Functional and molecular changes associated with intranasal buprenorphine in a healthy rodent model

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Abstract

Opioid addiction is a spiralling global epidemic associated with intense drug craving and the compulsive use of opiate drugs such as heroin, oxycodone, oxymorphone amongst others. Buprenorphine (BUP), commercially available as Subutex, is a partial opioid agonist that is used to treat opioid addiction and pain. It is associated with minimal risks of overdose and can be used outside of clinical care, making it the safest and most preferred choice of drug in the treatment of opioid addiction, over methadone and naltrexone. Literature suggests that opioids carry out their effects by altering the neurotransmitter systems of the brain *viz*. dopamine, norepinephrine, serotonin, glutamate and gamma-aminobutyric acid. Therefore, an ideal treatment drug should be able to counter these neurotransmitter changes in the brain. There is currently a lack of information on the pharmacodynamic effects of BUP in the brain, more specifically on how the drug affects brain neurotransmitter levels and its effect on the transcription factors Brain-derived neurotrophic factor (BDNF) and Cyclic AMP Response Element-Binding Protein (CREB).

This study evaluates the pharmacokinetics of BUP, its effect on neurotransmitter levels and the expression of BDNF and CREB at various time points following a single dose. Sprague-Dawley rats received 36 µL of 0.3 mg/mL of BUP via intranasal administration. Following dosing, animals were euthanised and brain tissues were collected at different time points. A rapid and sensitive liquid chromatograph-mass spectrometry (LC-MS method was developed for the quantification of BUP and neurotransmitters (dopamine, serotonin, glutamate, norepinephrine and gamma-aminobutyric acid) in brain tissue and the expression of CREB and BDNF was determined using qPCR. This thesis is divided into three chapters. **Chapter 1** contains a thorough background on BUP, opioid addiction and the role of neurotransmitters, BDNF and CREB. It also explains the principles of the quantification techniques used in this study i.e LC-MS and qPCR. **Chapter 2** is a manuscript that was submitted to Addiction Biology titled "Functional and molecular changes associated with intranasal buprenorphine administration in a healthy rodent model". Lastly, **Chapter 3** provides a general conclusion and future recommendations for the study.

The results in this present study indicate that BUP leads to significant changes in neurotransmitters, CREB and BDNF over time. Providing a better understanding of the mechanism of action of the drug, which could possibly improve the treatment of opioid addiction.

Declaration 1- Plagiarism

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Supervisor: Dr Sooraj Baijnath

Declaration 2- List of Publications

Sanelisiwe P. Xhakaza[a], Leon J. Khoza[a], Advaitaa M. Haripershad[a], Terisha Ghazi[b], Shanel Dhani[b], Cosmas Mutsimhu[c], Justice Molopa[c], Nithia P. Madurai[c], Lorna Madurai[c], Sanil D. Singh[a], Nirmala D. Gopal[d], Hendrik G. Kruger[a], Thavendran Govender[e], Anil Chuturgoon[b], Tricia Naicker[a], Sooraj Baijnath[a]* ***Addiction Biology*. Submitted 2020**. **ID: AB-10-2020-0268**

Author contributions:

- Sanelisiwe P. Xhakaza, Leon J. Khoza and Advaitaa M. Haripershad performed animal work and sample preparations. Sanelisiwe P. Xhakaza conducted data analysis and wrote the manuscript.
- Anil Chuturgoon Terisha Ghazi and Shanel Dhani assisted with PCR work.
- Cosmas Mutsimhu, Justice Molopa, Nithia P. Madurai and Lorna Madurai assisted with LC-MS analysis.
- Sooraj Baijnath designed and supervised the research project, and edited the manuscript.
- The remaining authors are co-supervisors and revised the manuscript.

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List of abbreviations

BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
CREB	cAMP Response Element Binding Protein
DA	Dopamine
DNA	Deoxyribonucleic acid
ESI	Electrospray ionisation
GABA	Gamma-aminobutyric acid
HPLC	High-performance liquid chromatography
iGluRs	Ionotropic glutamate receptors
LC	Locus ceruleus
LC-MS	Liquid chromatography- mass spectrometry
MAT	Medication assisted treatment
mGluRs	Metabotropic glutamate receptors
MOR	Mu-opioid receptor
NE	Norepinephrine
NAc	Nucleus Accumbens
NTs	Neurotransmitters
PCR	Polymerase Chain Reaction
PFC	Prefrontal cortex
PVDF	Polyvinylidene fluoride
RNA	Ribonucleic acid
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TOF	Time of Flight
VTA	Ventral Tegmental Area
C_{max}	Maximum concentration
T_{max}	Time of maximum concentration

CHAPTER 1

1.1 Introduction

Opioid addiction is a severe global health concern, according to CDC 46 802 deaths were recorded in the US alone due to opioid overdose, in 2018. Opioids are the main treatment for severe acute, perioperative, chronic pain in cancer and lower-back pain. [1,2] Their application has also been encouraged in the treatment of chronic diarrhoea, for example with Loperamide. [3,4] Using opioids to treat pain has the potential to lead to opioid addiction over time, meaning pain and opioid addiction are not mutually exclusive. [5] Society has the misconception of heroin being the only addictive opioid, but the misuse of prescription opioid medications, such as oxycodone, morphine or codeine does result in the number of people being dependent on opioids increasing dramatically and ultimately being a significant public health concern. [6] Opioids are described as chemicals that bind to mu (μ), delta (δ) and kappa (κ) receptors, which form members of the large family of G protein-coupled receptors (GPCRs). [7]

To help individuals struggling with opioid addiction, medication-assisted treatment (MAT) is often implemented; which comprises of pharmaceutical intervention and psychotherapy in the form of counselling and support from family and friends. [8] Psychosocial therapy involves patients controlling their urges to use drugs, the emotional strife and remain abstinent. [9] Buprenorphine (BUP) is one of four pharmaceutical agents used to treat opioid addiction, the others being methadone, naltrexone and naloxone. [10-12] Methadone is highly potent as a full opioid agonist, however it also comes with the increased likeliness of overdose. [13] Although methadone can be used by pregnant women, less treatment is required for Neonatal Abstinence Syndrome (NAS) for babies born of mothers treated with buprenorphine than methadone. [14] Naltrexone on the other hand requires a complete detox prior to treatment and cannot be taken by pregnant women or breastfeeding mothers. [15,16] Naloxone is an opioid antagonist that is recommended for patients with high overdose risk, to combat opioid overdose mortality.[17,18] BUP however has been found to be the safest and most preferred option in the treatment of opioid addiction as it carries lower risks of overdose and can be used without any supervision. [19,20] The above-mentioned disadvantages/limitations of methadone and naltrexone have, together with the lack of information surrounding its pharmacodynamic effects have led to BUP being selected as the drug of interest in this study. BUP, just like any other opioid carries

out its function by altering certain neurotransmitter pathways that control mood, appetite and reward.[21]

This highlights the importance of the quantification of neurotransmitters in the evaluation of the action of neuroactive drugs, in order to provide a better understanding of their pharmacodynamic effects and which diseases they would be most effective in treating. [21,22] Opioids are known to interact with three main neurotransmitter systems; the dopaminergic, glutamatergic and noradrenergic pathways [23] with literature also suggesting that GABA and serotonin being altered in during addiction. To date, there is no information regarding neurotransmitter level changes with time associated with BUP administration.

1.2 Opioids

Opioids are a group of substances derived from a the naturally occurring plant-based compound, opium .[24] Opioids include natural, semi-synthetic and synthetic chemicals which confer anti-nociception effects by acting at the opioid receptors in the central nervous system (*Table 1.1*). They are characterized by their ability to bind to the mu-, kappa- and delta-opioid receptors, followed by a subsequent alteration in neural signal transmission. [25] Opioids include both prescription drugs and illicit-narcotic agents. They can be divided into two classes; endogenous opioids, referring to those opioids that are produced by the body and exogeneous opioids that are introduced into the body. [2] Within the exogenous group are the naturally occurring opioids, semi-synthetic opioids and lastly fully synthetic. [26]

Table 1.1: Examples of different classes and subclasses of opioids

Classes of Opioids	Subclass	Examples
Endogenous opioids	N/A	dynorphins, enkephalins, endorphins, endomorphins and nociceptin/orphanin
Exogeneous opioids	Naturally occurring	morphine, codeine, thebaine and noscapine
	Semi-synthetic	diamorphine, oxymorphone, oxycodone, buprenorphine
	Fully synthetic	methadone and pethidine

1.3 Opioid Addiction

Opioid misuse is a spiralling global epidemic that is associated with high rates of mortality and morbidity due to fatal drug overdose, elevated health care costs, social harms, public disorder and crime. [27,28] This disorder affects both users of illicit drugs and patients abusing prescription opioids, along with their families. Approximately, 12-21 million people use opioids worldwide, with an annual death toll of 69 000 and the number of non-fatal overdoses many times higher. [29] There has also been a prominent rise in incidences where infectious diseases such as Hepatitis C and HIV are a consequence of promiscuous behaviour that occurs as a result of drug intoxication and the intravenous use of illicit opioids. [30,31] Dependence and addiction are used interchangeably, but there is a reasonable difference in these terms, which is why the American Academy of Pain Medicine, along with the American Pain Society and the American Society of Addiction Medicine developed a consensus document with their definitions as: "*Addiction is a primary, chronic, neurobiological disease with genetic, psychosocial, and environmental factors influencing its development and manifestations. It is characterized by behaviours that include 1 or more of the following: impaired control over drug use, compulsive use, continued use despite harm, and craving*". Whereas "*Physical dependence is a state of adaptation that is manifested by a drug class-specific withdrawal syndrome that can be produced by abrupt cessation of a drug, a rapid dose reduction decreasing blood level of the drug, and/or administration of an antagonist*". [32] Addiction is more intense as the cravings experienced lead to relapse or months or years after the patient is no longer opioid dependent. [33]

Interestingly, certain individuals' have a genetic disposition to developing opioid addiction, even when the medications are prescribed appropriately and taken as directed. [34] Opioid addiction becomes a chronic relapsing disease as opioids change the chemistry of the brain and lead to drug tolerance, meaning that the dose needs to be increased periodically to achieve the same effect. [35,36] On a molecular level, tolerance is thought to be a consequence of desensitization of the mu-opioid receptors leading to alterations in opioid receptor signalling. [32] Opioids are most addictive when you take them using routes of administration different from what was prescribed, such as crushing a pill so that it can be snorted or injected. [37] This alters the pharmacokinetic properties of the drug and causes rapid distribution of the opioid, resulting in an accidental overdose where there is respiratory depression or cessation, ultimately leading to unconsciousness or death if the overdose is not treated immediately. [38] Opioid misuse is associated with adverse effects such as severe respiratory depression, causing constriction of

the pupils (often referred to as pinpoint pupils), a decrease in oxygen saturation, loss in appetite, nausea, sedation, euphoria and constipation. [39,40] Upon discontinuation of opioid drugs, a severe withdrawal syndrome is experienced and is characterised by stomach cramps, diarrhea, rhinorrea, sweating, elevated heart rate, increased blood pressure and negative neurological effects including dysphoria, anxiety and depression. [41,42] There are currently three approved drugs that are used in the treatment of opioid addiction; methadone, naltrexone and BUP The focus of this study was to better understand the role of BUP in the treatment of opioid addiction by investigating the changes in neurotransmitter (NT) levels and the expression of transcription factors associated with its use.

1.4 Treatment of Opioid Addiction Using Buprenorphine (BUP)

Buprenorphine (BUP) is often prescribed in the treatment of opioid addiction due to its improved safety profile compared to other available treatment drugs, however its primary pharmaceutical indication is for the treatment of pain. [43] BUP is a semi-synthetic opioid derived from thebaine, a naturally occurring alkaloid of the opium poppy, *Papaver somniferum.* [44] The chemical structure of the BUP (*Figure 1.1*), is analogous to morphine, which is also a widely used opioid analgesic drug. [44] The distinguishing factor between the two is the presence of a cyclopropyl methyl on BUP and the C-7 side chain containing a t-butyl group which makes the drug highly lipophilic and enables easy diffusion across the blood brain barrier (BBB). [44,45] In the body, the drug is metabolized by the liver to an active metabolite norbuprenorphine, (*Figure 1.1).* [46]

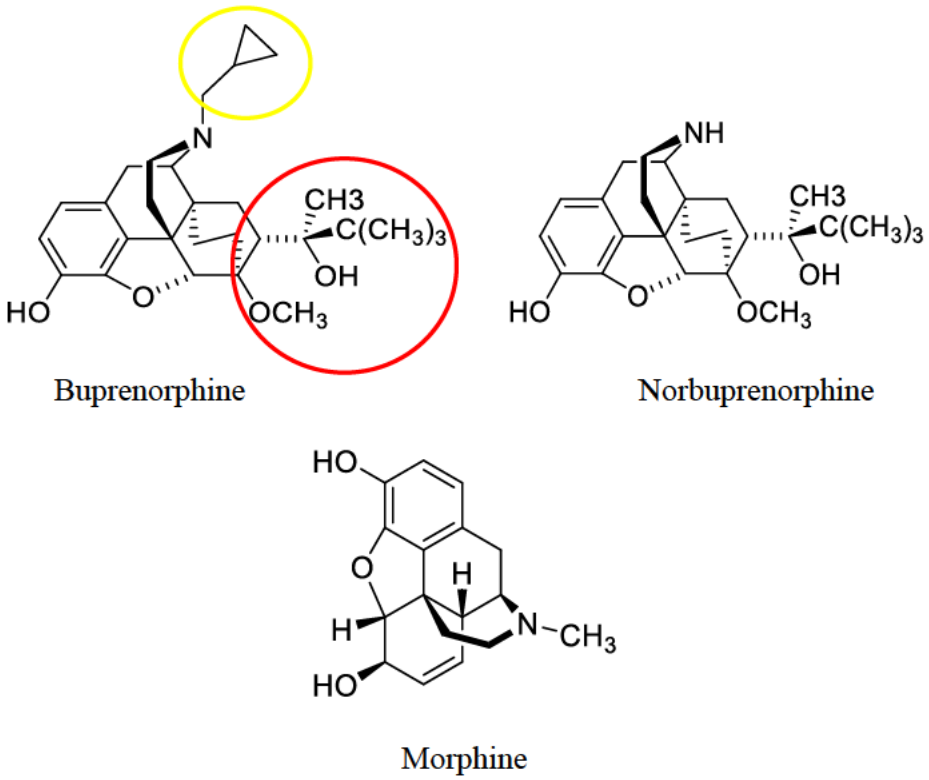

Figure 1.1: Chemical structures of buprenorphine; its metabolite, norbuprenorphine and morphine. The red and yellow circles represent the t-butyl and cyclopropyl methyl groups, respectively, which differentiate BUP from morphine. (prepared by author using ChemDraw).

Commercially available as Subutex, the drug is available as a sublingual tablet or in a filmstrip formulation for managing opioid addiction. [47] This opiate acts as a partial agonist at the mu-opioid receptor (MOR) and an antagonist to both the kappa- and delta-opioid receptors. [48,49] Upon receptor binding, BUP also produces familiar opioid effects such as; pain reduction, feelings of pleasure, and respiratory suppression albeit at a lower severity. [48] Prior to introducing the treatment, patients are instructed to stop taking their opioids with the aim of preventing precipitated withdrawal symptoms which may even lead to hospitalisation. [50] Although BUP is a partial agonist at mu-receptors, its strong receptor binding characteristics and slow rate of dissociation, result in prolonged clinical effects and limited physical dependence, in addition it also blocks exogenous full agonist opioids from reaching MORs. [32,51] This reduces the risks of toxicity and overdose, making BUP superior to other opioid addiction treatments such as methadone. [52] Nevertheless, there are drawbacks of using the sublingual form of BUP including the production of fluctuating blood concentration levels over

time, upon daily intake. [53] In addition, sublingual BUP is misused through parenteral administration and diversion to the illegal market. [54] This has led to the development of a buprenorphine/naloxone (4:1) formulation to prevent misuse, this is due to naloxone's MOR antagonistic properties. [46,55] BUP is well absorbed by patients with even significant renal dysfunction without any dose adjustment required. [32,56] BUP carries out its effects by altering neurotransmitters such as norepinephrine, serotonin, dopamine, glutamate and γ-aminobutyric acid (GABA).

1.5 The Role of Neurotransmitters in Opioid Addiction

The nervous system functions through the communication of cells using chemical messengers, known as neurotransmitters, that transmit signals across synapses from presynaptic cells to activate receptors on postsynaptic cells. [57,58] This interaction of cells is responsible for the modulation of motor movements, personality and behaviour of individuals. Therefore, any disturbances in this finely balanced system produces behavioural disorders and can promote certain neurobiological and psychiatric conditions. [21,59] According to their chemical structures, neurotransmitters (NTs) can be classified (*Table 1.2*) as amino acid transmitters including glycine, glutamate and gamma-aminobutyric acid (GABA); as monoamines/biogenic amine transmitters including dopamine, serotonin, epinephrine, norepinephrine; and lastly as neuropeptides including enkephalin, endorphin and substance P. [60]

Table 1.2: Different classes of neurotransmitters and their examples.

Class of neurotransmitters	**Examples**
Amino acid transmitters	Glycine, glutamate, GABA
Monoamines/ biogenic amine transmitters	Dopamine, serotonin, norepinephrine
Neuropeptides	Enkephalin, endorphins, substance P

Opiate drugs result in a feeling of pleasure through the indirect release of dopamine, the principal central nervous system neurotransmitter that is responsible for behaviour, control of movement, cardiovascular function, endocrine regulation and strongly associated with addiction. [59,61] The mesocorticolimbic dopamine (DA) system is known to be highly active in patients who suffer from drug addiction and is the major site of action for addictive drugs. [62] The system originates from the ventral tegmental area (VTA) with projections to the nucleus accumbens (NAc) and prefrontal cortex (PFC). [63,64] In the VTA, opiate drugs principally target GABAergic interneurons for two possible reasons: firstly, MORs are expressed on GABAergic and not on DA neurons or secondly, GABA neurons are more sensitive to the drug than DA neurons. [62] Upon binding, GABAergic neurons are inhibited (*Figure 1.2*), which leads to an indirect increase in activity of DA neurons, thus inducing the release of DA in NAc and PFC. [62,65]

Withdrawal symptoms in long term opioid users involves the locus coeruleus (LC) region of the brain *(Figure 1.2)*, where neurons produce norepinephrine (NE). [33] This neurotransmitter regulates alertness, breathing, blood pressure and mood. [66] Opioid binding to MORs in the LC neurons supresses the release of NE, resulting in drowsiness, slowed respiration and lower blood pressure. [33] Repeated exposure to opioids, leads to heightened activity of LC neurons; when opioids are no longer present in the system to supress the enhanced neuronal activity and the release of excessive amounts of NE. [67] This excessive release of NE is characterised by the physical symptoms of opioid withdrawal which include jitters, anxiety, muscle cramps and diarrhea. [67]

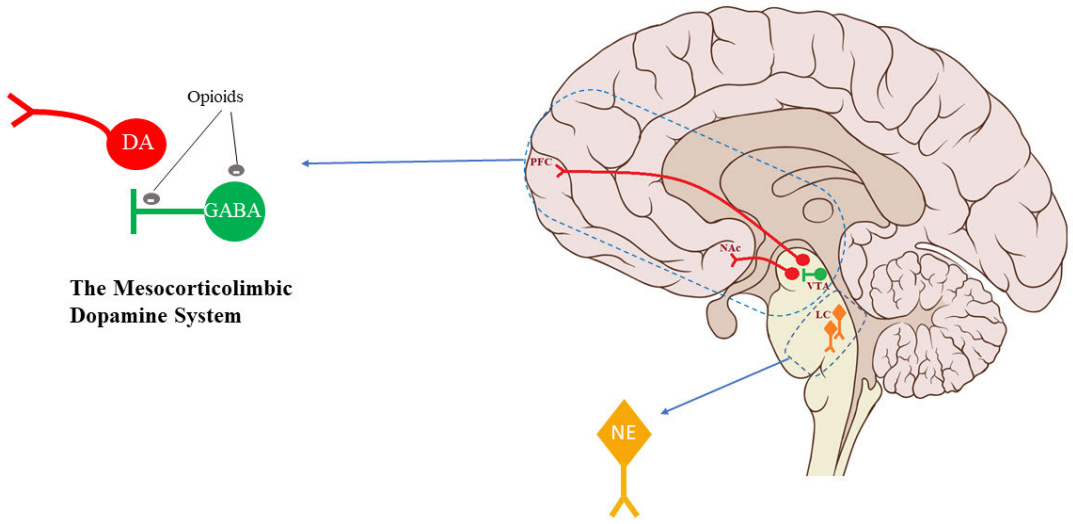

Figure 1. 2: The Mesocorticolimbic Dopamine System and the Locus Coeruleus (LC) as a target of opioid Drugs. Opioid drugs act indirectly via pre- and post-synaptic inhibition of GABAergic interneurons to activate the release of dopamine by DA neurons. The LC neurons release norepinephrine when opioids bind to the MORs (prepared by author).

Glutamate has been identified as the major excitatory neurotransmitter in the brain and plays an important role in opioid addiction. [68] In the reward and withdrawal aspects of consuming opiates, opioid memories are formed and maintained by glutmate.[69,70] Glutamate receptors are critically involved in the process of reward and withdrawal. [71] There are two categories of glutamate receptors, the ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs). [72] Within the iGluRs, a subtype known as the *N*-methyl-D-aspartic acid (NMDA) receptor stands out as most commonly implicated in the rewarding effects of opiates. [72] It has been suspected that glutamate may be involved in the processes of opioid addiction through its interaction with other neurotransmitters or neuropeptides such as, dopamine, GABA and substance P in the mesocorticolimbic dopaminergic regions. [68]

Serotonin (5-hydroxytryptamine, 5-HT) is a monoamine neurotransmitter that affects mood and cognition. [73,74] The neurotransmission of 5-HT plays a part in developing dependence and the expression of withdrawal from morphine. In addition, chronic morphine treatment is associated with an increase in expression of the 5-HT_{2C} receptor protein in the VTA, LC and NAc. [74,75] With the gathered evidence showing that neurotransmitters play a critical role in

opioid addiction this study will investigate the effects of BUP administration on dopamine, GABA, noradrenaline, serotonin and glutamate (*Figure 1.3*) levels in the brain.

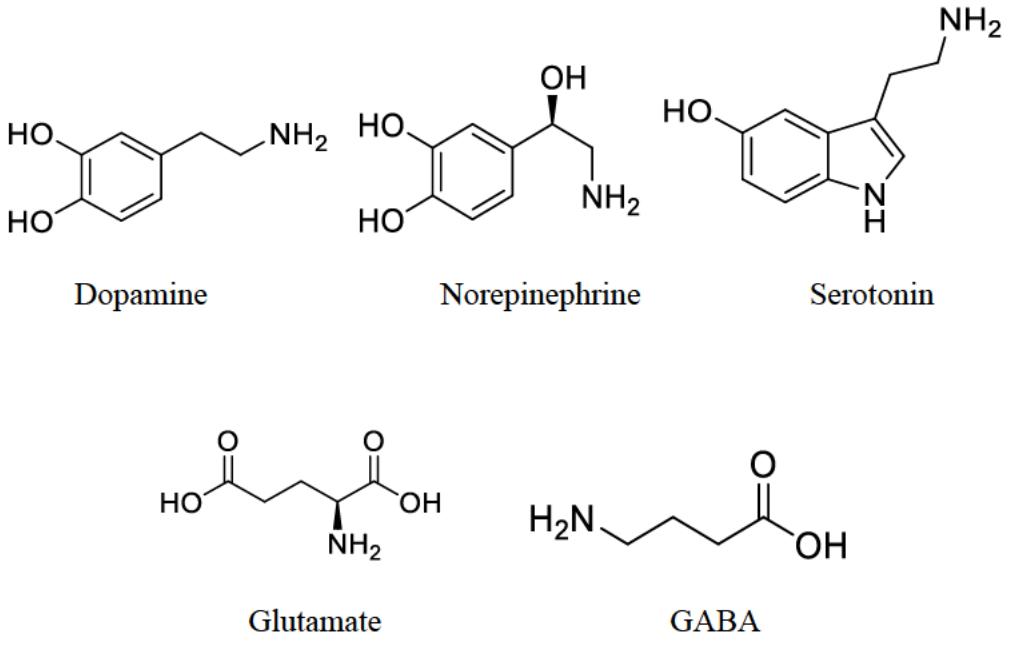

Figure 1. 3: Neurotransmitters (Dopamine, Norepinephrine, Serotonin, Glutamate and GABA) that are associated with mu-opioid receptor activation in the brain by buprenorphine. (prepared by author using ChemDraw).

1.6 Opioid addiction and the expression of CREB and BDNF

Opioids affect several neurobiological factors that are implicated in opiate-use disorder and the addiction process, two important factors being Brain-derived neurotrophic factor (BDNF) and Cyclic AMP Response Element-Binding Protein (CREB). [76,77] BDNF is a neurotrophic neuropeptide that is involved in the neural processes of growth, development, survival, maintenance, synaptic regulation, plasticity and anti-apoptotic regulation. [76,78] It also plays an important role in learning, memory formation, drug addiction and depression. [79,80] In the human genome, the BDNF gene has been mapped to be in chromosome 11. [81] It has been demonstrated that chronic morphine administration in mice supresses BDNF gene expression in the ventral tegmental area (VTA) with this blockade enhancing reward mediated by dopamine neuron activity .[64,82] Data from animal studies show that dopaminergic and serotonergic functions are modulated by BDNF. [81] BDNF is said to influence the survival of central serotonergic (5-HT) neurons, and when exogenously administered enhances 5-HT neurotransmission.[83] Although

endogenous BDNF is critical for the normal development and function of 5-HT neurons, there is no information on how endogenous BDNF influences neurotransmission of 5-HT following opioid intake.[84]

CREB is a transcription factor that is involved in neuronal survival, learning, long-term memory and drug dependence. [85,86] Research shows that acute administration of opiates inhibits the cAMP signalling pathway, thus decreasing locus coeruleus (LC) neuronal firing. [87] Contrary to acute, chronic exposure to opiates upregulates the cAMP system that leads to tolerance, dependence and drug-seeking behaviour. [77,88] Brain regions that have been observed to take part in this upregulation are the LC and NAc which have been shown an increase in the expression and phosphorylation of CREB. [86] Phosphorylated CREB activates transcription by binding to the CRE promotor region of target genes as a dimer and modulates their expression. [23,89] CREB signalling also regulates the expression of BDNF, this CREB-BDNF signalling is said to be critical in cell survival, synaptic structure and synaptic plasticity. [90,91]

To date there are no reports demonstrating the effect of BUP administration on the expression of CREB and BDNF and their potential role in the management of opioid addiction.

1.7 Liquid Chromatography- Mass Spectrometry (LC-MS)

Liquid Chromatography -Mass Spectrometry (LC-MS) is a powerful analytical technique used for the quantitative bioanalysis of pharmaceuticals and other biological analytes of interest. [92] This method is gaining high popularity in neuroscience research over other analytical methods such as enzyme linked immunosorbent immunoassay (ELISA), radioimmunoassay (RIA), gas chromatography, capillary electrophoresis as these methods are time consuming and costly, requiring pre and post-column derivatisation and time-consuming sample preparations with long chromatographic separations. [93,94] LC-MS has a high sensitivity and specificity, with a high applicability for complex biological matrices such as body fluids and tissues. [93,95]

A typical LC-MS system functions by combining the separating power of high-performance liquid chromatography (HPLC), with the detection power of mass spectrometry. *Figure 1.4* depicts the basic components of an LC-MS system, the first part being the liquid chromatography component, this is where a complex sample is injected to the column and separated based on the affinity of its individual components to the stationary phase. Secondly, the mass spectrometer where ionisation of compounds and detection occurs.

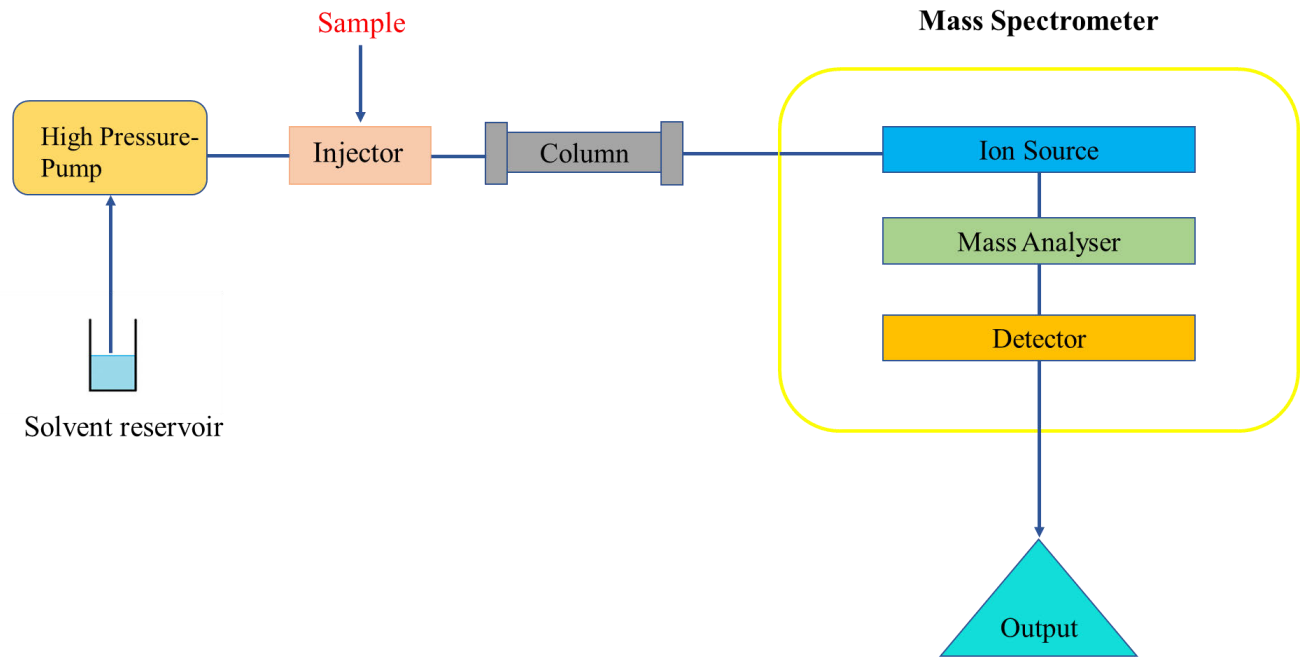

Figure 1. 4: Schematic representation of a Liquid Chromatography Mass Spectrometric (LC-MS) system (prepared by author).

1.7.1 **Liquid - Chromatography**

a) Solvent Reservoir

The solvent reservoir contains the mobile phase used to carry the sample through the system. [96] The solvents used depend on the different types of HPLC. Normal-phase HPLC, uses a nonpolar solvent and reverse-phase HPLC uses a mixture of water and a polar organic solvent. [97] To improve the chromatographic peak shape and signal of analyte, the mobile phase is spiked with an acid, most commonly acetic acid and formic acid, which provide a source of protons in reverse phase or act as ionising agents in the source of the mass spectrometer. [98] The solvent is propelled through the system by the pump

b) High Pressure – Pump

Pumps are regarded as the heart of the HPLC as they generate the high pressures required and provide the gradient to drive sample separation. Depending on the application; piston, electro-osmotic, diaphragm, reciprocating and syringe pumps are used. To entirely push samples, at a

uniform flow rate, through the system, the pressure of pumps typically range between 50-400 bar. [99]

c) Injector

An injector or autosampler, allows for precise sample volume introduction onto the mobile phase flow entering the column, without interfering with the set flow rate and pressure of the HPLC system. It is mandatory that the sample is introduced without and air bubbles that will disturb the pressure of the system. The most used injector is the rheodyne injector, with others being the septum and stop flow injector. Regardless of the type of injector, high switching precision, low dead-volume and minimal flow disturbance are key characteristics for obtaining in the reduction of band broadening and increasing resolution. [99]

d) Column

This is the first part of the system where separation occurs. The compounds or analytes in a sample are eluted in accordance to the degree at which they interact with the column stationary phase. A combination of a polar stationary phase and a non-polar mobile phase is referred to as a normal phase column. The polar silica packed around the column retains polar molecules, whereas non-polar molecules are eluted first with the mobile phase. [100] In a reversed phase column, the stationary phase is packed with modified silica to make it non-polar (silica-C18 molecule) and is compatible polar mobile phases. [101,102] This type of column can be applied to a wider range of organic molecules and uses aqueous based mobile phases, making it of high importance in biological research. [100,103-105] Once the sample has been separated accordingly it moves towards the mass spectrometer for detection.

1.7.2 Mass Spectrometry

*e) Ion*isation *Source*

The ionisation source is a compartment of the instrument where charged molecular species are produced. [106] Electrospray ionisation (ESI) is the most commonly used ionisation technique and has routinely been used in the study of biologically important analytes . [107] This is regarded as a soft ionisation technique, meaning that minimum internal energy is transmitted to the analytes during the ionisation process. [106] The sample is preferably soluble in a polar solvent and is introduced at atmospheric pressure through a needle at a potential difference. [108] The

applied voltage, usually in the range 3-4 kV, is dependent on the inner diameter of the needle and the solvents used. [106] This results in the formation of highly charged droplets, that are vaporised with the aid of a warm neutral gas such as nitrogen (known as the nebulizer gas). As the droplets progress through the ion source they become smaller and the coulombic forces between them increase and ultimately exceed the surface tension of the solvent generating ions. [108,109] The ions are then released to the gas phase and make their way to the mass analyser. [110] Unlike other ionisation methods, ESI can analyse non-volatile organic and inorganic compounds, with masses ranging from very low to extraordinarily high. [107,111]

f) Mass analyser

This part of the mass spectrometer is responsible for sensitivity through sorting and separating ions according to their mass-to-charge ratio (*m/z*). [112] The most commonly used types of mass analysers are the quadrupole and time of flight (TOF). The quadrupole analyser acts as a mass selective filter, consisting of four hyperbolic rods that are parallel to each other, when a voltage is applied, this allows the transmission of a narrow band of *m/z* values along the axis of the rods. Varying the voltage with time allows the transmission of a certain range of *m/z* values, resulting in a spectrum. [113] Quadrupole mass analysers are of great interest since they are relatively of low cost, tolerant to high pressures and are useful for the analysis of large biomolecules. [109,113]

The TOF mass analyser is known as the simplest and operates by accelerating ions at a fixed potential (1-20 kV) through a flight tube to the detector. [110] These ions travel a fixed distance of between 0.5-2.0 metres before colliding with the detector, depending on their *m/z* values. Higher *m/z* value ions have lower velocities in comparison to the lower *m/z* ions, meaning they are last to reach the detector. [110] Through measuring the time it taken to reach the detector after the ion is formed, the *m/z* of the ion can be determined. [110] This particular mass analyser has high mass accuracy, that enables the determination of molecular formulas for small molecules. [114] To improve the analyses of ions, tandem mass spectrometers that combine different mass analysers have been developed. Combining the quadrupole mass analyser with the TOF mass analyser produces a hybrid quadrupole time-of-flight (QTOF) mass analyser. [114] The QTOF has led to higher resolution mass spectrometers, [106] while triple quadrupole (QQQ) mass spectrometers allow for greater mass sensitivity.

g) Detector

When ions collide with the detector, the collision is recorded in the form of a mass spectrum, which is a plot of the relative abundance of ions versus their mass-to-charge ratio (*m/z*), which is the typical output (*Figure 1.4).* [115] There are two detectors that are normally used in mass spectrometry, *viz*. the electron multiplier (EM) and the Faraday cup. The EM is made up of a series of aluminium oxide dynodes with increasing potential. When ions strike the first dynode surface, electrons are emitted, which then move to the next dynode held at a higher potential and as a result more secondary electrons are generated. [56,116] Amplification is accomplished as secondary electrons are produced from dynode to dynode, this better known as a "cascading effect". [109]

The Faraday cup is a typical electrical detector, where a beam of positive ions impinging on the collector are neutralised by electrons. Ions strike the dynode surface, made up of BeO, GaP or CsSb, which causes the ejection of secondary electrons. [56] This detector can tolerate high pressures, has high accuracy, constant sensitivity and low electrical noise. Nevertheless, when compared to EM this detector is less sensitive as there is very little amplification of signal. [109]

1.8 Polymerase Chain Reaction (PCR)

The basic purpose of polymerase chain reaction (PCR) is to rapidly make numerous copies of a specific region of DNA or RNA. It is usually used to amplify and clone genes for gene expression studies, paternity testing, diagnosis of genetic diseases, forensics and detection of bacteria and viruses. [117-119] There are three principal steps in a PCR reaction; denaturation, primer annealing and extension. [120] These are often repeated over 25-40 cycles employing an automated thermal cycler. [121] The first step, denaturation occurs at 94-95˚C where the double-stranded DNA is separated into a single-stranded DNA, which serve as a template. [120] The second step, primer annealing, occurs at a temperature optimised for the specific primers according to their composition and length. [120] Primers, short oligonucleotides are complementary to the ends of the DNA sequence to be amplified, they attach to the template DNA and enable the polymerase enzyme to attach and copy the template. [121] The last step, extension, is where nucleic acid bases are added onto the template strand at72˚C. After each subsequent PCR cycle there is an exponential increase in the number of gene copies [120,121] Agarose gel electrophoresis is usually used to confirm the desired product of PCR. [121] Real-time quantitative reverse transcription PCR (RT-qPCR) is a sensitive, rapid and accurate technique that has become a method of choice in gene expression studies, which follows the

same principle as PCR. [117,122] The starting point is the RNA, which is used as a template to synthesise complementary DNA (cDNA), which is then used as a template for the quantitative PCR (qPCR) reaction. [117] When performing qPCR, a fluorescent dye-labelled probe or fluorescent DNA-intercalating dye is used as an indirect measure of the amount of nucleic acid present during each amplification cycle. [123] A standard curve can be achieved through measuring the PCR cycle for samples at which fluorescence reaches a certain threshold, known as the threshold cycle value (C_T). [123] qPCR allows for the determination of the expression of a particular gene when compared to a housekeeping gene.

1.9 Aim and objectives

Aim: To investigate the functional and molecular changes associated with BUP administration in a healthy rodent model.

Objectives:

I. To determine the pharmacokinetic properties of BUP in the rodent brain following the intranasal administration of a 0.3 mg/mL dose.
II. To monitor brain neurotransmitter changes at different time points post-BUP administration using LC-MS.
III. To determine changes in the expressions of CREB and BDNF genes at different time points following BUP administration, using qPCR.

1.10 Outline of thesis

Chapter 1 provides a thorough background on BUP, opioid addiction and how it effects neurotransmitters, BDNF and CREB. In addition, explains the principles of the quantification techniques used in this study; LC-MS and qPCR.

Chapter 2 is a manuscript which was submitted to Addiction Biology titled "Functional and molecular changes associated with intranasal buprenorphine administration in a healthy rodent model".

Chapter 3 provides a general conclusion and future recommendations for the study.

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CHAPTER 2

Functional and molecular changes in the rodent brain following intranasal buprenorphine administration

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ABSTRACT

Buprenorphine is an opioid drug used in the management of pain and the treatment opioid addiction. Like other opioids, it is believed that it achieves these effects by altering functional neurotransmitter (NT) pathways and the expression of important transcription factors in the brain, however there is a lack of scientific evidence to support these theories. This study investigated the pharmacodynamic effects of BUP administration by assessing neurotransmitter and molecular changes in the healthy rodent brain. Sprague-Dawley rats (150 – 200g) were intranasally administered buprenorphine (36 µL of 0.3 mg/mL) and sacrificed at different time points: 0.25, 0.5, 1, 2, 4, 6, 8 and 24 h post drug administration. LC-MS was used to quantify BUP and neurotransmitters (GABA, GLUT, DA, NE and 5-HT) in the brain; while *CREB* and *BDNF* gene expression was determined using qPCR. Results showed that BUP reached a C_{max} of 1.21 ± 0.0523 ng/mL after 2 h, with all neurotransmitters showing an increase in their concentration over time, with GABA, GLUT and NE reaching their maximum concentration after 8 h. DA and 5-HT reached their maximum concentrations at 1 h and 24 h, respectively post drug administration. Treatment with BUP resulted in significant upregulation in *BDNF* expression throughout the treatment period while *CREB* showed patterns of significant upregulation at 2 and 8 h, and downregulation at 1 and 6 h. This study contributes to the understanding of the pharmacodynamic effects of BUP in opioid addiction by proving that the drug significantly influences NT pathways that are implicated in opioid addiction.

Keywords: Buprenorphine, opioid addiction, brain-derived neurotrophic factor (BDNF), cyclic AMP Response Element-Binding Protein (CREB), neurotransmitters

2.1INTRODUCTION

Buprenorphine (BUP) is a semisynthetic opioid used for the treatment of opioid addiction and moderate to severe pain, [1]with doses in the range of 4-32 mg/day being required to treat opioid addiction in most patients.[2] Due to its lower risk of toxicity at higher doses, lower abuse potential, accessibility for office-based treatment and limited physical dependence, BUP is preferred over methadone and naltrexone in the management of opioid addiction. [3,4] Similar to other opioids, upon binding to the mu-opioid receptor, decreased pain, euphoria and respiratory suppression are experienced, however with limited potency since the drug acts as a partial opioid receptor agonist. [5,6]

When BUP binds to the mu-opioid receptors (MORs) in the brain, levels of the principal neurotransmitters such as dopamine (DA), γ-aminobutyric acid (GABA), serotonin (5-HT), glutamate (GLU) and norepinephrine (NE) are altered. [7] Neurotransmitters are chemical messengers that transmit signals from pre-synaptic to post-synaptic nerve cells, in addition they also play a role in immunoregulation. [8] They are involved in the regulation of moods, stress, learning and addiction. [9] Serotonin (5-HT) plays an important role in mood, sleep, appetite, sexual desire and neuroendocrine function. [10,11] Research shows that morphine (opiate) dependence and withdrawal is a result of serotonin neurotransmission. [12,13] Chronic administration of morphine increases the expression of the 5-HT_{2C} receptor protein, which suppresses the expression of nicotine-induced behaviour and depression-like behaviour during nicotine withdrawal. [14,15]

DA has also been showed to play an important role in drug addiction [16], DA is associated with emotion, food intake, locomotor activity, positive reinforcement, learning and memory. [17,18] GABA on the other hand acts as the principal mediator of synaptic inhibition. [19] When opiates activate MORs in the ventral tegmental area (VTA), GABA interneurons are inhibited, ultimately increasing the release of DA in its projecting regions *viz*. the nucleus accumbens (NAc) and prefrontal cortex (PFC). [20,21] Working in opposition to GABA, GLU is the major excitatory neurotransmitter in the mammalian brain, being involved in neuronal plasticity, learning, memory processes and plays an unquestionable role in opioid addiction. [22,23] In opioid reward, GLU is critical for the formation and maintenance of opioid memories that are formed after repeated drug use, which are simply a combination of memories regarding the opiate experience. [24] These opioid memories trigger cravings and relapse. Opioid withdrawal symptoms have been strongly associated with the locus ceruleus (LC), where NE is produced

and facilitates drowsiness, low blood pressure and respiratory depression. [25] In addition to changes in these chemical messengers, gene expression regulators such as cyclic AMP response element-binding protein (CREB) and brain-derived neurotrophic factor (BDNF) have been shown to be important transcription factors in modifying NT levels in response to extracellular stimuli. [26]

CREB is a transcription factor that is involved in neuronal survival, memory, learning and drug dependence. [27,28] The phosphorylation of CREB leads to its activation and enables it's binding to cAMP response element (CRE) sites which induces the expression of downstream genes. [29] This includes genes that alter neurotransmitter levels and genes encoding transcription, signal transduction factors and metabolic enzymes. [30] Opiates inhibit cAMP signalling pathways which decreases neuronal firing of the LC, which is implicated in opioid withdrawal. [31] In the chronic administration of opioids, there is an upregulation of the cAMP system that leads to tolerance, dependence and drug-seeking behaviour. [32] In addition, an increase in phosphorylated CREB is noted, binding to target genes and modulating their expression. [29] Biogenic amine neurotransmitters such as DA, 5-HT and NE have been shown to regulate CREB activation through G-protein receptors and G-protein mediated 2nd messenger signalling. [33] Evidence strongly suggests that the function and development of DA and 5-HT is a result of BDNF modulation.[34]

BDNF is a neurotrophic peptide that facilitates neuronal cell growth, maintenance and plasticity, has also been shown to be involved in drug addiction. [35] Previous studies show that dopaminergic, serotonergic and GABAergic development and functions are modulated by BDNF. [36-38] Pre-synaptically, BDNF regulates GLU release via the tyrosine kinase *Trk B* receptor and extracellular calcium mobilization. [39,40] Studies that have measured serum BDNF levels in heroin addicts have shown contradicting results. [36] Zang *et al*. and Angelucci *et al*. found BDNF serum levels lower than the control group ($p < 0.01$ and $p < 0.05$, respectively), whereas Zhang et al. found the opposite with serum BDNF levels of heroin addicts to be higher than in controls ($p = 0.001$). [38,41,42] In addition, a study where opiate dependent individuals were treated with heroin showed a significant increase in BDNF serum levels ($p = 0.009$) in these patients. [43] The evidence strongly implicates neurotransmitters and, CREB and BDNF, in the pathophysiology of opiate addiction, however there is a lack of information regarding how BUP alters these pathways in the management of opioid addiction. Therefore, we aim to investigate the functional (neurotransmitter) and molecular (*CREB/BDNF* expression) pharmacodynamic changes associated with BUP administration in a rodent model.

2.2MATERIALS AND METHODS

2.2.1 Experimental Animal Model

The animal experiments conducted in this study were approved by the Institutional Animal Research Ethics Committee, University of KwaZulu-Natal (approval reference: AREC/013/019M). Male Sprague-Dawley rats (150-200 g) were obtained from the Biomedical Resource Unit (BRU) of the University of KwaZulu-Natal (Durban, South Africa). Prior to the experiment, rodents were allowed to acclimatize for a period of one week. Animals were housed in clear polycarbonate cages, with a 12-h light/dark cycle at 21-24 ˚C, with environmental enrichment in the form of shredded paper and allowed *ad libitum* access to water and standard rodent feed.

The intranasal administration route is an effective route for rapid brain drug delivery and as a result has received attention for emergency administration in overdose situations, in addition this route is novel in the administration of BUP in rodent brain.[44,45] The rats (n = 3) were first anesthetized with isoflurane to minimize discomfort during intranasal administration and to prevent any unwanted movements during drug dosing. A previously reported method by our lab was implemented, where rats were placed in a supine position with their nose at an upright 90˚ angle to enable snorting of drops of 0.3 mg/mL of buprenorphine into the nasal cavity. A total of 36 µL/rat was administered using a micropipette (Eppendorf-P10), where 6 µL/nostril was dispensed in both naris with a hold time of 2 min between each dose. [46]

Animals were euthanized through decapitation at; 0, 0.25, 0.5, 1, 2, 4, 6, 8 and 24 h post drug administration. Blood was collected, the brain surgically extracted, quickly frozen using liquid nitrogen vapor and stored at -80 ˚C until time of LC-MS analysis. Tissue samples for gene expression studies and protein analysis were stored at -80 ˚C in Qiazol and cytobuster until time for analysis.

2.2.2 Chemicals and Reagents

LC-MS grade Acetonitrile and Methanol were purchased from Merck Ltd (Darmastadt, Germany) and Honeywell (Steinham, Germany), respectively. Ultrapure water was purified using a Milli-Q® water purifying system (Merck Millipore, Burlington, MA). All neurotransmitter standards; Dopamine hydrochloride, γ-Aminobutyric acid, L-Glutamic acid

monosodium salt monohydrate, Norepinephrine and Serotonin were supplied by Sigma Aldrich (St. Louis, USA). The deuterated internal standard was obtained from Clearsynth® (Ontario, Canada). IsoFor (Isoflurane) was obtained from Safeline Pharmaceuticals (Durban, South Africa) and BUP from Pharmed Pharmaceuticals (Durban, South Africa).

2.2.3 Sample Preparation for LC-MS

Brain tissue samples were homogenised in one volume of ultrapure water (1 mL/g) using a tissue homogenizer from OMNI International-The Homogenizer Company® (Kennesaw Georgia, USA). 850 µL of methanol was added to 100 µL aliquot of brain homogenate and 50 µL of internal standard, followed by vortexing for a minute. The mixture was centrifuged at 4˚C, at 4500 × *g* for 10 min for protein precipitation. A modified solid phase extraction was performed using a Discovery® DSC-18 (100 mg) cartridge (Merck, South Africa) to filter the resultant supernatant. The flow from the SPE was collected and transferred to LC-MS vials and dried using a ZipVap nitrogen evaporator (Gauteng, South Africa). The drying temperature was set to 55˚C for 15-20 min, with continuous nitrogen flow until dryness was achieved. The samples were resuspended in 200 µL of ultrapure water and ready for LC-MS analysis.

2.2.4 Liquid Chromatography - Mass Spectrometry

The LC-MS system consisted of a Thermo Scientific Vanquish Ultra-High-Performance Liquid Chromatography (UHPLC) (Waltham, MA USA) system coupled to TSQ Quantis Triple Quad mass spectrometer (Waltham, USA). The mass spectrometer was equipped with a heated electrospray ionization (H-ESI) source that was set to operate in the positive ionisation mode with a source spray voltage of 4809 V. Separation was achieved using a Poroshell 120 EC-C18 column (50 × 4.6 mm and 2.7 µm particle size) (Agilent Technologies, USA) which was maintained in a controlled column compartment with a temperature of 25˚C. The mobile phases used were; A: LC-MS grade water with 0.1% (v/v) formic acid and B: methanol with 0.1% (v/v) formic acid with a sample injection volume of 10 µL and a flow rate of 0.8 mL/min flow rate. The elution gradient consisted of an equilibration time of 1 min with 15% B, 0 min: 15% B, 1 min: 70% B, 1.1 – 5.5 min: 95% B, 5.1 – 5.6: min 15% with a total run time of 8 min. The mass spectrometer parameters used for mass isolation and ion quantification are shown in the Supplementary Information (SI-Table 1).

2.2.5 Total RNA isolation

Briefly, 100 µL of brain sample was added to 500 µL of Qiazol reagent (Qiagen, Hilden, Germany) and stored at -80˚C overnight for isolation of RNA. The samples were thawed at room temperature (RT) and a 100 µL of chloroform was added, centrifuged for 15 min at 12 000 × *g* at 4˚C. 250 uL of aqueous phase was transferred to a fresh 1.5 mL micro-centrifuge tube. Working on ice, 250 µL of isopropanol was transferred to the tube and sample incubated overnight at -80˚C. Samples were centrifuged for 20 min at 12 000 × *g* at 4˚C, the supernatant removed and the pellet washed with 500 µL of 75% cold ethanol. The sample was then centrifuged for 15 min at 7 400 × *g* at 4˚C, RNA pellets air dried for 30 min at RT and ethanol discarded. The RNA pellets were resuspended in 15 µL of nuclease-free water and incubated at RT for 3 min, before quantification of RNA. Total RNA was quantified and purified using Nanodrop™ 2000 spectrophotometer (Thermo-Fisher Scientific, SA). RNA purity was evaluated using the A260/A280 absorbance ratios. All samples were standardised to a final concentration of 1000 ng/µL.

2.2.6 *CREB* and *BDNF* mRNA expression

Reverse transcription of total RNA (1000 ng/µL) into cDNA was done using the Maxima H Minus First Strand cDNA Synthesis Kit (Thermo-Fisher Scientific, California, USA) as per manufacturer's instructions. PowerUp™ SYBR™ Green Master Mix (Thermo-Fisher Scientific, California, USA) was used to determine *CREB* and *BDNF* mRNA expression according to manufacturer's protocol. [*BDNF*: Sense: 5'-GAATTCATGACCATCCTTTTCCTTACTATG-3'; *CREB*: Sense: 5'-CCAAACTAGCAGTGGGCAGTATATT-3'] (1 uL), anti-sense primer (25 µM) [*BDNF*: Anti-sense 5'-AAGCTTTCTTCCCCTTTTAATGGTCAG-3'; *CREB*: Anti-sense 5'-GGTACCATTGTTAGCCAGCTGTATT-3'] was prepared. To normalise the expression of *CREB* and *BDNF* the *GAPDH* house keeping gene was used [GAPDH: Sense 5'-GGCACAGTCAAGGCTGAGAATG-3', Anti-Sense 5'–ATGGTGGTGAAGACGCCAGTA-3']. The Applied BioSystems ViiA 7 Real-Time PCR system (Thermo-Fisher Scientific, California, USA) was set to 95˚C for Initial denaturation (1 cycle) for 8 min, followed by PCR which consisted of 40 cycles of denaturation at 95˚C for 15 sec, annealing at 60˚C for 40 sec and extension for 30 s at 72˚C. Data analysis was done using methods described by Livak and Schmittgen (2001) and represented as a fold change relative to control.[47]

2.2.7 Statistical Analysis

All data is expressed as mean ± SD. Statistical analysis was performed using GraphPad Prism version 8.4.3 (GraphPad Software, USA). The unpaired t-test with Welch's correction was used to compare all data to the control group. Moreover, multiple t test- one per row was used to determine significance at different time points. $p<0.05$ was considered statistically significant. The pharmacokinetic parameters of BUP were calculated using Stata/IC 15.0 (StataCorp LLC, Texas, USA).

2.3RESULTS

2.3.1 Pharmacokinetics of BUP and associated brain neurotransmitter changes

The pK of BUP and the associated NT (GABA, GLUT, DA, NE and 5-HT) changes were monitored over a 24 h period following a single dose of 0.3 mg/mL BUP (*Figure 2.1*). The brain concentrations were determined in brain homogenates using an accurate and reproducible LC-MS method. The same method was used to determine the tissue pK of BUP over a 24-hour period (*Table 2.1*).

Table 2.1: Mean pharmacokinetic parameters after 0.3 mg/mL single dose of buprenorphine in rats.

Parameter	Mean
C_{max} (ng/mL)	1.21 (± 0.0523)
T_{max} (h)	2
$T_{1/2}$ (h)	6.27
K_{el}	0.11
$AUC_{0\text{-}inf}$ (ng × h/mL)	25.04
$AUC_{0\text{-}t24}$ (ng × h/mL)	24.18

C_{max} = maximum concentration; T_{max} = time of maximum concentration; K_{el} = elimination rate; $T_{1/2}$ = half-life, AUC = area under the concentration-time curve (n = 3).

BUP's brain tissue concentration increased rapidly reaching a C_{max} of 1.21 ± 0.0523 ng/mL, with a T_{max} of 2 h, with a half-life of 6.27 h and $AUC_{0\text{-}inf}$ of 25.04 ng × h/mL. In response to BUP administration, the tissue concentrations of GABA, GLU and NE followed the same trend

and reached their peak concentrations of 2060.85 ± 112.52 ng/mL, 1018.46 ± 49.60 ng/mL and 1389.94 ± 112.30 ng/mL, respectively after 8 h. 5-HT showed the lowest range of concentration (20.5-22.5 ng/mL) at all time points, with a peak concentration of 22.09 ± 0.190 ng/mL at 24 h. DA tissue concentrations increased rapidly in tandem with BUP and peaked after 1 h with a peak concentration of 42.33 ± 2.11 ng/mL and decreased close to baseline levels at 2 h when BUP was at its highest concentration.

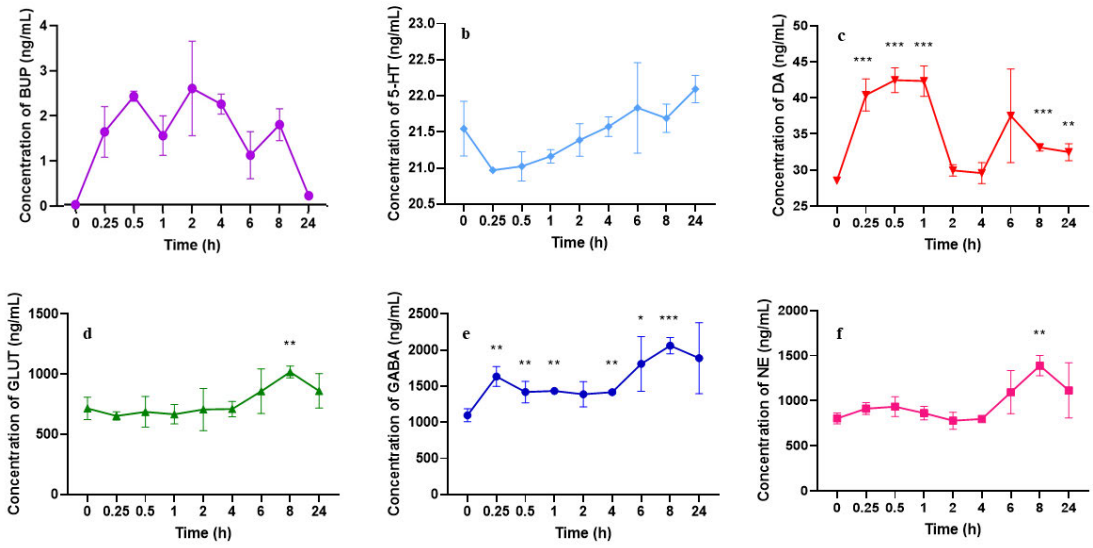

Figure 2. 1: Mean brain concentration at different time intervals of (a) BUP ; (b) 5-HT; (c) DA; (d) GLUT; (e): GABA; (f) NE following intranasal administration of a single dose of 0.3 mg/mL BUP in male Sprague-Dawley rats (n = 3). Values are expressed as mean ± SD. ($^{*}p<0.0332$, $^{**}p<0.021$ $^{***}p<0.0002$ $^{****}p<0.0001$).

2.3.2 Effect of Buprenorphine on CREB and BDNF expression

CREB and *BDNF* gene expression was measured using qPCR in the brain tissue of rodents sacrificed at different time points post a 0.3 mg/mL intranasal administration of BUP. *CREB* gene expression varied when compared to control, there was a significant downregulation at 1 and 6 h post dosing ($^{****}p<0.0001$ and $^{*}p<0.0332$ respectively). However, at 2 and 8 h the gene was significantly upregulated ($^{****}p<0.0001$ and $^{*}p<0.0332$ respectively) (*Figure 2.2*). *BDNF* expression was significantly upregulated ($p<0.0159$) with a maximum of 66.06-fold relative to control at 8 h post drug administration. (*Figure 2.3*).

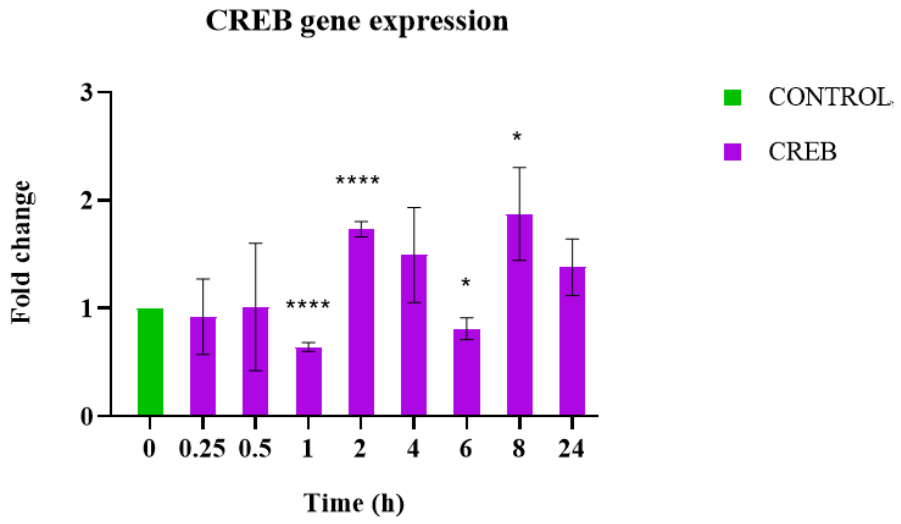

Figure 2. 2: CREB gene expression in half of male Sprague-Dawley rat brain at different time intervals following 0.3 mg/mL single dose of BUP. Data is represented as mean ± SD (n = 3). (*p<0.0332, **p<0.021 ***p<0.0002 ****p<0.0001).

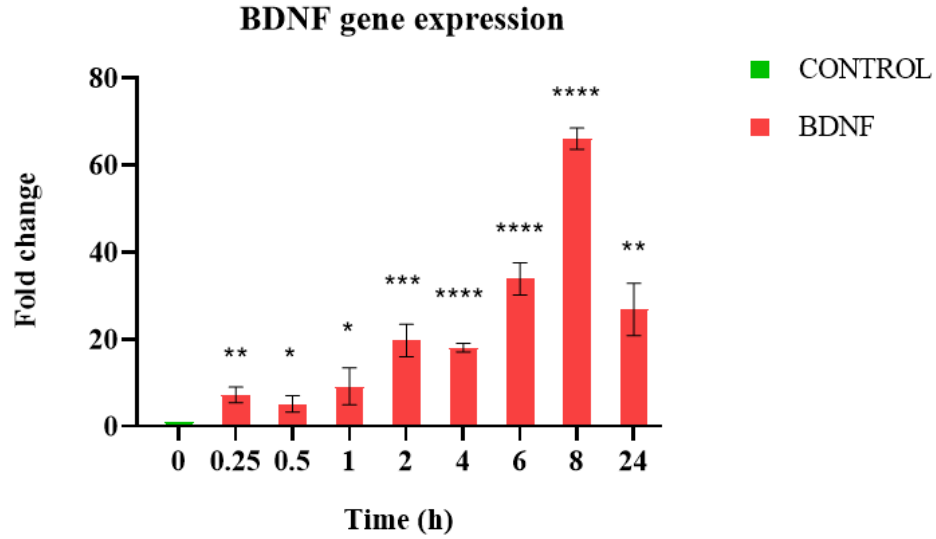

Figure 2. 3: BDNF gene expression in half of male Sprague-Dawley rat brain at different time intervals following 0.3 mg/mL single dose of BUP. Data is represented as mean ± SD (n = 3). (*p<0.0332, **p<0.021 ***p<0.0002 ****p<0.0001).

2.4 DISCUSSION

In this study, we investigated the pharmacodynamic effects of intranasally administered BUP (0.3mg/ml) on NT levels and *BDNF* and *CREB* expression in the rodent brain at 0, 0.25, 0.5, 1, 2, 4, 6, 8 and 24 h post drug administration. Mean tissue concentration-time profiles of BUP and NTs are shown in Figure 2.1a-f. The pharmacokinetic parameters of BUP are also summarized in Table 2.1. The results show that BUP entered the brain soon after administration and was detectable as early as 0.25 h post dosing, reaching a maximum tissue concentration (C_{max}) of 1.21 ± 0.0523 ng/mL after 2 h (T_{max}). There is a lack of literature information regarding the pK of BUP in the human or rodent brain as studies focus on plasma drug levels, however a study by Kendall *et al.* (2014) also obtained a T_{max} of 2 h in the brain after subcutaneously administering 0.3 mg/mL of BUP-HCl in a murine model.[48] While Gopal *et al.* (2002) were unable to completely characterise the pK profile of BUP following a 0.1 and 0.3 mg/mL dose which was intravenously administered to rats, due to very low tissue drug concentrations.[49] An 8 mg intranasal and sublingual administration of BUP in humans showed plasma C_{max} of 11.2 and 2.19 ng/mL, respectively with a T_{max} of 34.5 min and 0.67 h, respectively.[50,51]

All NTs (GABA, GLUT, NE, 5-HT and DA) in this study were altered by BUP administration when compared to the controls at various time points post drug administration (*Figure 2.1*). GABA levels steadily increased and reached a peak concentration of 2060.85 ± 112.51 ng/mL at 8 h post dose, then gradually decreasing to 24 h. GLUT and NE also reached peak concentrations at 8 hours post dose, however with lower concentrations of 1018.46 ± 49.60 and 1389 ± 118. 30 ng/mL respectively (*Figure 2.1*). GABA and DA were the most significantly affected by the administration of BUP with GABA levels being significantly increased and DA being significantly decreased throughout the duration of the treatment. Both of these NTs are known to play a critical role in the VTA, an area strongly associated with opioid addiction, where GABA interneurons are inhibited and cause and increase of DA in NAc and PFC.[21] The increase of GABA and decrease of DA, suggests that BUP is able to antagonize the neurotransmitter changes seen in opioid addiction. Decreases in DA reduces the risk of addiction while increases in GABA reduces the stimulation associated with drug use. The initial increase in DA is similar to the findings of Marquez *et al.* (2007) who showed increased locomotor activity in mice which received an acute dose of BUP (3 mg/kg, sc), since DA

regulates locomotor activity this may account for the initial increase observed in our study.[18,52] It is proven that opioid drugs supress NE, which results in respiratory depression, drowsiness and low blood pressure.[25] This study shows that BUP alters NE concentration in the brain by causing a gradual significant increase, which peaks at 8 h with a concentration of 1389 ± 112.30 ng/mL (p<0.021), this again suggests BUP's efficacy in opposing the neurotransmitter changes associated in opioid addiction and its potential to combat opioid induced respiratory depression. 5-HT was not significantly altered post BUP administration, however its concentration did show an upward trend. These findings suggest that the mechanism of action of BUP does not involve the modulation of 5-HT in the brain. The results may indicate that intranasal administration of BUP provides direct drug delivery to the central nervous system, possibly via the trigeminal nerve and olfactory tubes.[53] This is suggested by the rapid changes in neurotransmitter levels, as early as 1-2 h post drug administration.

Intranasal administration of a 0.3 mg/mL dose of BUP lead to significant upregulation of *BDNF* in the rodent brain at 8 h post drug administration. This is beneficial in opioid addiction since a study has shown that chronic morphine exposure increases the inhibition of the RNA polymerase II enzyme at the *BDNF* promotor region, thereby preventing RNA synthesis leading to decreased *BDNF* gene expression.[54] Porcher *et al.* also found that GABAergic development is modulated by BDNF,[37] this relationship was confirmed in our study since both GABA concentration (*Figure 2.1*) and BDNF expression (*Figure 2.2*) peaked at 8 h following drug dosing. CREB is known to regulate the expression of *BDNF*, which could account that decreased CREB levels reduce *BDNF* levels.[55] Our results show that *CREB* gene expression varied throughout the treatment period, being significantly upregulated at 2 h ($^{****}p$<0.0001) and 8 h ($^{*}p$<0.0332) and downregulated at 1 h ($^{****}p$<0.0001) and 6 h ($^{*}p$<0.0332).[56] With CREB levels reaching its maximum expression at 8 hours when BDNF expression was at its peak.

The results obtained from this study showed that BUP does significantly influence NTs (GABA, GLUT, NE, 5-HT and DA) and the gene expression of *BDNF* and *CREB*. Based on previous findings in literature, these changes are in direct opposition to those seen in models of opioid addiction. Therefore, this study greatly contributes to the understanding of the pharmacodynamic effects of BUP in the treatment and management of opioid addiction.

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AUTHORS CONTRIBUTION

SB and AC was responsible for the design of the research project, SPX, LJK and AMH performed animal work and sample preparations. TG[b] and SD assisted with PCR. CM, JM, NPM and LM assisted with LC-MS analysis. SDS assisted with animal experiments. SPX and SB analysed, interpreted the findings and wrote the manuscript. SB, NDG, TG[d], TN and HGK co- supervised the study.

CONFLICT OF INTEREST

The authors declare no conflict of interests.

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CHAPTER 3

3.1General conclusion and future recommendations

Buprenorphine (BUP) is a semisynthetic opioid derivative used to treat opioid addiction and pain, by acting as a partial agonist at the mu-opioid receptor and as an antagonist at delta- and kappa-opioid receptors contributing to its unique pharmacodynamic effects. In addition, BUP has a high opioid receptor affinity and slow dissociation rate from receptors resulting in a prolonged duration of action.[1] However, the exact mechanism by which BUP treats opioid addiction is unknown. This drug, like other opioids, is believed to have an effect on neurotransmitters, *BDNF* and *CREB.* The primary neurotransmitters believed to be altered by BUP include DA, GABA, GLU, NE and 5-HT. The mesocorticolimbic dopamine system is a neurotransmitter system that contributes significantly to opioid addiction. It originates from the VTA and involves indirect dopamine firing to the PFC and NAc, which is mediated via the inhibition of GABA neurons.[2] Opioid reward also requires glutamatergic neurotransmission, which involves GLUT, a major excitatory NT that is required for mu-opioid receptor activation.[3] During opioid withdrawal, NE is also supressed which results in drowsiness, low blood pressure and respiratory depression.[4] 5-HT which plays an important role in appetite, mood, memory, sexual behaviour and neuroendocrine function has also been shown to participate in the development of opioid dependence and withdrawal of the opioids. Chronic treatment of morphine has demonstrated an increase in the expression of 5-HT_{2C} receptor protein in NAc, VTA and LC.[5] Opioids also affect neurobiological transcription factors BDNF and CREB.[6,7] BDNF plays a crucial role in the modulation of neural and behavioural plasticity in drug abuse.[8] The transcription factor, CREB, facilitates in learning and memory, circadian rhythm, depression and addiction.[9] The present study, therefore, evaluated the effect of intranasal BUP on the neurotransmitters (GABA, GLUT, NE, 5-HT and DA) and the expression of CREB and BDNF.

In this study, 27 male Sprague-Dawley rats (150 – 200 g) were intranasally administered 0.3 mg/mL of BUP and euthanised at: 0.25, 0.5, 1, 2, 4, 6, 8 and 24 h post drug dosing. Thereafter, brain samples were collected and homogenised for analysis. The pharmacokinetics of BUP and

neurotransmitter (GABA, GLUT, NE, 5-HT and DA) levels were determined using LC-MS and gene expression of CREB and BDNF was quantified using qPCR.

The pharmacokinetic parameter of BUP show that a C_{max} of 1.21 ± 0.0523 ng/mL was achieved at a T_{max} of 2 h in the healthy rodent brain. The $AUC_{0\text{-}inf}$ was 25.04 ng × h/mL and the half-life ($T_{1/2}$) 6.27 h. These pharmacokinetic results were consistent with that of Kendall *et al.* who found a T_{max} of 2 h and C_{max} of 19.1 ng/mL, where they also administered 0.3 mg/mL of BUP-HCl in a murine model.[10] Post BUP dosing, the concentration of 5-HT decreases at 0.25 h and increases steadily reaching its peak concentration (22.09 ± 0.190 ng/mL) at 24 h. DA rapidly increases soon after BUP administration and concentration peaks at 42.33 ±2.11 ng/mL after an hour. However, its concentration is very low when compared to GABA which is initially above 1000 ng/mL and peaks at 8 h reaching a concentration of 2060.35 ng/mL. Literature states that the binding of opioids to mu-opioid receptors on GABA neurons, leads to the inhibition of these neurons and an indirect firing of DA neurons is achieved.[11] GLUT and NE also reached their maximum concentrations after 8 h, with their C_{max} being 1018.46 ± 49.60 ng/mL and 1389.94 ± 112.30 ng/mL, respectively. Gene expression results showed an overall significant upregulation ($^{*}p<0.0159$) in BDNF expression in the brain tissue in response to BUP administration. However, CREB expression varied showing significant upregulation (2 and 8 h, by $^{****}p<0.0001$ and $^{*}p<0.0332$ respectively) and downregulation (1 and 6 h, by $^{****}p<0.0001$ and $^{*}p<0.0332$ respectively). Overall, these results give an indication of the pharmacodynamic effects of BUP in the healthy brain.

This study demonstrated that BUP does significantly influence the levels of NTs (GABA, GLUT, NE, 5-HT and DA) and the gene expression of CREB and BDNF. An important finding in this study showed decreased levels of DA and higher levels of GABA with BUP administration. These pharmacodynamic effects are beneficial in treating opioid addiction since it is associated with higher levels of DA and lower levels of GABA. [12] In the future, we propose treating rats with different doses of BUP to clearly demonstrate the effect of the drug on the NTs (GABA, GLUT, NE, 5-HT and DA) and the gene expression of CREB and BDNF. In addition, treat the rats with BUP for longer periods with chronic doses to determine the long-term effects and addictive potential of BUP. Thereafter, studying neurobehavioral function, social interaction and locomotor activity by performing the novel object recognition test and social interaction tests to determine if there is any cognitive decline and social aversion due to BUP administration. Moreover, the analysis of other genes , such as the nerve growth factor (NGF) gene which belong to the same family of neurotrophins as BDNF and has been

previously studied together after methadone or BUP treatment in rats and have been shown to play a role in addiction and its treatment.[13,14] This will ultimately lead to a deeper understanding of the mechanisms of BUP's action and how they can be exploited by psychiatrists to better treat opioid addiction.

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Appendix I - Supplementary information

Functional and molecular changes associated with intranasal buprenorphine administration in a healthy rodent model

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1. Calibration sample preparation

In order to prepare calibration standards, 1 mg of each neurotransmitter standard was weighed and dissolved in 100 uL of water and 900 uL of methanol to produce a neurotransmitter multimix with a final concentration of 1mg/mL of each neurotransmitter. The multimix was then diluted with MeOH to 10 ug/mL and 1 ug/mL working solutions. An internal standard multimix was also prepared following the same procedure and diluted with MeOH to a 10 ug/mL working solution. Different concentrations of 50, 100, 250, 500, 750, 1000 ng/mL were prepared for the calibration curve. The Internal standard volume remaining constant throughout, 75 uL. Buprenorphine calibration samples were prepared as follows: 0.39, 0.78, 1.00, 1.56, 3.13, 5.00, 6.25, 10.00 ng/mL using methanol.

Table S 1: Mass spectrometer selected reaction monitoring and ion optics parameters used for the analysis of buprenorphine and neurotransmitters

Selected Reaction Monitoring (SRM) and Ion Optics Parameters							
Analyte	**Polarity**	**Precursor (m/z) $[M+H]^+$**	**Product (m/z)**	**Quantifier / Qualifier Ions (m/z)**	**Collison Energy(V)**	**RF Lens Voltage(V)**	**Dwell Time(msec)**
Dopamine	Positive	154.08	137	**Quantifier**	14.55	78	9.473
			90.946	Qualifier	27.51	78	9.473
			118.929	Qualifier	15	78	9.473
GABA	Positive	104.05	87	**Quantifier**	13.41	30	9.473
			46	Qualifier	55	30	9.473
			85.875	Qualifier	55	30	9.473
Glutamate	Positive	148.05	84.071	**Quantifier**	18.98	30	9.473
			131	Qualifier	23.72	30	9.473
Norepinephrine	Positive	170.088	152.054	**Quantifier**	10.23	79	9.473
			107.018	Qualifier	23.99	79	9.473
			135.018	Qualifier	14.63	79	9.473
Serotonin	Positive	177.000	159.982	**Quantifier**	10.23	30	9.473
			114.929	Qualifier	46.7	30	9.473
			132.929	Qualifier	55	30	9.473
D4-Serotonin	Positive	181.175	164.02	**Quantifier**	10.23	192	9.473
			78.557	Qualifier	34.57	192	9.473
			118.042	Qualifier	26.49	192	9.473
Buprenorphine	Positive	468.3	55.125	**Quantifier**	47.23	114	9.473
			396.3	Qualifier	35	114	9.473
			414.3	Qualifier	25	114	9.473

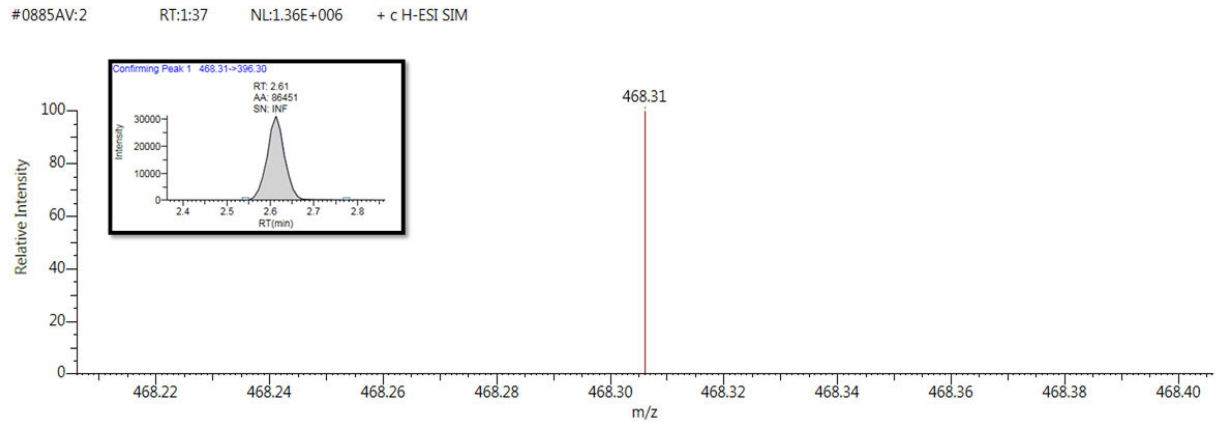

Figure S 1: Showing the precursor ion mass spectra of buprenorphine $[M+H]^+$ at 468.31 m/z embedded with its liquid chromatogram at 2.61 min as separated on a Poroshell 120 EC-C18 column (50 × 4.6 mm and 2.7 µm particle size).

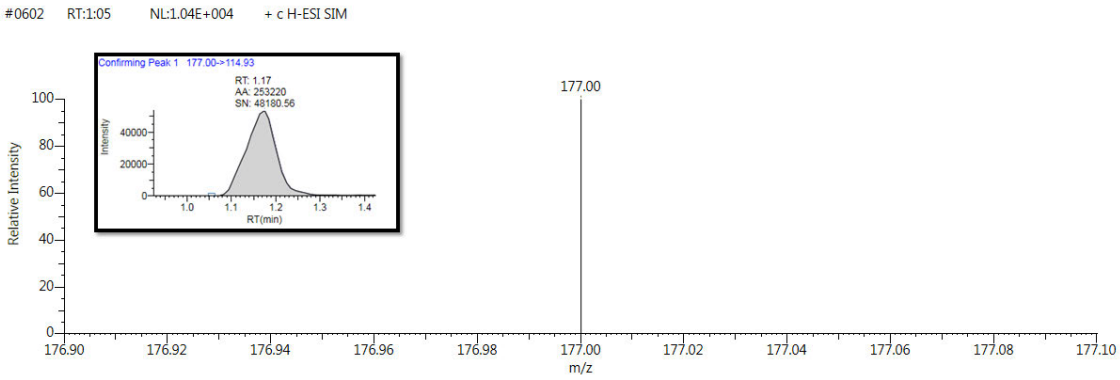

Figure S 2: Showing the precursor ion mass spectra of serotonin $[M+H]^+$ at 177.00 m/z embedded with its liquid chromatogram at 1.17 min as separated on a Poroshell 120 EC-C18 column (50 × 4.6 mm and 2.7 µm particle size).

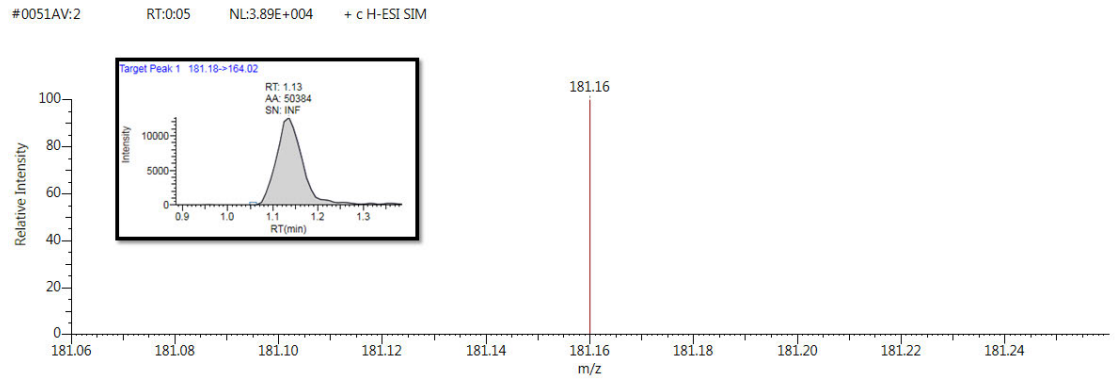

Figure S 3: Showing the precursor ion mass spectra of D4-serotonin $[M+H]^+$ at 181.16 m/z embedded with its liquid chromatogram at 1.13 min as separated on a Poroshell 120 EC-C18 column (50 × 4.6 mm and 2.7 µm particle size).

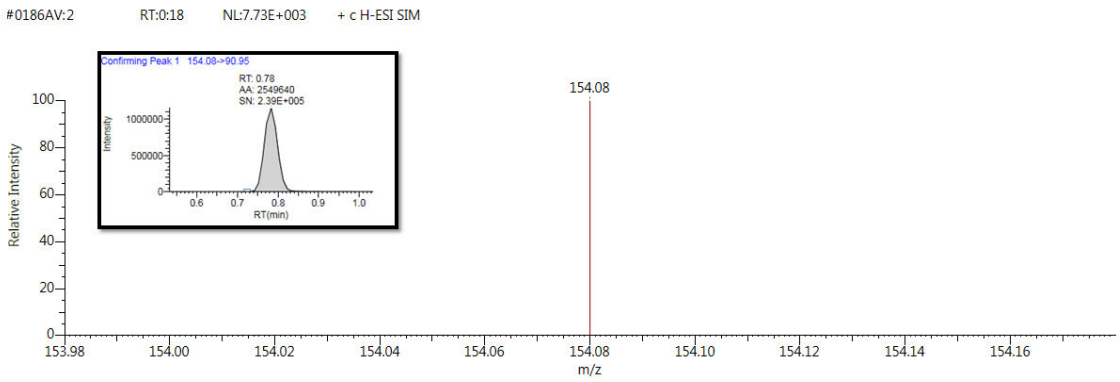

Figure S 4: Showing the precursor ion mass spectra of dopamine $[M+H]^+$ at 154.08 m/z embedded with its liquid chromatogram at 0.78 min as separated on a Poroshell 120 EC-C18 column (50 × 4.6 mm and 2.7 µm particle size).

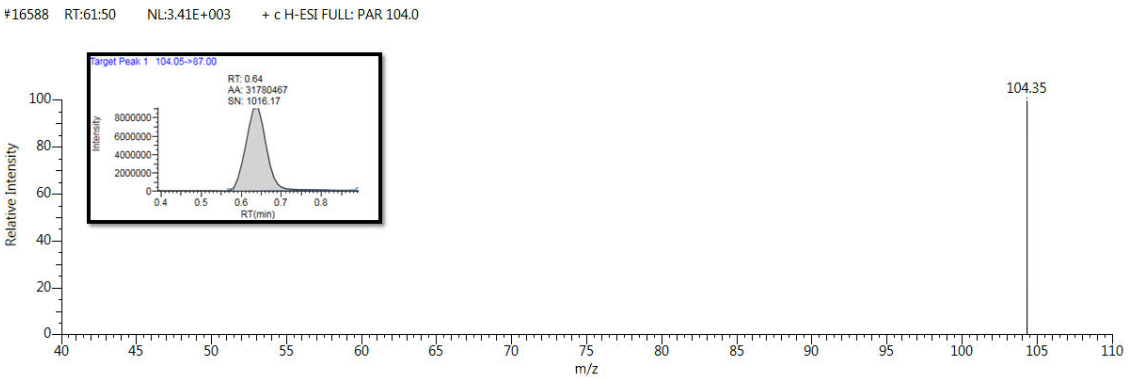

Figure S 5: Showing the precursor ion mass spectra of GABA $[M+H]^+$ at 104.35 m/z embedded with its liquid chromatogram at 0.64 min as separated on a Poroshell 120 EC-C18 column (50 × 4.6 mm and 2.7 µm particle size).

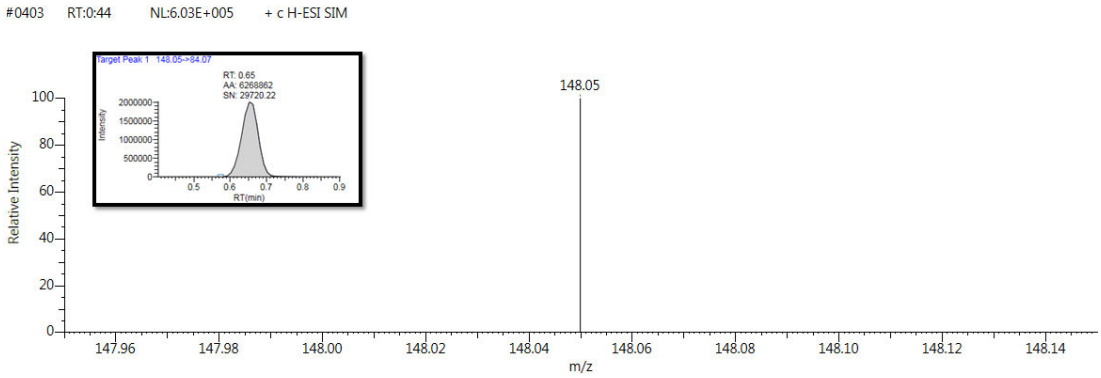

Figure S 6: Showing ion mass spectra of glutamate $[M+H]^+$ at 148.05 m/z embedded with its liquid chromatogram at 0.65 min as separated on a Poroshell 120 EC-C18 column (50 × 4.6 mm and 2.7 µm particle size).

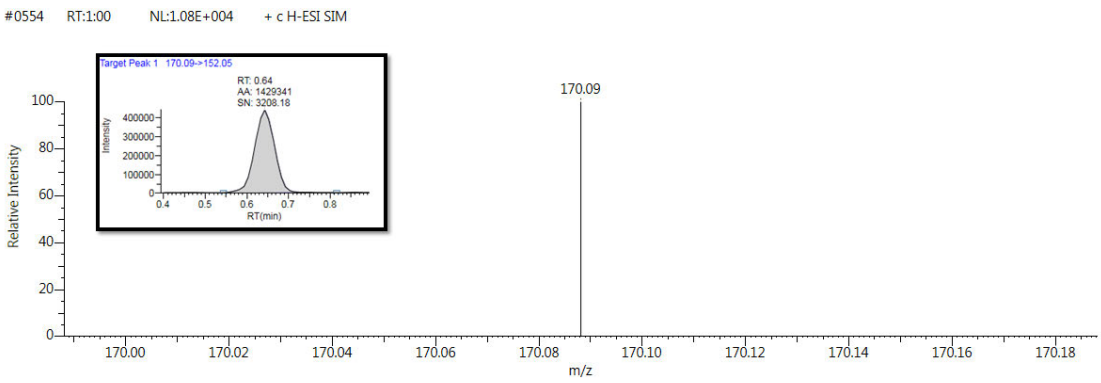

Figure S 7: Showing ion mass spectra of norepinephrine $[M+H]^+$ at 170.09 m/z embedded with its liquid chromatogram at 0.64 min as separated on a Poroshell 120 EC-C18 column (50 × 4.6 mm and 2.7 µm particle size) .

Table S 2: Concentration of BUP and NT's, with BDNF and CREB fold changes at different time points.

Time (h)	BUP (ng/mL)	BDNF (fold change)	CREB (fold change)	NT (ng/mL)	
0 (control)	0 0 0	1,00 1,00 1,00 **1,00 (± 0,00)**	1,00 1,00 1,00 **1,00 (± 0,00)**	GABA	1130,704 1166,397 994,431 **1097,18 (±90,75)**
				NE	820,906 854,837 737,805 **804,516 (±60,21292)**
				GLUT	735,364 797,395 615,614 **716,1243 (±92,40512)**
				DA	28,877 28,58 28,125 **28,52733 (±0,378756)**
				5-HT	21,242 21,97 21,425 **21,54567 (±0,378703)**
0.25	0.256 0.572 0.945 **0,591 (±0,344893)**	5,82 6,64 9,26 **7,24 (±1,80)**	1,30 0,62 0,84 **0,92 (±0,35)**	GABA	1714,913 1713,609 1475,246 **1634,59 (±137,9969)**
				NE	958,596 942,522 837,221 **912,7797 (±65,92744**
				GLUT	674,763 671,652 614,317 **653,5773 (±34,03601)**
				DA	41,984

					28,298 38,824 **36,36867** **(±7,165762)**
				5-HT	20,984 20,956 **20,97** **(±0,019799)**
0.5	1,093 2,271 1,167 1,510333 (±0,659795)	4,93 3,41 7,13 **5,16 (±1,87)**	1,61 0,97 0,43 **1,01 (±0,59)**	GABA	1314,309 1524,360 2026,726 **1621,80** **(±366,0672)**
				NE	891,663 852,915 1060,391 **934,9897** **(±110,3153)**
				GLUT	718,595 546,279 796,698 **687,1907** **(±128,1292)**
				DA	43,682 41,243 28,286 **37,737** **(±8,275157)**
				5-HT	20,909 21,257 20,904 **21,02333** **(±0,202377)**
1	0,574 0,133 0,865 **0,524** **(±0,368553)**	9,89 4,64 13,02 **9,18 (±4,23)**	0,59 0,66 0,67 **0,64 (±0,04)**	GABA	1966,510 1460,789 1405,932 **1611,08** **(±309,0336)**
				NE	948,542 814,18 823,413 **862,045** **(±75,05072)**
				GLUT	702,006 724,753 575,203

					667,3207 (±80,58291)
				DA	43,826 40,841 42,323 **43,333 (±2,115138)**
				5-HT	21,097 21,228 22,203 **21,50933 (±0,604293)**
2	1,559 0,863 1,208 **1,21 (±0.670)**	19,55 16,17 23,56 **19,76 (±3,70)**	1,74 1,66 1,80 **1,73 (±0,07)**	GABA	1265,585 1512,910 **1389,25 (±174,8852)**
				NE	712,061 845,074 **778,5675 (±94,05439)**
				GLUT	830,929 582,139 **706,534 (±175,9211)**
				DA	30,502 29,392 **29,947 (±0,784889)**
				5-HT	21,23 21,548 **21,389 (±0,22486)**
4	1,1 1,116 0,928 **1,048 (±0,104231)**	17,91 19,22 17,20 **18,11 (±1,02)**	2,00 1,23 1,23 **1,49 (±0,44)**	GABA	1397,553 1442,065 1416,253 **1418,62 (±22,35049)**
				NE	800,249 798,21 795,857 **798,1053 (±2,19787)**
				GLUT	754,546 636,724 738,991 **710,087**

					(±64,00849)
				DA	30,21 30,589 27,907 **29,56867** **(±1,451469)**
				5-HT	21,425 21,694 21,60 **21,57367** **(±0,13672)**
6	0,689 0,342 1,652 **0,894333** **(±0,678709)**	29,66 36,62 35,35 **33,88 (±3,71)**	0,87 0,87 0,70 **0,81 (±0,10)**	GABA	7422,942 1539,386 2077,298 **3679,88** **(±3252,729)**
				NE	925,163 26672,32 1263,92 **9620,468** **(±14768,31)**
				GLUT	727,916 1927,762 989,552 **1215,077** **(±630,9149)**
				DA	42,116 32,936 **37,526** **(±6,49124)**
				5-HT	21,391 22,276 24,247 **22,638** **(±1,462008)**
8	1,15 0,951 0,716 **0,939** **(±0,217249)**	66,04 68,51 63,62 **66,06** **(±2,45)**	2,24 1,98 1,41 **1,87 (±0,43)**	GABA	2093,174 1935,708 2153,665 **2060,85** **(±112,5166)**
				NE	1513,336 1293,718 1362,753 **1389,936** **(±112,304)**

				GLUT	1055,628 962,133 1037,613 **1018,458** **(±49,60357)**
				DA	33,548 32,599 33,232 **33,12633** **(±0,483244)**
				5-HT	 24,137 21,553 21,83 **22,50667** **(±1,418687)**
24	0,106 0,341 0,082 **0,176333** **(±0,14311)**	21,07 33,08 26,32 **26,82 (±6,02)**	1,24 1,22 1,69 **1,38 (±0,26)**	GABA	2234,811 1541,269 5619,704 **3131,93** **(±2182,206)**
				NE	1330,803 22841,82 898,16 **8356,927** **(±12546,15)**
				GLUT	962,073 760,234 1458,66 **1060,322** **(±359,4293)**
				DA	33,314 31,652 **32,483** **(±1,175211)**
				5-HT	21,96 22,229 **22,0945** **(±0,190212)**