Evaluation of the pharmacodynamic effects of Ketamine on neurotransmitter levels and CREB/BDNF expression in rat brain

By:

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A dissertation Submitted in fulfilment of the academic requirements for the degree of

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A thesis submitted to the School of Health Sciences, College of Health Sciences, University of KwaZulu-Natal, for the degree of Master of Medical Sciences in Pharmaceutical Chemistry.

This is the thesis in which the chapters are written as a set of discrete research publications that have followed the Journal of Biological Psychiatry format with an overall introduction and final summary. Typically, chapter two will be published in internationally recognized, peer-reviewed journal.

This is to certify that the content of this thesis is the original research work of Mr. Leon Joseph Khoza, carried out under supervision, at the Catalysis and Peptide Research Unit, Westville campus, University of KwaZulu-Natal, Durban, South Africa.

Supervisor: Dr. Sooraj Baijnath

Signature:  Date: 22/01/2021

Abstract

Mental disorders contribute to 13% of the global burden of disease. With major depressive disorder (MDD) expected to be the most significant contributor by 2030, the economic and social impact of this burden will be substantial. There have been various factors linked to the underlying pathophysiology of MDD, including a deficit in individual vital neurotransmitter connections between specific neurons, and alterations in the expression of the transcription factors cyclic AMP response element-binding protein (CREB) and the brain-derived neurotrophic factor (BDNF) in the brain. Ketamine, an N-methyl-D-aspartate receptor (NMDAR) rantagonist and an α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) stimulator has officialy been used as an anesthetic and analgesic drug since the 1970s, until recently when it was approved for the management and treatment of MDD. Ketamine works by altering and rebalancing the monoaminergic, glutamatergic and GABArgic sytems to stimulate new synaptic connections, better memory, and improved brain plasticity. However, there are limited published studies that demonstrate the direct relationship between ketamine, brain neurotransmitters levels, gene and protein expression in the management of MDD.

In this study, we investigated the pharmacodynamic effects of ketamine in the brain by assessing changes in monoaminergic, glutaminergic and GABAergic neurotransmitter levels using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Furthermore, we investigated the links between ketamine and the expression of transcription factors, cyclic AMP response element-binding protein (CREB) and brain-derived neurotrophic factor (BDNF) in treatment of depression using quantitative real-time polymerase chain reaction (qPCR) analysis. Twenty-one healthy male Sprague-Dawley (SD) rats were administered 15 mg/kg of ketamine via intraperitoneal administration at different time points (N = 3 per time point).

Experimental animals were euthanized by decapitation post-administration of ketamine, and brain samples were harvested for analysis.

As per LC-MS/MS and qPCR, the pharmacodynamic results demonstrated that ketamine's anti-depressive mechanism of action is due to alteration of the glutamatergic system which occurs via the disinhibition of glutamate release, which further boosts central nervous synaptogenesis, hence maintaining the in-balance neurotransmitters and genes associated with the pathophysiology of depression.

Declaration 1- Plagiarism

I, **Leon Joseph Khoza** declare that the research reported in this thesis, except where otherwise indicated, is my original work and has not been submitted for any degree or examination at any other university.

This thesis does not contain other person's data, pictures, graphs, or other information, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

a) Their words have been re-written, but the general information attributed to them has been referenced.
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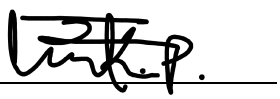
This thesis does not contain text, graphics or tables copied and pasted from the internet, unless specifically acknowledged, and the source being detailed in the thesis and in the reference's sections.

Signature: 

Leon J. Khoza

Declaration 2- List of publications

Investigations into the pharmacodynamic effects of Ketamine by evaluating its effect on brain neurotransmitter levels and CREB/BDNF expression. **Submitted October 2020**: **The Journal of Biological Psychiatry** (**Manuscript number: BPS-D-20-01782).**

Leon J. Khoza[a], Advaitaa M. Haripershad[a], Sanelisiwe Xhakaza[a], Terisha Ghazi[b], Shanel Dhani[b], Cosmas Mutsimhu[c], Molopa J. Molopa[c], Nithia P. Madurai[c], Lorna Mudurai[c], Sanil D. Singh[a], Thavendran Govender[d], Hendrik G. Kruger[a], Anil A. Churturgoon[b], Tricia Naicker[a], Sooraj Baijnath[a*]

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Contributions:

Leon J. Khoza contributed to the design of the study, conducted all experimental work, and prepared the manuscript.

Sooraj Baijnath supervised, conceptualised, reviewed the study and edited the manuscript.

Sanil D. Singh assisted with animal ethics clearance and advised on animal experimentation.

Sanelisiwe P. Xhakaza and Advaitaa M. Haripershad provided technical support, assisted with animal work (drug administration, tissue harvesting and collection) and sample preparation for analysis.

Terisha Ghazi and Shanel Dhani assisted with the running of quantitative real-time polymerase chain reaction analysis.

Cosmas Mutsimhu, Molopa J. Molopa and Nithia P. Madurai assisted with the running of LC-MS/MS and data processing.

Thavendran Govender, Hendrik G. Kruger, Anil A. Churturgoon and Tricia Naicker co-supervised the study.

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- I also wish to thank a countless number of people who contributed to the success of this thesis. I cannot count all of them; please accept my sincere gratitude.

- Above all, I thank my family for support and God for His sufficient grace and loving-kindness.

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List of Abbreviations

MDD	Major depressive disorder
WHO	World Health Organization
5-HT	Serotonin
NE	Norepinephrine
DA	Dopamine
MAOIs	Monoamine oxidase inhibitors
SSRIs	Selective serotonin reuptake inhibitors
TCAs	Tricyclic Antidepressants
PET	Positron emission neurotransmitters
TRD	Treatment-Resistant Depression
SERT	Sertraline
SARI	Serotonin Reuptake Inhibition
SERT	Serotonin Reuptake Transporter
NRIs	Selective Norepinephrine reuptake inhibitors
PCP	Phencyclidine
IV	Intravenous
US FDA	The United States Food and Drug Administration
IM	Intramuscular
NMDA	N-methyl- D-aspartate
HNK	Hydroxynorketamine
AMPARs	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors
BDNF	Brain-derived neurotrophic factor
TrkB	Tropomyosin receptor kinase B
LHb	Lateral habenula
LC	Liquid chromatography
GC	Gas chromatography
MS	Mass spectrometry
ESI	Electrospray ion
HPLC	High-pressure liquid chromatography
MALDI	Matrix-assisted laser desorption ionization
IP	Intraperitonially
Bw	Body weight
TOF	Time of flight
eEF2K	eukaryotic elongation factor 2 kinase
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
RT	Room temperature
RT	Reverse transcription
qPCR	Quantitative real-time polymerase chain reaction
CREB	cAMP response element-binding protein
APCI	Atmospheric Pressure Chemical Ionization
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
SPE	Solid phase extraction

Thesis outline

The presented Master's thesis is organized in discrete chapters, with each containing its references as follows:

Chapter 1: Presents the general introduction and literature review of major depressive disorder, various factors contributing to its pathophysiology, and its prevalence in Sub-Saharan African countries. Chapter 1 further outlines the use of ketamine and other anti-depressant medication in the treatment of the major depressive disorder. Moreover, chapter one describes in-depth the analytical techniques, study aims and objectives of this current study.

Chapter 2: Presents the full manuscript for this study, which was submitted for publication. The manuscript is titled; **Investigations into the pharmacodynamic effects of Ketamine by evaluating its effect on brain neurotransmitter levels and CREB/BDNF expression.**

Submitted to: The Journal of Biological Psychiatry (Manuscript number: BPS-D-20-01782)

Chapter 3: Presents a brief discussion of the thesis, conclusion, limitations, future recommendations, and appendix for this study.

CHAPTER 1

INTRODUCTION

1.1. Major depressive disorder (MDD)

Major depressive disorder (MDD), also known as clinical depression, is a chronic mental disorder that persists for at least two weeks, severe enough to cause significant problems in a patients' ability to uphold personal relations, meet school or work obligations, and partake in previously normal communal activities (1, 2). MDD is characterized by a high frequency of non-recovery and relapse, leading to suicidal ideation and morbidity (3-5). It is a multifaceted and common condition that poses significant challenges to both the patients and physicians who treat it and is habitually misdiagnosed as melancholic depression (6, 7). According to the new World Health Organization (WHO) report, it is projected that over 300 million people are affected by MDD, with an estimated 16.6% lifetime prevalence (2, 8, 9). This number is projected to increase, with an estimated projection of 46 million people in the United States being diagnosed with MDD by 2050 (2).

World Health Organization cites MDD as the leading cause of worldwide disability and is projected to become the second leading cause of worldwide disease and disability by 2030 (8, 10-13). Currently, the cause of MDD is suggested to be due to genetic, biological, environmental, and psychological factors (9). However, MDD frequently develops during young adulthood (14). Young adulthood is a developmental stage in life between the ages of 18 and 25 years, where an accumulative exposure to risk factors, such as uncertainty in finding employment, exploring identity, and enhanced self-focus increases the possibility of developing mental health problems (14). Since MDD is exclusively diagnosed by behavioral symptoms, there is a greater need to identify biological factors that may contribute to its pathogenesis (8).

1.2. Brain neurotransmitters and major depressive disorder

The cause of depressive illness has been linked to brain monoaminergic neuronal dysfunction (15, 16). Specific symptoms are allied with the increase or depletion of specific neurotransmitters (NTs), which suggest that specific symptoms of depression can be assigned to specific neurotransmitter or cascade of neurotransmitter changes (15, 17). The three central monoamine neurotransmitter systems associated with the pathophysiological changes in depression are serotonin (5-HT), norepinephrine (NE) and dopamine (DA) (15, 17-19). This led to the development of the monoamine theory of depression, which states that mental depression is due to deficit of brain monoaminergic activity and that depression is treated by drugs that increase this activity (11). **Figure 1** shows the chemical structures of 5-HT, NE, and DA.

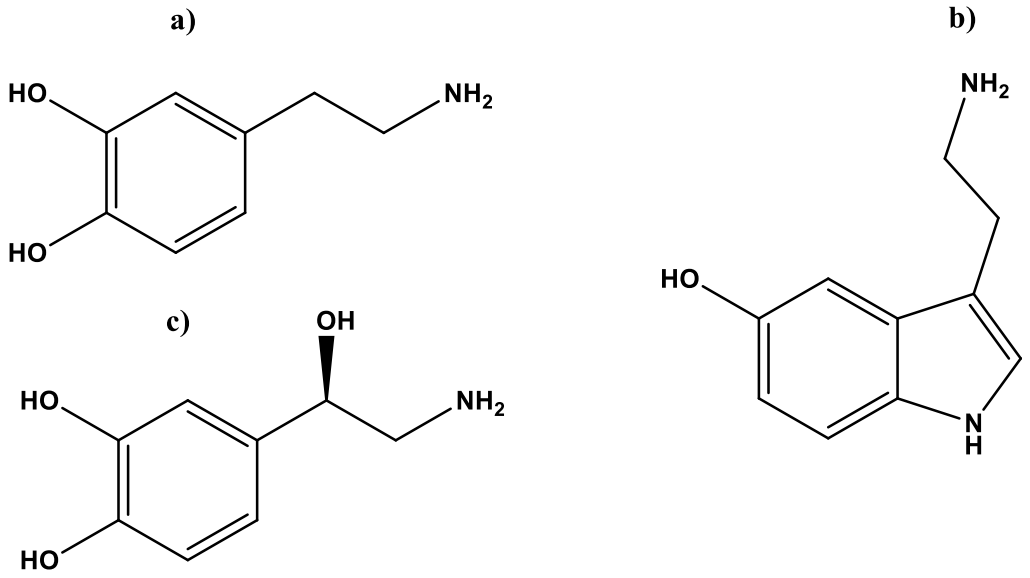

Figure 1: Monoamine neurotransmitters, a) Dopamine, b) Serotonin, and c) Norepinephrine. (Created by the author using ChemDraw Professional 17.1).

The monoamine neurotransmitter systems have mainly been linked in the pathophysiology of MDD not only due to their role in the therapeutic response that results from pharmacological targeting of these systems but also since their location in the brain overlap with the mood-

regulating pathways (20). **Figure 2** shows the role of monoamine neurotransmitters in regulating mood, attention, interest, alertness, energy, anxiety and obsession, and shows how they can contribute to the development of mental disorders due to monoamine deficits. Impairment of these functions is all prominent characteristics of MDD; thus, any changes in the monoamine neurotransmitter system may enhance negative emotions or depressive moods, hence aggravating MDD by potentiating these symptoms (20, 21). The changes or disturbance in the levels of the neurotransmitters may result from impairments in their synthesis, release, transportation and reuptake (21).

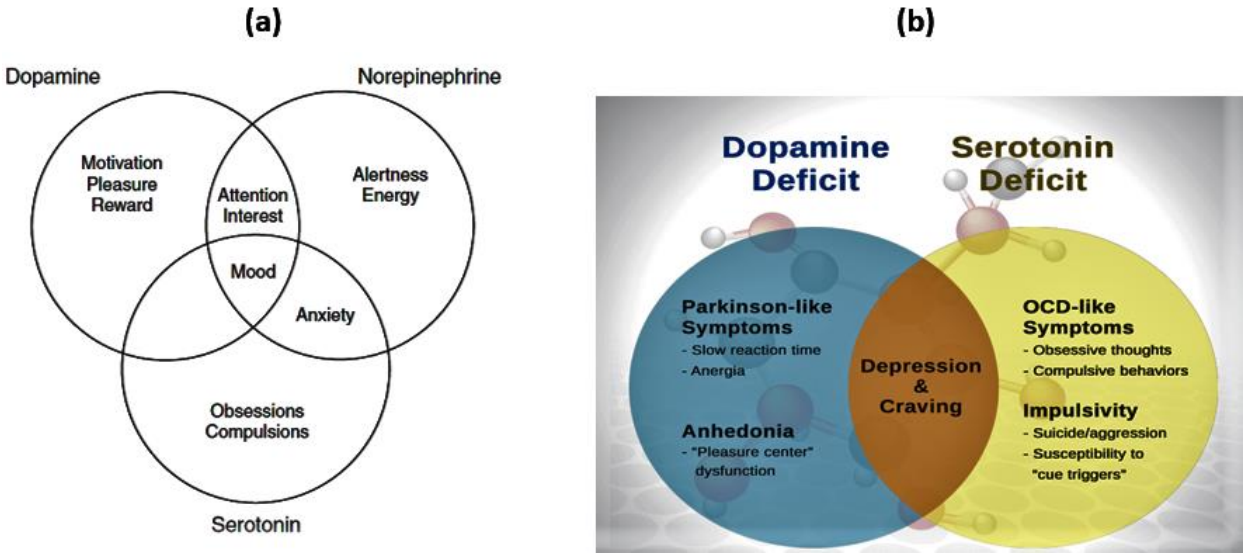

Figure 2: Monoamine regulation of mood and behavior. Adapted from Open Access literature https://drjockers.com/dopamine/ and (17).

In recent years, it has been proven that alteration of the monoamine neurotransmitters is not sufficient in explaining the pathophysiology underlying MDD (22). Hence, other brain neurotransmitters have also been studied and linked to the pathophysiology of MDD. These brain neurotransmitters include gamma-Aminobutyric Acid (GABA) and glutamate (Glut) (23, 24). GABA is the principal neurotransmitter that mediates neural inhibition, and it is mainly responsible for balancing and fine-tuning of excitatory neurotransmission of several neuronal systems, including the glutamatergic and monoaminergic neurotransmitter systems (22, 23).

Various studies have indicated that there is a significant decrease in GABA levels in various brain regions of MDD patients and stressed rodents (22, 23, 25). Similar observations have been made with the major excitatory neurotransmitter in the central nervous system (glutamate) (26, 27). Glut plays a critical role in significant brain functions such as plasticity, brain development, neuronal survival, and it serves as a metabolic precursor for GABA (25, 28). **Figure 3** shows the structure of glutamate and GABA.

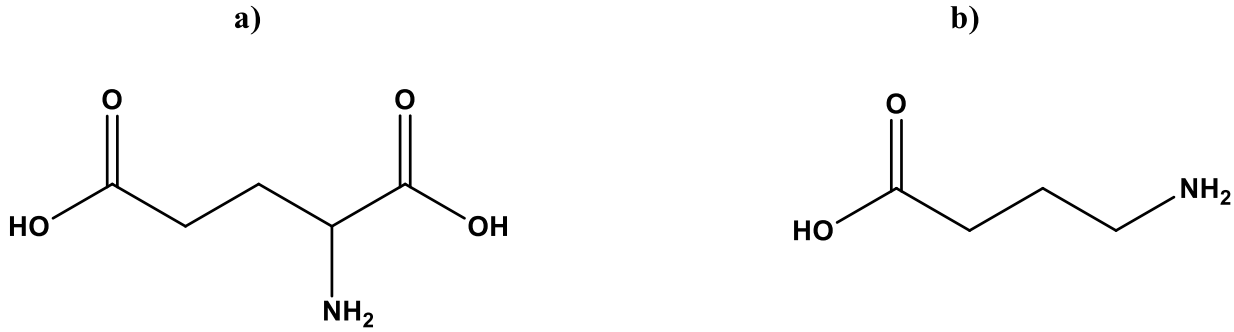

Figure 3: Chemical structure of a) Glutamate and b) GABA Neurotransmitters. (Created by the author using ChemDraw Professional 17.1).

1.3. Gene expression and major depressive disorder

Brain-derived neurotrophic factor (BDNF) a gene which encodes for BDNF protein along with cyclic adenosine monophosphate response element-binding protein (CREB) has been commonly implicated in the pathophysiology of major depressive illness (29, 30).

1.3.1. Brain-derived neurotrophic factor (BDNF) gene

BDNF is one of the many neurotrophic factors that regulate the formation and plasticity of neuronal networks in the brain and peripheral nervous system (31, 32). Hence, BDNF is crucial to the survival, growth and maintenance of neurons in critical brain circuitry involved in emotional and cognitive function (32). Its binding mediates the action of BDNF on specific receptors, such as the tropomyosin receptor kinase (Trk) receptors, which are responsible for controlling synaptic plasticity and strength of the mammalians nervous system (31, 33). Recent

in vivo studies have shown that stress reduces BDNF expression in the hippocampus and frontal cortex which leads to the development of depression in the later stages of life (31). A similar observation was made in MDD patients, where BDNF levels were reduced in the hippocampus of post-mortem samples taken from patients who have committed suicide (32). Different classes of antidepressants have been found to increase BDNF expression in the brain; furthermore increasing the levels of BDNF in the brain have shown antidepressant-like effects (31). These findings have made it plausible that decreased levels of BDNF induce a state of increased susceptibility to stress and depression (32). Based on this a neurotrophic hypothesis of depression was proposed, which states that the loss of BDNF plays a vital function in the pathophysiology of depression and that its restoration may represent a critical mechanism underlying antidepressant efficacy (34). **Figure 4** shows the proposed neurotrophic hypothesis of depression.

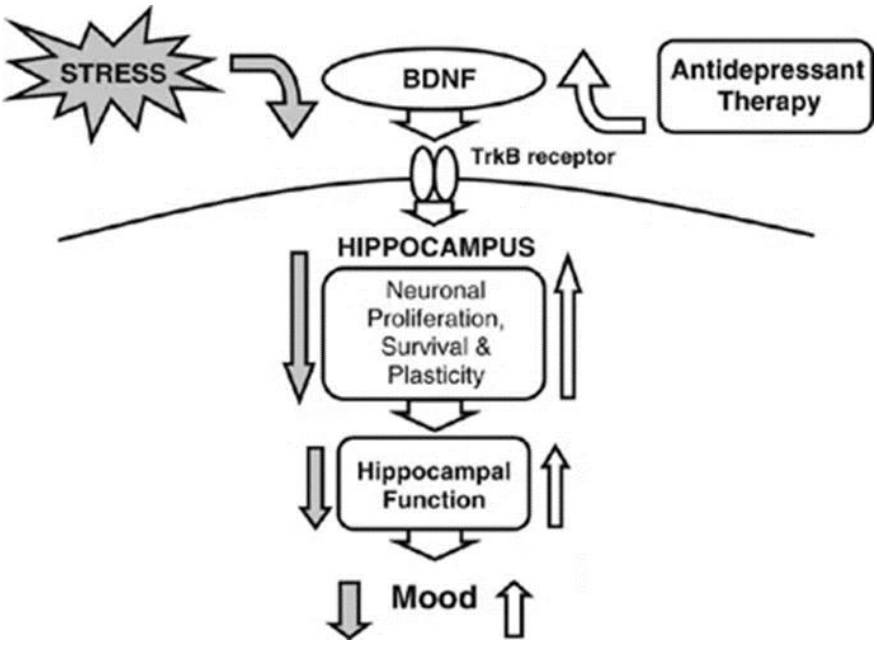

Figure 4: Schematic diagram outlining the proposed neurotrophic pathophysiology of depression. Reprinted from Open source literature (34).

1.3.2. Cyclic adenosine monophosphate response element-binding protein

Cyclic Adenosine monophosphate response element-binding protein (CREB) is a cellular transcription factor that binds to specific DNA sequences, thereby controlling the expression of certain genes (35, 36); with BDNF being one of the genes controlled by CREB transcription factor (36). CREB is responsible for various functions in different effector organs; some of its functions which have been studied concern the brain and the progression of neurodegenerative diseases (36, 37). Recent clinical studies have implicated CREB in signal pathways related to the pathogenesis and treatment of MDD (35). Studies have shown that the expression and functions of CREB are regulated by chronic antidepressant therapy and which is mediated by the expression of CREB in the hippocampus producing antidepressant-like effects, in behavioral models of depression (38, 39). Furthermore, post-mortem studies indicate that CREB levels are increased in subjects taking antidepressants at the time of death and decreased in the cerebral cortex of depressed patients (39). These findings shows that downregulation of CREB could contribute to the pathophysiology of depression and that upregulation of it could contribute to the therapeutic response (38).

1.4. Prevalence of depression in Sub-Saharan African countries

Statistical data regarding the prevalence rates of MDD in Sub-Saharan African countries is very scarce and inadequate (40, 41). Given the poor socioeconomic conditions of large segments of the population in many African countries, the prevalence of mental illness is expected to be high; with no exception to South Africa (40). Several factors such as racial discrimination, high rates of poverty, political violence, gender inequality, high crime rates and unpredictable acute injuries from mining to miners, their families and communities all suggest that majority of the South African population may be at high risk of developing MDD or other psychiatric disorders (40, 42). The sudden increase in suicidal rates across Southern African individuals has indeed confirmed that a large percentage of the population is living with

psychiatric disorders and are not receiving proper pharmaceutical or psychological interventions (43). Limited data on prevalence rates of mental disorders in South Africa demonstrates that critical aspects of mental health are not being prioritized and also not given the attention they require; as a result, affected individuals remain neglected (43). The negligence of mental health aspects has resulted in grave consequences where more than 100 mental health patients have lost their lives at Life Esidimeni – Life Healthcare in the Province of Gauteng in South Africa between 2015 and 2016 (43). Current studies that have been conducted used clinic attendance as a recruitment method, hence introducing a potential bias where members of the population who do not seek medical assistance or are unaware of their mental health status are not counted (41). A study by Tomlinson et al., (2009) in a small rural settlement in South Africa found an MDD prevalence rate of 9.7% among a population of 4351 adults South Africans of all racial groups (41). Due to limited psychopathological data, there is a great need to promote mental health and depression awareness as a significant health policy objective and increasing efforts to identify and treat depressive disorders. **Figure 5** shows prevalence of diagnosed clinical depression around the world.

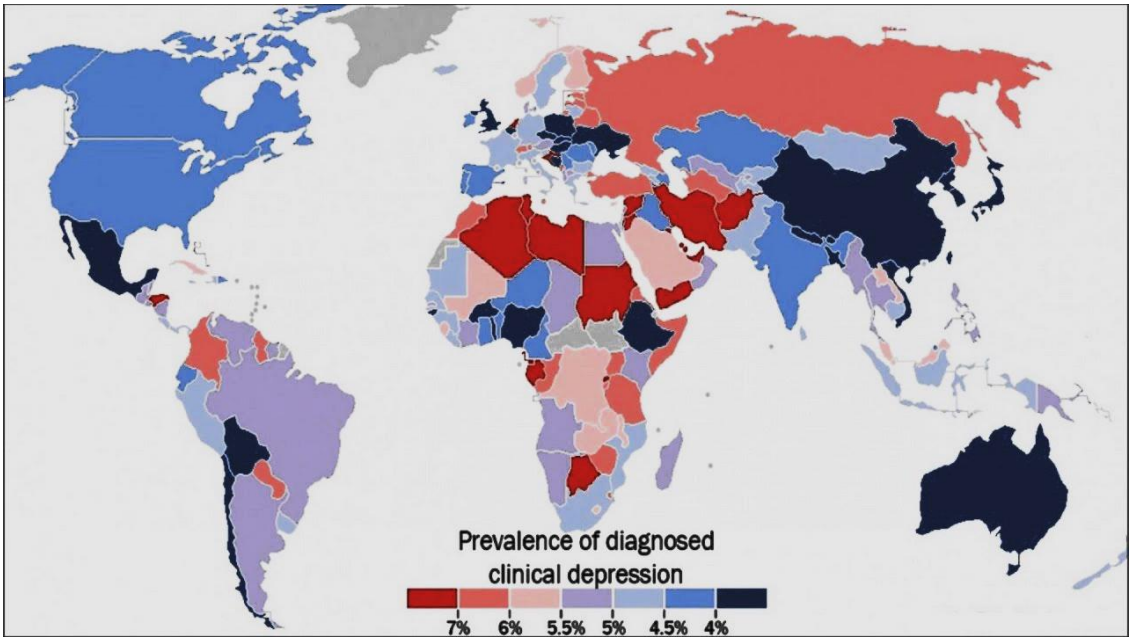

Figure 5: Depression rates around the world with the African continent, showing the high prevalence of the clinical disorder in the year 2010. Reproduced from Open Access Source; https://www.washingtonpost.com/news/worldviews/wp/2013/11/07/a-stunning-map-of-depression-rates-around-the-world/

1.5. MDD treatment using antidepressants

Identifying and treating depression is vital to the care of patients living with depressive disorder (44). Where antidepressants serve as a first-line treatment for moderate and severe depression regardless of environmental factors and depression symptom profile (44-46), this was observed in about two out of three outpatients with the current depressive disorder to receive antidepressant treatment (45, 47). In addition to treating the various types of depression, antidepressants are widely used in the treatment of depression, anxiety, eating disorders, phobia, obsessive-compulsive disorder, bipolar disorder and substance dependence (45). The medication is usually prescribed for depression of any severity of symptoms that have persisted for two years or more with all medication being considered equally effective; therefore, most clinicians select an agent-based upon its side effect and safety profile (44). The antidepressants are given at its therapeutic dose for 4–6 weeks before determining whether it is effective or not

and its often based on a long term treatment, which results in many common side effects, these include nausea, fatigue and drowsiness, weight gain, blurred vision and sexual dysfunction (44, 47, 48). There are various types of antidepressants, however only the following clinically prescribed drugs will be discussed; Tricyclic Antidepressants (TCAs), Monoamine oxidase inhibitors (MAOIs) and Selective serotonin reuptake inhibitors (SSRIs). They are mainly discussed since they are the first-line treatment clinically prescribed and have been widely available for over half a century (49). TCAs and MAOIs have been predominantly used from the 1950s till the1980s prior to the first introduction of the SSRIs (49).

1.5.1. Tricyclic Antidepressant (TCAs)

Tricyclic antidepressants were the first drug class used to treat depression and dominated treatment plans until the introduction of SSRIs in the 1980s and 1990s (50, 51). Tricyclic antidepressants are classified based on their structure with three benzene rings fused together, mainly due to its unknown mechanism of action at the time of discovery (50, 52). Therefore, TCAs are classified differently from other antidepressants, which are classified according to their mechanism of action (50, 52). TCAa have a different pharmacological profile with substantial pharmacological action at two reuptake transporters and three receptor proteins (50, 51); these include, a serotonin reuptake inhibitor, a norepinephrine reuptake inhibitor, a dopamine reuptake inhibitor, an anticholinergic-antimuscarinic drug and an α_1-adrenergic antagonist (50, 52). They carry out their therapeutic effects by blocking the reuptake pumps of serotonin and norepinephrine, resulting in increased concentrations of serotonin and norepinephrine in the synaptic cleft, which alleviate the symptoms of MDD (50-52). However, their adverse side effect profile limits their use, some side effect includes dizziness, memory impairments, drowsiness, decreased blood pressure, seizures and fatal if overdosed (50-55). Approximately 81% of deaths that occur in MDD patients are caused by TCA overdose (50-52, 55). Thus, TCAs are prescribed or reserved as a second-line treatment drug for the

treatment of severe and unresponsive depression, such as melancholic depression (50, 55). **Figure 6** shows some of the most commonly prescribed TCAs due to the efficacious therapeutic effects in the treatment of MDD.

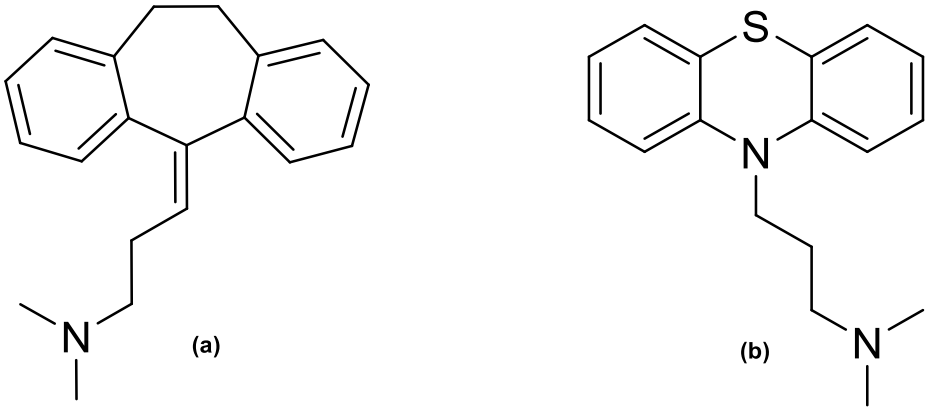

Figure 6: Common TCAs (a) Amitriptyline and (b) Imipramine. (Created by the author using ChemDraw Professional 17.1).

1.5.2. Monoamine oxidase inhibitors (MAOIs)

Clinical data shows that patients with MDD have symptoms that reflect changes in brain monoamine neurotransmitters, precisely serotonin, norepinephrine and dopamine (12). Patients with depression show high levels of the enzyme monoamine oxidase A (MAO-A) levels in brain regions, such as the hippocampus and prefrontal cortex (56). MAO-A enzyme is mainly responsible for the catalysis of the oxidative deamination of biogenic monoamines, a significant step in their synthesis (57). MAO-A limits the levels of monoamine neurotransmitters (serotonin, noradrenaline and dopamine) (56, 58). Positron emission tomography (PET) imaging has shown that higher MAO-A densities in the brain may contribute to the recurrence of depressive symptoms, signifying that inhibition of MAO-A may be an essential therapeutic intervention to prevent the recurrence of depression (56, 59). Monoamine oxidase-A inhibitors (MAOIs) were the first to be used as antidepressants and found to be active mainly for treatment-resistant depression (TRD) (12, 56). Monoamine oxidase-A inhibitors exhibit their effects by preventing the deamination of MAO-A in the brain

and body, thus enhancing central nervous system monoamine levels (59). However, their consumption has been reduced due to the severity of the adverse effects they have on central nervous system and peripheral organs; furthermore, reports of liver toxicity and in some cases, death resulted in reduced prescription of MAOI's (12, 56). Moreover, their reduced consumption is due to dietary restrictions, as they tend to interect with other drugs and food (tyramine reactions) (60). **Figure 7** shows candidate examples of MAOIs.

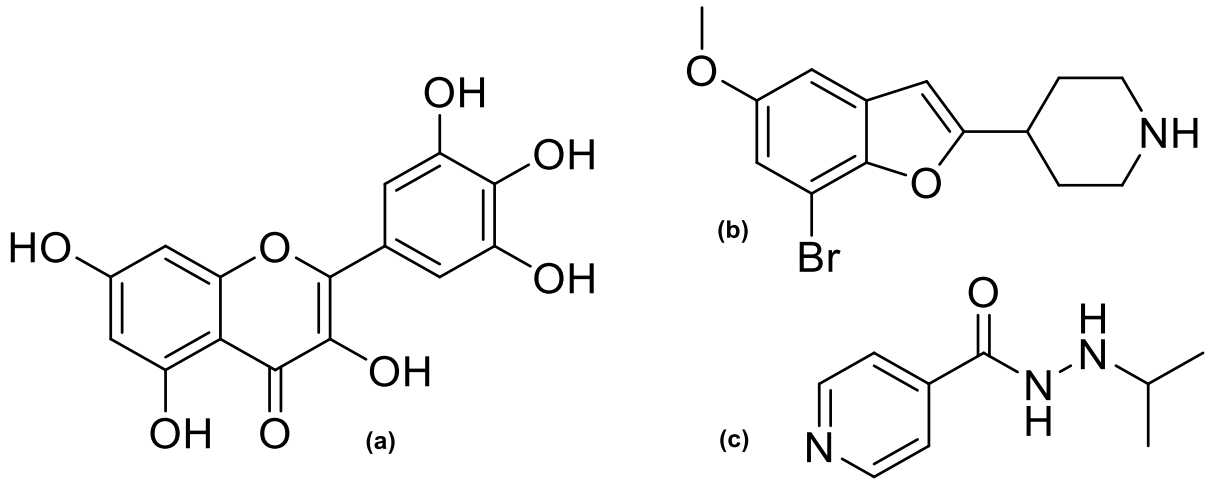

Figure 7: Examples of candidate MAOIs (a) Myricetin, (b) Brofaromine, and (c) Iproniazid. (Created by the author using ChemDraw Professional 17.1).

1.5.3. Selective serotonin reuptake inhibitors (SSRIs)

SSRIs are usually used as first-line therapy for treating MDD in both adults and children (5, 61, 62). SSRIs are increasingly being used due to their superior tolerability, safety profiles and their potential for improved medication compliance with once-a-day dosing schedules relative to other antidepressants, such as MAOIs and TCAs (61, 63). Thus MAOIs and TCAs are primarily reserved for moderate and severe MDD and SSRIs for milder forms of MDD (63). Furthermore, SSRIs are regularly used for maintenance therapy to prevent patient relapse (5). Although SSRIs are considered as the first-line treatment for MDD, only 50% of patients respond to the initial treatment, and even fewer patients achieve remission (64). Thus, a

stepwise clinical treatment approach is often exercised in the setting of SSRIs non-responsive patients to shift to a second antidepressant with an alternate mechanism of action (64). Commonly used SSRIs for treatment of MDD include sertraline (SERT) and paroxetine (**Figure 8**) (13).

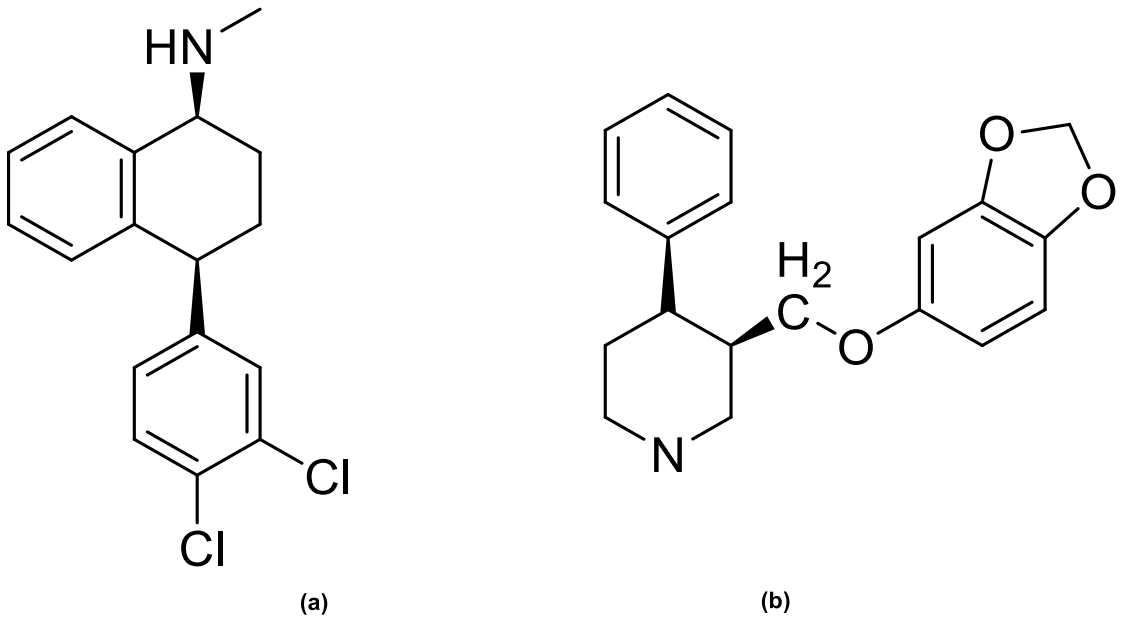

Figure 8: Common SSRIs (a) Sertraline and (b) Paroxetine. (Created by the author using ChemDraw Professional 17.1).

1.5.4. Alternative pharmacological therapy

Serotonin receptor Antagonists with serotonin reuptake Inhibitors (SARI) exhibit their therapeutic effects by moderately antagonising serotonin receptors with a weak serotonin reuptake transporter (SERT) inhibition. Hence, their primary pharmacodynamic effect and mechanism of action are not due to SERT inhibition (50). Typical SARIs include nefazodone, trazodone, and vortioxetine (50). Selective Norepinephrine Reuptake Inhibitors (NRIs) antidepressants are predominantly used for the treatment of MDD; however, they are also used for panic disorders, such as narcolepsy, bulimia, nervosa, and treating therapy-resistant pediatric nocturnal enuresis (50). Common NRIs include Reboxetine and atomoxetine. SARI

and NRIs have common side effects, such as SSRI-induced akathisia and sexual dysfunction (50).

Figure 9 shows a proposed model which illustrates the range of activity of antidepressants highlighting their positive and negative effects (17, 65). From the **Figure 9**, it can be deduced that use of norepinephrine/serotonin antidepressants can alleviate negative symptoms such as anxiety, fear and irritability, which are the hallmarks of depression (17, 65). In contrast, dopamine/norepinephrine antidepressants are shown to be more efficacious in treating depressive symptoms associated with the loss of positive affect such as loss of motivation, interest and pleasure (65). However, the latter drugs require chronic therapy to produce their desired clinical effects in MDD patients and hence more efficient, and rapid antidepressants are needed to provide more acute relief.

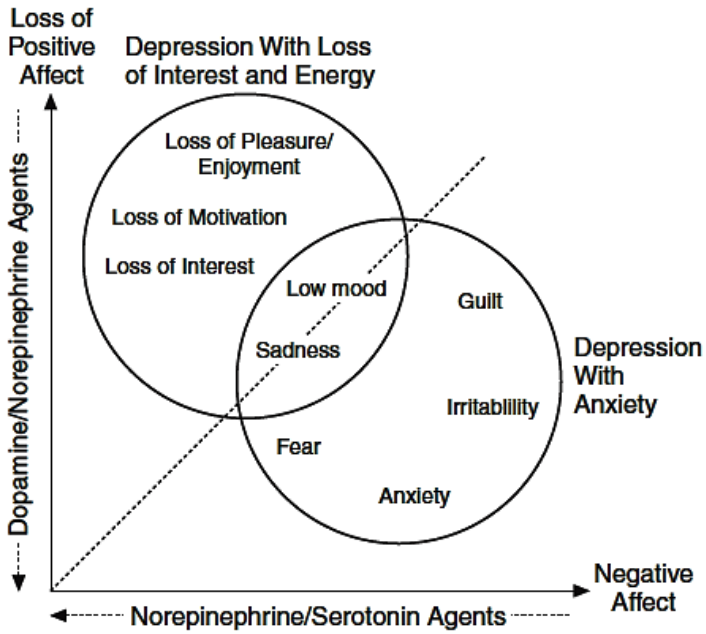

Figure 9: A Proposed model which shows the range of activity of antidepressants highlighting symptoms of positive and negative effects. Reprinted from Open Source literature (65).

1.6. Ketamine

Ketamine (RS-2-2-Chlorophenyl-2-methylamino cyclohexanone) is a racemic mixture that contains equal amounts of optical isomers that have nearly identical pharmacokinetic properties; however, possess different pharmacodynamic effects (66). Ketamine is a derivative of phencyclidine (PCP), and it was first administered to human volunteers in the 1960s as a human anesthetic drug (67-69). Due to its solubility in water and lipids, ketamine can be safely administered through multiple routes, such as intramuscular (IM), oral, rectal, subcutaneous, intravenous (IV) and epidural (70). Ketamine was primarily used for anaesthetic induction and is defined as a dissociative hypnotic dut to the cataleptic state of involuntary movement, maintenance of spontaneous respirations, profound analgesia, and unresponsiveness to surgical stimulation achieved with induction dose (69). The United States Food and Drug Administration (US FDA) officially approved ketamine as an anaesthetic in 1970 (67). Today ketamine is being repurposed and is being used off-label as an adjunct of multimodal analgesia (acute pain and chronic pain), and most recently as an antidepressant in the treatment of MDD (67, 71, 72). However, ketamine has some undesirable effects ranging from dissociative and psychotomimetic effects, memory and cognitive impairment, and direct and indirect peripheral effects (71). **Figure 10** shows the racemate structure of ketamine.

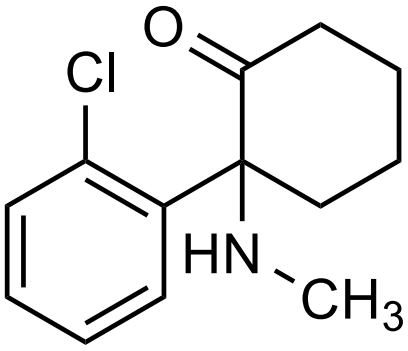

Figure 10: Structure of racemate Ketamine. (Created by the author using ChemDraw Professional 17.1)

1.6.1. Pharmacodynamic effects of ketamine as an antidepressant

Clinical studies have shown that despite the wide range of antidepressants available, approximately one-third of MDD patients do not to respond to first-line antidepressants regardless of adequate dosage and duration (3). Thus, placing an urgent need to develop novel and efficacious drugs to treat depression. Ketamine, a non-competitive glutamate N-methyl-D-aspartate receptor (NMDAR) antagonist, has recently become a focus of research for its antidepressant effects which occur within hours following administration of subanesthetic doses (3, 73). Ketamine has demonstrated fast-acting effects in patients treatment of patients with treatment-resistant depression (TRD) (74). Evidence of ketamine's antidepressant actions dates back to the 1970s, where preclinical studies have shown that ketamine exhibited similar effects to those observed following administration of classic antidepressant drugs (71). Ketamine exerts its effect based on a hypothesised direct inhibition of the NMDAR, which represents a target for faster-acting antidepressant actions (75). The following are the hypothesised ketamine's mechanisms of action in the treatment of depression during NMDAR inhibition (a) disinhibition of glutamate release, (b) blockade of extra-synaptic NMDARs, (c) blockade of spontaneous NMDAR activation, (d) the role of the ketamine metabolite (2R,6R)-hydroxynorketamine (HNK) and (e) inhibition of NMDAR- dependent bursting activity of lateral habeluna neurons. (75, 76). These hypothesised mechanisms are summarised below and illustrated in **figure 11**.

(a) Disinhibition of glutamate release:

According to the disinhibition hypothesis, ketamine selectively block NMDARs expressed on GABAergic inhibitory interneurons, leading to a disinhibition of pyramidal neurons and enhanced glutamatergic firing (75). The Induced released glutamate then binds to and activates post-synaptic α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPARs) which subsequently results in enhanced brain-derived neurotrophic factor (BDNF) release (75,

76). Activation of the tropomyosin receptor kinase B (TrkB) receptor and promotion of protein synthesis occurs through activation of the mechanistic target of rapamycin complex 1 (mTORC1) (75, 76).

(b) Blockade of extra-synaptic NMDARs:

Ketamine is suggested to selectively block/inhibit extra-synaptic GluN2B-containing NMDARs activated by low levels of ambient glutamate which are regulated by the glutamate transporter-1 that is located on the astrocytes (75, 76). This subsequently leads to the de-suppression of mTORC1 function, which then induce protein synthesis (75-77).

(c) Blockade of spontaneous NMDAR activation:

This hypothesis insinuates that ketamine hinders NMDAR-mediated neurotransmission, which leads to the inhibition of the eukaryotic elongation factor 2 kinase (eEF2K) activity, therefore preventing the phosphorylation of its eEF2 substrate (75). This effect then leads to an improvement or enhanced BDNF expression (75).

(d) Ketamine hydroxynorketamine (HNK) metabolites:

It is hypothesised that ketamine exerts NMDAR inhibition-independent antidepressant actions via the action of its metabolites, (*2R,6R*)-HNK and (*2S,6S*)-HNK (71). Ketamine is metabolised to HNKs after administration, and the HNK metabolites act to promote AMPAR- facilitated synaptic potentiation (71, 75).

(e) Inhibition of NMDAR-dependent burst firing activity of lateral habenula (LHb) neurons:

It is proposed that ketamine decrease excessive NMDAR-dependent burst firing activity of LHb neurons that is said to be associated with the depressive symptomatology (78). The observed mechanisms of ketamine are not mutually exclusive; hence, they may act complementary in bringing about the antidepressant actions of the drug as all hypotheses

suggest acute changes in synaptic plasticity, which leads to the sustained strengthening of excitatory synapses which are necessary for antidepressant responses (75).

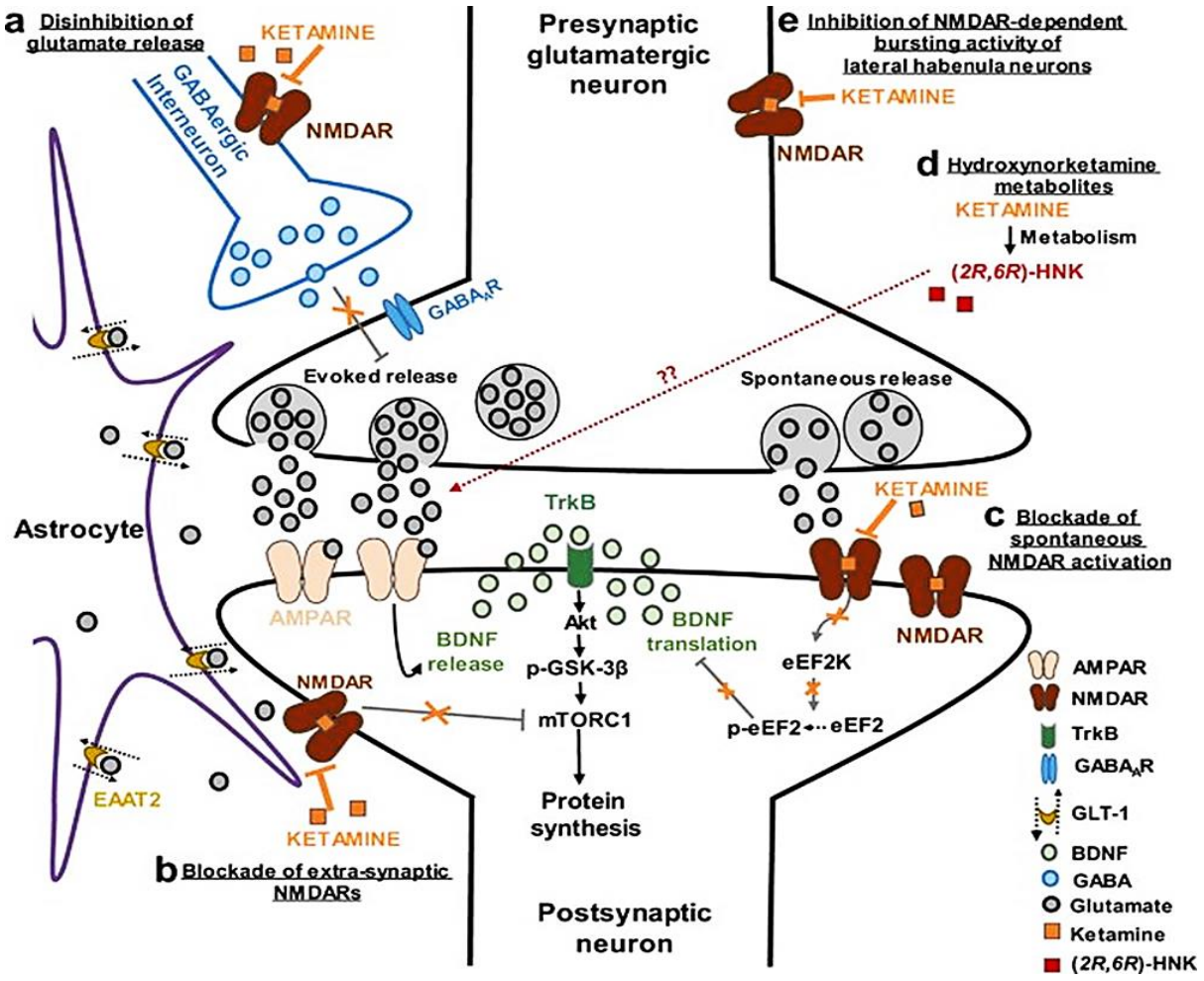

Figure 11: Proposed mechanisms of action for ketamine as an antidepressant. Reprinted from Open Access Literature (79).

1.7. Esketamine nasal spray for severe depression

Currently, there is an approved treatment for the emergency management of patients with a major depressive disorder who are assessed to be at imminent risk for suicide (80). Several clinical studies have demonstrated the robust and rapid therapeutic effects produced by ketamine at subanaesthetic dosages (81). However, most clinical studies that evaluated the anti-depressive effects of ketamine have been using its racemic mixture (81). The racemate of ketamine is comprised of R-(-)-ketamine enantiomer (Arketamine) and the S-(+)-ketamine

enantiomer (Esketamine) as shown in **Figure 12**. The ketamine racemate mixture can be separated by using chromatographic and electrophoretic techniques (82). Recent studies have shown that the analgesic potency of the S-(+)-ketamine is approximately two times higher than that of ketamine (racemate), subjects also presented with a lower incidence of impairment in concentration and memory with S-(+)-ketamine than with the racemate, depicting that S-(+)-ketamine is better tolerated than the racemic ketamine (81). Furthermore, studies have shown that S-enantiomer has approximately a 3-4 fold higher affinity for NMDARs than R-(-)-ketamine (83, 84). However, some studies have shown that R-(-)-ketamine has higher potency and more prolonged antidepressant effect than S-(+)-ketamine in animals, specifically rodents (83, 85). Thus, suggesting that NMDARs may not play a significant role in the sustained anti-depressive effects of ketamine (85).

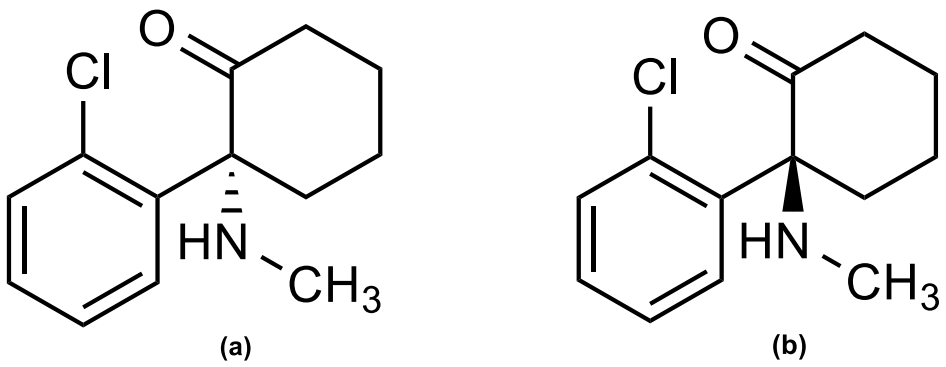

Figure 12: Enantiomers of ketamine (a) Esketamine and (b) Arketamine. (Created by the author using ChemDraw Professional 17.1).

Esketamine (S-(+)-ketamine) has most recently been approved for treating depression; it has been recently developed by Janssen Pharmaceuticals (United States) and approved by the United States Food and Drug Administration (US FDA) in March 2019 (86). The recently approved antidepressant makes use of an intranasal formulation, due to the instant onset of antidepressant effects observed in patients with TRD as early as 2 hours and 24 hours after single-dose of the intranasal formulation (80, 81, 84, 86). The first results that were published using the intranasal formulation were from a randomised, placebo-controlled, multiple-dose

study, which comprised of 67 participants with TRD, the study showed a dose-response relationship, favouring the highest intranasal dose of 84 mg (86). Recent clinical trials examined the use of Esketamine in individuals with MDD who have not responded to traditional antidepressant treatment, the primary outcome of the study showed a greater decrease in depression, with a significant difference from other patients receiving a traditional antidepressant treatment (86).

1.7.1. Esketamine usage guidelines

The approved esketamine nasal spray is only available through a controlled distribution system under a risk evaluation and justification strategy, according to the US FDA (87). Esketamine's associated adverse side effects includes: elavated blood pressure, dizness, sedation and psychotomimetic reactions (88). This means that both the patient and a physician must sign a patient enrolment form that states that the patient understands that s/he needs to have help safely leaving doctor's office and that the patient will not drive or operate heavy machinery the day after the use of the drug (87). Furthermore, Esketamine can only be taken under medical supervision (Doctor's office), although a patient would be able to self administer the drug after a doctor has demonstrated how to use it; hence ketamine remains the most readily available treatment of depression (87).

1.7.2. Esketamine Mechanism of Action

While the exact mechanisms underlying the anti-depressive effects of Esketamine remains unclear, the rapid antidepressant effects are said to occur via the blockage of NMDARs located on inhibitory interneurons, hence having a similar mechanism of action as racemate ketamine (85). This blockage results in disinhibition of pyramidal cells, which then leads to a burst of glutamatergic transmission (85). It is understood that the elevated increase of glutamate release activates AMPARs, as AMPAR antagonists block the antidepressant effects of ketamine and

its enantiomers (85). Collectively, AMPAR activation plays a substantial role in the antidepressant effects of ketamine and its enantiomers.

ANALYTICAL TECHNIQUES

1.8. Liquid Chromatography-Mass Spectrometry (LC-MS)

Chromatography is used to separate complex mixtures using the differences in the distribution coefficient between mobile and stationary phase (89, 90). Chromatography is separated into groups according to the state of the mobile phase, and it can be gas chromatography (GC), liquid chromatography (LC) or supercritical fluid chromatography (SFC) (89). Liquid chromatography uses a mobile phase in which the analytes are miscible (91). The LC separations are predominantly carried out in reversed-phase chromatography, where the mobile phase is polar then the stationary phase (non-polar) and the more polar analytes elute more rapidly than, the less or non-polar ones (91). A separation comprising a mobile phase of constant composition is referred to as an isocratic elution, while that in which the composition of the mobile phase is gradually changed is called gradient elution (91). Mass spectrometry (MS) is an analytical technique that measures the mass-to-charge (*m/z*) ratio of ionic species of an analyte (92). MS is broadly used in the pharmaceutical field due to favourable charactersistcs such as high selectivity, high sensitivity, and capability of providing information (relative molecular mass and structural characteristics) (89). Coupling of MS to chromatographic techniques has always been advantageous due to the sensitive and highly specific nature of MS detectors compared to other traditional chromatographic detectors (93). However, an interface is required to couple the two techniques together due to their incompatibilities; with its prime purpose being the removal of the chromatographic mobile phase and the generation of ionic species that can be detected using the mass spectrometer (91). The efficient coupling of MS with liquid chromatography (LC-MS) in this study was achieved

employing electrospray ion (ESI) sources which have generated a high drive in the development of LC-MS-based assays to be used in clinical chemistry (90).

LC with tandem MS (LC-MS/ MS) is now frequently used in clinical investigations (**Figure 13**), such as metabolic disorders, endocrinology, and therapeutic drug monitoring (90), this is mainly due to LC-MS being capable of both qualitative and quantitative analyses of complex biological matrices (89).

Figure 13: Schematic block diagram illustrating the basic workflow of a typical LC-MS system. (Created by Author).

1.8.1. Components of High-Pressure Liquid chromatography (HPLC)

A basic HPLC system comprises of the following components: binary/quarternary pump, an automated sample injector, column and detector.

1.8.1.1. Pump

The pump plays a role of delivering a high volume of mobile phase at a stable flow rate between 10 ul/min and 2 ml/min. It comprises of inert material towards solvents (91, 92). There are three main types of pumps: reciprocating, syringe and constant pressure pumps (92). These pumps are capable of delivering solvent at a constant composition or a changing composition.

1.8.1.2. Autosampler

The sample injector introduces a specified volume of sample into a flowing mobile phase liquid stream in the chromatographic system (91, 92). An injectable amount of sample volume range from 1µL to 100µL (92). There are two types of injectors used (automatic and manual

injectors). Automatic injectors are more user-friendly, accurate and precise, as compared to manual injectors (92). While injecting the sample into the system, the mobile phase is pumped at the chosen flow rate through the valve to the column to keep the column in equilibrium with the mobile phase and uphold chromatographic performance (91).

1.8.1.3. Columns or stationary phase

Commonly used HPLC columns contain a silica stationary phase fused with hydrocarbon chains, with various chemical modifications determining the polarity of the column (91, 92). Columns range between 50 to 300 mm in length, with particle sizes ranging between 5 to 3.5 μm and 3 μm internal diameter; and they consist of octyl (C8), octadecyl (C18), amino, cyano, and phenyl packing material (92). The nature of the compound being separated determines the type of column used (92).

1.8.2. Basic Components of a Mass spectrometry

The MS consist of three significant components, ionisation source and interface, a mass analyser and detector (92, 93).

1.8.2.1. Ionisation source and interface

The mixture of components (liquid) is transferred into the ion source of MS, where ion source is exposed to high vacuum (92). Due to the difference in the pressure, it is challenging to vaporise the liquid drops without losing a mixture of components; thus, interfaces are used (92). There are several ionisation sources and interfaces, such as Electrospray Ionisation (ESI), Matrix-assisted laser desorption ionisation (MALDI, Atmospheric pressure photo Ionisation (APPI) and Atmospheric Pressure Chemical Ionisation(APCI) (89-93).

Electrospray Ionisation (ESI):

ESI is the universally used ion source developed by John Bennett Fenn in 1980s (92, 93). ESI works well with moderately polar molecules; hence it is well suited for the analysis of a wide range of biological materials (93). Liquid samples are pumped across a metal capillary maintained at 3 - 5 kV and nebulised at the tip of the capillary to produce a fine aerosol of charged droplets as illustrated in **Figure 14** (94). The charged droplets undergo rapid evaporation by the assistance of heat and dry nitrogen where they are continuously reduced in size until they become unstable upon reaching Rayleigh limit (95, 96). When the charge exceeds the Rayleigh limit, the droplet undergoes Coulomb explosion, where it dissociates, leaving a stream of positively charged ions, as seen in **Figure 14** (96, 97). The remaining electrical charge on the droplets is transferred to the analytes, which are ionised then transported into the high vacuum of the MS via a series of small apertures and focusing voltages (90, 92, 93). The ions (postive/negative) are then detected through operation of the ion source and ion optics (93). LC-MS with ESI is largely used in biological research for medical analysis (92).

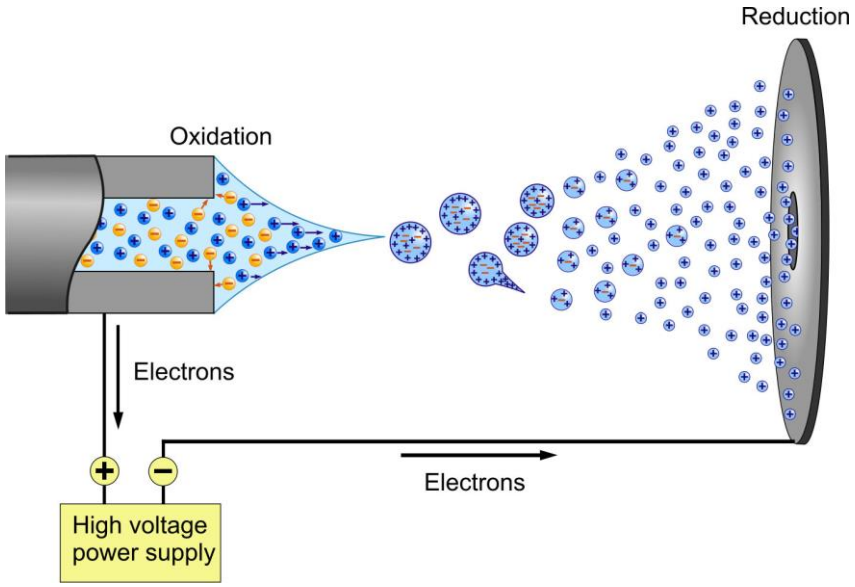

Figure 14: Electrospray ionization source principle of ion generation. (Adapted from https://commons.wikimedia.org/w/index.php?curid=72802277).

1.8.2.2. Mass Analyser

Mass analyser is responsible for separation of ionized according to their mass to charge (m/z) ratio; use of mass analyser depends on its speed, time, rate and its reaction (93). MS allows for the analysis of multianalyte panels using a single method, which saves time and resources (90). Several technologies available allow for the combination of ion source and MS detectors to detect ions, mainly quadrupole, time of flight (TOF), and hybrid analyzers (90).

Quadrupole Mass Analyser:

The quadrupole analyser primarily contains four set of parallel metal rods (**Figure 15**); where the combination of constant and alternating voltages allows the transmission of a narrow band of *m/z* values along the axis of the rods (93). Voltage alternation with time allows scanning across a range of *m/z* values, which results in a mass spectrum (93). Most quadrupole analysers can measure above 4000 *m/z* with scan speed going up to 1000 *m/z* per sec, and it can allow monitoring of a specific *m/z* value; achieved by stepping the voltages (93). This technique is advantageous because it improves the detection limits of desired analytes; this is achieved by direct scanning of to specific ions instead of scanning across ions that are not produced by the analyte. Furthermore, ions can undergo fragmentation by collisions with an inert gas (nitrogen or argon) in a process know as collision-induced dissociation (93).

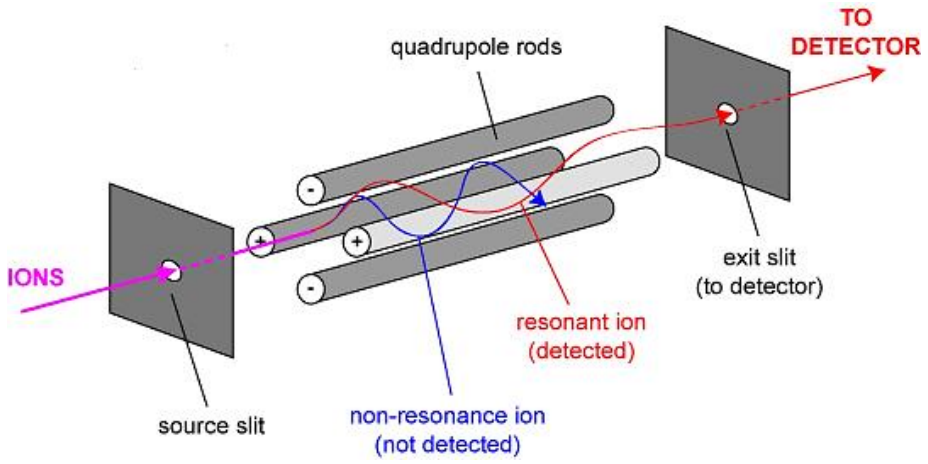

Figure 15: Schematic diagram of quadrupole mass analyzer. Adapted from open source: http://www.bris.ac.uk/nerclsmsf/techniques/gcms.html

1.8.2.3. Detector

Detector is an essential part of MS that produces a current that is proportionate to the number of ions striking it (92). The formed ions pass from the analyser to the detector for detection and transformation into a digital signal that can be analysed (92).

Point Ion Collectors Detector: The ion collector are placed at a predetermined point in MS, and focused on the detector positioned at a single point; the data is recorded when the electric current flow caused by the arrival of ions is proportional to the ions arriving at point ion detector (92).

Array Detector: array detector is a collection of point collectors placed in detector where mass to charge (m/z) of ions are separated and are recorded using point ion collector (92). Spatially separated ions with the mass range are simulteneously detected at the same time in array detector (92).

Electron multiplier detector: this is the most common MS detector, due to its high signal amplification and relatively low cost (94, 98). Most multipliers are cone-shaped and can amplify the relatively weak ion beam signal to the order of 10^5 to 10^6 (94, 98). It is made of

glass that is coated with semiconductor substance that emits secondary electrons when impacted by charged particles (98). Ions from the mass analyser are fast-tracked to the outer end of the multiplier where they colllide the multiplier surface, resulting in emission of several electrons, and these electrons are then attracted further into the multiplier, undergoing further collisions with the surface (94, 98). This results in the ion beam leaving the mass analyser to be converted into a cascade of electrons (current) is digitized in an analogue-to-digital converter and sent to a computer system where the mass spectrum is generated (98).

1.9. Quantitative real-time polymerase chain reaction (qPCR)

Quantitative real-time polymerase chain reaction (qPCR) is a reliable modern quantitative nucleic acid technique that is widely used for gene expression analysis (99). It is primarily employed in several vital areas, including diagnosis of infectious diseases, human genetic, microbiology and biotechnology analysis (99). qPCR enables simultaneous analysis of different samples from as little as one cell in the same experiment, and it has high sequence specificity, increased sample throughput, cost-effective and highly accurate (99-101). Furthermore, it does not require post-amplification processing (99). The accuracy of qPCR is determined by several factors, such as variability in efficiencies of reverse transcription and PCR reaction (101). During gene expression analysis, number of variables are taken into considering when using qPCR; this includes: quality of ribonucleic acid (RNA), the efficiency of amplification, starting material, and suitable reference gene which serves as an elementary prerequisite for reliable results (99). During gene expression analysis by qPCR, the messenger RNA found in the sample is copied to complementary DNA by reverse transcription (RT) (102). The RT step is crucial for ensuring accurate quantification and higher sensitivity; **Figure 16** shows a summarized protocol of a typical qPCR experiment (102).

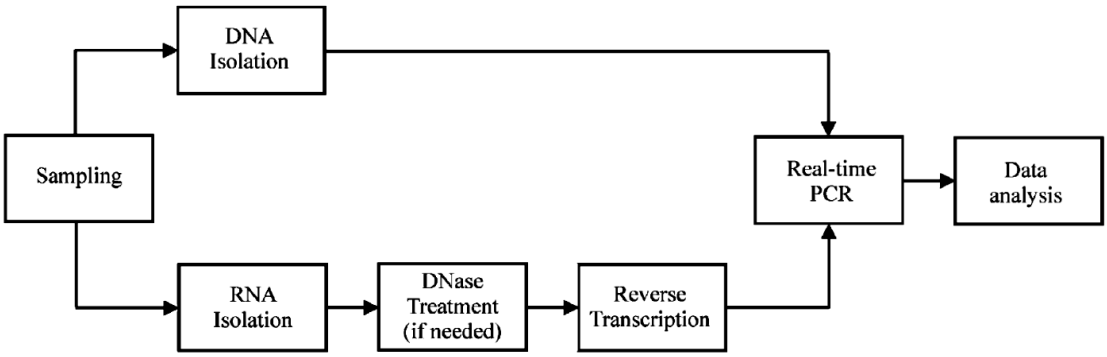

Figure 16: Scheme of the QRT-PCR analysis process. Adapted from Open-Source Literature (102).

1.10. Motivation for the study

Researchers have established that deficits in vital neurotransmitter networks between specific neurons in the brain may contribute to the development of depression (17). Ketamine, an NMDAR antagonist and an AMPAR stimulator have been as an off-label in the management and treatment of MDD until the US FDA approved esketamine nasal spray as a treament for MDD (73, 86). Ketamine has been used as an offlabel drug due to its ability to stimulate the generation of new receptors and synaptic pathways in the brain, which helps patients regulate their mood, sleep better, and experience better focus . Ketamine works by altering and rebalancing the excitatory and inhibitory system (glutamate and GABA) to stimulate new synaptic connections, better memory, and improved brain plasticity (103). However, there are limited published studies that demonstrate the direct relationship between ketamine, brain neurotransmitters levels, and gene expression in the management of MDD. This study will contribute to the understanding of the role of Ketamine in the treatment and management of MDD.

1.12. Aims and objectives

The research aims to study the pharmacodynamic effects of ketamine in the brain by assessing changes in neurotransmitter levels using LC-MS/MS and to investigate the effect of ketamine

in transcription factor cyclic AMP response element-binding protein (CREB) and the neurotrophin brain-derived neurotrophic factor (BDNF) in treatment of depression using quantitative real-time polymerase chain reaction analysis.

Objectives:

1. To develop and optimise an LC-MS/MS method for the simultaneous quantification of neurotransmitters (serotonin, norepinephrine, dopamine, glutamate and γ-aminobutyric acid) in the brain homogenates of rodents treated with ketamine.
2. To establish the effects of ketamine on gene expression of BDNF and CREB in the brain of rats treated with ketamine.
3. To determine the anti-depressive mechanism based on the pharmacodynamic changes observed during LC-MS and qPCR analysis.

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CHAPTER 2

Investigations into the pharmacodynamic effects of Ketamine by evaluating its effect on brain neurotransmitter levels and CREB/BDNF expression

Investigations into the pharmacodynamic effects of Ketamine by evaluating its effect on brain neurotransmitter levels and CREB/BDNF expression

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Abstract

Background: Ketamine has recently been approved for the management and treatment of major depressive disorder (MDD); however, its mechanism remains unknown. In this study, we examined the pharmacodynamic effects of Ketamine in rat brain by assessing changes in neurotransmitter levels and investigating the links between Ketamine and the transcription factors, brain-derived neurotrophic factor (BDNF) and cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB).

Methods: Twenty-one healthy male Sprague-Dawley (SD) rats were administered 15 mg/kg of Ketamine intraperitoneally at 0 (control), 5, 15, 30, 60, 120, and 240 minutes (n = 3 per time point). Liquid chromatography tandem mass spectrometry (LC-MS/MS) was employed to assess neurotransmitter levels in rodent brain samples and the gene expression of *BDNF* and *CREB* was determined using quantitative polymerase chain reaction (qPCR).

Results: Ketamine reached C_{max} (351.33 ± 108.38 ng/mL) in the brain at 5 minutes post-administration. At Ketamine's T_{max}, Glutamate and gamma-aminobutyric acid (GABA) showed a significant increase (p = .005 and p = .007, respectively) compared to the control and other monoamine neurotransmitters in the brain. At 5 minutes, *BDNF* showed significant (p < .0001) downregulation of 0.96-fold compared to the control, while *CREB* showed no significant changes. At low Ketamine concentrations (0.428 ± 0.042 ng/mL), *BDNF* showed an increase of 0.25-fold compared to 5 minutes (Ketamine's, C_{max}).

Conclusion: The findings show that ketamine may alleviate depression by altering the glutamatergic system which occurs via the disinhibition of glutamate release. This enhances central nervous synaptogenesis and balances the deficit of other neurotransmitters and proteins associated with the pathophysiology of depression.

2.1. Introduction

Major depressive disorder (MDD) is one of the most common psychiatric disorders affecting over 264 million people worldwide (1, 2); World Health Organization's (WHO) estimated demographics have shown that approximately 1 in every 23 people in the world suffers from depression (3). Depression has led to severe socioeconomic and health consequences, making MDD the costliest mental disorder worldwide (2, 4, 5), with it projected to become the most significant contributor to the global disease burden by 2030 (3). Cognitive and affective deficits combined with anhedonia for more than two weeks are the main symptoms of MDD (6, 7). Several clinical studies have linked various factors to the underlying pathophysiology of depression; these factors include brain monoaminergic, GABAergic, and glutamatergic neuronal dysfunction (8-12). This has led to the monoamine hypothesis of depression, which predicts that the underlying pathophysiologic basis of depression is a depletion in the levels of dopamine (DA), serotonin (5-HT) and norepinephrine (NE) in the central nervous system (13); however, depression remains a multi-etiological disease.

A similar hypothesis was developed regarding the transcription factors, brain-derived neurotrophic factor (BDNF) and cAMP response element-binding protein (CREB), which states that loss of BDNF and CREB plays a significant role in the pathophysiology of depression and that restoration of their activities may represent a critical target underlying the effectiveness of antidepressants (14, 15). This was supported by various studies that assessed neurotransmitter alterations associated with MDD; these studies demonstrated that neurotransmitters such as gamma-aminobutyric acid (GABA), glutamate (Glut), DA, 5-HT and NE were deficient in both the depressed rodent and human brain (11, 16-20). Furthermore, clinical studies have shown reduced BDNF and CREB levels in the hippocampus in post-mortem samples taken from MDD patients who have committed suicide (21). Many of the currently available antidepressants are known to potently alter the central monoaminergic

system and increase BDNF and CREB expression in the brain (15, 22-24). However, they require chronic therapy (prophylactic treatment) to produce their desired antidepressive effects which increase the risk of severe adverse effects; hence more efficient and rapid-acting antidepressants are needed to manage the disorder.

Some clinical studies have shown that Ketamine possesses effective, rapid antidepressant effects as early as 2-24 hours against treatment resistant MDD (25-27). Ketamine, a widely used anaesthetic agent, has been used as an off label treatment for MDD until March 2019, when the United States Food and Drug Administration (US FDA) approved Esketamine (the S-enantiomer of Ketamine) in a nasal spray formulation for the management of treatment-resistant MDD (28). However, Esketamine use is well controlled and it is only made available under a strict risk evaluation and mitigation strategy due to its adverse side effects (28). In addition, the cost of this intranasal treatment is high and maybe inaccessible for patients in low- and middle-income countries (29). The high cost is associated with healthcare cost and logistics due to availability under healthcare supervision and must be imported to other countries. Hence, Ketamine remains the most readily available efficacious antidepressant in the treatment of severe depression, treatment-resistant depression, and MDD. Currently, there are limited studies investigating the direct relationship between the expressions of BDNF and CREB as well as brain neurotransmitter changes associated with Ketamine use for the treatment of MDD.

This study therefore aimed to investigate the pharmacodynamic effects of ketamine in the brain by assessing changes in monoaminergic, glutaminergic and GABAergic neurotransmitter levels using liquid chromatography tandem mass spectrometry (LC-MS/MS). Further, the relationship between Ketamine and the expression of the transcription factors, *CREB* and *BDNF* was investigated using quantitative polymerase chain reaction (qPCR). These findings

will contribute to our understanding on the role of Ketamine in the treatment and management of MDD.

2.2. Methods and Materials

2.2.1. Chemicals and Reagents

All chemicals and reagents utilised in this study were of analytical grade: Serotonin (5-HT), (-)-Norepinephrine (NE), Dopamine hydrochloride (DA), y-Aminobutyric acid (GABA), and L-Glutamic acid monosodium were all sourced from Sigma-Aldrich (St. Louis, MO). Serotonin D4 hydrochloride, an internal standard for quality control, was purchased from Clearsynth (Mississauga, Canada). Ketamine-HCL solution (100 mg/ml) was purchased from Fresenius Kabi Manufacturing (SA) Pty. Ltd, and LC-MS grade methanol (MeOH) was procured from Sigma Aldrich (Steinham, Germany). Analytical grade formic acid was purchased from Merck Millipore (Johannesburg, South Africa). Discovery® DSC-18 solid-phase extraction (SPE) cartridge (wt. 100 g, volume 1 mL) was purchased from Merck, South Africa. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), CREB, and BDNF primer sequences were purchased from Inqaba Biotec™ (Pretoria, South Africa). Water was purified using a Milli-Q water purification system (Bedford, MA, USA). All other chemicals utilised in this study were of analytical grade.

2.2.2. Animals and Ethics

The use of animals, including experimental procedures, handling and treatment were all approved by the University of Kwazulu-Natal Animal Research Ethics Committee (UKZN, AREC) with approval reference **AREC/003/018M**. Twenty-one male Sprague-Dawley (SD) rats ranging from 120-130 g were sourced from the University of Kwa-Zulu Natal Biomedical Resource Unit (Durban, South Africa). All animals were housed under appropriate ethical standards approved by the Biomedical Resource Centre in a well-ventilated room with

humidity control systems, a 12-hour light/dark cycle, *ad libitum* access to standard rat chow and water, and the recommended environmental enrichment.

2.2.3. Drug administration and sample collection

Twenty-one animals were administered a dose of 15 mg/kg body weight (bw) of Ketamine-HCL via intraperitoneal (IP) injection per time point, with three animals serving as the control. The dose was based on a previous study conducted at the University of KwaZulu-Natal, Catalysis and Peptide Research Laboratory, which showed that IP administration of 15 mg/kg.b.w of Ketamine-HCL exhibited more favourable brain drug delivery in order to produce maximum therapeutic concentrations when compared to other routes of administration (30). Animals (n = 3 per group) were euthanized by decapitation using a rodent guillotine at 0 (control), 5, 15, 30-, 60-, 120-, and 240-minutes post Ketamine administration. Brain tissues were surgically removed post-termination, frozen gradually using liquid nitrogen, and stored at -80 °C until further analysis.

2.2.4. Biological Samples

Brain samples were weighed and cut into small pieces with a surgical blade before being homogenized using an OMNI tissue homogenizer (Kennesaw Georgia, USA) in ultra-pure water in a 1 g: 1 mL ratio. The homogenised samples were then stored at -80 ˚C until further processing. Brain samples for qPCR were stored (−80 °C) in Qiazol lysis reagent (500 µL; Qiagen, Hilden, Germany).

2.2.5. LC-MS/MS Method

Instrumentation:

The liquid chromatography (LC) system used for analysis consisted of a Thermo Scientific Vanquish Ultra-High-Performance Liquid Chromatography (UHPLC) (Waltham, MA USA) coupled to Thermo Scientific TSQ Quantis Triple Quadrupole Mass Spectrometer (Waltham,

MA USA). All the data obtained was analysed and processed using Thermo Trace finder General (4.1 SP5) and Thermo Scientific SII Xcalibur 1.3 (3.0.20389) software.

Chromatographic conditions:

Chromatographic separation was achieved using a Poroshell 120 EC-C18 (50 mm x 4.6 mm, 2.7 μm) (Agilent Technologies, California, USA) with a gradient mobile phase composition consisting of 0.1% v/v Formic Acid in ultra-pure water (A) and 0.1% v/v Formic Acid in Methanol (B). The gradient elution was as follows: The gradient started with a 1 min pre-injection equilibration step held at 15% B, then increased linearly from 15-70% B in 1 min, followed by a further increase to 95% B in 0.1 min, then held at 95% B for 4.4 min, followed by a decrease to 15% B in 0.1 min and finally held for 2.4 min at 15% B. The flow rate was set at 0.800 mL/min, with an injection volume of 10 μL and a total run time of 9 min.

Mass spectrometric condition:

Quantitative studies were conducted using Tandem MS (MS/MS) via a Heated Electrospray Ionization (H-ESI) interface in positive mode, with the following source parameters: spray voltage, 4.8 kV; sheath gas, 50 Arb. Unit; auxiliary gas, 15 Arb. Unit; sweep gas, 1.2 Arb. Unit; vaporizer temperature, 400 ˚C; and ion transfer tube 325 ˚C. Other conditions, such as collision energy per analyte of interest, are summarized in **Table 1**.

Table 1: Selected Reaction Monitoring (SRM) and Ion Optics Parameters

Compound	Precursor (m/z) $[M+H]^+$	Product *(m/z)*	Quantifier/ Qualifier Ions (m/z)	Collison Energy(V)	RF Lens Voltage(V)	Dwell Time(msec)
Dopamine	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	104.05	87	**Quantifier**	13.41	30	9.473
		46	Qualifier	55	30	9.473
		85.875	Qualifier	15	30	9.473
Glutamate	148.05	84.071	**Quantifier**	18.98	30	9.473
		131	Qualifier	23.72	30	9.473
Norepinephrine	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	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
Serotonin D4 hydrochloride	181.175	164.020	**Quantifier**	10.23	192	9.473
		78.557	Qualifier	34.57	192	9.473
		118.042	Qualifier	26.49	192	9.473
Ketamine	238.1	124.989	**Quantifier**	47.23	85	9.473
		220.030	Qualifier	22.85	85	9.473
		162.911	Qualifier	31.65	85	9.473

2.2.6. Sample preparation for LC-MS/MS analysis

Samples for LC-MS/MS analysis were prepared by adding 100 µL of brain homogenate to 850 µL of Methanol (MeOH), spiked with 50 µL of internal standard (10 µg/mL) and vortex mixed briefly. The mixture was then centrifuged at 10 000 x *g* for 10 minutes at 4 ˚C. The resulting supernatant was filtered through a DSC-18 SPE cartridge (wt. 100 g, volume 1 mL) and the eluent was collected in an autosampler vial. 400 µL of the SPE eluent sample was then dried or evaporated under a stream of nitrogen using a flow of 2 bars using Zipvap nitrogen

evaporator (Glass-Col, Terre Haute, USA). The dried sample was then reconstituted in 400 µL ultrapure water and vortex mixed for 30 seconds, before LC-MS/MS analysis

2.2.7. RNA isolation

RNA was extracted from brain tissues using Qiazol reagent (Qiagen, Hilden, Germany). Briefly, 100 µL brain homogenate was incubated overnight (-80 °C) in 500 µL Qiazol reagent. Thereafter, the samples were thawed at room temperature (RT) and chloroform (100 µL) was added. The samples were centrifuged (12 000 x *g*, 15 min, 4 °C) and the aqueous phase containing crude RNA was transferred to fresh 1.5 mL micro-centrifuge tubes. Isopropanol (250 µL) was added to the aqueous phase followed by overnight incubation at -80 °C. The samples were then centrifuged (12 000 x *g*, 20 min, 4 °C) and the RNA pellets were washed in 500 µL of 75 % cold ethanol. The samples were further centrifuged (7 400 x *g*, 15 min, 4 °C) before discarding the ethanol and allowing the RNA pellets to air dry for 30 min at RT. The RNA pellets were resuspended in 15 µL nuclease-free water, followed by incubation at RT for 3 min before RNA quantification. Quantification and purity of RNA samples was determined using the Nanodrop™ 2000 spectrophotometer (Thermo-Fisher Scientific, SA). The purity of the RNA was assessed using the A260/A280 absorbance ratios. All samples were standardized to a final concentration of 1,000 ng/µL.

2.2.8. *CREB* and *BDNF* mRNA/gene expression

Total RNA (1,000 ng/µL) was reverse transcribed into cDNA using the Maxima H Minus First Strand cDNA Synthesis Kit (Thermo-Fisher Scientific, California, USA) as per the manufacturer's instructions. Thereafter, the mRNA expression of *CREB* and *BDNF* (See **Table 2** for primer sequences) was determined using the PowerUp™ SYBR™ Green Master Mix (Thermo-Fisher Scientific, California, USA) as per the manufacturer's protocol. The reaction was amplified using the Applied BioSystems ViiA 7 Real-Time PCR System (Thermo-Fisher

Scientific, California, USA) with the following cycling conditions: initial denaturation (95°C, 8 min), followed by 40 cycles of denaturation (95°C, 15s), annealing (60°C, 40s), and extension (72°C, 30s). *GAPDH* was used as the housekeeping gene to normalize differences in mRNA expression. The data was analysed according to the methods described by Livak and Schmittgen (2001) and represented as a fold change relative to the control (31).

Table 2: Primer sequences of *CREB*, *BDNF*, and *GAPDH*

Gene	Primer Sequence
CREB	Sense: 5'-CCAAACTAGCAGTGGGCAGTATATT-3' Anti-sense: 5'-GGTACCATTGTTAGCCAGCTGTATT-3'
BDNF	Sense: 5'-GAATTCATGACCATCCTTTTCCTTACTATG-3' Anti-sense: 5'-AAGCTTTCTTCCCCTTTTAATGGTCAG-3'
GAPDH	Sense: 5'-GGCACAGTCAAGGCTGAGAATG-3' Anti-Sense: 5'-ATGGTGGTGAAGACGCCAGTA-3'

2.2.9. Statistical analysis

Statistical analysis was carried out using GraphPad Prism 8.4.3. (GraphPad Software Inc., La Jolla, USA). Statistical significance (p) was determined using the Holm-Sidak t-test method, with alpha = 0.05. Each row was analyzed individually without assuming a consistent standard deviation (SD). Results were expressed graphically as mean ± SD. A $p < 0.05$ was considered significant. Pharmacokinetic (pK) parameters were determined using STATA/IC 15.0 (StataCorp LLC, Texas, USA).

2.3. Results

2.3.1. Regulation of neurotransmitters by Ketamine

At clinical doses, Ketamine is known to directly affect a wide range of brain neurotransmitters; hence, we investigated the pharmacodynamic effects of Ketamine on five major brain neurotransmitters associated with MDD at different time intervals (5, 15, 30, 60, 120, and 240 min). Ketamine levels were also quantified *(for spectra and chromatograms: See Appendix*) after administration of 15 mg/kg.b.w (IP) at each time interval (**Fig. 17A**). Ketamine reached its highest concentration (C_{max}) in the brain at 5 min post-administration (351.33 ± 108.38 ng/mL) (**Table 3**). At 15 min, Ketamine concentration significantly decreased by five-fold when compared to the drug concentration at 5 min post dosing (p = .050) (**Fig. 17A**). From 15 min, onwards Ketamine levels decreased gradually, as shown in **Fig. 17A.**

Glut and GABA showed a similar trend with respect to their concentration changes post Ketamine administration (**Fig. 17C and 17F)**. At 5 min post administration of Ketamine both Glut and GABA were significantly increased (1167.98 ± 108.31 ng/mL, p = .005 and 1458.65 ± 82.70 ng/mL, p = .007 respectively) compared to the control group at 0 min (716.1 ± 92.41 ng/mL, p > .9999, and 1097 ± 90.75 ng/mL, p > .9999 respectively) (**Fig. 17C and 17F**). Similarly, after 15 min, they were significantly increased (Glut: 1218.37 ± 212.39 ng/mL, p = .0198; GABA: 1619.70 ± 47.36 ng/mL, p < .001), respectively. At 30 min, Glut (909.93 ± 47.81 ng/mL, p = .0321) and GABA (1345.77 ± 157.68 ng/mL, p = .0771) were decreased when compared to the concentrations observed at 15 min and increased at 60 min. A significant increase compared to the control (716.1 ± 92.41 ng/mL, p > .9999, and 1097 ± 90.75 ng/mL, p > .9999 respectively) group was also observed at 60 min for Glut and GABA (1120.75 ± 76.65 ng/mL, p = .0043 and 1393.10 ± 52.34 ng/mL, p = .0081), respectively (**Fig. 17C and 17F**). Further, Ketamine significantly increased Glut levels at 120 (1071.42 ± 128.25 ng/mL, p = .0176) and 240 (1028.30 ± 46.72 ng/mL, p = .0064) min when compared to the control (716.1

± 92.41 ng/mL, $p > .9999$) whilst GABA had no significant change at those time points compared to the control (**Fig. 17C and 17F)**.

NE displayed a significant increase (1452.44 ± 134.64 ng/mL, $p = .00160$) at 5 min compared to the control (804 ± 60.21 ng/mL, $p > .9999$); this was followed by a sharp decline at 15 min (1035.10 ± 184.10 ng/mL, $p = .1083$) (**Fig. 17B**). At 30 min, NE was significantly increased (1367.29 ± 221.80 ng/mL, $p = .0321$) when compared to the control group (804 ± 60.21 ng/mL, $p > .9999$). This significant increase in NE was also observed at 120 (1237.07 ± 134.26 ng/mL, $p = .0176$) and 240 (1189.44 ± 126.26 ng/mL, $p = .0064$) min post Ketamine administration (**Fig. 17B**).

DA showed no significant increase at 5 min (29.79 ± 1.25 ng/mL, $p = .1696$), 15 min (29.91 ± 1.71 ng/mL, $p = .2432$), and 30 min (28.79 ± 2.06 ng/mL, $p = .8355$) respectively (**Fig. 17D**). At 5- and 15-min post Ketamine administration, DA concentration remained constant, with a slight decrease at 30 min. This was followed by a significant increase in DA at 60 min (32.35 ± 2.32 ng/mL, $p = .0081$) compared to the control (28.53 ± 0.38 ng/mL, $p > .9999$). DA levels decreased slightly at 120 min; however, it was still significantly (29.62 ± 0.50 ng/mL, $p = .0394$) increased when compared to the control group (28.53 ± 0.38 ng/mL, $p > .9999$). Between 120 min (29.62 ± 0.50 ng/mL, $p = .0394$) and 240 min (29.12 ± 0.52, $p = .1829$), the concentration levels remained constant (**Fig. 17E**).

5-HT showed a significant increase (22.76 ± 0.56 ng/mL, $p = .0362$) at 5 min compared to the control group (21.55 ± 0.88 ng/mL, $p > .9999$) (**Fig. 17E**). At 15 min (22.42 ± 1.00 ng/mL, $p = .2311$), 5-HT levels were lower than that at 5 min. This was followed by an increase in 5-HT levels at 30 min (23.25 ± 1.60 ng/mL, $p = .1456$) and 60 min (23.13 ± 0.81 ng/mL, $p = .0367$) compared to the control (21.55 ± 0,378703 ng/mL, $p > .9999$). From 60 min onwards, a slight decrease was observed; however, these levels remained constant from 120-240 min (**Fig. 17E**).

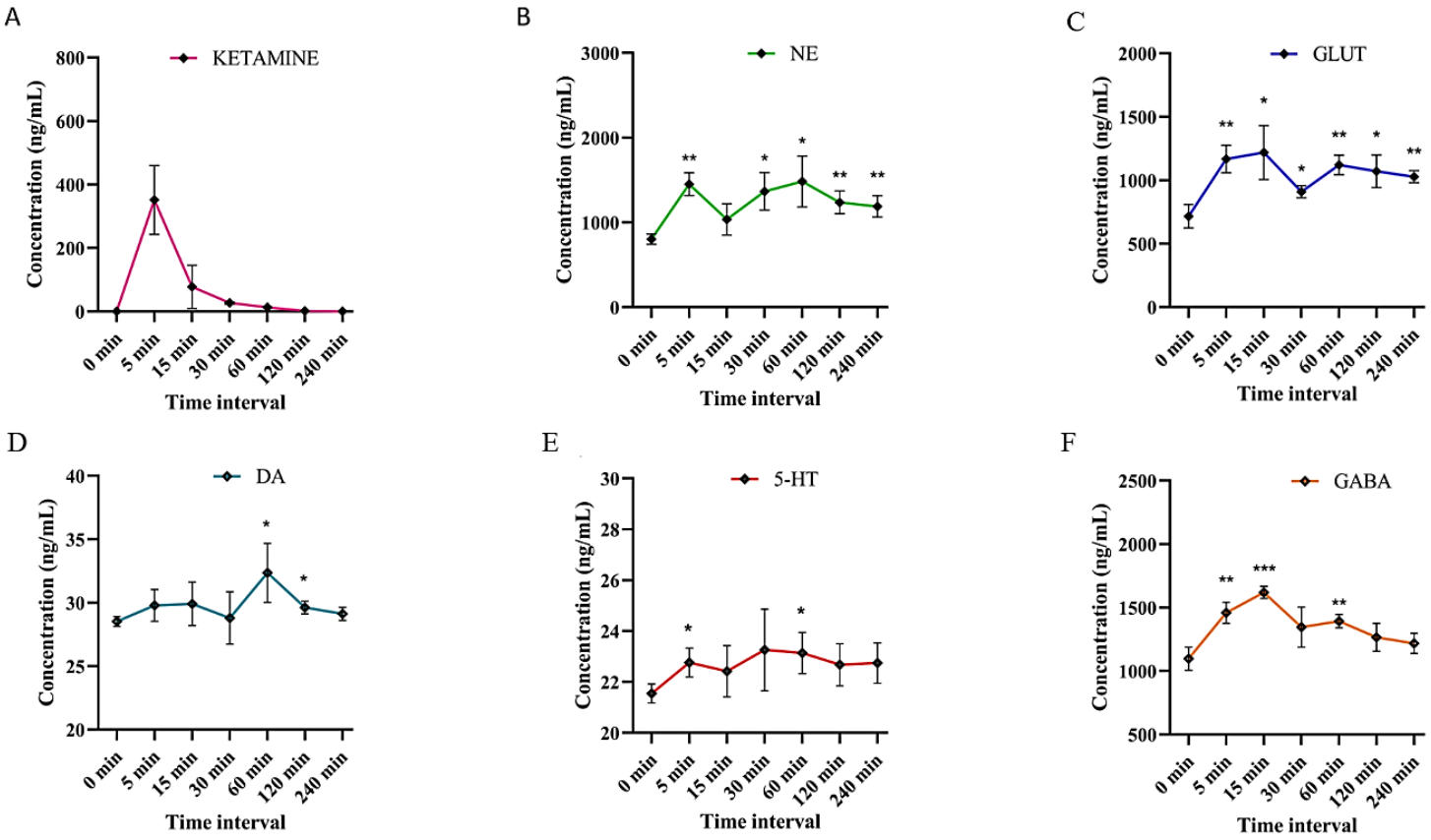

*Figure 17: Brain concentrations (ng/mL) of; A) Ketamine; B) NE; C) Glut; D) DA; E) 5-HT and F) GABA at various time points following a single dose of Ketamine (15 mg/kg; IP). The data is presented as mean ± SD obtained from experiments performed in triplicate (N = 3). (****p < .0001; **p < .00100; *p < .05).*

Table 3: Summary of the pharmacokinetic parameters of Ketamine following a single dose (15mg/kg b.w.) of ketamine

C_{max} (ng/mL)	T_{max} (min)	$T_{1/2}$ (min)	K_{el}	$AUC_{0\text{-}inf}$ (ng min/mL)
351.33 ± 108.38	5	38.8968	0.0178	5464.343

2.3.2. Effect of Ketamine on *CREB* and *BDNF* expression

We further investigated the pharmacodynamic effects of Ketamine on *BDNF* and *CREB* gene expression at different time intervals during the treatment period. At 5 min post drug administration, *BDNF* was significantly downregulated (decrease in expression) by 0.96-fold

relative to the control ($p < .0001$). At 15 min, *BDNF* expression was upregulated (increase in expression) compared to that at 5 min (**Fig. 18A**).

At 30 min, *BDNF* showed significant downregulation of 0.69-fold relative to the control ($p = .0055$). *BDNF* expression was downregulated by 0.91-fold at 60 min compared to the control ($p < .0001$), while no significant difference was observed at 120 min (**Fig. 18A**). However, at 240 min, *BDNF* was significantly downregulated by 0.71-fold relative to the control ($p < .0017$) (**Fig. 18A**).

CREB showed no significant change compared to the control group at 5 and 15 min (**Fig. 18B**), with 30 min showing a non-significant increase of 0.14-fold relative to the control ($p = .4866$). At 60 min, *CREB* was significantly downregulated by 0.47-fold compared to the control ($p < .0001$) (**Fig. 18B**). At 120 min, Ketamine decreased *CREB* expression by 0.13-fold compared to the control ($p = .0418$) (**Fig. 18B**). Similarly, at 240 min post dosage, there was significant downregulation of *CREB* with a 0.25 relative fold change (**Fig. 18B**).

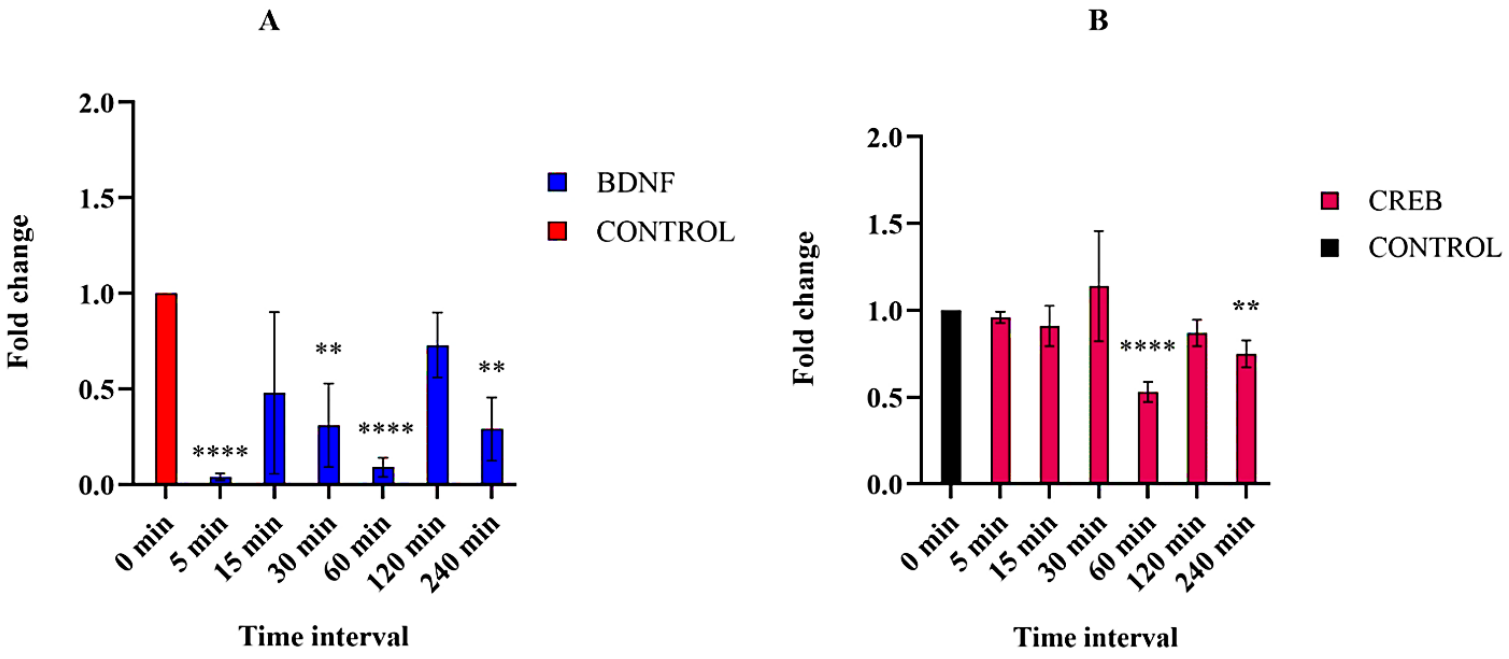

*Figure 18: Effect of Ketamine exposure on A) BDNF and B) CREB mRNA expression in brain tissue analyzed using qPCR. The data is presented as mean ± SD obtained from experiments performed in triplicate (N = 3). (****p < .0001; **p < .00100). 0 min was a control (Untreated).*

2.4. Discussion

In this study, we investigated the pharmacodynamic effects of Ketamine in rat brain by assessing changes in the levels of neurotransmitters that are usually associated with depression. Ketamine levels increased rapidly to reach C_{max} at 5 min followed by a gradual decrease at 15 min to 240 min. This was consistent with a previous study conducted in our laboratory, which showed Ketamine's C_{max} at 5 min post-administration(30) (**Fig. 17A**). After 5- and 15-min post dosing of ketamine, glutamate and GABA showed a sharp, significant increase in concentration when compared to other neurotransmitters. The increase of glutamate levels was consistent with various reports in literature, which indicated that ketamine increases glutamate neurotransmission by both increased glutamate release and increased α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPAR) expression (32-35). The observed phenomenon occurs via the ketamine disinhibition hypothesis, which proposes that Ketamine selectively blocks N-methyl-D-aspartate receptors (NMDARs) on GABAergic inhibitory interneurons, leading to the disinhibition of pyramidal neurons and enhanced glutamatergic release (32-35).

GABA showed a similar trend to glutamate, where there was an increase in its concentration in the brain, this is consistent with a study conducted by Ghosal et al. (2020), which showed an increase in GABA levels post ketamine administration (36). The upregulation of GABA is driven by a concurrent increase in glutamate levels and stimulation of the NMDARs, which also proceeds via the Ketamine disinhibition hypothesis. However, it is important to note that the mechanism of GABA upregulation remains unclear. This may occur due to increased glutamate levels since glutamate serves as a precursor for GABA (19); or via an unknown ketamine mediated mechanism.

The sharp increase in Glutamate and GABA levels at 5 and 15 min is in direct correlation with Ketamine concentration in the brain, where at 5- and 15-min Ketamine levels were at their

highest in brain samples (**Fig. 17A, C and 17F**). At 30 min post-administration, glutamate and GABA decreased in the brain when compared to 5 and 15 min respectively; this decrease is directly associated with the decreased concentration of Ketamine in the brain (**Fig. 17A)**.

The three monoamine neurotransmitters associated with depression (NE, DA, and 5-HT) were also investigated. At 5 min post-administration, when ketamine concentrations were at their highest, DA showed no significant changes compared to NE and 5-HT, which showed a significant increase compared to the control group (**Fig. 17A-17F**). Previous studies also reported increased levels of 5-HT and NE in brain regions such as the hippocampus and the cortical and midbrain upon administration of an acute dose of Ketamine (37-41). The increase of 5-HT is assumed to occur when Ketamine enhances glutaminergic neurotransmission in the brain, via disinhibition hypothesis of Ketamine (blockade of NMDARs and stimulation of AMPARs), this is supported by a study conducted by López-Gil, X. et al. (2019); which suggest that "*antidepressant-like effects of ketamine are caused by the stimulation of the prefrontal projection to the dorsal raphe nucleus and locus coeruleus caused by an elevated glutamate in the medial prefrontal cortex, which would stimulate release of serotonin*" (37). This was further supported by a study conducted by Nishitani, N. et al (2014) (42) . Although ketamine increased NE regulation in the brain, there is no direct link between the two; Russell and Wiggins (2000) demonstrated that enhanced glutamate levels stimulate NE to be released in the rat brain (43). In addition, the upregulation of NE cannot be linked to enhanced 5-HT regulation because projections of 5-HT neurons have an inhibitory effect on NE neurons (44).

DA showed no significant increase at other time points except at 60 min post Ketamine administration, where a significant surge in levels of DA was observed (**Fig. 17D**). This observation was consistent with the study conducted by Kokkinou et al. (2018), which showed significantly increased levels of DA post administration of Ketamine (45). Similarly to glutamate, the upregulation of DA is said to occur via the disinhibition hypothesis of Ketamine,

where NMDAR blockade on GABAergic interneurons leads to a projected regulation of midbrain dopamine neuronal cell bodies, which subsequently leads to an increase in pyramidal cell firing and increased excitation of dopamine neurons (45).

The expression of *BDNF* and *CREB* gene was also investigated at similar time intervals as the neurotransmitters to determine the link between ketamine concentrations, neurotransmitter levels and expression of these genes. Several studies have shown that subanesthetic doses of Ketamine (10-30 mg/kg) induces *BDNF* expression in the brain by selectively blocking NMDAR expressed on GABAergic inhibitory interneurons which leads to a disinhibition of pyramidal neurons and enhanced glutamatergic release. The released glutamate then binds to and activates AMPAR resulting in enhanced BDNF expression and release, subsequently resulting in the activation of the tropomyosin receptor kinase B (TrkB) receptor and promotion of protein synthesis (46-50). Our study showed a significant decrease in *BDNF* mRNA expression after 5 min post Ketamine (15 mg/kg) administration (**Fig. 18A**). A recent study by Kim and Monteggia (2020), found that low dose of ketamine (5 mg/kg, IP) produces rapid antidepressant responses as well as critical molecular and synaptic effects that were not observed at higher doses (20 and 50 mg/kg, IP) (49). A similar study conducted by Wu et al. (2020) also found that low-dose Ketamine (10 and 30 mg/kg) induced antidepressant effects, while high-dose Ketamine (100 mg/kg) induced cognitive impairment and pro-depression behavior (50). The study further indicated that 100 mg/kg Ketamine increased *BDNF* mRNA expression after 24 hours (50). Therefore, a possible explanation for the observed suppression of *BDNF* mRNA at 5 min post-administration of Ketamine (15 mg/kg), could be linked to higher concentration levels of Ketamine in the brain (**Fig. 17A and Fig. 18A**); as it is observed that ketamine mediated increase in BDNF expression have an inverse relationship with ketamine, since BDNF expression increases as Ketamine concentrations begin to decrease (**Fig. 17A and Fig. 18A)**.

At 5-30 min post administration of ketamine, there was no significant changes in the expression of *CREB* when compared to the control. A study conducted by Réus et al. (2011), proved that *CREB* expression in the brain is not associated with the antidepressant response of Ketamine (51). However, it has been shown that *BDNF*, via activating its tropomyosin receptor kinase B, can induce the activation of the mammalian target of rapamycin (mTOR) signaling and *CREB* signaling, which could explain the fluctuations observed in *CREB* expression (**Fig. 18B**) at 30-240 min post-administration of ketamine (52, 53).

The implications of this study are significant with several experimental and clinical studies advocating the use of Ketamine in the treatment of MDD. There are various hypothesized mechanisms of ketamine in alleviating depression, such as disinhibition of glutamate release, blockade of extra-synaptic NMDARs, blockade of spontaneous NMDAR activation, the role of (2R,6R)-hydroxynorketamine (ketamine metabolite) and, inhibition of NMDAR- dependent bursting activity of lateral habeluna neurons (34, 54). This study showed that the rapid anti-depressive effect of ketamine is due to its influence on the glutamatergic pathway, which leads to alterations in various synaptogenic pathways in the brain, and enhances the release of glutamate, GABA, and the principal monoamine neurotransmitters (**Fig. 19**).

In addition, it was observed that ketamine has inverse relationship with *BDNF* gene expression. The relationship between ketamine and *CREB* could not be established in this acute study; however, literature suggests that enhanced *BDNF* expression induces *CREB* signaling (52, 53). Therefore, this study found that out of many hypothesized Ketamine's mechanisms of action, the disinhibition of glutamate hypothesis was the primary mechanism of action that subsequently enhances synaptogenesis in the central nervous system, allowing the other hypothesized mechanisms to be initiated. Hence, ketamine's disinhibition of glutamate hypothesis was found to be the central mechanism in the rapid amelioration of MDD.

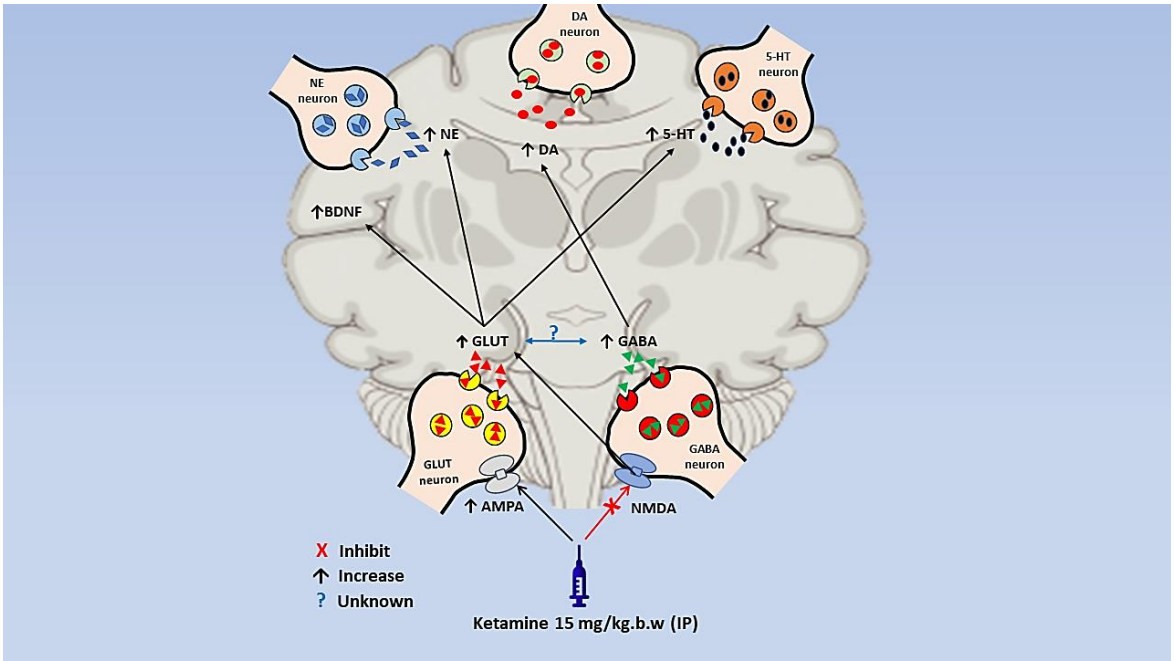

Figure 19: Schematic diagram summarizing the pharmacodynamic effects of Ketamine (15 mg/kg.b.w) as found in this study. Created by the Author.

2.5. Conclusion

This study demonstrated that ketamine alleviates depression by significantly altering the glutamatergic system which occurs via the disinhibition of glutamate release, this enhances central nervous synaptogenesis, correcting the imbalance of neurotransmitters and proteins associated with the pathophysiology of depression. This study shows that the anti-depressive action of ketamine may be two-fold in that its initial alleviation of depressive symptoms is due to its effect on the glutamatergic system, which is latter followed by an increase in BDNF expression, a target of traditional antidepressants. However, the findings of this study are not completely inclusive, hence, more studies are still needed.

2.6. Acknowledgements

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2.7. Disclosures

The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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CHAPTER 3

SUMMARY AND CONCLUSION

3.1. Summary and Conclusion

Ketamine is currently aproved by the US FDA as anesthetic drug, that is being used off-label for pain manangement (acute and chronic), and most recently as an antidepressant in the treatment of MDD (1-3). Several clinical studies have indicated that subanesthetic doses of ketamine infusions provide rapid relief of depressive symptoms in people suffering from MDD and TRD (4-6). This study investigated the pharmacodynamic effects of ketamine on several vital brain neurotransmitters in the treatment of MDD. These included the monoamine neurotransmitters (DA, 5-HT, and NE) and amino acid neurotransmitters (Glut and GABA), which have been shown to be deficient or altered in MDD patients and in depressed animal models (7-12). The study further investigated the effects of ketamine on transcription factors, *BDNF* and *CREB* expression in the brain as they have been linked to the pathophysiology of MDD and that are targets of conventional antidepressant drug classes (13-15).

Twenty-one healthy male SD rats in a mass range of 120-130 g were administered 15 mg/kg of ketamine intraperitonially at different time intervals. LC-MS/MS was used to quantify or assess concentration levels of neurotransmitters, whilst gene expression was achieved using qPCR. The pharmacokinetic results showed that ketamine reached a maximum concentration of 351.33 ± 108.38 ng/mL at 5 min post drug administration (**Fig. 17A**). This finding was similar to a previous study conducted in our laboratory which showed that ketamine concentrations in the brain increased rapidly to its C_{max} in 5 min thereafter decreased gradually to its trough concentrations (16).

The results obtained showed that at C_{max} of ketamine, Glut and GABA had a significant increase in their concentration compared to the monoamine neurotransmitters. Throughout the study, Glut and GABA had a higher concentration in the brain compared to the monoamine neurotransmitters (**Fig. 17A-17F**). These findings indicated that ketamine mainly affects the glutamatergic and GABAergic neurotransmission systems compared to the monoaminergic

neurotransmission system. This is due to ketamine's disinhibition of glutamate hypothesis, which indicates that ketamine selectively blocks NMDRs on GABAergic inhibitory interneurons, which results to a disinhibition of pyramidal neurons and improved glutamatergic release, which further enhances GABAergic neurotransmission (**Fig. 19**). The observed increase in monoamine neurotransmitters could be linked to the same hypothesized disinhibition mechanism of ketamine. During NMDAR blockage and AMPAR stimulation, there is an increase in new synaptic connections and improved brain plasticity, which subsequently results in increased monoamine neurotransmitters and alleviation of depressive symptoms (17).

The study also found that at high ketamine concentration levels, the gene expression of *BDNF* was significantly downregulated while *CREB's* gene had no significant change compared to the control groups. However; as concentration levels of ketamine declined, there was an upregulation in the expression of the *BDNF* gene , indicating that there is a delay in ketamine's effect in the expression of *BDNF*, and that upregulation of *BDNF* is responsible for the signaling of *CREB* (**Fig. 18A and B**); this is supported by literature which indicates that enhanced *BDNF* expression induces *CREB* signaling (18, 19)

This study showed that the rapid anti-depressive effect of ketamine is due to its influence on the glutamatergic pathway, which leads to alterations in various synaptogenic pathways in the brain, by enhancing the release of glutamate, GABA and altering the principal monoamine neurotransmitters (**Fig. 19**).

In conclusion, the study demonstrated that ketamine's disinhibition of glutamate hypothesis is the vital key mechanism of action in the treatment of MDD since it enhances central nervous synaptogenesis and corrects the imbalance of neurotransmitters, and genes associated with the

pathophysiology of depression. In addition, this study indicated that there is a delay in *BDNF's* response to ketamine administration (Inverse relationship).

This research had some limitations in that we did not investigate the long-term effects of ketamine administration, it is therefore recommended that a similar study is carried out over a long period of time. This study should also include the use of depressed animal models. Depression can will be induced in rats by subjecting them to several manipulations of chronic unpredictable stress (CUS) over a period of a month. This will allow for animal behavioral tests to be conducted and compare behavioral outcomes in response to ketamine therapy in both depressed animal models and healthy animals. Furthermore, it will further allow us to determine if chronic administration of ketamine impacts the expression of transcription factor *CREB* which was inconclusive in this study.

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APPENDIX

Investigations into the pharmacodynamic effects of Ketamine by evaluating its effect on brain neurotransmitter levels and CREB/BDNF expression

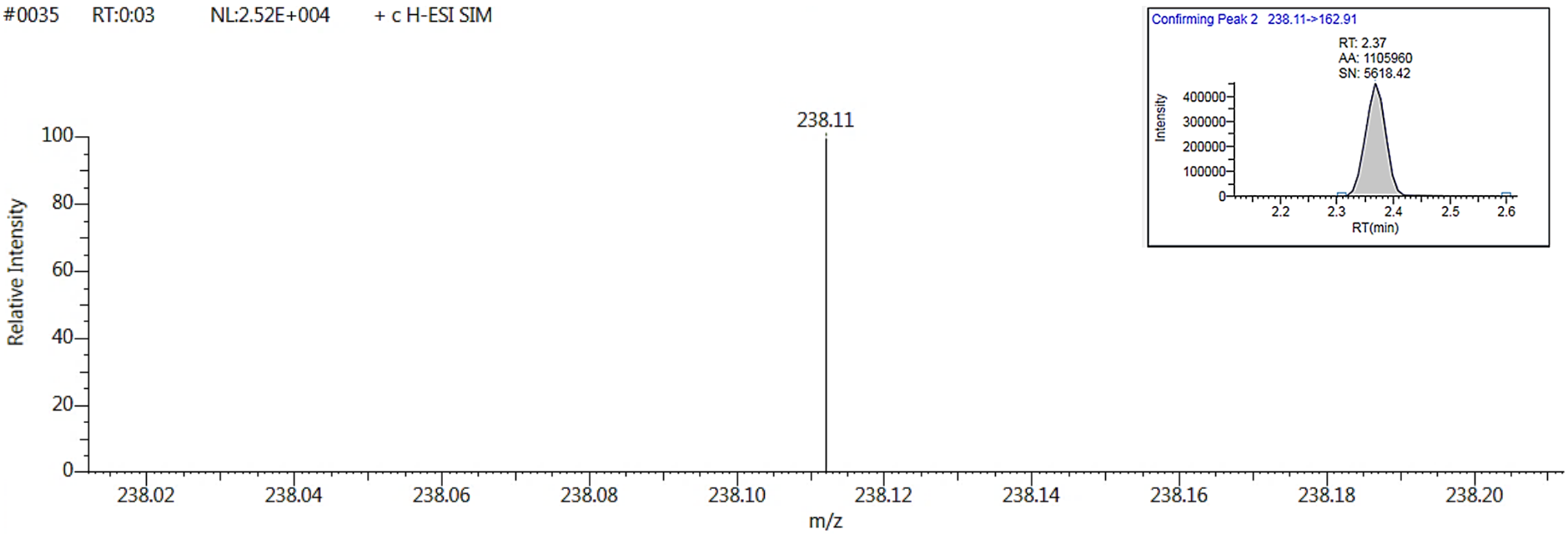

Figure 20: A) LC-MS/MS chromatogram of Ketamine's target ions at RT 2.37 min; B) Ketamine precursor spectrum showing $[M+H]^+$ at 238.11 m/z.

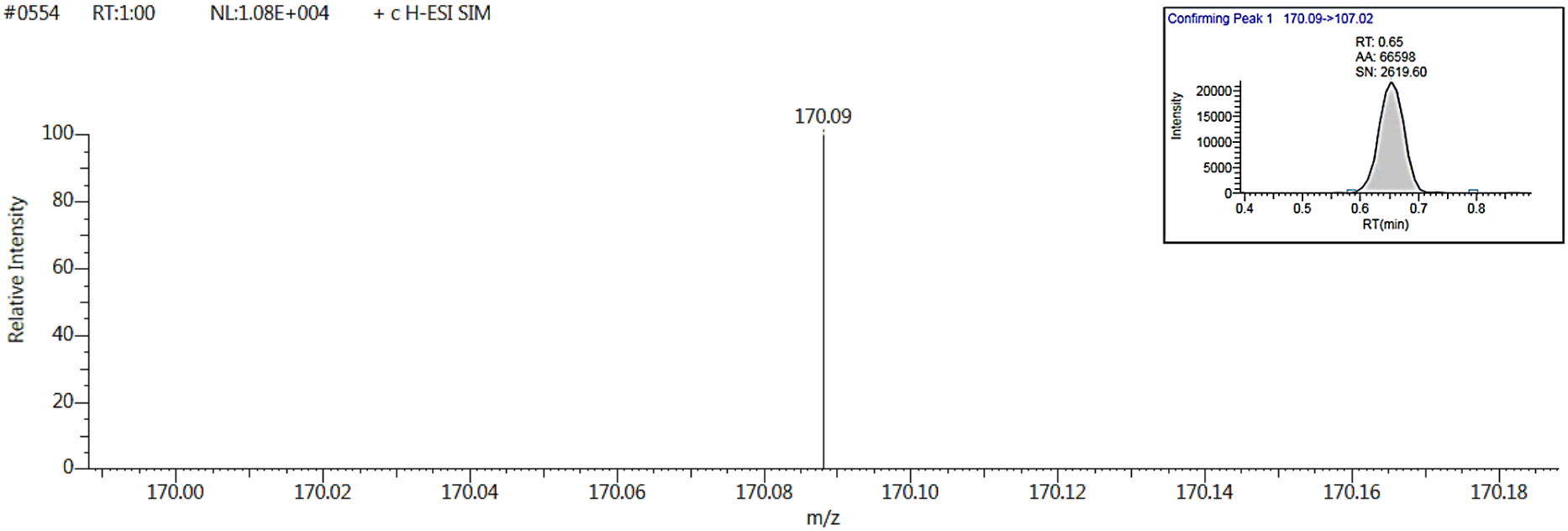

Figure 21: A) LC-MS/MS chromatogram of NE's target ions at RT 0.65 min; B) NE precursor spectrum showing [M+H]+ at 170.09 m/z .

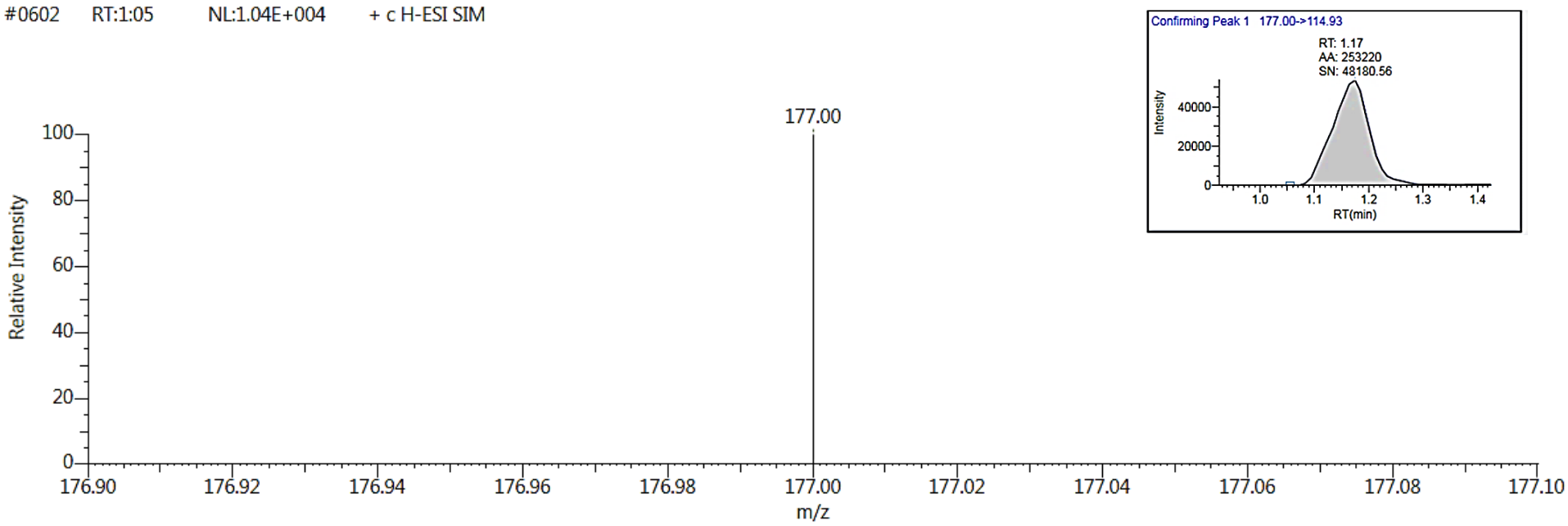

Figure 22: A) LC-MS/MS chromatogram of 5-HT's target ions at RT 1.17 min; B) 5-HT precursor spectrum showing [M+H]+ at 177.00 m/z .

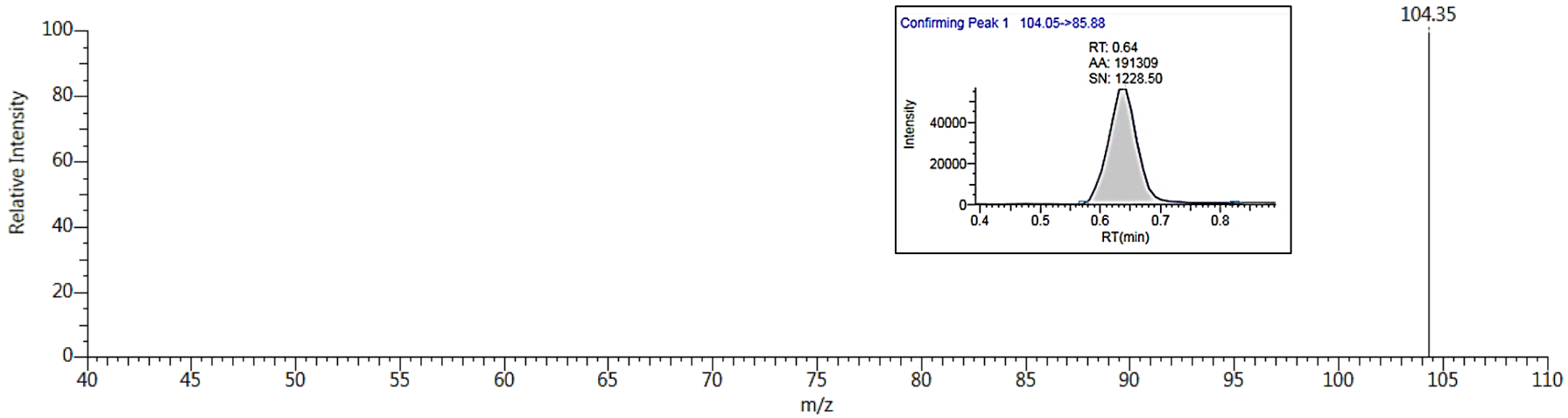

Figure 23: A) LC-MS/MS chromatograph of GABA's target ions at RT 0.64 min; B) GABA precursor spectrum showing [M+H]+ at 104.35 m/z .

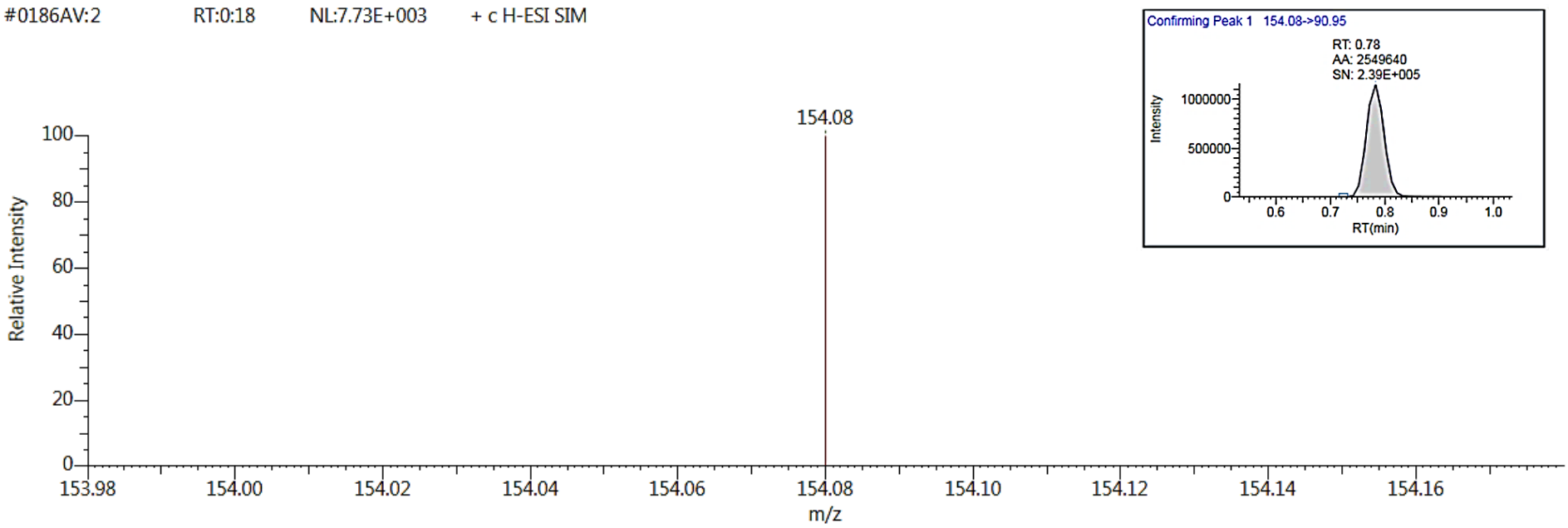

Figure 24: A) LC-MS/MS chromatogram of DA's target ions at RT 0.78 min; B) DA precursor spectrum showing [M+H]+ at 154.08 m/z .

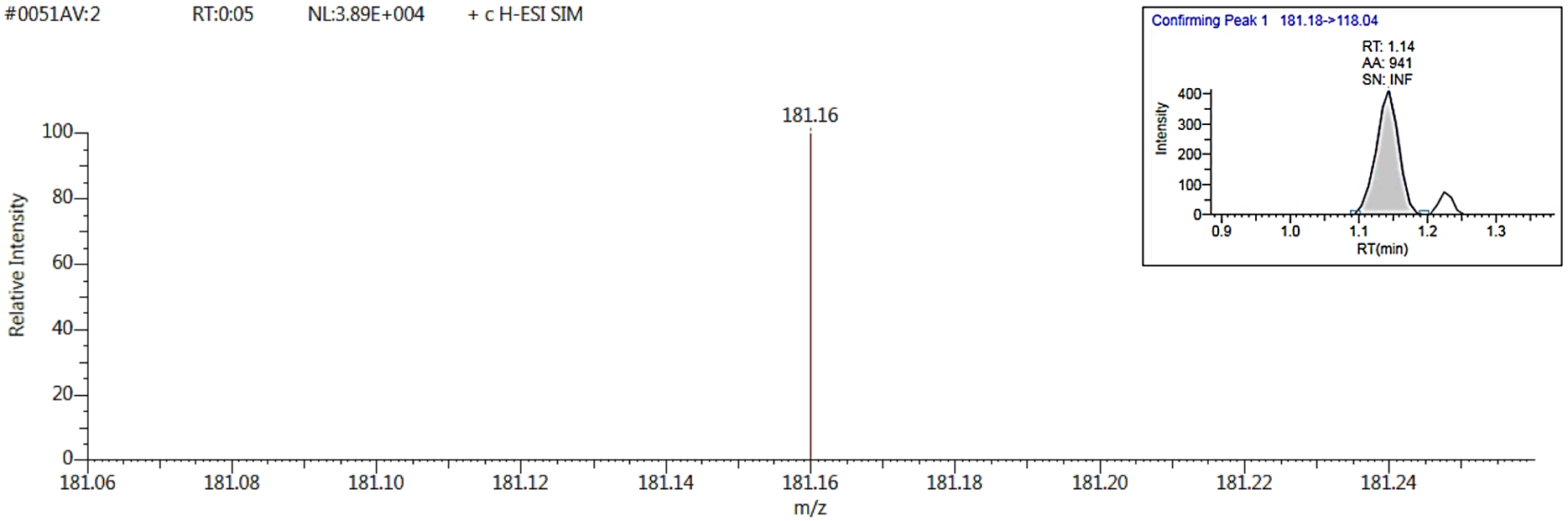

Figure 25: A) LC-MS/MS chromatograph of IS's target ions at RT 1.14 min; B) IS precursor spectrum showing [M+H]+ at 181.16 m/z .

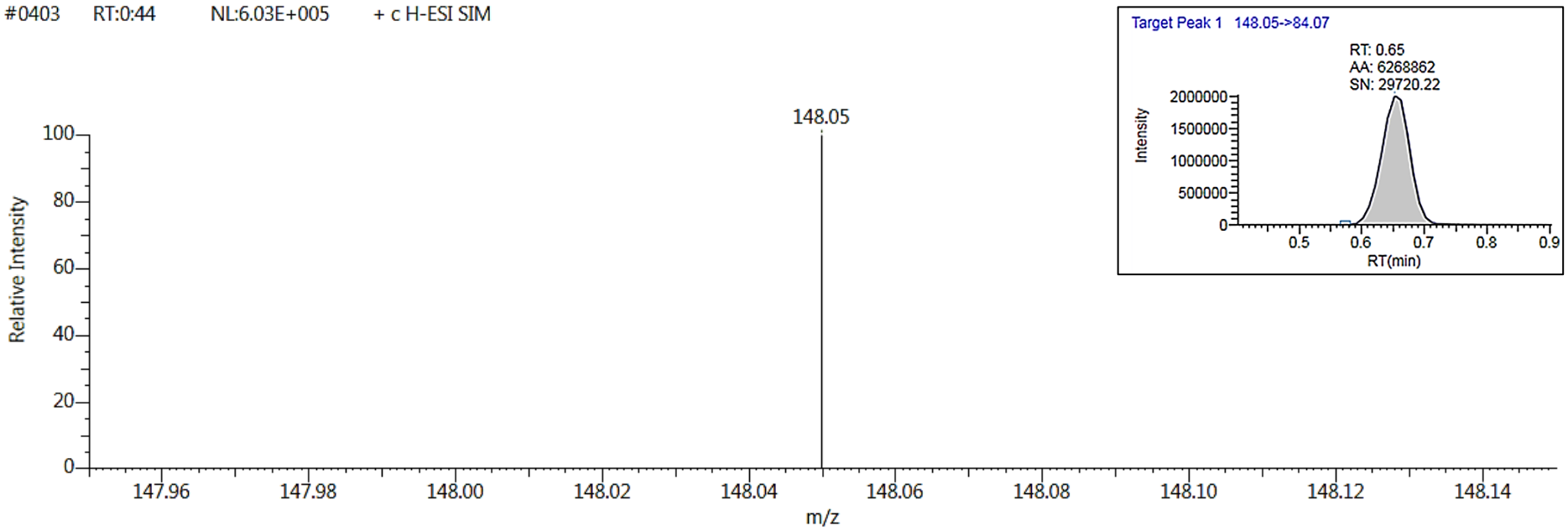

Figure 26: A) LC-MS/MS chromatograph of GLUT's target ions at RT 0.65 min; B) GLUT precursor spectrum showing [M+H]+ at 148.05 m/z .

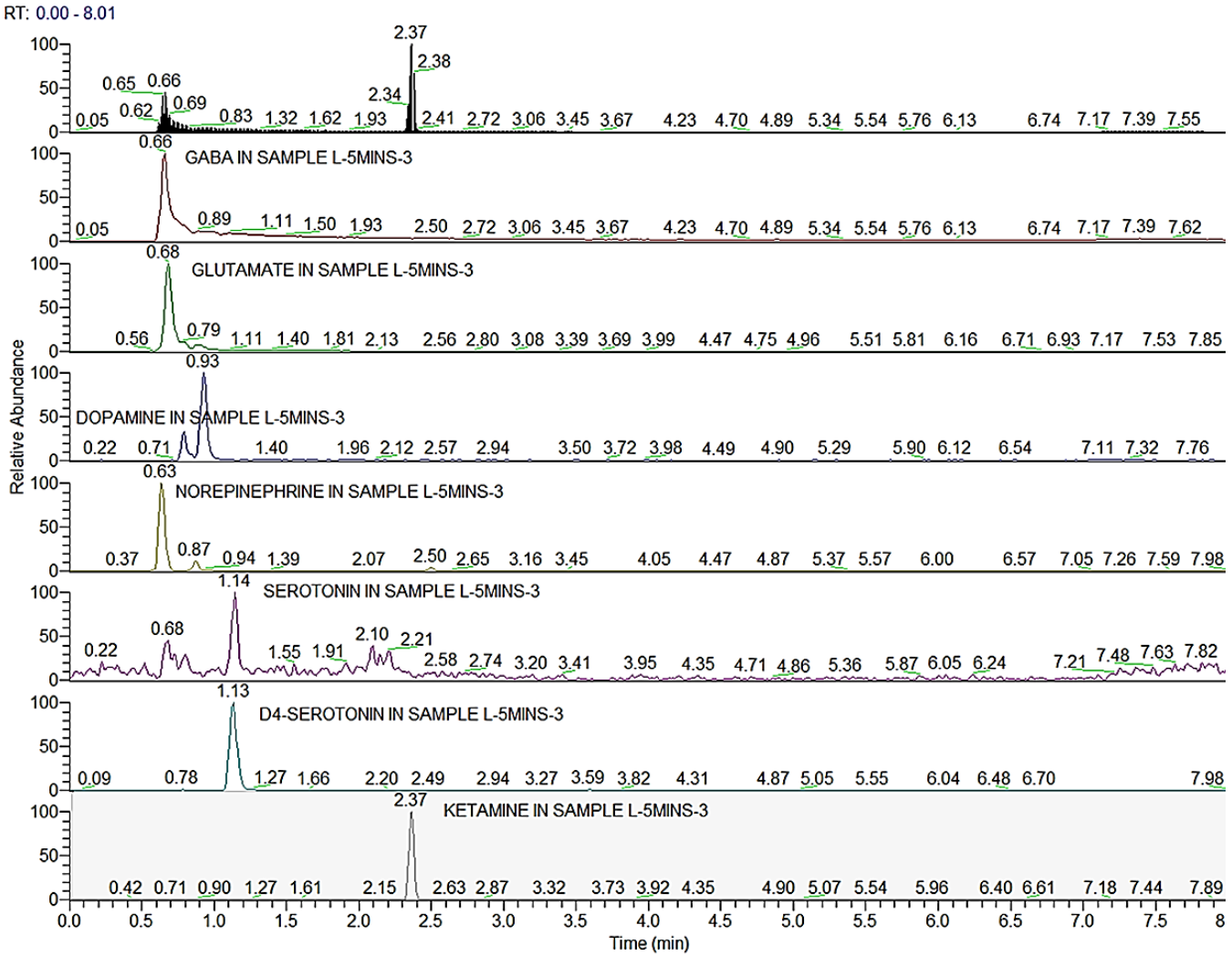

Figure 27: LC-MS/MS chromatogram showing DA, GABA, GLU, NE, 5-HT, IS and ketamine in brain sample at 5 min post-administration of 15 mg/kg ketamine.