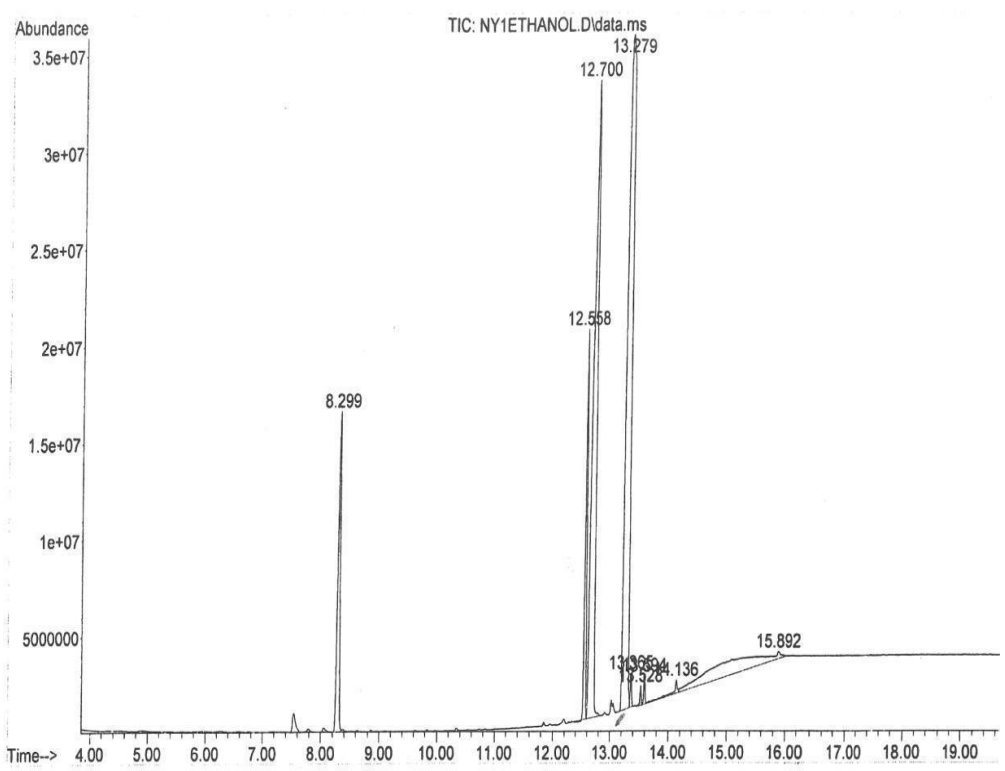

Figure 4.2: Chromatograph of nyaope samples extracted with ethanol

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Table 4.2: Compounds identified in nyaope samples extracted with ethanol.

Retention time (min)	Library / ID	Reference no	CAS #	Quality (%)
8.299	Caffeine	191	000058-08-2	94
12.558	Codeine	224	006703-27-1	93
12.700	Heroin-M (6-acetyl-morphine)	525	059833-14-6	95
13.279	Morphine 2AC	225	000561-27-3	95

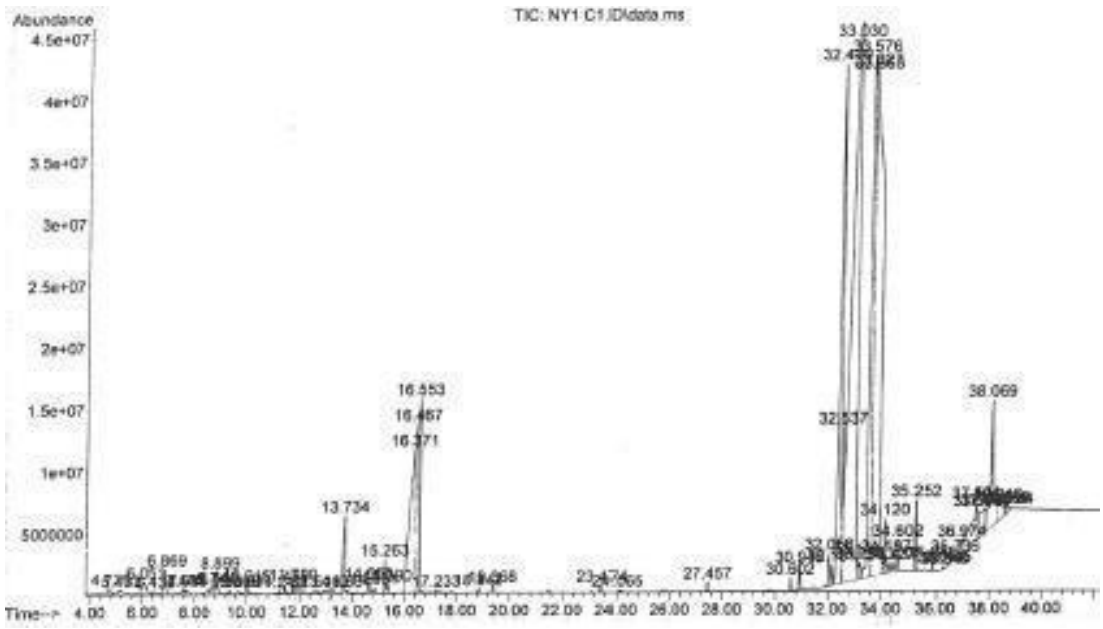


Figure 4. 3: Chromatograph of nyaope samples extracted with methanol.

Table 4.3: Compounds identified in nyaope samples extracted with methanol.

Retention time (min)	Library / ID	Reference no	CAS #	Quality (%)
13.734	1(3H)-isobenzofuranone	52288	000569-31-3	99
16.371	1H-purine-2,6-dione 3,7dihydro-1,3,7-trimethyl- acetamide	53168	000058-080-2	97
16.457 (same as 16.371)	1H-purine-2,6-dione 3,7dihydro-1,3,7-trimethyl- acetamide	53168	000058-080-2	97
16.553 (same as 16.371)	1H-purine-2,6-dione 3,7dihydro-1,3,7-trimethyl- acetamide	53170	000058-080-2	97
32.479	acetylcodein	148310	1000153-00-2	98
32.537	6-monoacetylmorphine	140705	002784-73-8	99
33.033 (same as 32.537)	6-monoacetylmorphine	140705	002784-73-8	99
33.575	diacetylmorphine	160913	000561-27-3	99
33.627 (same as 33.575)	diacetylmorphine	160913	000561-27-3	99

4.2 Determination of the appropriate dose of nyaope

Behavioural scientists generally investigate the effects of psychoactive drug usage at a predetermined dose when studying drug-related disorders (Higgins *et al*., 2004). The drug dose plays a significant role in behavioural changes and an overdose could lead to death.

I embarked on a pilot study to determine an appropriate dose of nyaope for our experiments since there is no literature describing the therapeutic and lethal dose of this drug. As a pilot study, I used an initial dose of 10 mg/kg administered intraperitoneal (ip) to 12 male and 9 female rats. The animals were observed for 60 minutes post nyaope injection. Figure 4.4 reports the number of rats (21) who died after administration at specific time points on their own. The dose of 10 mg/kg of nyaope affected the other 11 rats in such a way that they were euthanized at various time points after the nyaope injection, following the advice of a qualified veterinary surgeon. I was then left with 40 rats hence I needed re-apply for the modification of ethical approval and redesign the experimental model with the 40 rats (20 males and 20 females).

A substantial number of male rats (67%) were terminated within 25 minutes of the nyaope injection (Figure 4.4). The remaining animals survived for at least 50 minutes. Female rats tolerated the nyaope treatment slightly better and showed a more gradual termination frequency. Several hallmark symptoms were monitored including physical appearance (quality of coat, bulging of eyes, and colour of feet) and physiological functioning (breathing). Figure 4.5 showed the quality of the coat of two control rats, while the overall appearance of nyaope-treated animals can be seen in Figure 4.6. The visual comparison clearly shows that drug-treated animals displayed a ruffled coat exhibiting piloerection and bulging eyes typical of exophthalmos. The feet of nyaope-treated animals had a cyanotic discolouration (Figure 4.7). Nyaope-treated animals struggled to breathe, exhibiting hyperventilation, mainly through the mouth, with accelerated inspiration and expiration (Figure 4.8). The other rats were euthanized.

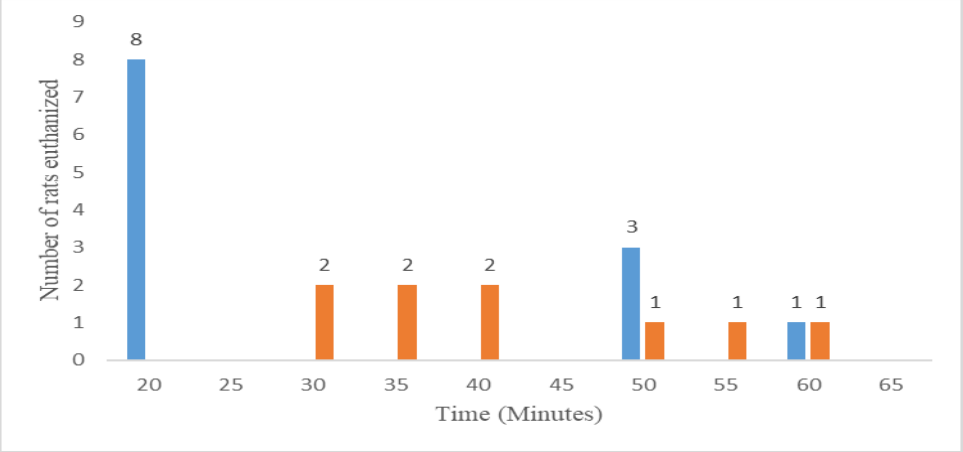
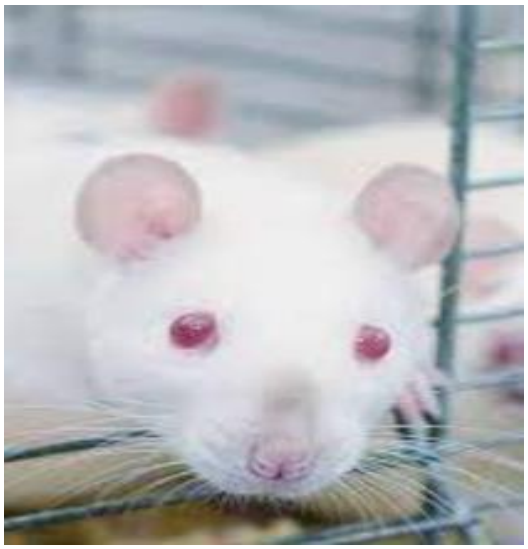


Figure 4.4: The number of rats that were euthanized at different time points over the 60 minutes following the administration of 10 mg/kg nyaope.

Figure 4.5: The quality of the coats of two control animals.

A 

B 

Figure 4.6: The drug-treated animal with 10mg/kg of nyaope clearly shows

(A) Polioerection and (B) Exophthalmos

(A) (B)

Figure 4.7: The drug-treated animal with 10mg/kg of nyaope displayed discolouration (A) and a pale appearance (B).

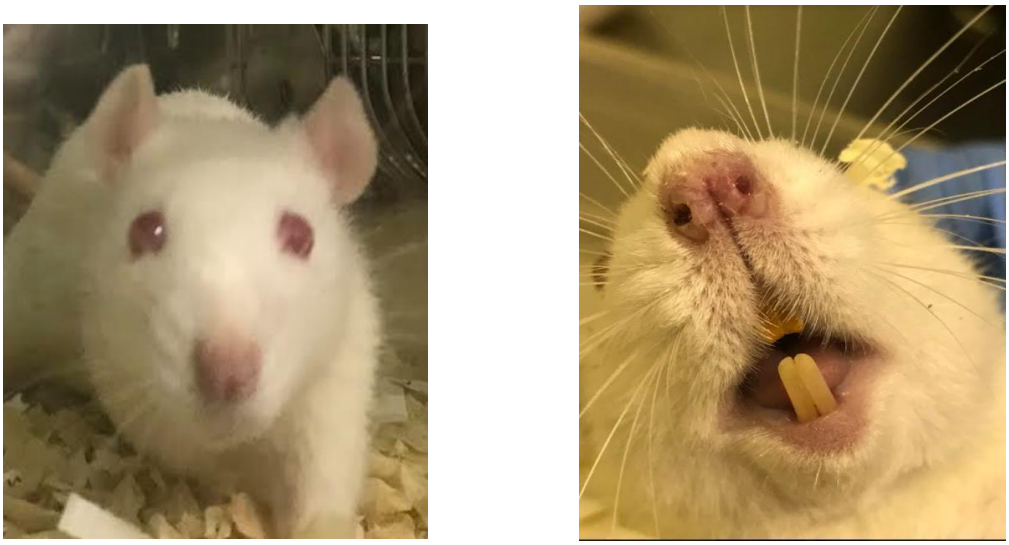


Figure 4.8: The appearance of a rat breathing normally before (a) and then struggling after (b) treated with nyaope (10 mg/kg).

4.3 Animal welfare

Based on the observations of the pilot study, the nyaope dose was decreased to 1 mg/kg. The body mass of this group of rats was monitored weekly (Figure 4.9). Monitoring occurred from the time of receipt of the animals from the university's animal facility until the end of the experiment and lasted for nine weeks. There was no significant difference in the body mass of control and drug-treated animals over the entire monitoring period for either males

or females (Figure 4.10). Male rats, in general, had a greater body mass 320.0g, 61; SD: 9.99) than female rats ($\bar{x}$:196.57g; SD: 9, 89) (Figure 4.10). None of the previously recorded phenotypic features was observed in these animals. Consequently, this dose (1 mg/kg) was used in subsequent experiments.

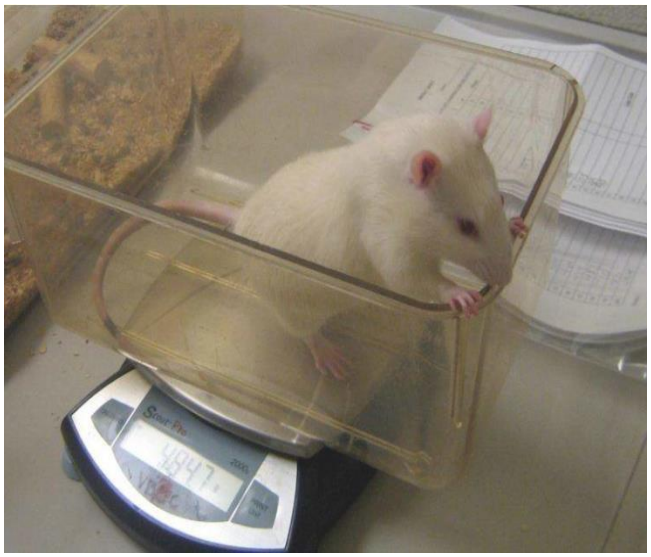


Figure 4.9: A rat being weighed.

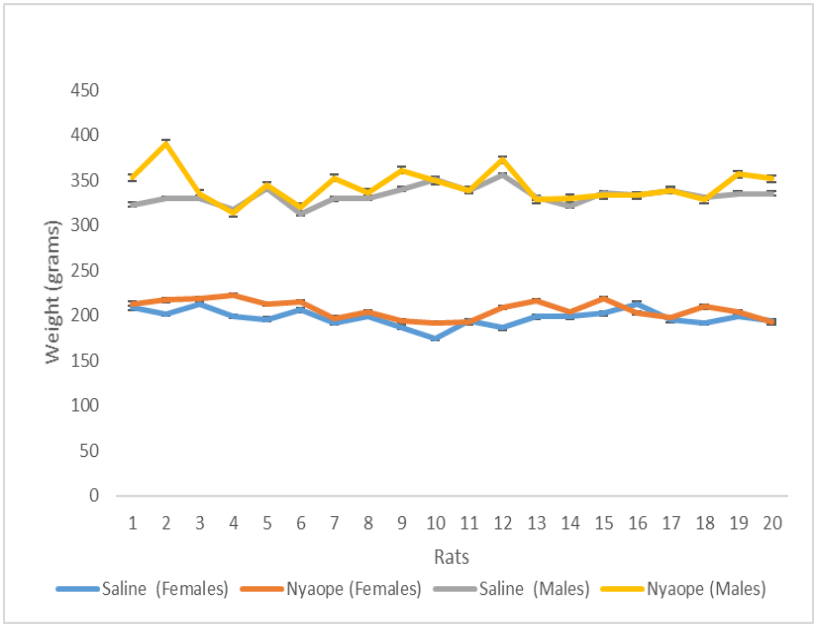


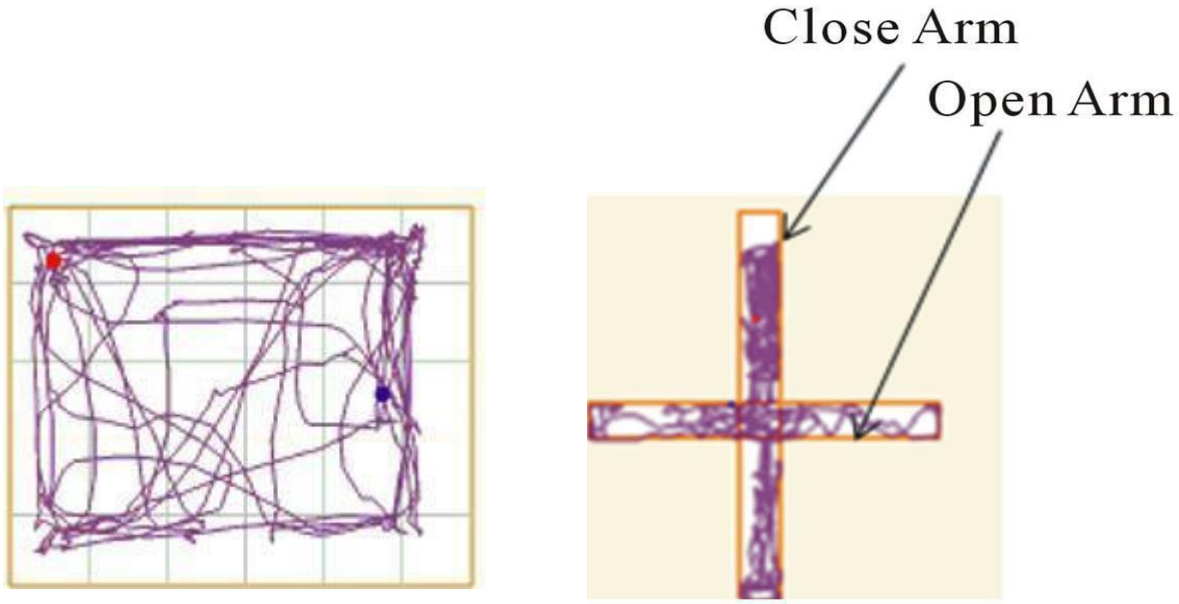
Figure 4.10: The body mass of saline-treated and nyaope-treated male and female rats over nine weeks. Data is represented by the mean ± SD (n=40).

4.4 Behavioural assessments

Behavioural assessments were performed to evaluate the impact of nyaope treatment on the locomotor activity, mood and cognitive behaviour of the animals. For this purpose, the OFT and EPM were used to monitor locomotor and anxiety-like behaviour, while the NOR test and the Y-maze were used to evaluate cognitive function.

4.4.1 The impact of nyaope on the locomotor activity of rats

Scoring videos of small animals enables the measurement of their locomotor activity, their position in an arena, their interactions with an object, and their engagement in defensive behaviours. During all tests, large individual differences were found as animals had different movement patterns in the different areas (Figure 4.11).



A B

Figure 4.11: A typical recording track of a rat moving within the open field arena (A) and the elevated plus-maze (B).

4.4.1.1 Head distance travelled in the open field

Figure 4.12 depicts the head distance travelled in 5 minutes in the open field test of saline (n=12) and nyaope-treated (n=12) animals. Data were normally distributed (Shapiro-Wilk test). A repeated measures two-way ANOVA was used to analyse grouped data that was followed by unpaired t-tests. There were significant differences in head distance travelled in both saline-treated and nyaope- treated animals. For the saline group, the animals displayed significantly lower distances post-treatment (Figure 4.12). Similarly, nyaope-treated animals showed a significant decrease in head distance travelled post-treatment (Figure 4.12). However, the post-nyaope head distance travelled was significantly lower than the post-saline head distance travelled (Figure 4.12).

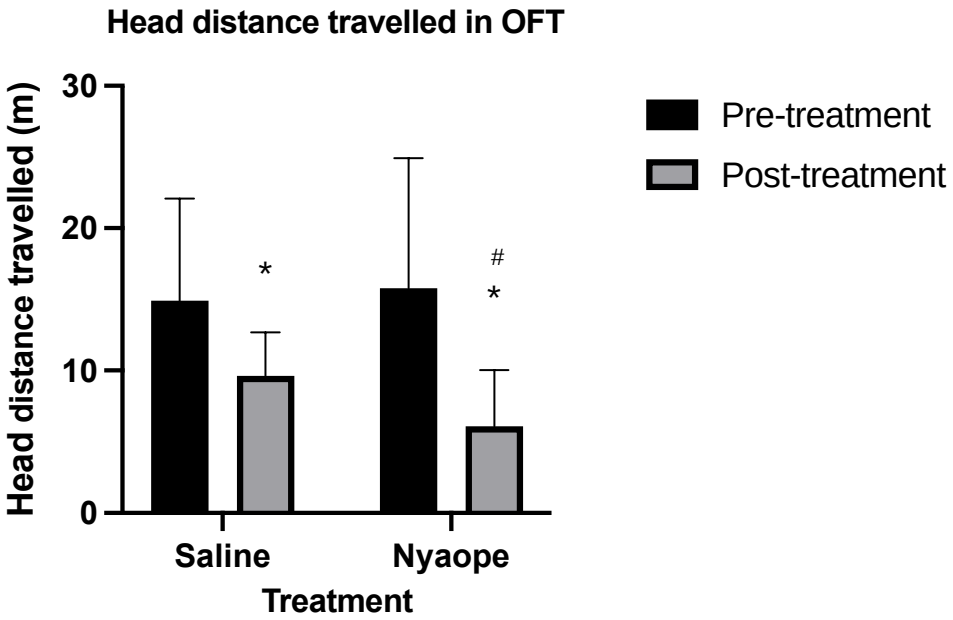


Figure 4.12: Head distance travelled in the open field test (Saline, n=12; Nyaope, n =12).

*p< 0.0002, Post-treatment groups vs Pre-treatment groups
p= 0.0221, Post-treatment nyaope vs Post-treatment saline

The data of the saline and nyaope-treated groups were then analysed according to gender. Data from female rats mirrored that of the total group of animals. Data were normally distributed (Shapiro- Wilk test). A repeated measure two-way ANOVA was used to analyse grouped data and this was followed by unpaired t-tests. Data are presented as mean ± SD. The female rats showed a significant decrease in head distance travelled following both saline (n=6) and nyaope (n=6) treatment (Figure 4.13), with nyaope-treated female rats exhibiting a significant lower head distance travelled post nyaope treatment than post saline treatment (Figure 4.13). In contrast, male rats yielded no significant differences between saline (n=6) and nyaope (n=6) treatment (Figure 4.14). Data were normally distributed (Shapiro-Wilk test). A repeated measure two-way ANOVA was used to analyse grouped data and this was followed by unpaired t-tests. Data are presented as mean ± SD.

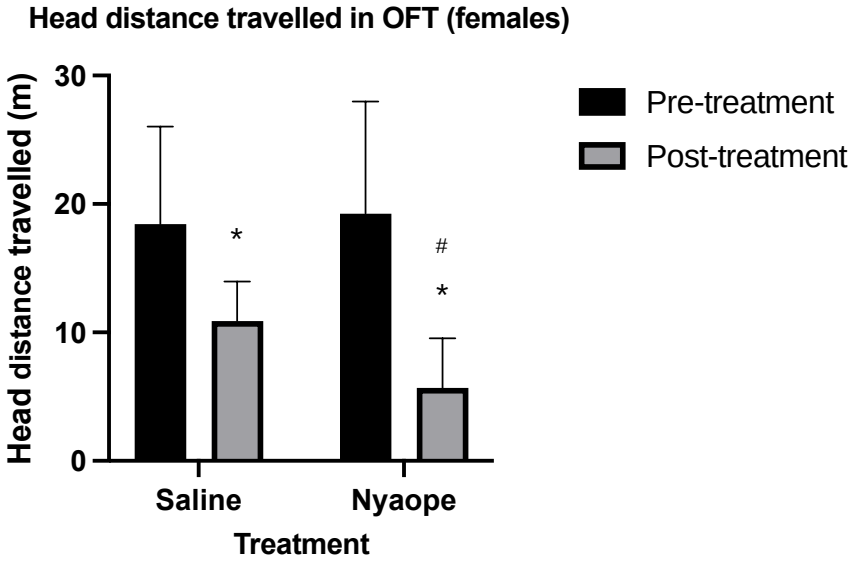


Figure 4. 13: Head distance travelled in the open field test by female rats

(Saline, n=6; Nyaope, n=6)

*p<0.0005, Post-treatment groups vs Pre-treatment groups:
#p=0.027, Post-treatment nyaope vs Post-treatment saline

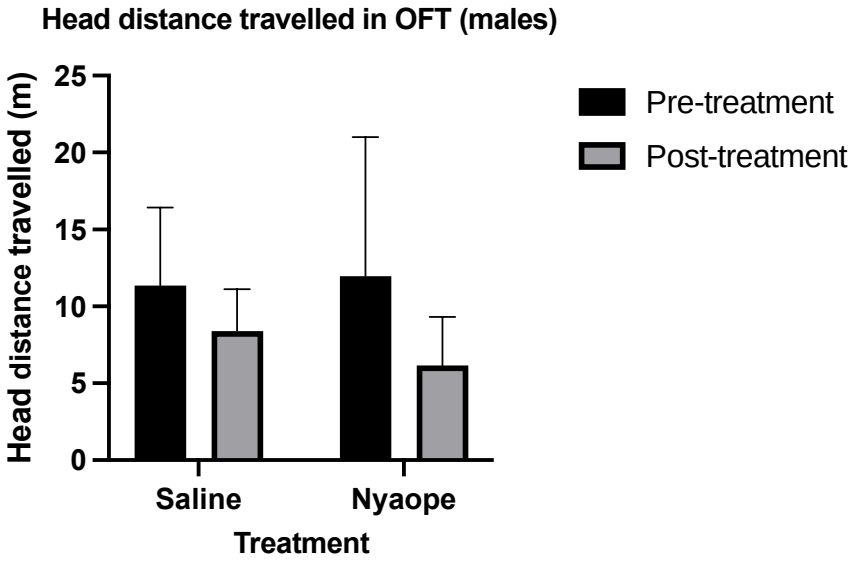


Figure 4.14: Head distance travelled in the open field test by male rats (Saline, n=6; Nyaope, n =6).

4.4.1.2 Mean speed of animals in the open field

The mean speed of rats in the OFT was recorded to determine whether the decrease in head distance travelled was a result of animals attaining lower speeds following treatment. Data had a normal distribution (Shapiro-Wilk test). Data were analysed using repeated measures two-way ANOVA and unpaired t-tests. Animals that were treated either with saline (n=12) or nyaope (n=12) showed a significant decrease in mean speed (Figure 4.15) and there was also a significant decrease in the mean speed of nyaope-treated animals compared to saline-treated animals post-treatment Figure 4:15).

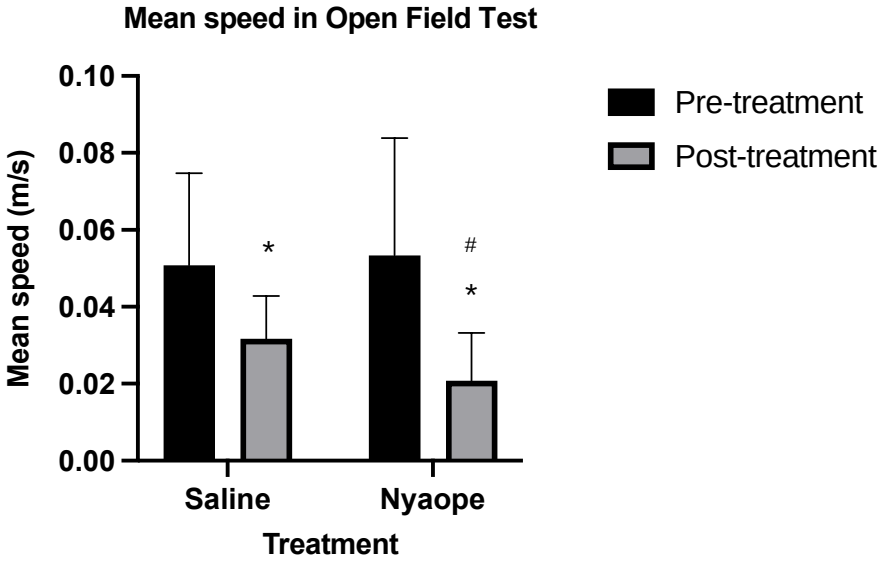


Figure 4.15: Mean speed travelled by rats in the open field test (Saline, n=12; Nyaope, n =12).

*p<0.0001, Post-treatment groups vs Pre-treatment groups;

#p=0.034, Post-treatment nyaope vs Post-treatment saline

Further analysis of mean speed showed that there was a decrease in mean speed that occurred in both the area against the wall (Figure 4.16) and in the centre (Figure 4.17) of the open field. The data depicted a decrease in mean speed in nyaope treated as compared to saline-treated rats.

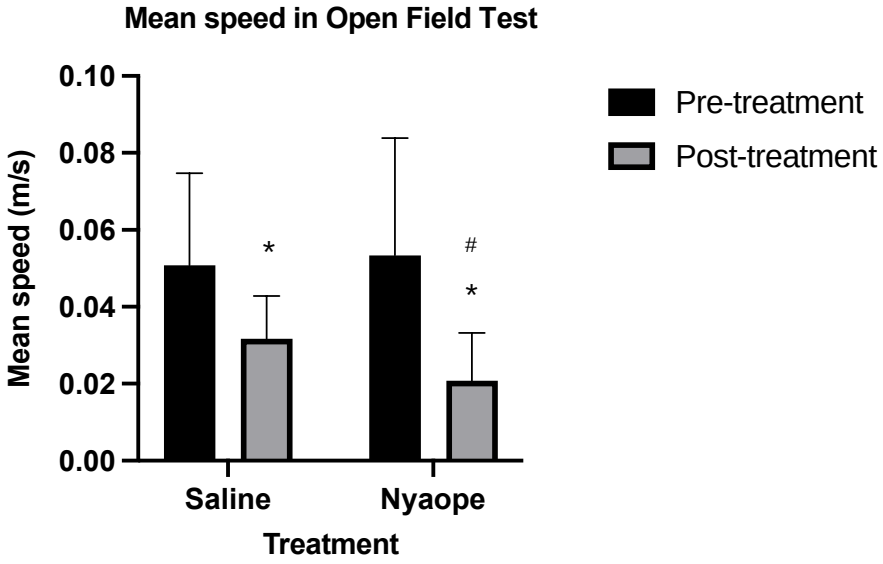


Figure 4.16: Mean speed travelled by rats against the wall of the open field test (Saline, n=12; Nyaope, n =12).

*p<0.0001, Post-treatment groups vs Pre-treatment groups;

#p=0.0195, Post-treatment nyaope vs Post-treatment saline

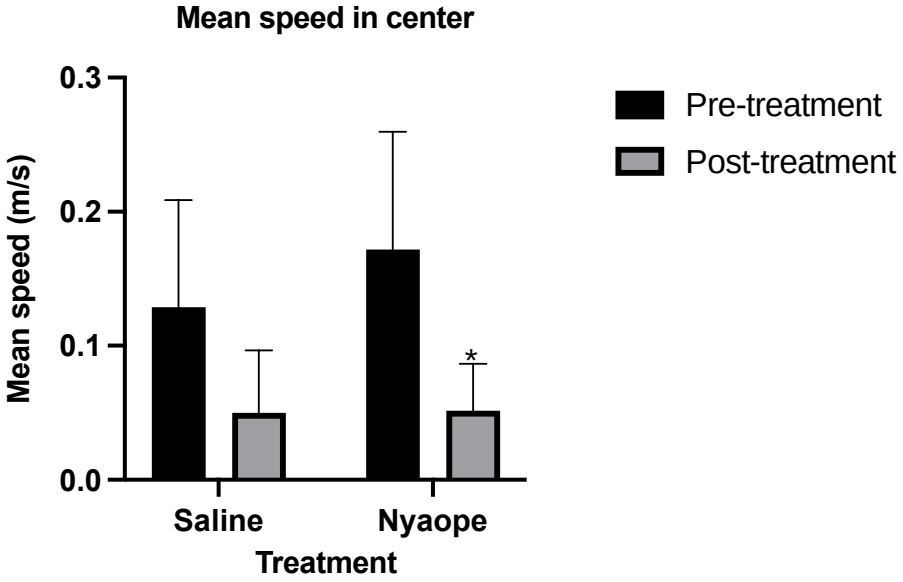


Figure 4.17: Mean speed travelled by rats in the centre of the open field test (Saline, n=12; Nyaope, n =12).

*p<0.0001, Post-treatment nyaope vs Pre-treatment nyaope.

4.4.1.3 Distance travelled in the EPM by both genders

There were no significant differences in the distance travelled in the elevated plus-maze by rats that were treated with saline (n=12) or nyaope (n=12) (Figure 4.18). Data were normally distributed (Shapiro-Wilk test). Data were analysed using repeated measures two-way ANOVA. Data are presented as mean ± SD.

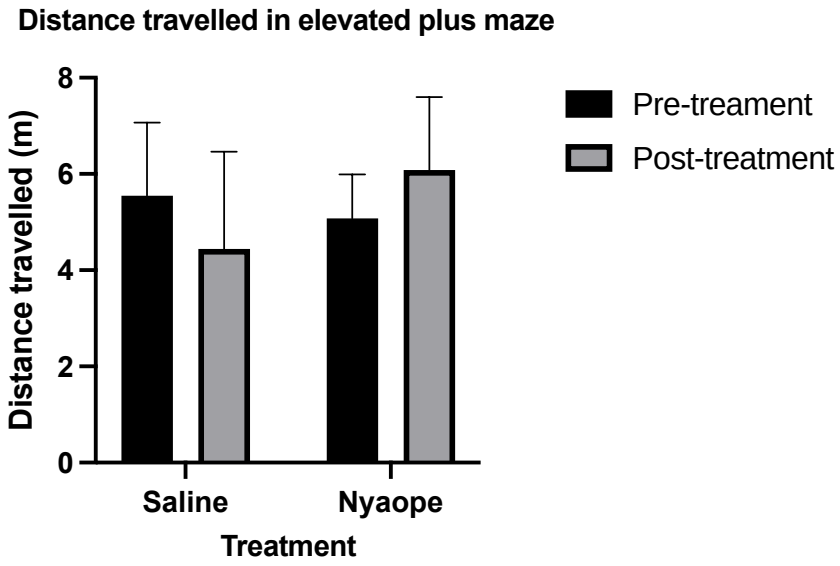


Figure 4.18: Distances travelled by rats in the elevated plus-maze (Saline, n=12; Nyaope, n=12).

4.4.1.4 Mean speed of animals in the elevated plus-maze

There were no significant differences in the mean speed of rats in the elevated plus-maze that were treated with saline (n=12) or nyaope (n=12) (Figure 4.19). Data were normally distributed (Shapiro- Wilk test). Data were therefore analysed using a repeated measure two-way ANOVA. Data are presented as mean ± SD.

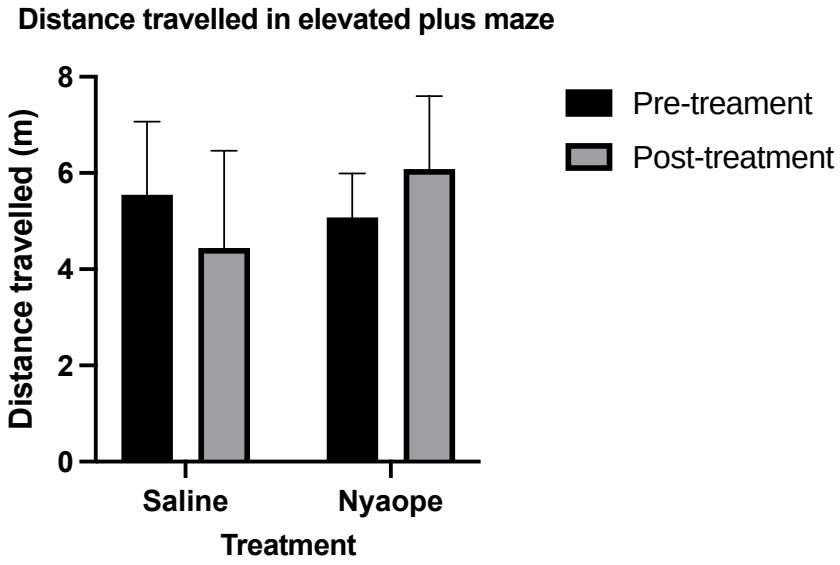


Figure 4.19: Mean speed travelled by rats in an elevated plus-maze

(Saline, n=12; Nyaope, n=12)

In summary, rats treated with nyaope showed decreased locomotor activity as indicated by a reduction in distance travelled in the open field test. This was most evident in female rats. The decrease in distance travelled was associated with a decrease in speed attained by nyaope-treated rats, occurring mainly in the centre area of the open-field arena.

4.5 The impact of nyaope on the mood of rats

4.5.1 Time spent against the wall of the open field

Both saline (n=12) and nyaope-treated (n=12) animals spent significantly more time against the wall of the open field arena compared to pre-treatment (Figure 4.20). Data were normally distributed (Shapiro-Wilk test). Data were therefore analysed using a repeated measure two-way ANOVA followed by unpaired t-tests. Data are presented as mean ± SD. Nyaope-treated female rats (n=6) spent significantly more time against the wall of the open field arena post-treatment than pre-treatment (Figure 4.21). Male rats (saline, n=6; nyaope, n=6) showed no significant differences between pre-treatment and post-treatment time (Figure 4.21).

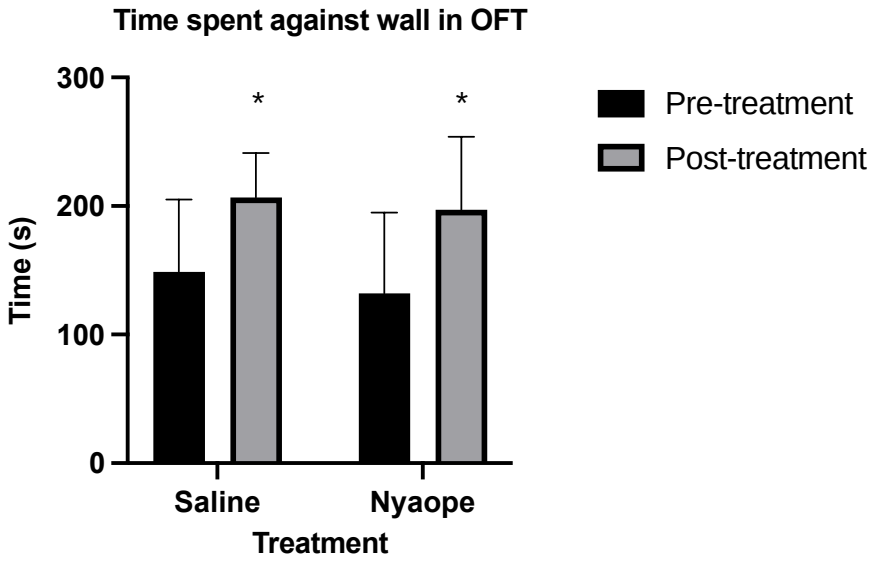


Figure 4.20: Time spent by rats against the wall in the open field test (Saline, n=12; Nyaope, n =12).

*p=0.014, Post-treatment groups vs Pre-treatment groups.

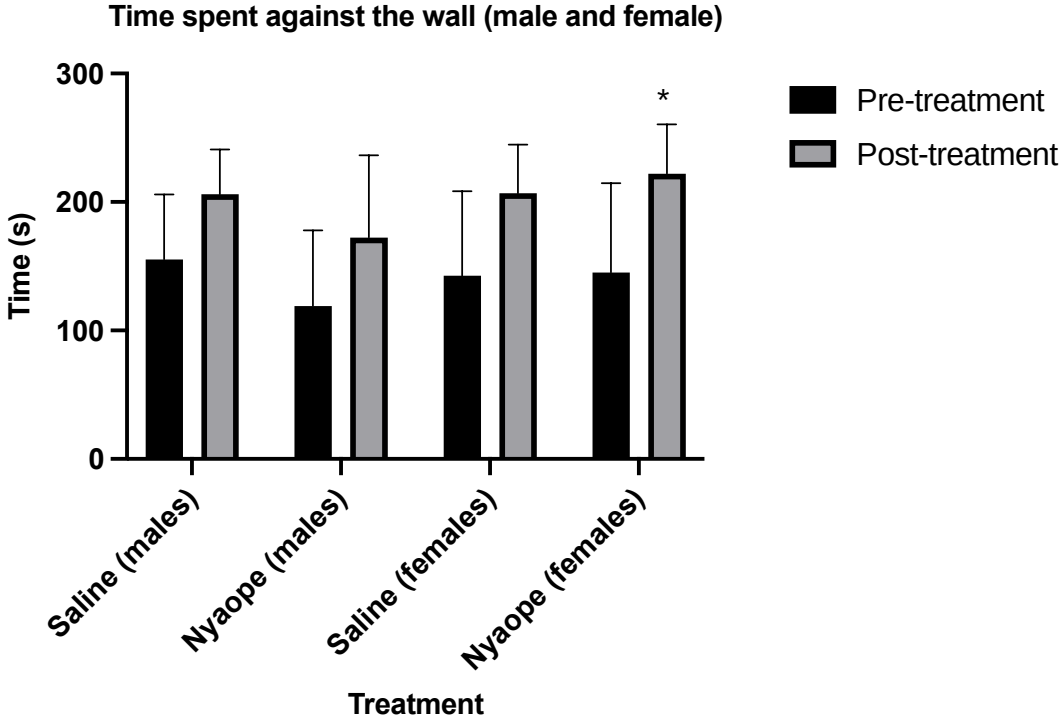


Figure 4.21: Time spent by rats against the wall

(Males: Saline, n=6; Nyaope, n=6 and Females: Saline, n=6; Nyaope, n=6).

*p=0.04, Post-treatment nyaope-treated female rats vs Pre-treatment nyaope-treated female rats.

4.5.2 Time spent in the centre area of the open field

Saline-treated rats (n=12) spent significantly more time in the centre of the open field arena than before treatment (Figure 4.22). There was no significant difference in the saline and nyaope treated animals as per gender. This difference was not evident in nyaope-treated rats (n=12, Figure 4.22). Data were normally distributed (Shapiro-Wilk test). Data were analysed using a repeated measure two-way ANOVA followed by unpaired t-tests. Data are presented as mean ± SD.

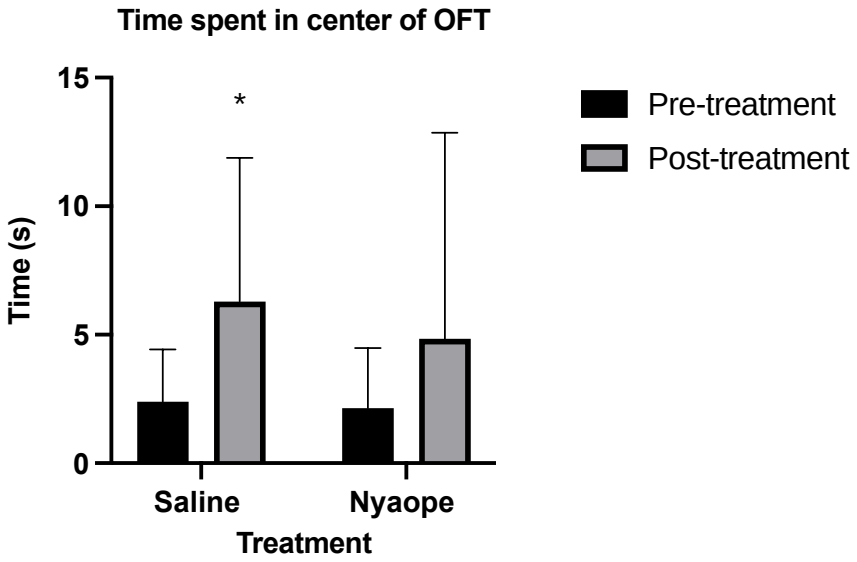


Figure 4. 22: Time spent by rats in the centre of the open field test (Saline, n=12; Nyaope, n =12).

*p=0.0219, post-treatment saline vs pre-treatment saline.

4.5.3 Time spent in the open and closed arms of the elevated plus-maze

In general, rats spent more time in the closed arms than in the open arms of the EPM. The amount of time spent in the closed arm was significantly less after saline (n=12) and nyaope (n=12) treatment compared to pre-treatment (Figure 4.23). There were no significant differences in any treatment group concerning time spent in the open arms of the EPM (Figure 4.23). For all groups, the amount of time spent in the open arms was significantly reduced compared to the time spent in the closed arms. Data were normally distributed (Shapiro-Wilk test). Data were therefore analysed using a repeated measure two-way ANOVA followed by unpaired t-tests. Data are presented as mean ± SD.

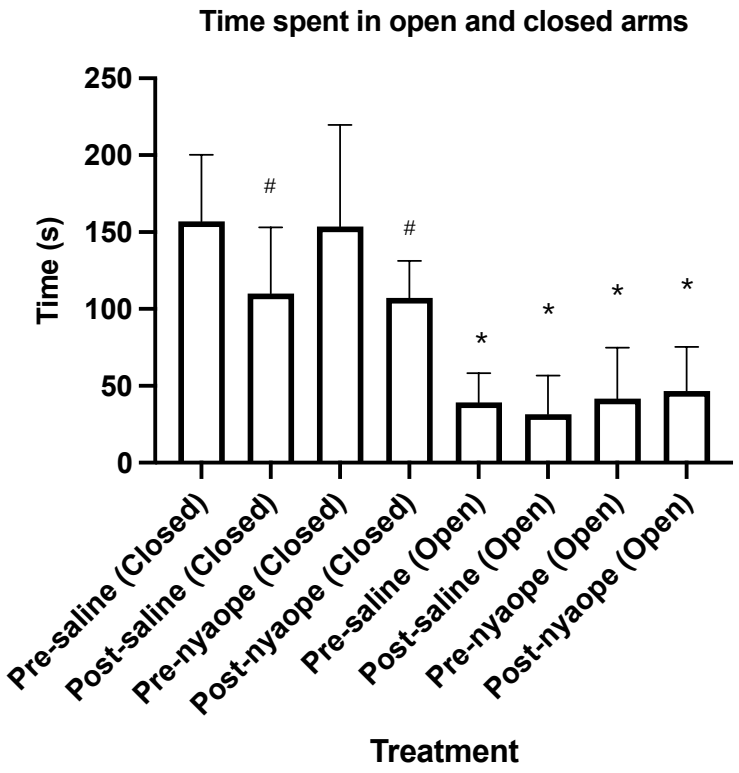


Figure 4.23: Time spent by rats in the open and closed arms of the elevated plus-maze (Saline, n=12; Nyaope, n =12).

*p<0.0001, respective groups (Closed) vs respective groups (Open), i.e. Pre-saline (Closed) vs Pre-saline (Open), Post-saline (Closed) vs Post-saline (Open), and Pre-nyaope (Closed) vs Pre- nyaope (Open); #p<0.0001,

Post-saline (Closed) vs Pre-saline (Closed) and Post-nyaope (Closed) vs Pre-nyaope (Closed)

4.5.4 The number of head entries into the open and closed arms

No significant differences were found in the number of head entries into the open and closed arms of the EPM (Figure 4.24). Neither saline (n=12) nor nyaope-treated (n=12) animals showed any significant differences in head entries. Data were normally distributed (Shapiro-Wilk test). Data were therefore analysed using a repeated measure two-way ANOVA followed by unpaired t-tests. Data are presented as mean ± SD.

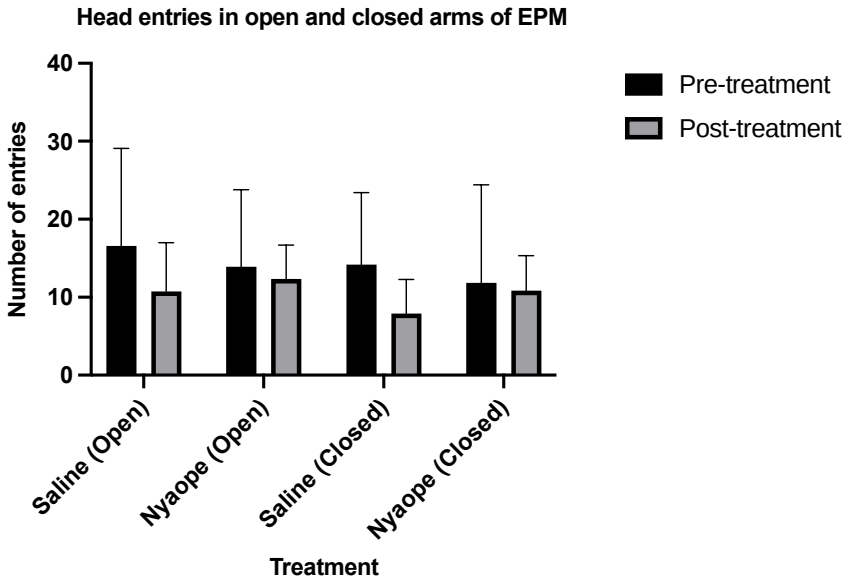


Figure 4. 24: Head entries into the open and closed arms of the elevated plus-maze. (Saline, n=12; Nyaope, n =12).

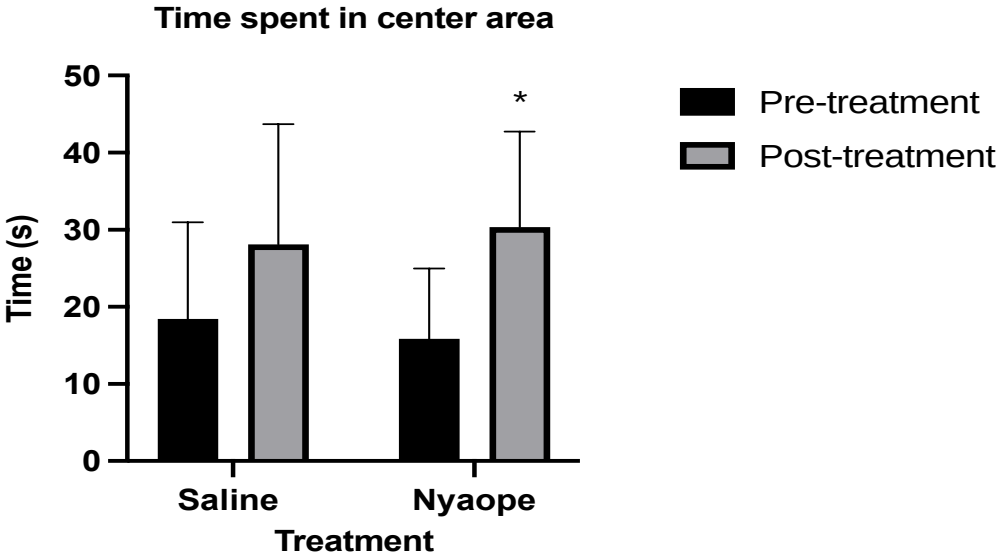


Figure 4.25**:** Time spent by rats in the centre areas of the elevated plus-maze (Saline, n=12; Nyaope, n =12).

*p<0.05, Post-nyaope treatment vs Pre-nyaope treatment

Figure 4.25 shows the time spent in the centre area of the elevated plus-maze by rats treated with either saline (n=12) or nyaope (n=12), but there was a significant difference in time spent in the centre zone between pre-and post-nyaope-treated animals (Figure 4.25). Data were normally distributed (Shapiro-Wilk test). Data were therefore analysed using a repeated measure two-way ANOVA and presented as mean ± SD.

In summary, during the open field test (OFT), both saline and nyaope-treated animals spent more time against the wall after treatment and this effect was most prominent in female rats. Saline-treated animals spent more time in the centre of the open field and this increase was not seen in nyaope-treated animals. In the EPM, animals spent more time in the closed arms of the apparatus than in the open arms. The time spent in the closed arms was reduced after rats were treated with either saline or nyaope. There was no significant difference in the number of head entries by the rats into the various arms of the EPM.

4.6 The impact of nyaope on the cognitive behaviour of rats

4.6.1 The novel object recognition test

During the experiment, the movement of animals was tracked using one of the features of the ANYmaze software for both the NOR test (Figure 4.26A) and the Y-maze (Figure 4.26B). These tracing images provide evidence that animals were moving inside the apparatus before and after being treated with saline and nyaope.

A B

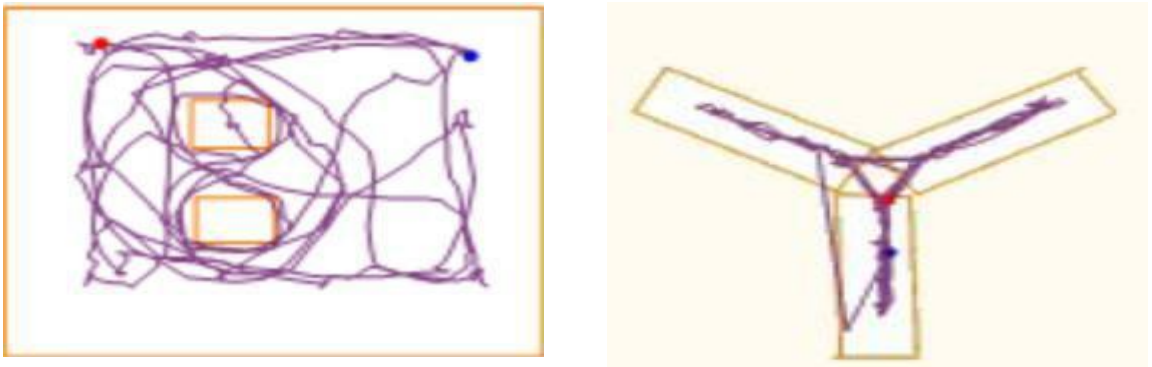


Figure 4.26: A representative locomotion-tracking pattern of a rat moving inside the novel object recognition test (A) and the Y-maze (B)

Two parameters were measured to indicate alterations in cognitive behaviour in the novel object recognition test. These were the number of head entries into the novel object expressed as a percentage of total entries (Figure 4.27), and the head time spent with the novel object as a percentage of the total time (Figure 4.30). Data were normally distributed (Shapiro-Wilk test). Data were therefore analysed using a repeated measure two-way ANOVA. Data are presented as mean ± SD. Rats that received saline (n=12) displayed significantly fewer head entries to the novel object post-treatment compared to pre-treatment (Figure 4.27, p=0.0307). This significant decrease in time spent was not observed in the nyaope-treated animals (n=12, Figure 4.27). When males and females were analysed separately, no significant changes were observed in males (n=6, Figure 4.28) or females (n=6, Figure 4.29). Evaluation of the head time spent with the novel object yielded no significant results (saline, n=12; nyaope, n=12, Figure 4.30).

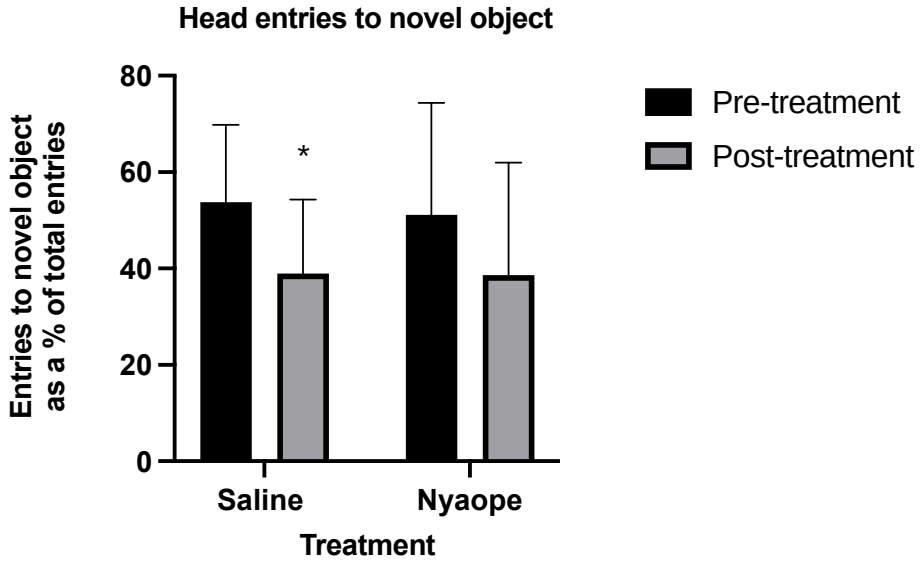


Figure 4. 27: Head entries into the novel object (Saline, n=12; Nyaope, n =12).

*p=0.0307, Post-saline vs Pre-saline treatment

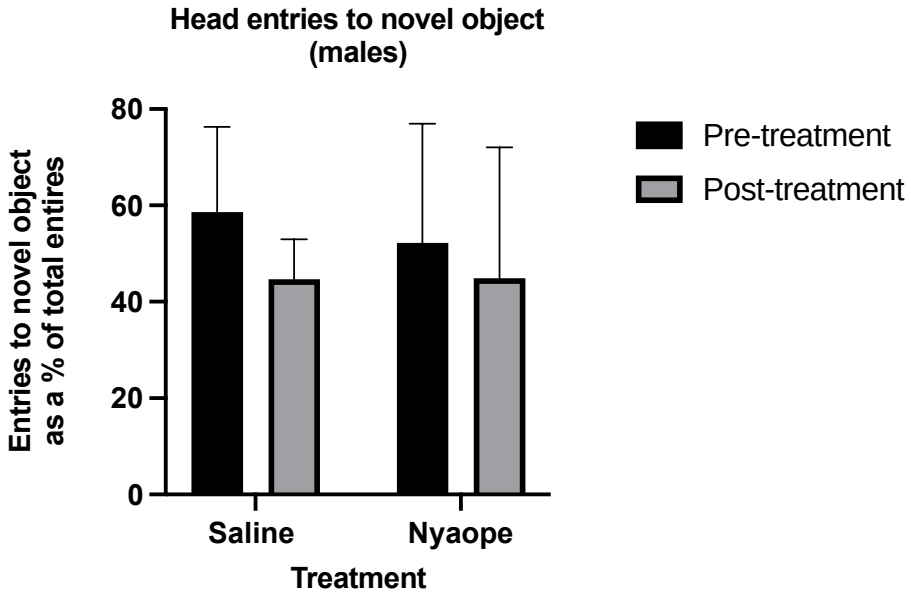


Figure 4.28: Head entries into the novel object by male rats (Saline, n=6; Nyaope, n =6).

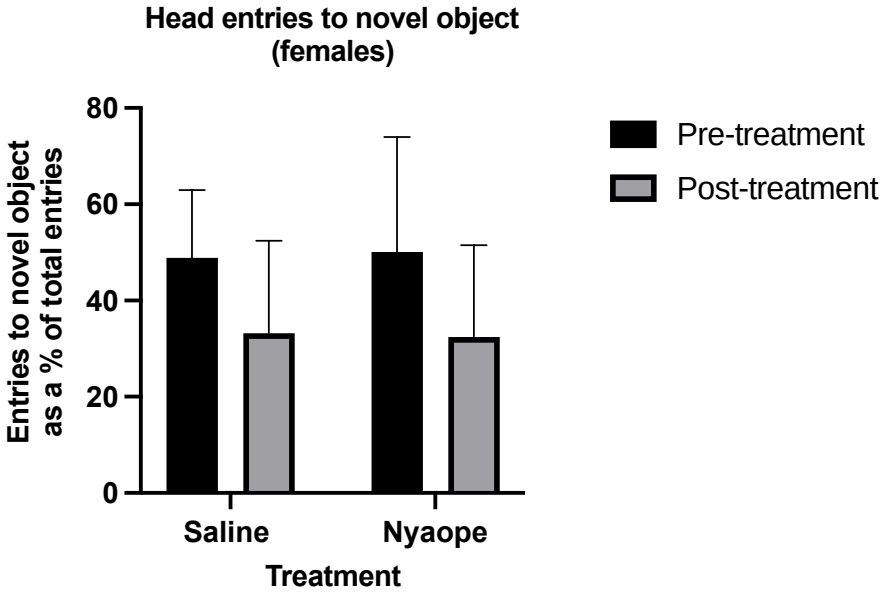


Figure 4.29: Head entries into the novel object (females) (Saline, n=6; Nyaope, n =6).

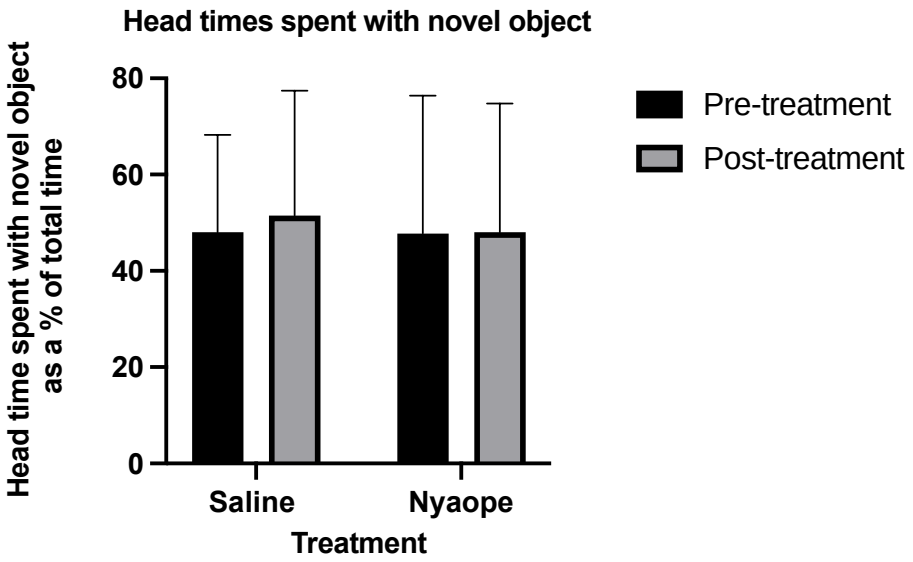


Figure 4. 30: Head time spent with the novel object (Saline, n=12; Nyaope, n =12).

4.6.2 The Y-maze

There were no significant differences in distance travelled between saline-treated (n=12) and nyaope-treated (n=12) rats in the Y-maze (Figure 4.31). Data were normally distributed (Shapiro- Wilk test). Data were therefore analysed using repeated measures two-way ANOVA. Data are presented as mean ± SD.

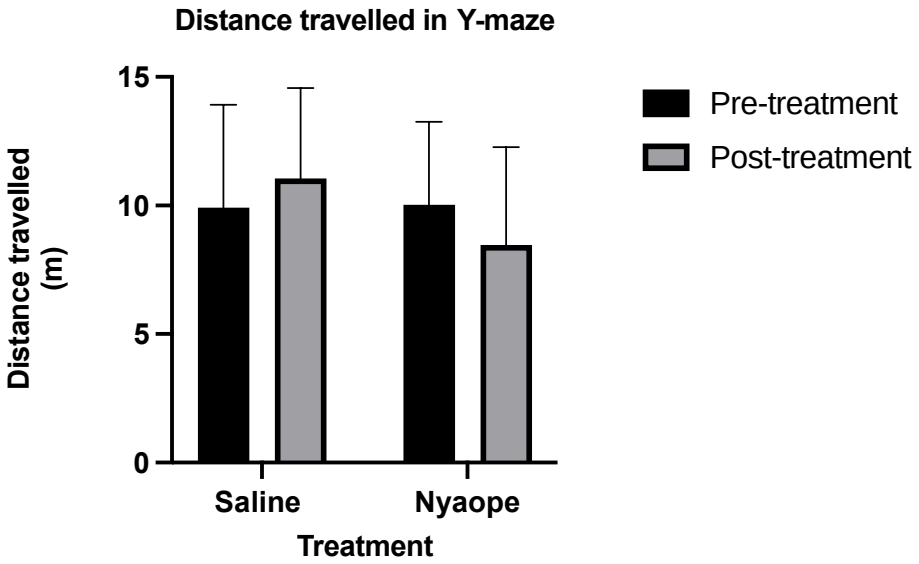


Figure 4. 31: Distance travelled by rats in the Y-maze (Saline, n=12; Nyaope, n =12).

Rats treated with saline (n=12) showed a significant increase in the number of triads completed post-treatment compared to pre-treatment (Figure 4.32). This increase seen in saline-treated animals was absent in nyaope-treated animals (n=12, Figure 4.32). Data were normally distributed (Shapiro-Wilk test). Data were therefore analysed using repeated measures two-way ANOVA followed by an unpaired t-test. Data are presented as mean ± SD.

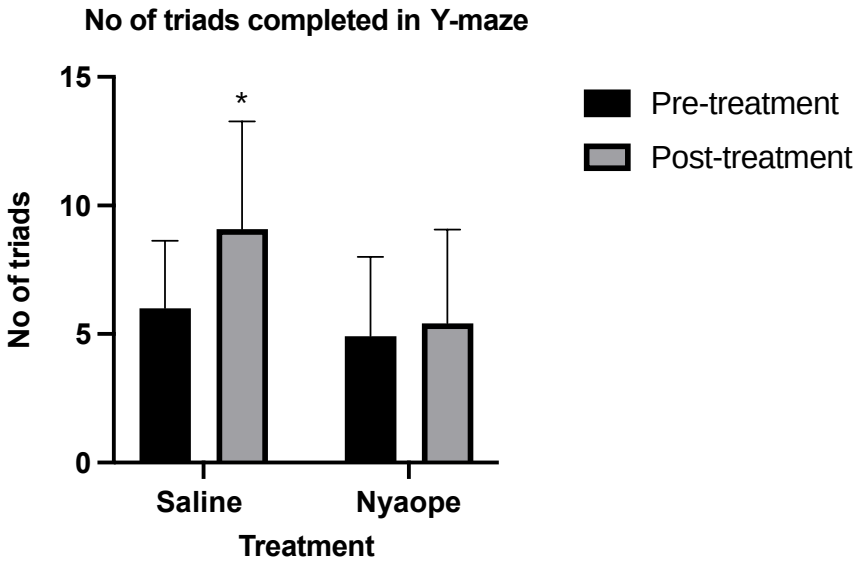


Figure 4.32: Number of triads completed by rats in the Y –maze
(Saline, n=12; Nyaope, n =12).
*p=0.0419, Pre-saline vs Post-saline

Separating the group of animals according to sex showed that the increase in the number of triads observed in the Y-maze was due to an increase elicited by female rats (Figure 4.33). Data were normally distributed (Shapiro-Wilk test). Data were therefore analysed using repeated measures two-way ANOVA followed by an unpaired t-test. Data are presented as mean ± SD.

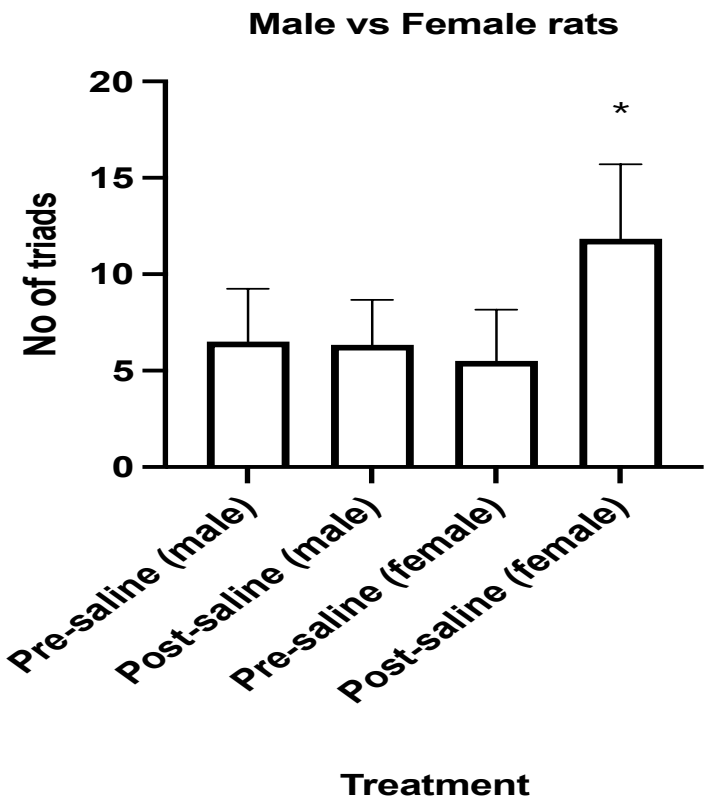


Figure 4. 33: Number of triads completed in the Y-maze by male (n=6) and female (n=6) rats treated with saline.

*p=0.008, Pre-saline (female) vs Post-saline (female)

4.7 Effects of nyaope treatment on opioid signalling

A semi-quantitative ELISA was used to investigate the effects of nyaope treatment on opioid signalling in the FC. No significant changes were observed in the expression of the total or phosphorylated JNK (Figure 4.34) or Erk1/2 (Figure 4.35) between saline and nyaope-treated animals. Figure 4.34 shows the levels of total and phosphorylated JNK in the FC of saline (n=12) and nyaope (n=12) treated animals in comparison to an internal standard. Since these values were lower than the internal standard, the true reflection thereof should be interpreted with caution. Whilst Figure 4.35, shows the levels of total and phosphorylated Erk 1/2 in the FC in saline (n=10) and nyaope (n=12) treated animals in comparison to an internal standard.

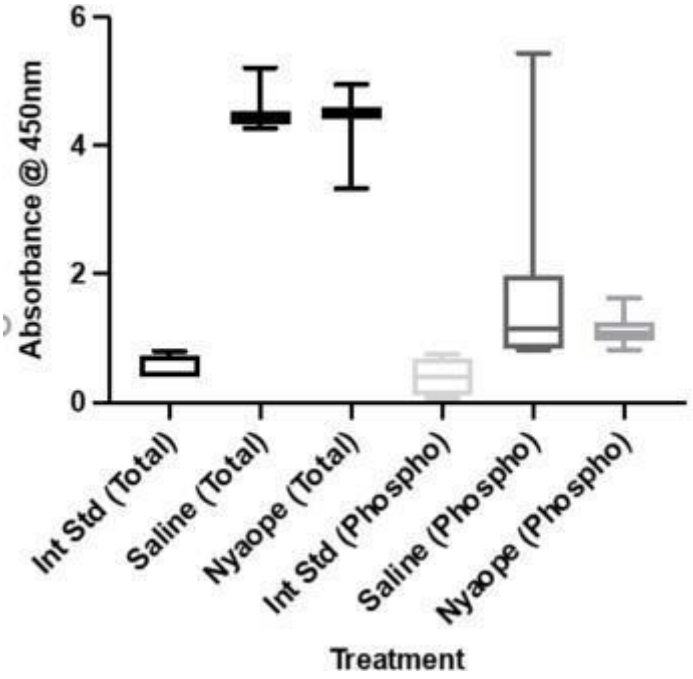


Figure 4. 34: The c-Jun N-terminal kinase levels in the frontal cortex at an absorbance of 450 nm.

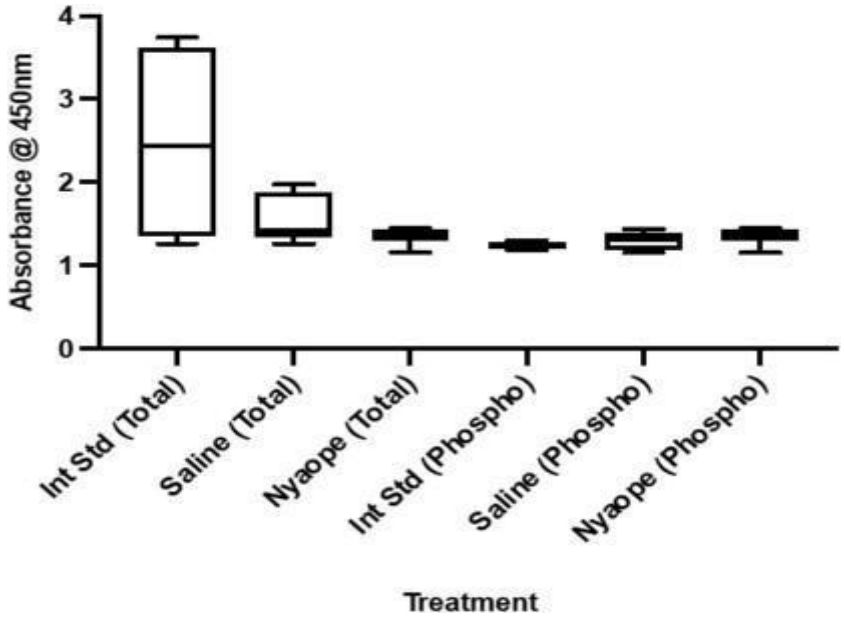


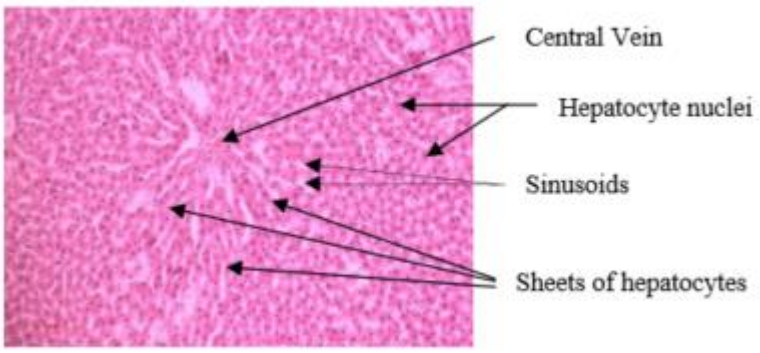
Figure 4. 35: The extracellular signal-regulated kinase 1/2 levels in the frontal cortex at an absorbance of 450 nm

4.8 The effect of nyaope treatment on the morphology of the liver

4.8.1 Histological analysis of the liver tissue of rats treated with saline or nyaope

Figure 4.36 shows the morphology of the liver of a saline-treated rat. The photomicrographs show the normal structure at 10X (A) and 40X (B) magnification. The central vein, sinusoids and sheets of healthy-looking hepatocytes can easily be identified. Rats treated with nyaope displayed a different morphology. There was collagenous thickening around the central vein and a reduction in sinusoidal space/sizes compared to the saline-treated animals (Figure 4.37A). The Masson trichrome stain indicated the presence of connective tissue and fibrosis around the portal tract and central vein of nyaope-treated animals, as well as intense staining of collagen (Figure 4.37 B and C).

A



B

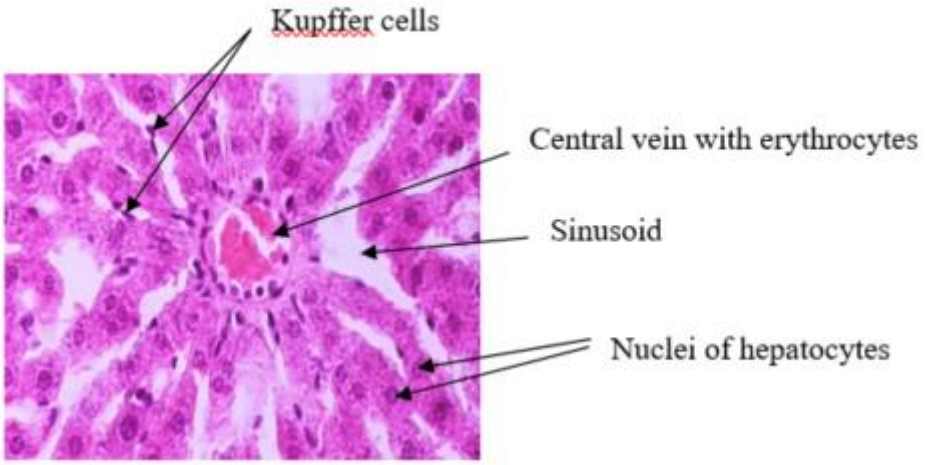
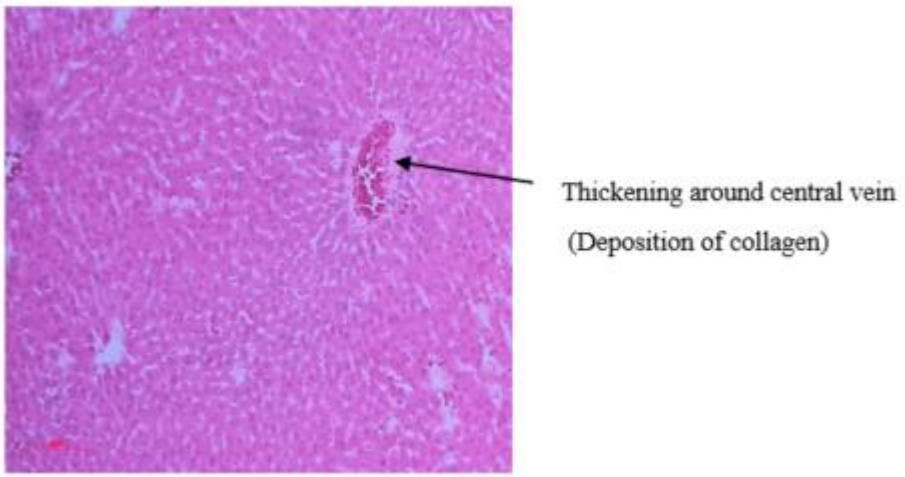


Figure 4. 36: A representative photomicrograph of the liver tissue of a saline-treated rat at 10X (A) and 40X (B) magnification**.**

(A)



B C

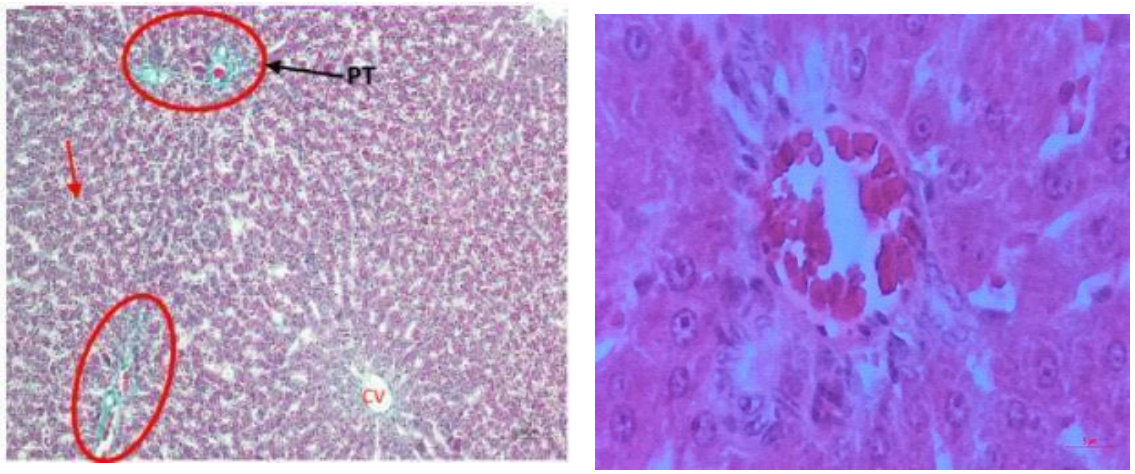


Figure 4.37: Representative photomicrographs of haematoxylin and eosin-stained (A) and Masson trichrome stained (B and C) tissue of the liver of a nyaope-treated rat at 10X (A and B) and 40X (C) magnification. PT: portal tract, CV: central vein.

4.8.2 Analysis of plasma albumin and liver enzyme levels of rats treated with either saline or nyaope

Plasma levels of albumin, alanine transaminase and lactate dehydrogenase were assessed to investigate the effects of nyaope treatment on liver function. Animals treated with nyaope (n=12) had significantly lower plasma albumin levels compared to saline-treated animals (n=12, Figure 4.38). Data were normally distributed (Shapiro-Wilk test). Data were therefore analysed using an unpaired t-test. Data are presented as mean ± SD.

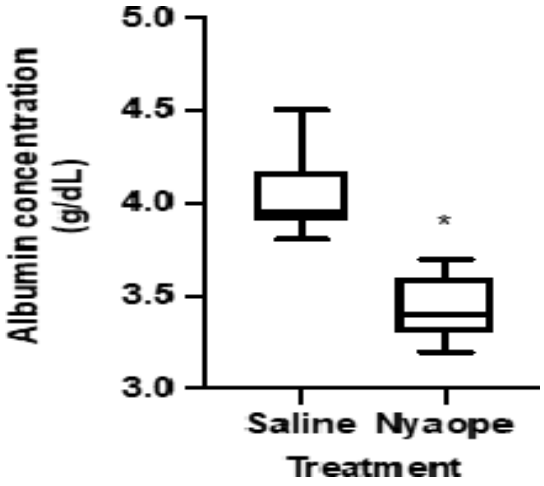


Figure 4. 38: Plasma albumin levels of saline (n=12) and nyaope (n=12) treated animals.

*p<0.0001, Nyaope vs Saline

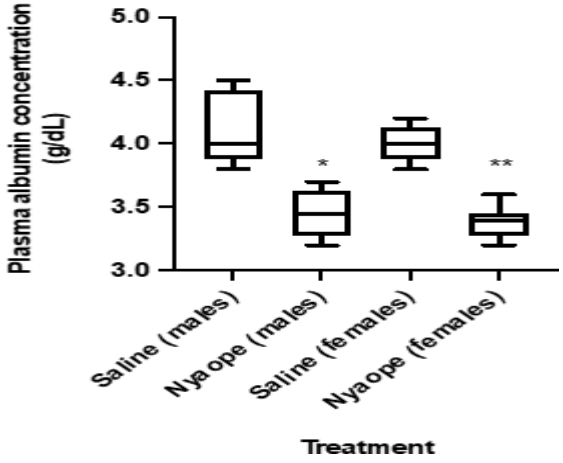


Figure 4. 39: Plasma albumin levels of saline (n=6) and nyaope (n=6) treated male and female rats.

*p<0.001, Nyaope (males) vs Saline (males); **p<0.0001, Nyaope (females) vs Saline (females)

Figure 4.39 shows the plasma albumin levels of male (n=6) and female (n=6) rats treated either with saline or nyaope. Data were normally distributed (Shapiro-Wilk test). Data were therefore analysed using a one-way ANOVA followed by an unpaired t-test. Data are presented as mean ± SD. A significant decrease in plasma albumin levels was observed in both male and female animals (Figure 4.39). Treating rats with nyaope (n=12) had no significant effect on plasma alanine transaminase levels (Figure 4.40). Nyaope treatment did however cause a significant increase in plasma lactate dehydrogenase levels (Figure 4.41) and these significantly elevated plasma lactate dehydrogenase concentrations were observed in both males (n=6) and females (n=6) rats (Figure 4.42). Data were normally distributed (Shapiro-Wilk test). Data were therefore analysed using the unpaired t-test. Data are presented as mean ± SD.

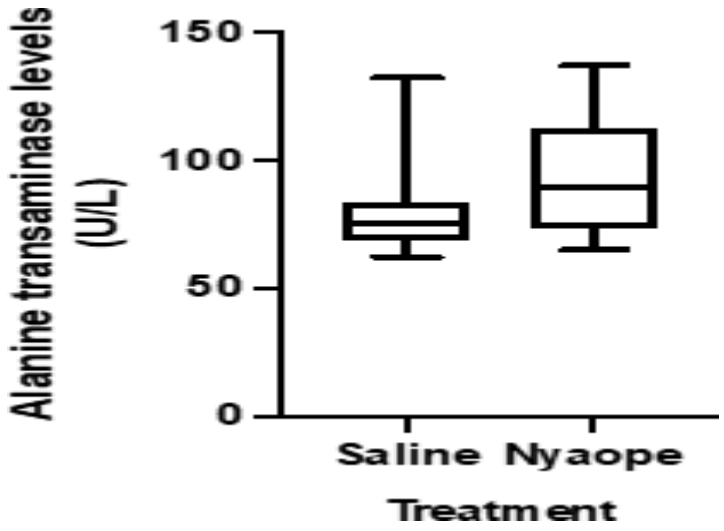


Figure 4. 40: The plasma alanine transaminase of saline (n=12) and nyaope (n=12) treated rats.

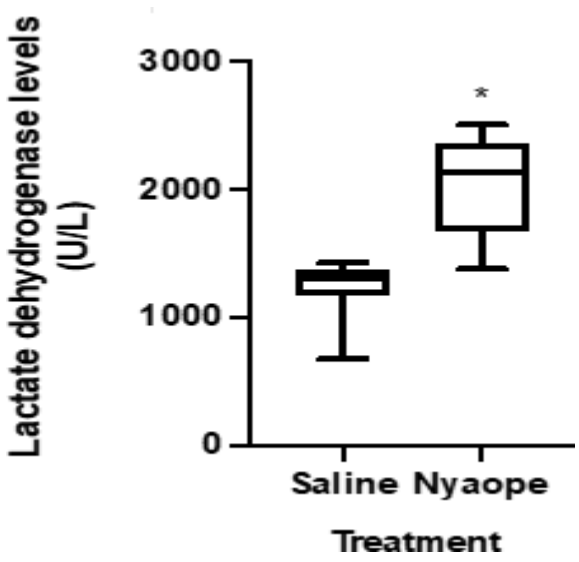


Figure 4. 41: The lactate dehydrogenase level of saline (n=12) and nyaope (n=12) treated rats.

*p<0.0001, Nyaope vs Saline

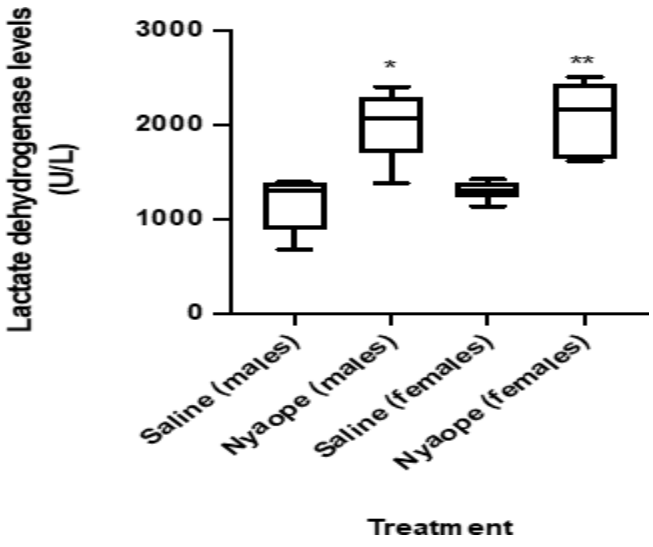


Figure 4. 42: Lactate dehydrogenase (LDH) levels of males (n=6) and females (n=6) treated with saline and nyaope

*p<0.0015, Nyaope (males) vs Saline (males); **p<0.0006, Nyaope (females) vs Saline (females)

CHAPTER 5: Discussion

5.1 Introduction

Substance used disorder (SUD) continues to be a major public health concern worldwide with its incidence escalating at alarming rates (WHO, 2019; World Drug Report 2019). Part of this concern stems from the impact of the chronic use of illicit substances on the behaviour of its consumers (Tidey & Miczek, 1992; Malanga & Kosofsky, 1999). Although intake of opiates is associated with experiences of pleasure and euphoria, its long-term use may result in several health complications (Kosten & George, 2002). Nyaope is one of the street drugs commonly consumed by the youth in South Africa, especially in the northern provinces of the country. Due to its devastating effects on the individual consumer, the family, and the community at large, the present study set out to identify the chemical composition of this street drug and to investigate its effects on several behavioural and physiological parameters.

5.2 Characterization of nyaope

Characterization or profiling of street drugs is important to gain insights into the active ingredients that mediate its effects. Therefore, drug profiling provides the chemical basis that underpins the pathophysiological impact of the drug. The profiling exercise simultaneously identifies additional substances that are added during the preparation or production of the drug by the dealer. Identifying the composition of a street drug is therefore vital to understanding its mechanism of action and to informing remedial strategies to address harmful effects. Nyaope has previously been described as a low-grade heroin to which various other compounds have been added as bulking agents (Khine *et al*., 2015; Mthembi *et al*., 2019). In the current study, nyaope samples were subjected to three different extraction solvents, namely dichloromethane, methanol and ethanol. The three methodologies provided comparable data that unequivocally showed that the nyaope samples contained significantly high levels of 6-acetyl-morphine (6-AM) and morphine. In contrast to previous reports, we found nyaope to consist mainly of heroin and heroin intermediates of exceptionally high quality and purity. My data, therefore, do not support the description of nyaope as a low-grade version of heroin. Indeed, my study highlights this misconception and encourages scientists and health care professionals to refer to nyaope in the same vein as heroin.

Heroin, or diamorphine, is a diacetyl derivative of morphine. In the body, it is rapidly converted to 6-AM or monoacetylmorphine (MAM). In my analysis of nyaope, this major heroin derivative appeared in significant amounts together with 3-acetyl morphine (3-AM). 3-AM is a less active metabolite of heroin compared to the more active 6-AM. A study by Avvisati *et al*. (2019) showed that 6-AM displays effects similar to that of pure heroin. For instance, it exhibits robust reinforcing properties in a rat self-administration setting akin to heroin, and evokes drug-seeking behaviour equal to heroin. 6-AM is further metabolised to morphine (morphine 2AC) and hence the presence of high concentrations of this compound was not surprising. The study by Avvisati *et al*. (2019) showed that the heroin metabolite 6-AM resembles heroin in the ability to sustain self-administration behaviour and to precipitate relapse into drug seeking after a period of abstinence. As a result 6-AM has reinforcing effects and may contribute to addictive behaviour.

Besides heroin, notable amounts of codeine and caffeine were also identified in the nyaope samples, yet in much smaller quantities. Since these compounds by themselves have addictive properties (Norman *et al*., 2016; Addicott, 2014), their presence adds to the potential of nyaope to induce SUD. Codeine, a "weak" opioid (Adler *et al*., 1955; Middleton & Nielsen, 2019; Chen *et al*., 1990), has been suggested to be a gateway substance to other opiates including morphine and heroin. It is well known that the administration of codeine relieves pain and, when ingested in higher doses, may lead to pleasurable, euphoric sensations. The physiological mechanisms of codeine-induced euphoria are well documented. Codeine binds to μ-opioid receptors to elicit its effects on the central nervous system (Chidambaran *et al*., 2017). Codeine is normally O-demethylated to form morphine via the activity of the cytochrome P450 enzyme CYP2D6 (Goldsack *et al*., 1996). However, studies have shown that codeine can also be converted to codeine-6-glucuronide and that this metabolite can generate opioid effects (Susce *et al*., 2006). The combined action of morphine and codeine-6- glucuronide may therefore lead to an escalated nyaope-mediated pleasurable experience.

The effects of caffeine on the central nervous system are varied. Clinical studies have shown an association between chronic caffeine ingestion and negative mood states such as anxiety, restlessness, insomnia, tachycardia (Herz, 1999), and impaired cognitive function (Ritchie *et al*., 2007; Santos *et al*., 2010). On the other hand, preclinical experiments reported caffeine administration to stimulate motor activity in rodents (Acevedo *et al*., 2016). The effects of caffeine are proposed to be mediated via activation of adenosine A1/A2 receptors that influence DAergic

neurotransmission and subsequent protein kinase A (PKA)-dependent mechanisms (Acevedo *et al*., 2016). Caffeine, in conjunction with heroin, may have synergistic effects that may result in a greater deleterious impact on the wellbeing of nyaope consumers. For instance caffeine, which is present in many products, modulates neurotransmitter systems in mesocorticolimbic brain regions. Studies have found that caffeine induces positive effects in animal models of certain neurological diseases, in part by modulating dopaminergic signalling. It is also worth noting that caffeine, at high doses, induces undesirable health responses on other systems, including cardiovascular, skeletal and muscular systems (Alasmari, 2020).

Unlike those of others (Khine *et al*., 2015; Mthembi *et al*., 2019), my extractions showed no significant traces of bulking agents. A reason for this discrepancy could be differences in methodology. Khine and co-workers used two independent methods to characterise their nyaope samples that included both gas chromatography-mass spectrometry (GC-MS) as well as time-of-flight direct sample analysis mass spectrometry (TOF DSA MS). These authors reported nyaope to contain antiretroviral drugs, antidepressants and benzodiazepines, in addition to morphine, caffeine, codeine and amphetamine. Despite variations in experimental procedures, the data collected shows that nyaope has a strong opiate base and therefore an extremely unsafe substance to consume. Alternatively, variations in composition of nyaope could also be attributed to differences in additive ingredients used in the production of the drug in the various provinces (Mthembi *et al*, 2029; Mokwena, 2015; Khine *et al*., 2015). Nevertheless all studies show that nyaope consists mainly of heroin and its metabolites.

5.3 Body mass measurements

The body mass of the animals were recorded weekly for two reasons; firstly, to monitor the wellbeing of the animals, and secondly, to assess whether the administration of nyaope would affect this physiological parameter. While there was a marked difference in the body mass of females and males, with males weighing more, no significant differences were observed over time, or between treatment groups.

My results were somewhat surprising, as heroin dependence has been associated with significant reductions in body mass (Nazrul Islam *et al*., 2002). It has been proposed that heroin suppresses the appetite, inhibiting food cravings. This behavioural change is likely because substance users often sacrifice elementary requirements, such as food, to purchase the substance of abuse (Van

Buskirk & Potenza, 2010). One of the negative outcomes of continuous substance use (heroin, morphine and other depressant drugs) are associated with awful nausea (Rock *et al*., 2018) that in turn leads to severe emesis and eventual loss of essential nutrients (Parker *et al*., 2011).

Interestingly, substance users can occasionally experience an increase in body mass, particularly under circumstances of poly-drug consumption. Combining heroin with cannabis (such as is the case with nyaope) allows the cannabis to neutralise the nausea induced by heroin intake (Rock *et al*., 2018). Furthermore, cannabis products can promote appetite, which could lead to body mass gain (Nazrul Islam *et al*., 2002; Parke, *et al*., 2011). A preclinical study where experimental animals displayed signs of opiate withdrawal, also demonstrated an amplified desire for the intake of carbohydrates during a rehabilitation program (Lieblich *et al*., 1991). In support of this observation, a postmortem investigation of Swedish IV substance abusers showed that approximately 36% of the substance users were overweight with a body mass index (BMI) >25 (Rajs *et al*., 2004). It is therefore important that substance users be educated in healthy eating behaviours so that their general medical condition is not further compromised (Hendricks & Gorbach, 2009; Santolaria- Fernandez *et al*., 1995).

5.4 Determination of dose measurements

Part of the danger of an overdose, is the unpredictability of how much it takes to overdose. Two people can use the same amount of the same drug and one may overdose while the other is fine. During the initial phase of the project, 72 rats were requested from CAS for the assessment of nyaope on the opioid signalling pathway in the rat's brain. The initial dosage was 10 mg/kg to the animals, and we unfortunately lost 21 treated rats in the process natural and the euthanized 10 rats (Figure 4.4). The first dose of 10mg/kg was the pilot study that resulted in reducing the dose to 1mg/kg as an effective dosage. We have now ascertained that a dose of 1 mg/kg was a suitable dose for my study. In a study by Hutto and Crowder (1997), administration of morphine dosages of 0.32, 1.0, 3.2, and 10 mg/kg could not show a dosage consequence on reinforcement ability amongst 75% of four dosages (Hutto & Crowder, 1997) of 50 -70 days old male Sprague–Dawley rats. The animals were implanted in the right external jugular vein with Weeks-type catheters of silicone rubber and polyethylene. The drug compartments were separated by a common wall, one end of which bisected the open end of the choice compartment. Therefore, for varying morphine dosages that occurred between subjects, the first set of 10 dosage of the trial showed a slight difference amongst trained groups administered with three lower dosages. Most animals preferred

the higher dosage more than three times as often as the lower dose. Preliminary of my current experiment showed that nyaope at 10 mg/kg was an extremely a toxic substance. At the current state of losing so many rats due to high dosage, tissues samples were collected, for the local veterinarian to execute visual post-mortem examinations. These included kidney, lung and heart tissues.

The normal physiological function of the brain in mammals is strongly dependent on the unharmed neurovascular connection process (Attwell *et al*., 2010). However, neurovascular coupling may be impaired by the administration of drugs (Franceschini *et al.,* 2010; Masamoto & Kanno, 2012). Neurovascular coupling refers to the mechanism that links the transient neural activity to the subsequent change in cerebral blood flow, and is regulated by both chemical signals and mechanical effect. The administration of drugs is proficient in modulating cerebrovascular autoregulation and blood-brain barrier (BBB) reliability under various circumstances (Lu *et al*., 1998; Te´trault *et al*., 2008). These circumstances may include exposure to drugs such as morphine and heroin, as well as psychostimulants such as amphetamine (Pimentel *et al*., 2020).

Heroin use is associated with significant levels of morbidity and mortality (Darke *et al*., 1996; Degenhardt *et al*., 2011; Darke *et al*., 2005). It was estimated that people who use heroin are up to six times more likely to die prematurely due to overdose as compared to alcohol consumers (Klingemann, 1992). It was estimated that 10 mg/kg of heroin injected in monkeys lead to dependence behaviour. This was evident when the administration of the drug was terminated and monkeys showed withdrawal signs but did not die until the end of the study (Gerak *et al*., 2009). Despite several studies reporting on the dose and toxicity of heroin, no publication describes the appropriate dosage of nyaope to be used in experiments. Therefore, the scientific literature used to populate this section of the study was mainly based on data used in the administration of heroin, since heroin was the main active ingredient used during the preparation of nyaope (Khine *et al*., 2015; Mthembi *et al*., 2018). The study by Hutto and Crowder, (1998) was used as the reference to support the use of 1mg/kg of nyaope. Therefore, the use of 1 mg/kg was considered the appropriate dose to use in the current study, as no rat died following the administration of this dose.

5.5 Behavioural assessment

A battery of tests, designed to assess rodent behaviour under various paradigms, was utilised in the present study to investigate the impact of nyaope on some brain function domains. In doing so, we determined the effects of nyaope on locomotor activity, exploratory behaviour, anxiety-like behaviour and cognitive behaviour in rats.

5.5.1 The effect of nyaope on locomotor activity

The OFT and the EPM are commonly used to measure locomotor activity in rodents (Seibenhener & Wooten, 2015; Sestakova *et al*., 2013). Software tracking the behaviour of the animals in the test arena provided information about the behaviour of animals after they were released into, or entered on their own accord, an open, novel environment. Using ANYmaze software, we recorded parameters indicative of the locomotor behaviour of our animals. Animals treated with both saline and nyaope displayed head distances travelled in the OFT at significantly reduced levels post-treatment compared to pre-treatment. However, the post-treatment distance for nyaope-treated animals was significantly lower than that of the saline controls. This effect was predominantly observed in female animals. The reduction in travel distance over time is likely due to familiarisation or habituation to the environment (post-treatment vs pre-treatment).

The exploratory drive in these animals was therefore diminished and their sense of novelty likely decreased. Interestingly, this reduction in distance travelled over time was more pronounced in nyaope-treated rats than in saline-treated rats. This finding suggests that nyaope induced a hypo-locomotor effect. Such a suggestion is quite plausible since nyaope also induced a significantly greater reduction in mean speeds obtained by the rats in both the wall area and the centre of the arena. One can therefore argue that the nyaope-treated rats travelled at significantly lower speeds, resulting in decreased distances travelled.

Data from the EPM showed no effects of nyaope on locomotor activity. This was surprising as the EPM is commonly used to measure this parameter. Since the EPM test is based on exploratory behaviour one would have expected results that were in line with the OFT data. The EPM results may therefore suggest that the OFT is a more sensitive test to identify differences in locomotor activity than the EPM.

The OFT result of a nyaope-induced reduction in locomotor activity is in line with a previous study indicating that heroin consumption may lead to increased passivity (Gutierrez *et al*., 2021). These authors exposed female rats to vaporized heroin (100 mg/ml) and found decreased activity 60 minutes post-exposure. Interestingly, the activity of the rats increased with time (Gutierrez *et al.*, 2021). It may therefore be possible that in our study where the rats were treated once a day for 5 days only, presented an acute heroin effect, that might change with longer treatment times. Other studies have also reported heroin to increase locomotor activity (Andersen *et al*., 2009; Eriksen *et al*., 2014). In their experiments, Schlussman and co-workers assessed locomotor activity measuring the number of crossings between two compartments of a conditioned place preference chamber (Schlussman *et al.*, 2008). The group of Kvello and co- workers used activity chambers to measure heroin-induced activity in their mice to determine locomotor activity (Kvello *et al*., 2016), compared to our study, where distance travelled and speed obtained in the OFT were used to assess locomotor activity. These authors injected heroin at a dose of 1.05 mg/kg subcutaneously and studied locomotor activity immediately for 20 minutes. They too documented a heroin-induced increase in activity that was reduced by pre-treating the animals with antibodies against 6-monoacetylmorphine (6-MAM), the first product in heroin metabolism.

Similarly, clinical studies show that the intake of many drugs, such as amphetamine, caffeine, nicotine, and cocaine, may increase locomotor activity after intake administered over 21 days (Frankel *et al*., 2007; Pautassi *et al*., 2012; Mizoguchi *et al*., 2010) to such an extent that the enhanced locomotor activity was one of the major side effects of morphine analgesia (Hough *et al*., 2014). The importance of sex concerning SUDs has gained increasing attention as evidence highlights significant sex differences in prevalence rates, health service utilization, treatment outcomes, and physiological consequences following alcohol and drug use (Brady *et al*., 2009; Darke *et al*., 2003). A study by Back *et al*. (2011) reported that females presented with a broader range of symptoms including more psychiatric comorbidities, medical problems, and psychosocial difficulties. Comparably, Lynch & Carroll (1999) demonstrated that female rats acquired both cocaine and heroin self-administration more rapidly than their male counterparts, and that this acquisition occurred in a greater percentage of females compared to males. These findings concurred with reports from other studies using similar animal models (Schlussman *et al*., 2008; Andersen *et al*., 2009; Zhang & Kong, 2017).

In line with the above-mentioned reports, we also found more pronounced effects of nyaope in female rats than in males. This finding is in supported of the work of Kvello *et al*. (2016) that also reported sex differences. In their study female mice treated with heroin showed an increase in locomotor activity, while this increase was not observed in male mice. The reason for the sex discrepancy may be attributed to differences in hormone composition between males and females. Interestingly, in a recent study conducted by our laboratory, more than 300 nyaope users seeking assistance at health care facilities in Gauteng Province in South Africa, were interviewed and evaluated. Most of these consumers (more than 80%) were men (Morgan *et al.,* 2018). A reason for this strong bias in the study population was that this outcome could be because of cultural reasons where females are less likely to attend rehabilitation facilities in fear of being stigmatized and out casted from their communities (Busza, *et al.,* 2018).

In a healthy individual, the locomotor activity is mainly centred and controlled in the brain. A member of the nerve growth factor (NGF)-related family of neurotrophins called brain-derived neurotrophic factor (BDNF), supports the physiological function of midbrain DA neurotransmitters *in vivo* and *in vitro* (Shults *et al*., 1994).This due to the fact that BDNF has been shown to stimulate the release of dopamine in the mesolimbic dopamine system. BDNF mostly employs the power of TrkB receptor–protein tyrosine kinase as the principal resource of indicator transduction and mRNA. Therefore, both BDNF and TrkB are commonly present in rodents' forebrains. This includes DA cell body regions and their terminal fields (Numan & Seroogy, 1997). Numerous behavioural research stated that confined management of BDNF or other neurotrophic factors can supplement the nigrostriatal DAergic physiological functioning and locomotor behaviour pattern (Hebert *et al*., 1996; Horger *et al*., 1998). However, it is unknown whether the effect of BDNF on behaviour is mainly allied with the mesolimbic DA system.

Humans, as well as other organisms engage in behaviours that are rewarding; the pleasurable feelings provide positive reinforcement so that the behaviour is repeated. Acute morphine increases dopamine release in the NAc (Di Chiara & Imperato, 1988; Johnson & North, 1992) by inhibiting GABAergic neurons in the VTA and rostromedial tegmental nucleus (RMTg) that synapse on dopaminergic neurons (Tepper & Martin, 1995; Jalabert *et al*., 2011 ; Jalabert *et al*., 2011); (Appendix 6 & 7). NAc dopamine is known to play a role in motivational processes, and dysfunctions of mesolimbic dopamine may contribute to motivational symptoms of depression and other disorders, as well as features of substance abuse. Experimental studies have shown that

DAergic neuronal bursting is associated with a greater degree of DA release than an equivalent tonic activity pattern. The mesolimbic dopaminergic system is a key component of the reward pathways in the mammalian (Salamone & Correa, 2012). The soma (cell body of a nerve cell) and dendrites of dopaminergic neurons of this pathway are located in the ventral tegmental area (VTA) of the midbrain. Therefore, drugs of abuse produce their reinforcing effects through actions in the limbic component of the basal ganglia, a circuit of nuclei that is responsible for the influence of motivational, emotional, contextual and affective and information on behaviour. This interaction between the opioids in the mesolimbic DA system has been concerned with the strengthening and locomotor-stimulating qualities of adulterated drugs (Koob, 1992; Kalivas *et al*., 1993).

Furthermore, modifications in the mesolimbic DA system following multiple exposures to adulterated drugs of abuse have been proposed to inspire the motivational aspects of SUD (Nestler & Aghajanian, 1997). Fascinatingly, numerous collaborations of research had illustrated that BDNF and lifestyle modifications in response to chronic drug exposure were very common (Chang *et al*., 2009). The chronic drug exposure appears to impact BDNF levels in both human and rodents and innate differences in BDNF have been associated with the development of addiction for multiple substances of abuse. Therefore, the administration of BDNF directly into the VTA may prevent numerous distinguishing biochemical modifications normally provoked by the substance of abuse in the VTA and NAcc (Berhow *et al*., 1995) (Appendix 6 & 7). The Intra-VTA BDNF may also avert the chemical effects of chronic administration of substances of abuse on the morphology of VTA-DA neurons (Sklair-Tavron *et al*., 1996). In addition, chronic administration of substances of abuse may modify the physiological functioning of precise proteins in the intracellular signalling cascades that may intercede the BDNF action (Berhow *et al*., 1996). Together, this evidence suggests that BDNF and drugs of abuse, such as cocaine, may control the mesolimbic DA system in part through converging cellular pathways and that BDNF may thereby influence the reinforcing and locomotor activating properties of substances of abuse. Therefore, and as stated earlier, it is possible that the nyaope-induced decrease in locomotor activity may be an acute effect and that this effect may not involve BDNF. Since BDNF has been implicated in the pathophysiology of heroin dependence (Zhang *et al*., 2016), it may be that its role only comes into play at advanced stages of drug consumption.

An alternative explanation, suggested by several previous studies, is that locomotor activation was an acute behavioural response to opiate administration (Mørland *et al*., 1994; Oliverio & Castellano, 1975; Castellano *et al*., 1976; O'Neal *et al*., 2001). This behaviour has been linked to opiate receptor stimulation as it was completely inhibited by opiate antagonists (Joyce & Iversen, 1979). Opioid- induced stimulation of motor activity independently of DA production has been confirmed by other studies (Cornish *et al.*, 2001). These findings demonstrate the interaction between the DAergic mesocortical and mesolimbic systems in an individual consuming a substance of abuse (Spanagel & Weiss, 1999).

5.5.2 The impact of nyaope on the mood of rats

The OFT, originally developed by Calvin Hall in 1934, is commonly used to assess emotionality in rodents (Kraeuter *et al.*, 2019). In general, an increase in the percentage of time spent in the central area of the apparatus compared to the total time spent in the apparatus is usually interpreted as an anxiolytic effect, while an increase in time spent in the peripheral area is associated with an anxiogenic effect. The effects of many different drugs have been investigated in the OFT, including compounds with effective or potential anxiolytic effects (benzodiazepines, serotonin ligands, neuropeptides), and compounds with stimulant (amphetamine, cocaine), sedative (neuroleptic) or prostration-inducing (epileptogenic drugs) activities.

A task, using an elevated apparatus that included an elevated open alley, which produced a strong approach-avoidance conflict, and an enclosed alley, which did not, was first described by Montgomery (1958). This task was modified into an elevated maze with four arms (two open and two enclosed) arranged to form a plus shape (Handley & Mithani, 1984). The EPM has subsequently become one of the methods of assessing anxiety-like behaviour in rodents by using the ratio of time spent on the open arms compared to the time spent on the closed arms (Pellow *et al*., 1985). The EPM, therefore, relies upon the innate preference of rodents for dark, enclosed spaces (approach) and an unconditioned fear of heights and open spaces.

In the present study, all rats spent more time against the wall of the OFT post-treatment compared to pre-treatment. However, when the genders were analysed separately, only the female rats that were treated with nyaope showed a significant increase in time spent against the wall post-treatment compared to pre-treatment. These results suggested that there may be a time effect where animals become more anxious and this effect was more evident in nyaope-treated females. The

female spend more time not far from the starting point. Whereby there is more time and less locomotor activity , Such an explanation is plausible as saline-treated rats spent significantly more time in the centre post-treatment compared to pre-treatment, and this difference was absent in the nyaope-treated group. Combined, the data points to nyaope eliciting an anxiolytic effect, especially in female animals. Heroin is known to be a mood enhancer eliciting feelings of pleasure. It is therefore plausible for nyaope to have anxiolytic effects rather than being anxiogenic. The prominence of effects in females can be ascribed to differences in the metabolism of nyaope in the two species. Differences in pharmacokinetics that include drug transporters and drug metabolizing enzymes between sexes have been documented (Meibohm *et al*., 2012).

In our observations in the EPM corroborated the OFT data to some extent. At pre-treatment, all animals spent significantly more time in the closed arms compared to the open arms of the EPM.

While the time spent in closed vs open arms was significantly different between pre-saline and post-saline treatments, this difference was not significant in the nyaope-treated group. It was found that nyaope-treated animals spent significantly more time in the neutral centre area of the EPM. Interestingly, there were no significant differences in the number of head entries into the various arms, suggesting that the animals did not differ in terms of exploratory behaviour, but were rather undecided as to which arm to enter. Collectively, the data once again suggests an anxiolytic effect induced by nyaope.

In an eloquent study by Farah Naquiah *et al*., (2016), adult male rats were treated with increasing doses of heroin (3 – 13.5 mg/kg) for 14 days. Their male offspring were subjected to the OFT and EPM where the animals displayed high levels of anxiety-like behaviour. It is therefore highly likely that nyaope can induce mood changes that may include anxiety-like behaviour.

Since mood is regulated by certain areas in the brain, performing the OFT and EPM tests allow one to gauge the functioning of structures such as the hippocampus, amygdala, dorsal raphe nucleus (Gonzalez & File, 1997; Silveira *et al*., 1993), as well as underlying mechanisms, for example, neurotransmission, hypothalamic–pituitary–adrenal axis function or neurotransmitter signalling, relevant to anxiety-like behaviour behaviour (Rodgers *et al*., 1992; Silva & Brandao, 2000). How nyaope impacts these systems are not yet known, hence our investigation into the JNK and Erk pathways.

Drugs interfere with the way neurons send, receive, and process signals via neurotransmitters. This binge intoxication stage of the addiction cycle is characterized by a disruption of executive function caused by a compromised prefrontal cortex. The activity of the neurotransmitter glutamate is increased, which drives substance use habits associated with craving, and disrupts how dopamine influences the frontal cortex. Therefore is known that substance dependence is marked by abnormal hypothalamic-pituitary-adrenal (HPA) axis function (Kiefer & Wiedemann, 2004; Walter *et al*., 2006). An atypical stress response occurs in both heroin and cocaine dependence (Kling *et al*., 2000 ; Schluger *et al*., 2003)).

It is known that not all individuals who are exposed to addictive drugs develop an addiction. However, psychostimulant sensitivity is only one of many factors that could predispose an individual to addiction vulnerability. Most of the adulterated depressants drugs elicit aversive reactions (Paine *et al*., 2002; Rogerio & Takahashi, 1992) that compete with its well-known reinforcing effects (Geist & Ettenberg, 1990). In an elegant study, Bush & Vaccarino (2007) investigated whether an individual difference in anxiety-like behaviour levels could predict susceptibility to addictive behaviour. These authors found a strong correlation between EPM behaviour and intake of cocaine. Animals that displayed open arm avoidance were also the ones that showed increased cocaine consumption in a self-administration chamber. These findings suggest that anxiety-like behaviour may predispose individuals to substance abuse and therefore predict individual differences in motivation to respond to adulterated drugs. More excitingly, this could offer a behavioural screening procedure to identify individuals at risk for SUD (Bush & Vaccarino, 2007).

5.5.3 The impact of nyaope on the cognitive behaviour of rats

The NOR test is widely used to assess learning and memory functions in rodents (Belcher *et al*., 2005; Kamei *et al*., 2006; Antunes & Biala, 2012; Lueptow, 2017) and is based on the rodent's innate preference for novelty. Thus, the test is based on the premise that animals will spend more time with a novel object than with a familiar object.

The Y-maze is another behavioural assay that has been commonly used to assess spatial working memory in rodents (Prieur & Jadavji, 2019; Row *et al*., 2007). Here, the principle of the animal's innate preference for a novel environment is once again applied. Since the Y-maze has three arms,

animals with intact cognitive function will alternately visit the three arms, completing more triads than animals with impaired cognitive function.

In the present study, two parameters were measured to indicate cognitive performance in the novel object recognition test. These were head entries into novel objects and head time spent with a novel object. The data showed that saline-treated rats displayed significantly more head entries pre-treatment compared to post-treatment. This significant difference was absent in rats that were treated with nyaope. These observations suggest that saline-treated rats preserved their memory of the NOR test performed pre-treatment and therefore the degree of novelty of the test at post-treatment was notably diminished. The fact that this difference was not present in nyaope-treated rats could indicate that the same level of memory retention was not maintained in this group. However, there were no significant differences in head time spent with the novel object between the groups, suggesting that the impact of nyaope on cognitive function as measured in the NOR test, should be interpreted with caution. Alternatively, since the engagement with the novel object was high in nyaope-treated animals, it may also be possible that nyaope increases treated animals' sensitivity to novelty.

Observations from our Y-maze experiments revealed that saline-treated rats completed significantly more triads post-treatment compared to pre-treatment. Once again, nyaope-treated animals did not exhibit this difference, suggesting that the ability to complete a meaningful number of successful triads was compromised in rats that received nyaope and that the spatial working memory could have been compromised. Such a suggestion has validity as the distance travelled by both groups was equal, showing that their locomotor competency was not challenged in this test. Alternatively, since the comparative saline group displayed a significant increase in the number of triads completed post-treatment vs pre-treatment, this could indicate that the saline-treated animals have "learnt" the maze and therefore could increase their performance during the second (post-treatment) assessment. It may be possible that the nyaope-treated animals could not "learn" the maze to the same extent, hence the poorer performance, suggesting reduced cognitive function.

In the current study there was no significant difference between pre and post nyaope treated animals. Brain-imaging studies in humans and neuropsychological studies in nonhuman animals have shown that repeated drug use causes disruptions in the brain's highly evolved frontal cortex,

which regulates cognitive activities such as decision-making, response inhibition, planning and memory (Pau *et al*., 2002 ; Todd, 1999; Mitrović *et al*., 2011). These were the finding of this project but her is adequate evidence that shows substance with heroin does affect the spatial working memory) Such a suggestion has validity as the distance travelled by both groups was equal, showing that their locomotor competency was not challenged in this test.

Looking collectively at the data of the two tests, some evidence exists that suggest that nyaope may have a negative impact on cognitive behaviour (more likely in females), albeit moderate in the current experimental paradigm. Zhang and co-workers recently reported impaired cognitive functioning in heroin users when compared to age-matched healthy controls (Zhang *et al*., 2021). Similar findings were reported previously where cognitive impairments were associated with chronic heroin use (Ma *et al.*, 2019). These clinical results are supported by preclinical observations showing rats exposed to heroin also display deficits in cognitive function (Lu *et al*., 2014; Zhu *et al.*, 2019). For instance a study by Eisch *et al*., (2000) demonstrated in heroin self-administering adult, male Sprague–Dawley 275–300 g rats, receiving 60 mg/kg per injection for two weeks, opiate-induced suppression of hippocampal neurogenesis that was associated with cognitive deficits. Our data are therefore in line with these reports and underline the potential of nyaope to affect learning and memory in those who consume the drug.

Repeated heroin use changes the physical structure (Wang *et al*., 2012) and physiology of the brain, creating long-term imbalances in neuronal and hormonal systems that are not easily reversed (Ignar & Kuhn, 1990; Kreek *et al*., 1984). Studies have shown deterioration of the brain's white matter due to heroin use, which may affect decision-making abilities and the ability to regulate behaviour (Li *et al*., 2016). Also, individuals suffering from SUD exhibit alterations in the anterior cingulate, orbitofrontal, and dorsolateral prefrontal cortices, where abnormalities are linked to impaired emotion regulation and inhibitory control (Goldstein & Volkow, 2011). Abnormalities in these brain areas are said to underlie the development of opioid dependence.

Impairments in learning and memory, as well as the presence of mood changes in heroin users, created an interest in the role of limbic structures in chronic substance use. Regardless of the hippocampal area lesion (CA3, CA4, or dentate gyms), it was found that rats with bilateral damage performed more poorly on the Y-maze than the controls (Conrad *et al.*, 1996). Findings from experiments conducted in a radial maze expanded the importance of other limbic structures in

addition to the hippocampus. These included the dorsal striatum and amygdala (McDonald & White, 1993). The hippocampus mediates a cognitive/spatial form of memory, whereas the dorsal striatum mediates stimulus-response (S–R) habit memory. The amygdala mediates Pavlovian and stimulus affect-associative relationships (Maren, 2001), while also subserving the modulatory role of emotional arousal on other types of memory (Schwabe, 2013). The frontal cortex is known to play an essential role in heroin addiction (Schmidt, et al., 2013). In an animal study on juvenile rodents exposure to morphine (0.4 mg/kg, subcutaneously twice a day for 21 days), induced severe astrocytosis and neuronal death in the medial prefrontal cortex. These alterations in fronto-cortical cytoarchitecture were associated with poor performance in the Morris water maze, passive avoidance and NOR tests (Adedayo, *et al*., 2018). It is, therefore, possible that nyaope-induced effects observed in our study could have been mediated by any single or combination of these brain areas.

5.6 The effects of nyaope treatment on opioid signalling

Endogenous opioids represent one of the primary natural reward systems in the body that play a central role not only in health but also in the development of disease states, including drug addiction (Bodnar, 2012). Exogenous opioids ranging from opiate derivatives to prescription opioid analgesics, interact with opioid receptors in the body to produce effects ranging from therapeutic to pathological. Members of the MAPKinase pathway have been implicated in the production of some of these effects. For instance, ERK isoforms are present in multiple brain regions including those relevant to addiction, suggesting that ERK may act as the intracellular interface for signalling the intersection of drug rewards and related contextual information (Zamora-Martinez & Edwards, 2014). Furthermore opioid activation of the MAPKinase pathway has been demonstrated in the frontal cortex (Li *et al*., 2010). Studies have also shown that animals receiving chronic administration of morphine exhibited noticeable rostral-caudal gradients in ERK distribution with the FC showing an ERK1/ERK2 ratio of 0.16, while the pons/medullar region had a ratio of 1.5 (Ortiz *et al*., 1995).

Opiate addiction can be approached as a form of neuroplasticity, where the lasting and aberrant adaptations in the brain play major roles in the development of the principal features of this chronic medical disorder: opiate tolerance and dependence, behavioural sensitization, and compulsive drug use (Hyman, 2005; Christie, 2008). Preclinical studies, using rats, have shown that chronic morphine exposure can result in structural changes of neurons compatible with the induction of

synaptic plasticity. Signalling pathways, including the MAPKinase pathway, have been associated with morphine-induced changes in neuronal size, synaptic connectivity and behavioural plasticity (e.g. Mazzucchelli *et al*., 2002; Girault *et al*., 2007; Russo *et al*., 2007. Therefore, several signalling pathways could link opioid receptor activation and the induction of neural plasticity in the frontal cortex.

The present study investigated whether the effects of nyaope treatment on behaviour could have been mediated by activation of the opioid signalling pathway. Opioids exert their functions by acting on four different opioid receptors namely mu (μ) (MOR), delta (δ) (DOR), kappa (κ) (KOR) and nociceptor/orphanin FQ (nociceptin) receptor (NOR) previously known as opioid receptor-like -1 (ORL1) (Dhawan *et al*., 1996; Waldhoer *et al.,* 2004). Ligand binding to these receptors leads to the activation of the mitogen-activated protein kinase (MAPKinase) pathway (Rauch *et al*., 2016; Lake *et al*., 2016). Subsequently, the levels of phosphorylated c-JNK and Erk 1/2 was measured in the FC. This brain area was specifically chosen as it is intimately involved in manifesting heroin- induced addictive behaviours (Perry *et al*., 2011; Volkow *et al*., 2019). Chronic exposure to opiates reduces the birth of new neurons in the dentate gyrus of the adult hippocampus, and this is known to affect learning and memory (Shors, 2004; Eisch *et al*.,2000)

Our data showed that there was no significant difference between frontal cortical levels of phosphorylated c-JNK and Erk 1/2 of saline-treated and nyaope-treated animals. This was surprising as previous studies have demonstrated a clear link between opioid receptor activation and its impact on a widespread of pathophysiological parameters including respiratory depression (Withey *et al*., 2017), neuronal apoptosis (Pu *et al.*, 2015) and abnormal locomotor activity, mood and cognitive behaviours (Azzam *et al.*, 2019). All these effects are said to be mediated via the MAPKinase pathway and the associated activation of Erk1/2 and c-JNK signalling.

These findings, therefore, indicate a prominent role for Erk 1/2 and c-JNK in eliciting the negative consequences of opioid intake. One possible explanation for the Erk 1/2 and c-JNK results that were obtained in our experiment, could be the presence of differences in methodology. Many of the previously mentioned studies were performed using cell culture systems (Pu *et al.*, 2015) or a different rodent model (Withey *et al.*, 2017), or the levels were measured in brain tissues other than the FC (Gao *et al.*, 2001). It may also be that the duration or dose of nyaope exposure, although enough to cause behavioural effects, were not severe enough to induce significant molecular

aberrations in the FC. Of note is that nyaope- treated animals did perform poorer in the Y-maze than the saline-treated controls. The Y-maze evaluates the rat's spatial working memory (Kraeuter *et al.*, 2019), a function that involves a significant contribution from the FC (Bahmani *et al*., 2019). It may therefore be useful to include additional behavioural tests such as the spatial delayed response test or attention and control tests (Chudasama, 2011) to evaluate FC functioning in more detail.

5.7 The effect of nyaope treatment on liver structure and function

As a major site of metabolism, the liver has been central to many studies investigating the toxic potential of xenobiotics. As such, liver structure and function has been extensively studied in substance abusers. For instance, a test centre in Singapore reported the prevalence of hepatic dysfunction in approximately 75 % of 1500 parenteral substance users (Chia *et al*., 1973; Beattie *et al.*, 1975). Similarly, the intake of illicit substances accounted for approximately 20-40% of all instances of severe and sudden-onset hepatic failure admitted in a drug rehabilitation centre in New York (Edland, 1972). Epidemiological statistics such as these clearly show the vulnerability of the liver to exogenous drugs.

Morphological changes of the liver associated with extended exposure to substances of abuse, included the development of steatosis and inflammation (Paties *et al*., 1987; Osna *et al*., 2017). Paties *et al*. (1987), after completing a clinical study in Italy, reported that in a cohort of 851 patients suffering from substance dependence, steatosis was observed in about 70% and portal inflammation in 93% of cases. At the ultrastructural level, it seems as if mitochondria (Begriche *et al*., 2011) hyperplasia and hypertrophy of the smooth endoplasmic reticulum (Ilic *et al*., 2010; Aly & Kleiner, 2011), can occur with chronic drug intake as evidenced in heroin addicts. Morphological changes to the sinusoid system of the liver include thickening of the sinusoidal wall and fibrosis of perisinusoidal space (or space of Disse) - a location in the liver between a hepatocyte and a sinusoid (Trigueiro de Araújo *et al.*, 1993). In the current study organelle modification was observed in the liver of nyaope treated animals. The walls of the sinusoids are lined with phagocytic cells, called Kupffer cells that digest old red blood cells and clear the bloodstream of toxins or hazardous substances. The results obtained in the current study agree with the histological findings described in the paragraphs above. The rats treated with nyaope displayed diminished sinusoidal spaces compared to saline-treated rats. In addition, collagen deposits appeared around the central vein and there was a presence of connective tissue and fibrosis in the

proximity of the portal tract in the livers of nyaope-treated animals. These structural abnormalities are in line with the observations of comparable clinical and preclinical studies and confirm opioid-mediated increases in liver collagen fibres and the development of liver fibrosis (Gorodetzky *et al*., 1968; Litt *et al*., 1972).

The morphological abnormalities invoked by substances of abuse are often associated with physiological function disturbances. According to Atlanta (2014), drug users frequently exhibit hepatocyte damage resulting in impairments in liver protein and enzyme synthesis, location and activity. Injury to hepatocytes, therefore, is commonly reflected in the release of liver enzymes into the bloodstream (Giannini *et al*., 2005). Long-term use of opioids has acute effects on homeostasis of the body. It is for this reason that the present study also compared the plasma concentrations of albumin, alanine transaminase and lactate dehydrogenase between nyaope-treated animals and their saline-treated controls. The data showed that the plasma albumin levels were significantly lower in nyaope-treated rats compared to saline-treated animals for both males `and females. Hence in other studies nearly 56% out of total 25 patients of 25-45 years, who were heroin addicts, showed level of ALT higher than the normal level (Farooqi *et al.*, 2016). While plasma alanine transaminase levels were not significantly different between the two groups of animals, the plasma concentration of lactate dehydrogenase was significantly higher in rats that were treated with nyaope compared to saline-treated rats.

Together, these biochemical results support the morphological findings, showing nyaope to markedly affect liver structure and function. The fact that the plasma levels of alanine transaminase did not change significantly suggests that the nyaope-induced effects were moderate and not severe enough to cause extensive liver damage. Such an explanation is plausible and underpins the modest effects of nyaope on the behaviour of the animal. Nevertheless, the nyaope-induced changes observed in the current study are in accordance with existing literature reporting opioid dependence to be associated with elevated plasma levels of liver enzymes such as lactate dehydrogenase (Panchenko *et al*., 1999; Atici *et al*., 2005).

Chapter 6: The potential of *Moringa oleifera* to reverse nyaope-induced deleterious effects

6.1 Introduction

The data in chapter 4 reports the effects of nyaope exposure on the wellbeing of rats. The main findings were that repetitive nyaope administration led to behavioural abnormalities and structural and functional aberrations in the liver of the animals. More specifically, nyaope-treated animals displayed signs of hypo-locomotion, increased anxiety, cognitive impairment, abnormal liver morphology, decreased plasma albumin levels and increased plasma lactate dehydrogenase concentrations. Traditional Chinese herbal medicines may be used to complement current treatments for drug addiction, including withdrawal and relapse. As the molecular mechanisms of traditional Chinese herbal medicines are elucidated, further advances in their use for the treatment of drug addiction are promising (Zhu *et al*., 2017). Chinese herbal medicine is part of a larger healing system called traditional Chinese medicine (TCM), which also includes acupuncture, massage, dietary advice and exercise. Extracts of *Moringa oleifera* is frequently used by rural communities as a remedy for a variety of ailments. As such, concoctions of the plant are helpful for conditions of inflammation (Minaiyan *et al*., 2014), hyperglycemia (Lalas & Tsaknis, 2002), hypertension, cancer, nausea, (Paliwal *et al*., 2011) and liver abnormalities (Oyagbemi *et al*., 2013). *Moringa oleifera* may also be beneficial to central nervous system-related impairments (Gupta *et al*., 1999; Sutalangka *et al*., 2013). Many nervous disorders have been known to show positive results against the use of moringa leaves. *M. oleifera* leaves extract possesses the neuroprotective and memory enhancing effects (Sutalangka *et al*., 2013). Due to these latter reports of *Moringa oleifera*, the current study investigated whether extracts of this plant were able to reverse the detrimental effects induced by nyaope.

6.2 Methodology

In lieu of COVID-19 restrictions, together with ethical considerations (Replacement, Refinement, Reduction), this part of the study mainly focused on the effects of *Moringa oleifera* on saline and nyaope-treated animals. However, for ease of comparison, we repeat the data reported in Chapter 4. This experiment followed the same design as described in Table 3.1. In brief, a total of 16 animals were first treated with saline (four males and four females) or nyaope (four males and four females), after which they were all treated with an aqueous extract of *Moringa oleifera*. As described in section 3.3.1, one gram of commercially obtained *Moringa oleifera* leaves were crushed, boiled in 10 ml distilled water for ten minutes, cooled to room temperature, filtered and then administered to the rats (5 ml intraperitoneal injection for five consecutive days).

Behavioural assessments were performed before saline and nyaope treatments, and then again immediately after the *Moringa oleifera* administration. Animals were sacrificed and liver tissues were collected for histological analysis. Blood was collected to measure plasma albumin, and lactate levels.

6.3 Results

Several promising results were obtained when saline and nyaope-treated animals were given *Moringa oleifera* injections. While the distance travelled by rats treated with nyaope (n=8) was significantly lower than that of saline-treated (n=8) controls (Figure 6.1, which is similar to Figure 4.12) this reduction was absent when nyaope-treated animals were given the aqueous extract of *Moringa*. This result suggested that the administration of *Moringa* has the potential to reverse nyaope-induced hypo-locomotor activity. Data were normally distributed (Shapiro-Wilk test). A repeated measures two-way ANOVA was used to analyse grouped data followed by unpaired t-tests. Data are presented as mean ± SD.

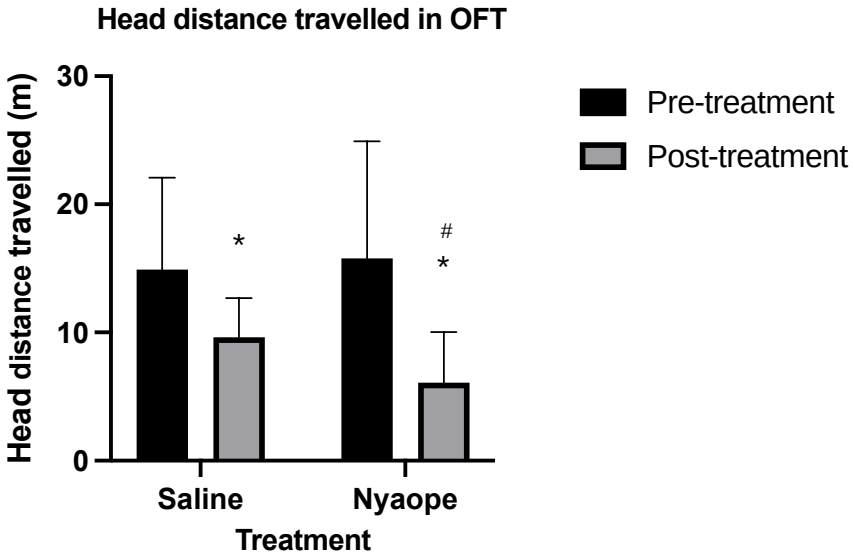


Figure 6.1: Head distance travelled in the open field test by rat's pre and post-treatment with saline and nyaope.

*p< 0.0245, post-*Moringa* treatment vs pre-*Moringa* treatment

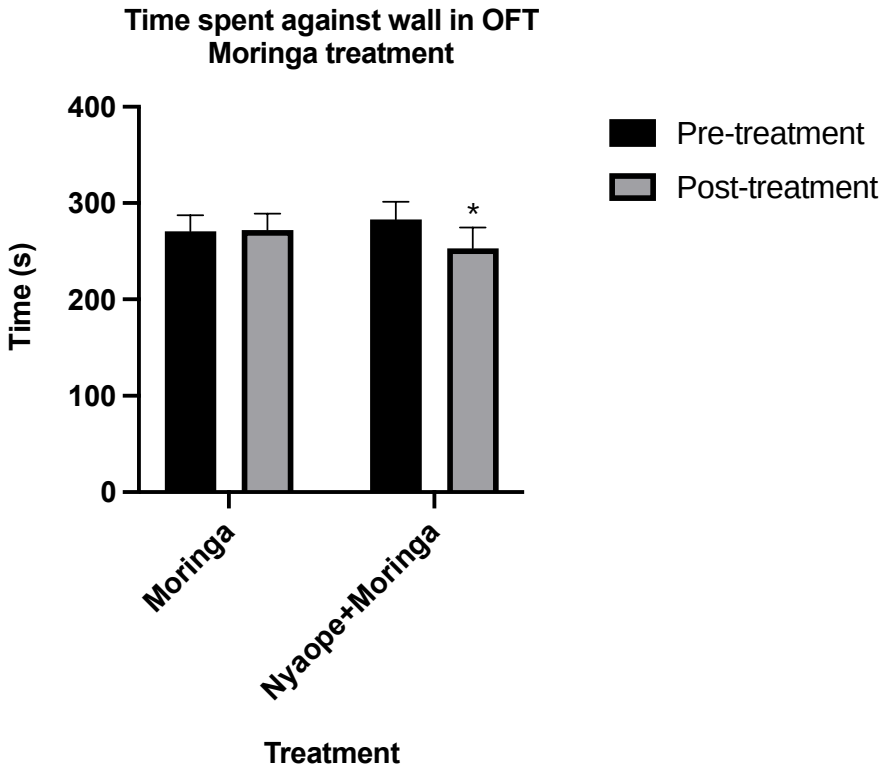


Figure 6.2: The time rats spent against the wall in the open field test pre and post-treatment with *Moringa* and nyaope plus *Moringa.*

*p<0.0095, Post-nyaope + *Moringa* treatment vs Pre-nyaope plus *Moringa* treatment

Nyaope-treated animals spent significantly more time against the wall in the open field test (Figure 6.2), while this increase was absent in nyaope-treated animals receiving *Moringa* treatment. This observation suggested that *Moringa* may also have anxiolytic properties. Data were normally distributed (Shapiro-Wilk test). A repeated measures two-way ANOVA was used to analyse grouped data followed by unpaired t-tests. Data are presented as mean ± SD.

Figure 6.3 (see also Figure 4.20) depicts the number of head entries the rats made to engage the novel object in the novel object recognition test. Here we observed that rats treated with saline displayed a significantly lower number of head entries post-treatment compared to pre-treatment. In contrast, rats that were treated with nyaope and *Moringa oleifera* showed a significantly higher number of head entries post-treatment compared to pre-treatment. Data were normally distributed (Shapiro-Wilk test). Repeated measures two-way ANOVA was used to analyse grouped data that was followed by unpaired t-tests. Data are presented as mean ± SD.

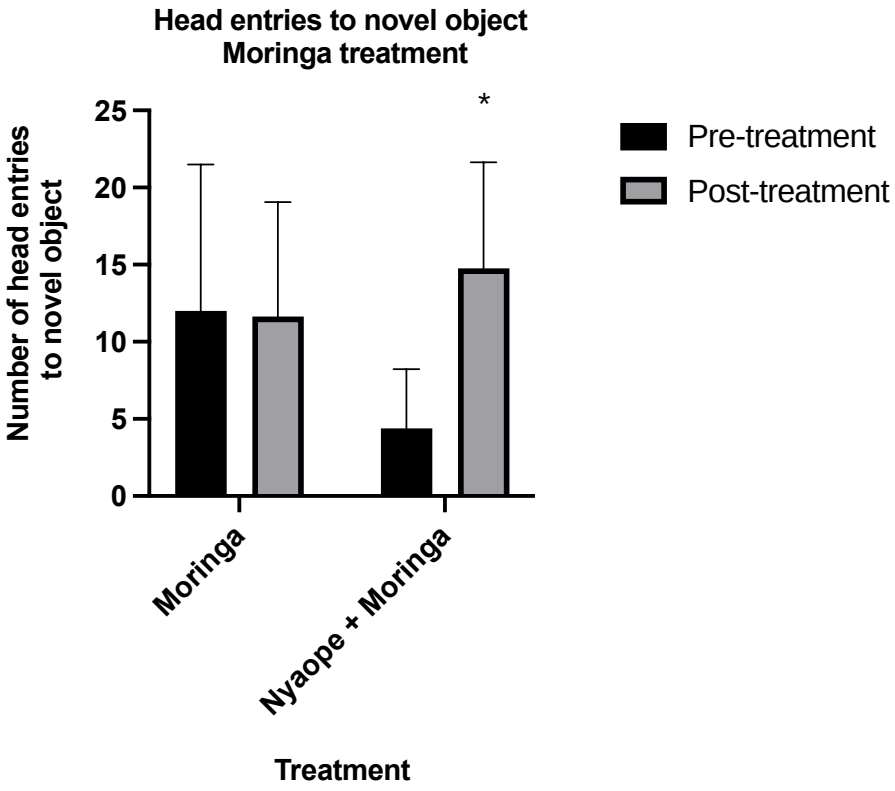


Figure 6.3: Head entries into a novel object by rats pre-, and post-treatment with *Moringa* and nyaope plus *Moringa.*

*p<0.0023, post-nyaope + *Moringa* treatment vs pre-nyaope + *Moringa* treatment

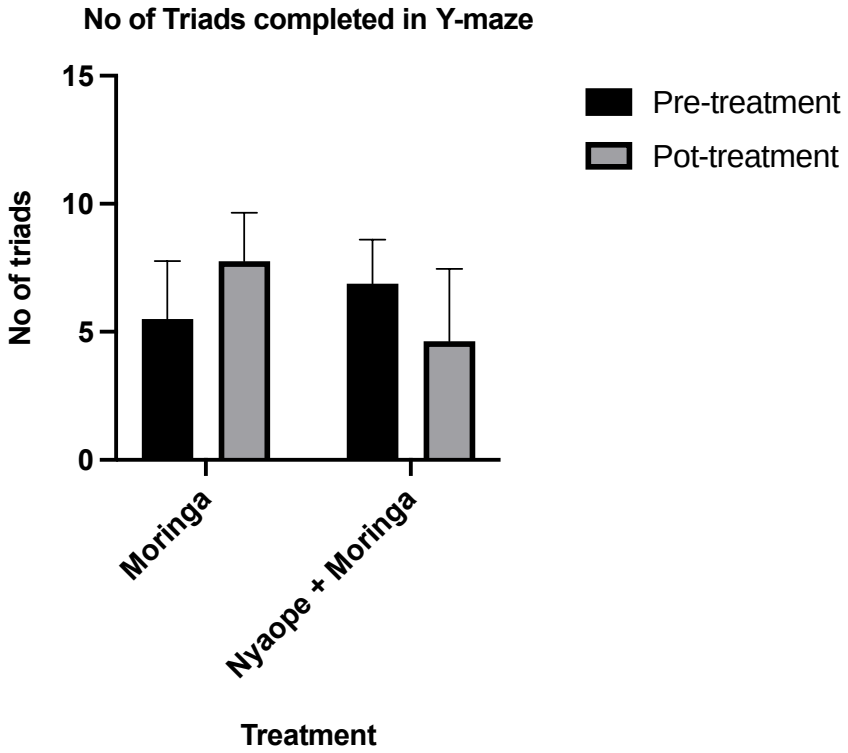


Figure 6.4: Number of triads completed by rats in the Y-maze pre-, and post-treatment with *Moringa* and nyaope plus *Moringa.*

While saline-treated rats exhibited an increase in the number of triads in the Y-maze post-treatment vs pre-treatment (Figure 6.4), this increase was not observed in *Moringa* or nyaope plus *Moringa* treated animals. Data were normally distributed (Shapiro-Wilk test). A repeated measures two-way ANOVA was used to analyse grouped data. Data are presented as mean ± SD.

Measurements of liver enzymes revealed interesting results. Rats treated with nyaope showed a significant decrease in plasma albumin levels compared to saline controls Treating the animals with nyaope and *Moringa* generated results that showed an opposite direction in comparison the that obtained from animals treated with nyaope alone (Figure 6.5, see also Figure 4.27). Rats treated with nyaope and those that were treated with nyaope and *Moringa*, had significantly elevated concentrations of plasma lactate dehydrogenase levels compared to saline-treated and *Moringa*-treated animals respectively (Figure 6.5).

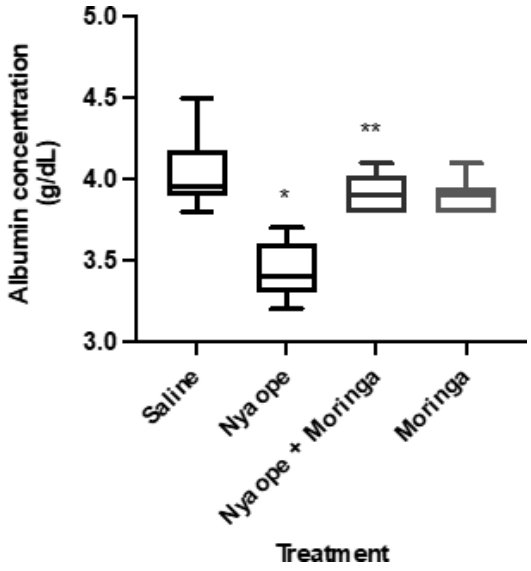


Figure 6.5: The plasma albumin levels of all the treated blood samples.

*p < 0.0001, Nyaope vs Saline; **p < 0.001, nyaope vs nyaope + Moringa.

Data were normally distributed (Shapiro-Wilk test) and subsequently, a one-way ANOVA followed by unpaired t-tests were used to analyse the data. Data are presented as mean ± SD.

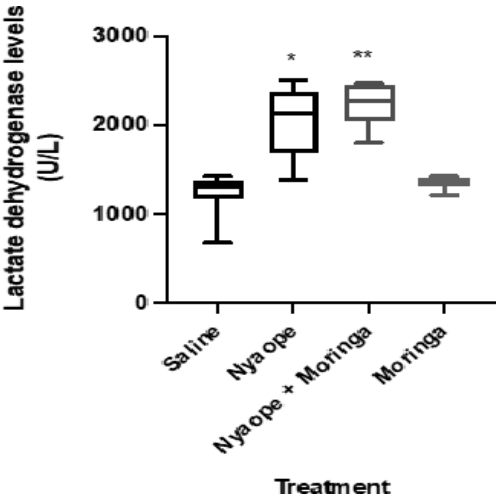


Figure 6.6: The plasma lactate dehydrogenase levels of all treatments.

*p < 0.0001, Nyaope vs Saline; **p < 0.001, nyaope + Moringa vs Moringa

Figure 6.6 shows the plasma lactate dehydrogenase concentrations of rats treated with saline (n=12), nyaope (n=12), nyaope + *Moringa oleifera* (n=6) as well as *Moringa oleifera* (n=6) alone. Data were normally distributed (Shapiro-Wilk test) and subsequently, a one-way ANOVA followed by unpaired t-tests were used to analyse the data. Data are presented as mean ± SD.

6.4 Discussion

The most common pharmacotherapeutic approach to treat opioid dependence includes the administration of methadone and buprenorphine (Dole & Nyswander, 1965; Donald *et al*., 1978). The main outcomes of the treatment interventions are to stabilize the individual and decrease or even eliminate the opioid use behavioural pattern. Despite these favourable outcomes, these drugs have side effects. For instance, unwanted effects associated with methadone include drowsiness, bigheadedness, weakness, dry mouth, urinary retention, constipation and slow or troubled breathing (Giacomuzzi *et al*., 2001), while buprenorphine may cause headaches, mild dizziness, insomnia, vomiting, constipation, and somnolence (Soyka, 2017; Sadek, 2021). Both treatments have addiction potential when taken long term, and a likelihood of death if administered incorrectly (Saloner *et al*., 2018). Given the undesirable consequences of current therapies, the search for alternative remedies continues. The inefficiency of conventional drugs has encouraged scientific investigation into the pharmaceutical potential of plant extracts. Moreover, there is a green movement that is changing the attitude of the general population to prefer naturally derived substances and extracts in the wake of them being inherently safer and more desirable than synthetic chemical products (Pandey *et al.*, 2011).

The use of phytomedicine for pathological conditions, including addictive behaviours, has been in practice in China for many years and continues to be improved with well-designed clinical trials seeking greater efficacy and efficiency (Wang et al., 2001). The results of these clinical trials suggest that mixtures of herbs, including Radix Aconiti Lateralis Preparata, Radix Astragali, and Rhizoma Cimicifugae, may be considered to treat opioid-dependent patients (Franke *et al*., 2001). Other reports also support phytomedicine's promise to manage SUD. Extracts of *Panax ginseng* provided positive outcomes in a cohort of opioid-dependent sufferers (Cheng *et al*., 1980; Takahashi & Tokuyama, 1998; Gillis, 1997; Kim *et al*., 1999). Similarly, an extract from *Piper methysticum* Forst reduced drug cravings and promoted abstinence in a group of substance abusers from the Pacific Islands and indigenous Australians (Savage *et al*., 2015; Baum *et al*., 1998;

Botello *et al*.,. 2020). Despite these advances made in the east of the globe, alternative strategies to treat substance abuse are relatively new to western medicine (Manheimer *et al*., 2003).

In the present study, the administration of an aqueous extract of *Moringa oleifera* was shown to reverse several effects induced by nyaope. For example, the *Moringa oleifera* extract was able to (i) reverse the decrease in head distance travelled in the OFT, (ii) decrease the time spent against the wall in the OFT, (iii) increase the number of head entries into the novel object in the NOR test, and (iv) reverse the decrease in plasma albumin concentration. These observations speak to the potential of *Moringa oleifera* to redress abnormalities associated with anxiety, cognitive impairment and liver malfunction. The *Moringa oleifera* extract did not affect the number of triads completed in the Y-maze, or the nyaope-mediated increase in plasma lactate dehydrogenase levels.

Nevertheless, the results do suggest that the extract may hold some benefit with respect to the negative consequences following nyaope intake. *Moringa oleifera* (Lamarck) belongs to the Moringaceae family and is one of 13 species of tree and shrubs that can be found in several countries (Anwar *et al*., 2007; Olson & Fahey, 2011). It is recognized for its nutritional properties (Ferreira *et al*., 2008; Posmonteir, 2011) as research had shown that *M. oleifera* contains vitamins, minerals, amino acids, beta carotene, antioxidants and omega-3 and -4 fatty acids (Fahey, 2005; Saini *et al*., 2016). In addition to its nutritional value, the leaves of *M. oleifera* have almost twice the total phenolic and three times the flavonoid content of that of various vegetables (Pakade *et al*., 2013). Furthermore, *M. oleifera* has fascinating medicinal capabilities (Luqman *et al*., 2012), including anti-inflammatory effects (Newton *et al*., 2010), anti- hyperglycemic properties (Lalas & Tsaknis, 2002), and mitigating memory impairment and neurodegeneration in animal models (Sutalangka *et al.*, 2013). Since many of the impairments of the central nervous system involve oxidative stress including psychiatric disorders such as anxiety (Salim, 2017), the property of *Moringa* as an anti-oxidant as a mechanism for its beneficial effects is of particular relevance to the present study.

The positive results obtained in our experiments therefore add to the growing literature advocating the use of herbal medicine in the treatment of pathological states including mental disorders. Herbal remedies have been used by many people suffering from anxiety-like behaviour or depression (Ernst, 2007)

Chapter 7: Conclusions

7.1 General

The use of illegal substances amongst the youth is continuously escalating. In the northern provinces of South Africa, the most common substance of choice is an inexpensive street drug, called nyaope. Current literature indicated that nyaope was a concoction of heroin, antiretroviral drugs, antidepressants, and other bulking agents that included rat poison and powdered detergents. The most common practice of nyaope intake is by smoking it with cannabis. One of the first aims of our study was to determine the exact chemical composition of nyaope. We subsequently collected nyaope samples from three regions that were geographically and provincial distinct from each other. Chemical analysis of the nyaope samples showed that methanol was a suitable solvent for sample preparation for the identification, comparison and profiling of nyaope samples. Our gas chromatography/mass spectrometry (GC-MS) data showed that nyaope consisted of very high levels of good quality heroin and heroin derivatives. We also found reasonable quantities of codeine and caffeine. These compounds all have significant effects on the central nervous system (CNS) that led us to believe that nyaope is an extremely dangerous drug that may have seriously damaging consequences for its consumers. Since there is no literature reporting on in vivo experiments with nyaope, and being aware of its high heroin content, a pilot study was performed to establish a suitable dose of nyaope for our actual experiment. We found that our initial dose of 10 mg/kg was lethal to the animals and most died within 60 minutes post-injection. A dose of 1 mg/kg was subsequently found to be tolerable for our experimental rats.

For the main experiment, male and female rats were treated either with saline or nyaope for five consecutive days. The impact of this treatment on behaviour and liver structure and function was evaluated. In terms of behaviour, we focused on locomotor activity, mood status and cognitive function. The liver structure was histologically assessed, while the liver function was gauged by measuring the plasma levels of albumin, alanine transaminase and lactate dehydrogenase. In summary, our data showed nyaope-induced impairments in locomotor activity, signs of anxiety-like behaviour, and abnormalities in cognition. Interestingly these effects were more pronounced in females. While the results obtained may be considered moderate, it does point to the potential

danger of nyaope affecting behaviour significantly. In addition, nyaope treatment led to structural damage of the liver that was reflected in its functional impairments. This finding showed that nyaope can negatively affect many organs in the body, emphasizing its toxic capability.

Investigations into the possible mechanism of action of nyaope yielded non-significant results. Neither c-JNK nor Erk1/2 signalling was affected by nyaope administration, suggesting that the effects of the drug could have been mediated through means other than opioid receptor activation.

Alternatively, because of the modest behavioural results, it may also be possible that our treatment paradigm was not sufficient to invoke a strong signal that was identifiable with the ELISA technique adopted in this experiment. A semi-quantitative assay was used, and it may therefore be possible that its level of sensitivity was unable to detect differences between experimental and control animals.

Extracts of *Moringa oleifera* are often used in rural communities as a remedy for pathological conditions involving inflammation, the cardiovascular system, cancer and even liver disease. Given these reports, we investigated if similar positive outcomes could be obtained in our nyaope-treated rats. Administration of an aqueous extract of *Moringa oleifera* for five days was able to partially reverse nyaope-induced hypo-locomotion, anxiety-like behaviour, cognitive disturbance and liver function. The extract, therefore, showed promise as a therapeutic agent that could be considered in the treatment of nyaope-induced abnormalities.

As mentioned, our data are suggestive and the effects observed were moderate and not convincing. Several limitations of the study were therefore identified, that when addressed, could lead to more definitive conclusions. Firstly, the sample size of the various groups should be increased. Parameters of behaviour are by nature highly variable, and this variability impacts the statistical significance of differences between groups. Experience has taught that solid behavioural results are obtained when samples sizes are at minimum 15 animals per group. As indicated in the text, investigations into the effects of Moringa were severely curtailed by the conditions imposed by COVID 19 restrictions. Ideally the group sizes should have been much bigger, the investigation into the potential beneficial effects of Moringa should have included control and nyaope only treated groups, to make the results more meaningful. Investigations into other members of the opioid signalling pathway as well as the confirmation

by opioid receptor inhibitors (e.g. naloxone), together with studying additional brain areas (e.g. hippocampus), would have strengthened the study significantly.

The incongruity of the behavioural data between the various behavioural tests, could also be the result of variations in experimental protocols. For instance the OFT and Y-maze test were performed over a period of 5 minutes, while the EPM and NOR tests were conducted over a period of 3 minutes. Studying the behaviour over 1 minute bins would therefore have been desirable. Also the effects of nyaope in the NOR test or Y-maze were measured at least 3 days after the last nyaope was administered. This could imply that the observed effects may be that of drug withdrawal rather than intoxication. Secondly, since the animals tolerated the dose of 1 mg/kg nyaope well, the duration of treatments should be increased to at least three weeks. This may provide more time for the nyaope-mediated effects to surface more prominently. Thirdly, adopting Western blotting technology may be a better biochemical method to assess the concentration or activation of signalling proteins.

In addition to addressing the above limitations, it is further recommended that future studies include biochemical investigations on other brain structures (e.g. the hippocampus), a comparison between the effectiveness of herbal extracts and opioid antagonists, and an investigation into the impact of cannabis on nyaope-induced toxicity (since nyaope is consumed together with cannabis). In conclusion, our study has demonstrated that nyaope may be toxic to the body, and that phytotherapeutics has the potential to reverse some of the deleterious effects of the drug.

7.2 Clinical implications

Nyaope is taken South Africa constituency by storm. The higher number of people that use nyaope is increasing at a very alarming rate every day. The major challenges that are facing the scientist is the ability to eradicate nyaope in the street. Knowledge about the effects of nyaope on physiology is limited. The model used in the current study matches the human condition reasonably well. After consumption nyaope users often present with changes in locomotor activity that manifests as sleepiness and blurry speech. In addition to its effects on the central nervous system, my study also shows nyaope to affect the normal physiological functions of the liver. On the brighter side, my data suggest that phytomedicine has the potential to reverse the physiological changes induced by drugs.

Appendix 1: Molecular neurocircuits as focal points for neuroplasticity in addiction

Binge/intoxication	
Brain section	Neurotransmitter
Ventral tegmental area (circuit 1)	Glutamate
Ventral tegmental area (circuit 2)	γ-aminobutyric acid
Dorsal striatum (circuit 3)	Dopamine
Dorsal striatum (circuit 4)	Glutamate
Withdrawal/negative affect	
Brain section	Neurotransmitter
Ventral tegmental area (circuit 5)	Corticotropin-releasing factor
The central nucleus of the amygdala (circuit 6)	Corticotropin-releasing factor
BNST (circuit 7)	Norepinephrine
Nucleus accumbens shell (circuit 8)	Dynorphin
Habenula (circuit 9)	Acetylcholine
The central nucleus of the amygdala (circuit 10)	Neuropeptide Y
The central nucleus of the amygdala (circuit 11)	Endocannabinoids
Preoccupation/anticipation	
Prefrontal cortex (circuit 12)	Glutamate
Prefrontal cortex (circuit 13)	γ-aminobutyric acid
Hippocampus (circuit 14)	Glutamate
Basolateral amygdala (circuit 15)	Glutamate
BNST (circuit 16)	Corticotropin-releasing factor
BNST (circuit 17)	Norepinephrine
Insula (circuit 18)	Corticotropin-releasing factor

Appendix 2: Dosage calculation

In order to prepare a nyaope concentration of 1mg/ml

The stock solution of 10ml Nyaope at a concentration of 1mg/kg

1mg nyaope was dissolved in 1ml saline

10 mg of nyaope in 10 ml

To calculate the dose in milligrams use the following formula:

Weight (g) X dosage (1mg/kg) = dose

If the rats weighed 300 g, they received a volume of 0.3 ml to give a dose of 1 mg/kg

Appendix 3: Neurotransmitter systems involved in the neurocircuitry of addiction stages and functional domains

Binge/intoxication	
Dopamine	Increase
Opioid peptides	Increase
Serotonin	Increase
γ-aminobutyric acid	Increase
Acetylcholine	Increase
Withdrawal/negative affect	
Corticotropin-releasing factor	Increase
Dynorphin	Increase
Norepinephrine	Increase
Hypocretin (orexin)	Increase
Substance P	Increase
Dopamine	Decrease
Serotonin	Decrease
Opioid peptide receptors	Decrease
Neuropeptide Y	Decrease
Nociceptin	Decrease
Endocannabinoids	Decrease
Oxytocin	Decrease
Preoccupation/anticipation	
Dopamine	Increase
Glutamate	Increase
Hypocretin (orexin)	Increase
Serotonin	Increase
Corticotropin-releasing factor	Increase

Appendix 4: **Nyaope permit**

health
Department:
Health
REPUBLIC OF SOUTH AFRICA

DEPARTMENT OF HEALTH
Private Bag X828
PRETORIA, 0001
Republic of South Africa

UMNYANGO WEZEMPILO
LEFAPHA LA MAPHELO

PERMIT IN TERMS OF SECTION 22A(9)(a)(i) OF THE MEDICINES AND RELATED SUBSTANCES ACT 1965, (ACT 101 OF 1965) TO ACQUIRE, POSSESS AND USE SCHEDULE 6 AND 7 SUBSTANCES FOR THE PURPOSE OF EDUCATION

Date of Issue: 03 May 2018	Expiry Date: 02 May 2019	Permit No: POS 140/2018/2019

Authority is hereby granted in terms of Section 22A(9)(a)(i) of the above-mentioned Act to **Mr M M Sekhotha** of the **University of Limpopo, School of Molecules + Life Science, Department of Physiology + Environmental Health** to acquire, possess and use, subject to the conditions stated, the under-mentioned Schedule 6 and Schedule 7 substances in respect of which the quoted quantity should not be exceeded during the period **07 February 2018** to **06 February 2019.**

Name of Scheduled Substance(s)	Schedule	Total quantity of substance(s) and/or preparation(s) allocated per calendar year
Nyaope	Schedule-7	500 g [five hundred grams]

Total Items: 1

The acquisition, possess and use the relevant substances are subject to the following conditions:

1. The substances shall be used for **Analytical Purposes** only.
2. The control over the substances shall be the responsibility of:

   Full Name & Surname: **Mr M M Sekhotha**

   ID Numbers: **680207 5461 080**
3. Complete details of the substances acquired and used shall be recorded in registers designed specifically for this purpose in accordance with the provisions of the relevant regulations to the Medicines and Related Substance Act, 1965.
4. Orders for the substances shall be signed for by:

   Full Name & Surname: **Mr M M Sekhotha**

   ID Numbers: **680207 5461 080**
5. When the substances are acquired, the name and address of the supplier, the date supplied, the quantity supplied and the number of the relevant invoice shall be recorded on this permit.
6. The register referred to in paragraph 3, as well as copies of orders and invoices pertaining to the supply of the substances, shall be available at the offices of the **University of Limpopo, School of Molecules + Life Science, Department of Physiology + Environmental Health** for a period of at least three years and shall be subject to inspection by Inspectors appointed in terms of the Medicines and Related Substances Act, 1965.
7. This permit expires on **03 May 2019** and shall on expiry be returned to the Department of Health for cancellation and shall be accompanied by a statement reflecting the quantity of substances on stock at expiry.

DIRECTOR-GENERAL:
DEPARTMENT OF HEALTH
DATE: 03 May 2018

Appendix 5**: Wits Ethic certificate**

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2018/09/40/C

APPLICANT: Mr M Sekhotha

SCHOOL: Physiology
DEPARTMENT:
LOCATION:

PROJECT TITLE: Characterizing the effects of a street cocktail drug (nyaope) on the opiod signalling system in the Wistar rat brain

Number and Species

72X 40-45 days/150 -200g male Wistar Rats

Approval was given for the use of animals for the project described above at an AESC meeting held on 2018/09/25. This approval remains valid until 2020/10/16.
Unreported changes to the application may invalidate the clearance given by the AESC
An annual progress report must be provided

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and is subject to any additional conditions listed below:

The researcher should specify the concentration/dilution of the active to be used in the rats once the in vitro experiments have been conducted

Signed: _____ (Chairperson, AESC) Date: 22nd OCTOBER 2018

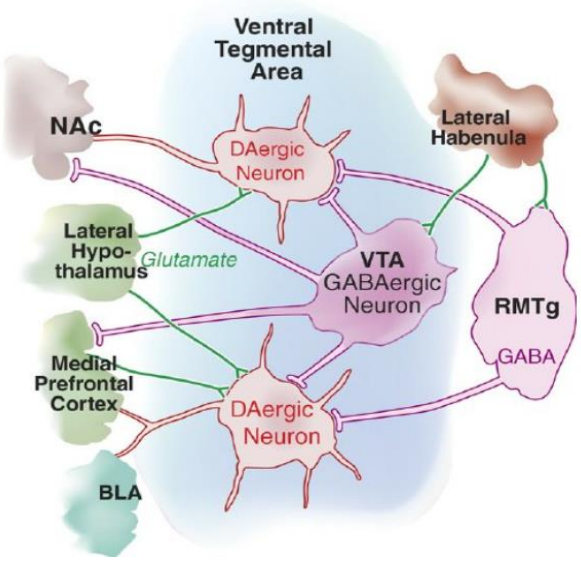
I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ (Registered Veterinarian) Date: 18 October 2018

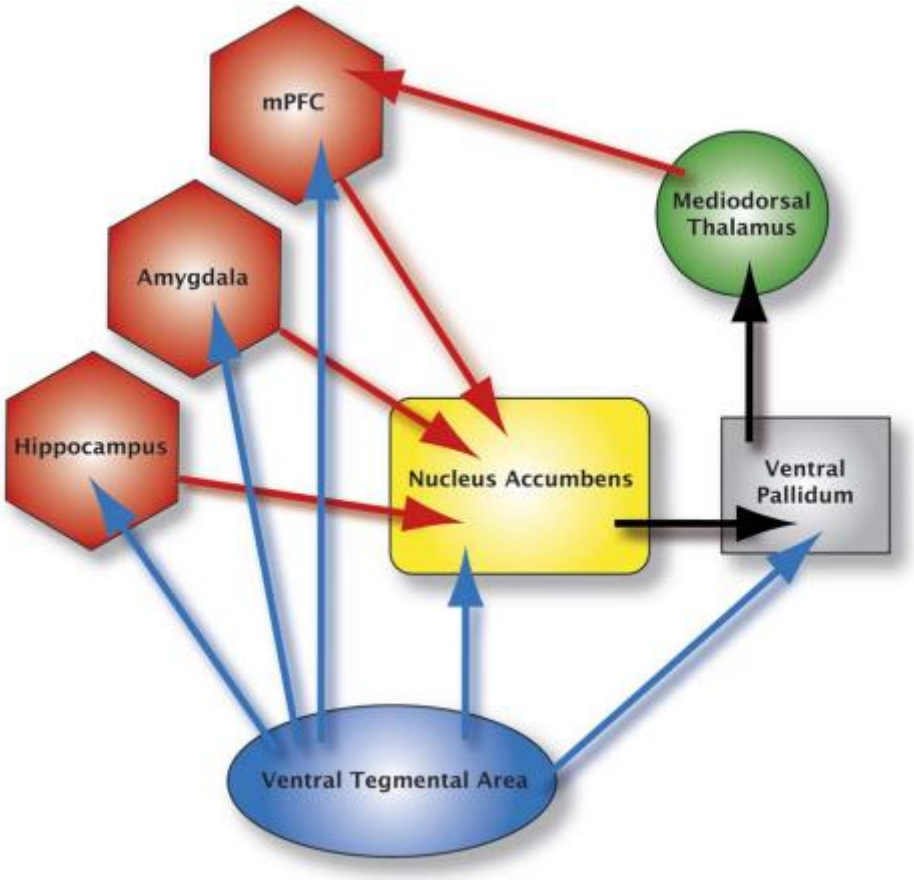
cc: Supervisor: Professor W Daniels
Director: CAS

Works 2000\lain0015\AESCCert.wps

Appendix 6: **The relation between different components in the brain**



Appendix 7**: Illustration of the interaction between different components with VTA in the brain**



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