Bio-behavioural effects of novel glutamate active compounds in a rodent model of depression

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Abstract

Major depressive disorder is a universal neuropsychiatric disorder affecting individuals on a global scale. It causes major disability independent of age, gender, ethnicity, sociological or economic status with an increasing prevalence and morbidity rate. The precise aetiological basis for this condition remains under investigation due to its complexity and several causalities thought to be related to its origin. Multiple hypotheses have been formulated in clarifying the existence of this disease which involves several systems, such as monoamines, glutamate regulation, neurotrophic factors, HPA-axis regulation, and several others. Not only are neurochemical imbalances associated with MDD; structural changes within the brain have also been documented. These changes have been investigated for involvement in depression-induced cognitive aberrations relating to memory and learning processes. The glutamatergic pathway is one of the systems suggested to be involved in the aforementioned deviations, linking a variety of other pathways, viz. the tryptophan metabolic pathway and N-methyl-D-aspartate (NMDA) receptor modulation. Currently, a vast array of treatment modalities are in place for treating MDD and treatment is more often focussed on the reversal of monoamine imbalances within the depressive brain. Though these compounds are effective in treating MDD, nearly 40% of patients never experience therapeutic effects and only 30 – 50% undergo successful remission. Consequently, the investigation into other biological targets and novel treatment options for MDD has been necessitated.

Due to the structural brain alterations that co-present with MDD, several depression-induced cognitive abnormalities, viz. disrupted attention and concentration, declarative memory insufficiencies, disrupted thought processes and impaired neurogenesis have been unearthed and are now established as phenotypical of the condition. The aforementioned may stem from abnormal glutamate firing and NMDA receptor overexcitation in various brain regions for which supporting evidence does exist in both animal and human studies. However, no antidepressant compounds directly target the glutamatergic system and all of its involved components in order to reverse these irregularities. Therefore, it is essential to explore biological targets and new treatment options capable of exerting antidepressant-like and/or procognitive actions within the glutamatergic system.

Preclinical research has provided evidence in support of drug compounds exerting antidepressant-like effects via the glutamate pathway, either directly or indirectly, e.g. ketamine, memantine, allopurinol and sodium benzoate. However, evidence pertaining to the latter two compounds is in short supply. Therefore, the aim of this study was to investigate whether chronic treatment with allopurinol and sodium benzoate proved capable of reducing depressive-like behaviours and/or depression-induced cognitive impairments within the FSL

model as well as their effects on monoaminergic - and BDNF concentrations in different brain areas linked to depression.

Confirmation of the depression-like phenotype of the FSL rat model compared to its healthy cohort, the Flinders resistant line (FRL) rat, was accomplished using the FST. As a result, the face validity of the model was reaffirmed allowing its application in investigating the plausible antidepressant-like capabilities of allopurinol and sodium benzoate. However, the presence of depression-induced cognitive impairments could not distinctly be confirmed in this model using the MWM test even though a small difference was observed.

The acute dose-ranging analysis with allopurinol and sodium benzoate proved effective in reducing depressive-like behaviours in the FSL rat in the FST.

Consistent with earlier studies, administration of a chronic fixed dose protocol using allopurinol proved successful in significantly reducing depressive-like behaviours in the FSL rat using the FST. Similar outcomes were observed for sodium benzoate using the same protocol, although not to the same extent. Furthermore, the effects of fluoxetine drifted toward reduced immobility in the FST though no significant results were obtained. Ketamine and memantine similarly reduced immobile behaviour, although the latter not significantly so. Neither allopurinol nor sodium benzoate proved capable of significantly reducing depression-induced memory impairments (viz. memory retrieval) in the FSL rat during MWM testing. Yet, both compounds promoted memory consolidation over the 5 days of acquisition training. Interestingly, fluoxetine significantly impaired memory retrieval whereas ketamine visibly enhanced it. Memantine appeared to have had similar effects to that of fluoxetine. Only sodium benzoate proved capable of significantly enhancing striatal dopamine levels, though it may appear as if allopurinol had a positive effect in this regard. Predictably, fluoxetine enhanced prefrontocortical noradrenaline and serotonin levels. Data obtained for the rest of the compounds in the various brain regions proved inadequate and could not be used to corroborate the findings in the FST. With the exception of memantine, none of the other treatment options proved successful in enhancing brain BDNF concentrations.

Though these results may appear inconclusive, current literature in association with the findings in this study support the antidepressant-like capabilities and potential procognitive effects of allopurinol and sodium benzoate and, therefore, the practical implications of the results from this study should not be overlooked. However, further studies are necessary in order to clarify the investigated effects surrounding allopurinol and sodium benzoate in the treatment of depression and associated depression-induced cognitive impairments.

Keywords: major depressive disorder (MDD), cognitive impairment, N-methyl-D-aspartate (NMDA); monoamine, brain-derived neurotrophic factor (BDNF); allopurinol, sodium benzoate, Flinders sensitive line (FSL)

Opsomming

Major depressiewe versteuring (MDV) is ’n universele neuropsigiatriese afwyking wat talle individue wêreldwyd affekteer. Dit veroorsaak groot fisiese en psigiese inperking onafhanklik van ouderdom, geslag, etnisiteit, sosiologiese en ekonomiese status met ’n toename in voorkoms en morbiditeitskoers. Die etiologiese basis vir MDV word steeds ondersoek vanweë die kompleksiteit van die toestand. Veelvuldige hipoteses is geformuleer met die doel om die bestaan van die toestand te verklaar en behels verskeie neurofisiologiese sisteme onder andere monoamien transmissie, glutamaat en HPA-as regulering, neurotrofiese faktore en nog vele meer. Buiten vir neurochemiese wanbalanse wat met MDV geassosieer word, is strukturele veranderinge met betrekking tot brein fisiologie ook opgemerk. Hierdie veranderinge word ondersoek vir moontlike betrokkenheid in depressie geïnduseerde kognitiewe inkorting met betrekking tot geheue en leerprosesse. Die glutamaat stelsel, verantwoordelik vir die koppeling van verskeie ander transmissie bane insluitend die triptofaan metaboliese weg en N-metiel-D-aspartaat-(NMDA)-reseptor modulering, is een van die sisteme wat vermoedelik betrokke is in die voorafgenoemde afwykings. ’n Groot verskeidenheid behandelingsmodaliteite is tans beskikbaar vir die behandeling van MDV en is dikwels gefokus om, met betrekking tot ’n depressiewe breinmodel, monoamien wanbalanse te herstel. Alhoewel hierdie behandelingsmodaliteite effektief is, ervaar sowat 40% van pasiënte geen terapeutiese effekte nie en slegs 30-50% bereik suksesvolle remissie na behandeling. Gevolglik is die ondersoek en navorsing na ander biologiese teikens en nuwe behandelingsopsies vir MDV genoodsaak.

As gevolg van die teenwoordigheid van strukturele breinveranderinge in MDV, is verskeie depressie-geïnduseerde kognitiewe inkortings, onder andere aandagafleibaarheid, ingeperkte geheue en konsentrasievermoë, verswakte neurogenese en ontwrigte denkprosesse, aangetoon en word dit tans beskou as fenotipiese merkers van MDV. Bogenoemde kognitiewe inkortings kan herlei word na abnormale glutamaattransmissie en NMDA-reseptor oorstimulering in verskeie breindele waarvoor heelwat bewyse in beide dier- en mense studies bestaan. Daar is egter geen antidepressiewe middels wat direk op die glutamaatsisteem inwerk om sodoende die onreëlmatighede in hierdie sisteem en sy betrokke komponente om te keer nie. Daarom is dit nodig om biologiese teikens en nuwe behandelingsmodaliteite te ondersoek wat die glutamaatsisteem teiken ten einde antidepressiewe en/ of pro-kognitiewe effekte te veroorsaak.

Bewyse verkry uit pre-kliniese navorsing, ondersteun die gebruik van geneesmiddelverbindings wat antidepressiewe effekte via die glutamaatbaan, direk of indirek, bewerkstellig. Dit sluit onder andere middels soos ketamien, memantien, allopurinol en natriumbensoaat in. Bewyse met betrekking tot allopurinol en natriumbensoaat se antidepressiewe effektiwiteit, is egter beperk en daarom was die doel van hierdie studie om

te bepaal of kroniese behandeling met die twee verbindings daartoe in staat is om depressiewe gedrag en/of depressie- geïnduseerde kognitiewe inkorting in die Flinders sensitiewe lyn (FSL) rotmodel te verminder, asook om die effekte op monoamien- en BDNF konsentrasies in verskillende breindele wat met depressie geassosieer word, te ondersoek.

Bevestiging van fenotipiese depressiewe gedrag van die FSL rotmodel vergeleke met die gesonde kontrole, die Flinders weerstandige lyn (FRL) rot, is gedoen deur gebruik te maak van die FST (geforseerde swem toets). Die geldigheid van die model is hiermee herbevestig en derhalwe kon dit aangewend word in die ondersoek na moontlike antidepressiewe effekte van allopurinol en natriumbensoaat. Die teenwoordigheid van depressie-geïnduseerde kognitiewe inkorting kon nie duidelik in hierdie model bevestig word deur gebruik te maak van die “Morris water maze” (MWM) toets nie, alhoewel klein veranderinge wel waargeneem is.

Die akute dosis-reeks analise met allopurinol en natriumbensoaat was effektief met betrekking tot vermindering van depressiewe gedrag in die FSL model deur gebruik te maak van die FST.

Die chroniese toedienning van ’n vaste dosis allopurinol, het ’n beduidende vermindering van depressiewe gedrag in die FSL rotmodel in die FST veroorsaak, in ooreenstemming met vorige studies. Soortgelyke, dog minder dramatiese resultate is waargeneem vir natriumbensoaattoediening volgens dieselfde chroniese toedieningsprotokol. Fluoksetien het aanleiding gegee tot ’n nie-statisties-betekenisvolle verminderde immobiliteit in die FST. Ketamien en memantien het soortgelyk immobiliteit verminder, alhoewel memantien se vermindering nie statisties betekenisvol was nie. Nie allopurinol of natriumbensoaat kon daarin slaag om ’n betekenisvolle vermindering in depressie-geïnduseerde geheue inkorting (herroep van geheue) gedurende MWM toetsing te veroorsaak nie. Tog het beide verbindings geheue konsolidasie bevorder tydens die vaslegging van geheue gedurende die vyf-dag opleidingsperiode. Interessant genoeg het chroniese fluoksetientoediening ’n aansienlike vermindering in die herroeping van geheue veroorsaak terwyl ketamien dit sigbaar verbeter het. Memantien het soortgelyke effekte as fluoksetien teweeg gebring. Slegs natriumbensoaat was daartoe in staat om striatale dopamien vlakke beduidend te verhoog alhoewel dit voorkom of allopurinol ook ’n positiewe effek op hierdie uitkoms kon gehad het. Soos verwag kon word, het fluoksetien prefontale kortikale noradrenalien- en serotonienvlakke verhoog. Data wat verkry is vir die res van die verbindings in die verskillende breindele was onvoldoende en kon nie gebruik word om bevindinge in die FST te staaf nie. Met die uitsondering van memantien, het geen ander behandelingsmodaliteit ’n suksesvolle verhoging in BDNF konsentrasie teweeg gebring nie.

Alhoewel die resultate onbeslissend voorkom, ondersteun literatuurbevindinge die resultate van hierdie studie met betrekking tot die antidepressiewe en potensiële kognitiewe effekte van allopurinol en natriumbensoaat. Dus moet die praktiese toepaslikheid van hierdie studie se resultate nie oor die hoof gesien word nie. Verdere studies is egter nodig om die effekte van allopurinol en natriumbensoaat in die behandeling van depressie en geassosieerde kognitiewe inkorting uit te klaar.

Sleutelwoorde: Major depressiewe versteuring (MDV), kognitiewe inkorting/ afwyking, N-metiel-D-aspartaat (NMDA), monoamien, brein-afkomstige neurotrofiese faktor (BDNF), allopurinol, natriumbensoaat, Flinders sensitiewe lyn (FSL)

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ADDENDUM A

A

ACTH	-	Adrenocorticotropic releasing hormone
AD	-	Aldehyde dehydrogenase
AESD	-	Acid-extraction sample diluent
AIDS	-	Acquired immune deficiency syndrome
ALLOP	-	Allopurinol
AMPA	-	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	-	Analysis of variance
AR	-	Aldehyde reductase

B

BDNF	-	Brain-derived neurotrophic factor
BPD	-	Borderline personality disorder
BSA	-	Bovine serum albumin
BST	-	Brain stimulation therapy

C

Ca^{2+}	-	Calcium
CaMKII	-	Calmodulin-dependent protein kinase II
cAMP	-	Cyclic adenosine monophosphate
CBT	-	Cognitive-behavioural therapy
cFos	-	Proto-oncogene (human homolog of the retroviral oncogene v-fos)

cGMP	-	Cyclic guanosine monophosphate
CNS	-	Central nervous system
CO	-	Carbon monoxide
COMT	-	Catechol-O-methyltransferase
CRF	-	Corticotropin-releasing factor
CREB	-	cAMP response element-binding
CREB-P	-	cAMP response element-binding protein
CSF	-	Cerebrospinal fluid

D

DA	-	Dopamine
DAAO	-	D-amino acid oxidase
DBH	-	dopamine-β-hydroxylase
DF	-	Dilution factor
DFP	-	Diisopropyl fluorophosphates
DHBA	-	3, 4-dihydroxy-benzylamine
DHMA	-	3, 4- dihydroxymandelic acid
DHPG	-	3, 4-dihydroxyphenylglycol
DNA	-	Deoxyribonucleic acid
DOPAC	-	Dihydroxyphenylacetic acid
DSM	-	Diagnostic and Statistics Manual of Mental Health

E

ECT	-	Electroconvulsive therapy

ED	-	Electrochemical detection
e.g.	-	Exempli gratia (for example)
ELISA	-	Enzyme-linked immunosorbent assay

F

FC	-	Frontal cortex
FDA	-	Food and drug administration
FKBP5	-	FK506 binding protein 5: Protein coding gene for depression
FLX	-	Fluoxetine
FRL	-	Flinders-resistant Line
FSL	-	Flinders-sensitive Line

G

GABA	-	Gamma-aminobutyric acid
GAD	-	General anxiety disorder
GLT	-	Glutamate transporter
GSK	-	Glycogen synthase kinase

H

HEPA	-	High-efficiency particulate arrestor
HIV	-	Human immunodeficiency virus
HPA	-	Hypothalamic-pituitary-adrenal
HPC	-	Hippocampus

HPLC	-	High-performance liquid chromatography
HVA	-	Homovanillic acid

I

IDO	-	Indolamine-2, 3-dioxygenase
IGF	-	Insulin-like growth factor
IL	-	Interleukin
i.p.	-	Intraperitoneally
IPT	-	Interpersonal therapy
IS	-	Internal standard

K

K^+	-	Potassium
KET	-	Ketamine
KMO	-	Kynurenine 3-monooxygenase
KYNA	-	Kynurenic acid

L

L1CAMS	-	L1 cell adhesion molecule
LPS	-	Lipopolysaccharide
LTP	-	Long-term potentiation

M

MAO	-	Monoamine oxidase

MAOI	-	Monoamine oxidase inhibitor
MAPK	-	Mitogen-activated protein kinases
MCH	-	Melanin-concentrating hormone
MDD	-	Major depressive disorder
MEM	-	Memantine
Mg^{2+}	-	Magnesium
MHPG	-	3-methoxy-4-hydroxyphenylglycol
miRNA	-	Micro ribonucleic acid
mPFC	-	Medial prefrontal cortex
mTOR	-	Mechanistic (mammalian) target of rapamycin
MWM	-	Morris water maze

N

Na^{+}	-	Sodium
Na^{+}/K^{+}-ATPase	-	Sodium-potassium-adenosine-trisphosphatase
n/a	-	Not applicable
NA	-	Noradrenaline
NAc	-	Nucleus accumbens
NCAM	-	Neural cell adhesion molecule
NIMH	-	National Institute of Mental Health
NMDA	-	N-methyl-D-aspartic acid
NK	-	Neurokinin
NO	-	Nitrous oxide
NOS	-	Nitric oxide synthases

NORT	-	Novel object recognition test
NPY	-	Neuropeptide Y
NR2B	-	Subunit of the NMDA receptor
n/s	-	Non-significant
NT	-	Neurotrophin

O

OCD	-	Obsessive compulsive disorder
OFT	-	Open-field test

P

PAF	-	Platelet activating factor
PFC	-	Prefrontal cortex
P-gp	-	Permeability glycoprotein
PKA	-	Protein kinase A
PKC	-	Protein kinase C
PKG	-	Protein kinase G
PMDD	-	Premenstrual dysphoric disorder
PMNT	-	Phenylethanolamine N-methyltransferase
PPD	-	Post-partum depression
PTSD	-	Post-traumatic stress disorder

Q

QA	-	Quinolinic acid

R

REM	-	Rapid eye movement sleep
rTMS	-	Repetitive transcranial magnetic stimulation

S

5-HT	-	Serotonin
§	-	Subsection
SAD	-	Seasonal affective disorder
SB	-	Sodium benzoate
SEM	-	Standard error of the mean
SNRI	-	Serotonin and norepinephrine reuptake inhibitors
STR	-	Striatum
SSRI	-	Selective serotonin reuptake inhibitor

T

TCA	-	Tricyclic antidepressant
TDO	-	Tryptophan 2, 3 dioxygenase
TNF	-	Tumour necrosis factor alpha
TREK-1	-	Potassium channel subfamily K member 2
TrkB	-	Tropomyosin receptor kinase B
TRD	-	Treatment resistant depression

V

VEGF	-	Vascular endothelial growth factor
VEH	-	Vehicle
Viz.	-	Namely
VMA	-	Vanillylmandelic acid
VNS	-	Vagus nerve stimulation
VTA	-	Ventral tegmental area

W

WHO	-	World Health Organisation

Chapter 1 : Introduction

1.1 Problem statement

Major depressive disorder (MDD) is one of several neuropsychiatric conditions to plague the world, being further subcategorised under affective disorders (NIMH, 2011). MDD is a universal condition (O'Donnell & Shelton, 2011) that is characteristically capricious, incapacitating and has a low remission rate prompting substantial socio-relational impairment worldwide (DSM-5™, 2013). Nearly 400 million individuals suffer from this complex disease (WHO, 2012), which may well be an underestimation due to insufficient diagnostics and/or underreporting. Both local and international estimates have reported lifetime prevalence for major depression in South Africa nearing 10%, with disease onset ranging between 22-26 years of age (Kessler & Bromet, 2013; Tomlinson *et al.*, 2009). Individuals with MDD experience a vast array of physical and psychological symptom manifestations that may be chronic or persistent (Kemp *et al.*, 2012; O'Donnell & Shelton, 2011) and of environmental or genetic origin (Nestler *et al.*, 2002; Kiyohara & Yoshimasu, 2009). Furthermore, MDD may co-exist or be triggered by other comorbidities such as anxiety disorders (e.g. phobias), obsessive compulsive disorder and other chronic disease states (e.g. myocardial infarction, HIV/AIDS, diabetes mellitus and Parkinson's disease) (Ménard *et al.*, 2015). The co-presentation of the aforementioned disease conditions substantially reduce patient recovery rate (DSM-5™, 2013). Various brain regions have been examined for involvement in MDD and its related symptomatologies (NIMH, 2011). Evidently, researchers were able to unearth morphological brain alterations in the form of amygdala enlargement, hippocampal shrinkage, neurodegeneration and brain tissue atrophy which has been implicated in the manifestation of impaired hippocampal and prefrontocortical activity, neurocognitive abnormalities such as impaired memory, concentration loss and indecisiveness (Kemp *et al.*, 2012; Pittenger & Duman, 2008). These changes and symptoms may also persist well after depressive symptoms have subsided (Solé *et al.*, 2015).

To date, several hypotheses have been suggested to underlie depression and involve an array of physiological and neurological systems that include cholinergic, monoaminergic, GABAergic and glutamatergic pathways, neuropeptides, the hypothalamic-pituitary-adrenal axis, circadian rhythms and neuroplasticity pathways (Chapter 2, §2.4.2). However, for this study the focus was placed on the glutamate hypothesis (Chapter 2, §2.4.2.4) of depression and how areas within this system viz., N-methyl-D-aspartate (NMDA) receptors (Chapter 2,

§2.4.4) and the kynurenine pathway (Chapter 2, §2.4.5), may present as potential targets in treating MDD and associate cognitive abnormalities. The glutamate system has long been investigated for its role in the development of depression based on evidence of NMDA receptor antagonism inducing antidepressant-like effects (Trullas & Skolnick, 1990). Additionally, researchers were able to establish that brain and plasma glutamate concentrations were substantially higher in depressed individuals (Pittenger & Duman, 2008). This surplus leads to toxic glutamate-induced structural and neurochemical changes in the brain affecting crucial neurological constructs as well as monoamine regulation and function leading to the development of depression and accompanying depression-induced cognitive anomalies (Sanacora *et al.*, 2012).

There are manifold treatment options available for MDD and associated memory and cognitive-impaired conditions. Unfortunately, few, if any, produce the desired therapeutic effect. Antidepressants effectively reduce the impairments caused by depression, but present with various safety and efficacy issues (especially when combined) as well as delayed onset of action (O'Donnell & Shelton, 2011; Sadaghiani *et al.*, 2011), necessitating novel treatment options. Ketamine, memantine, sodium benzoate and allopurinol are compounds of relevance that are all capable of regulating the glutamatergic system by antagonising (or modulating) the NMDA receptor either directly or indirectly, amounting to a reactive up-regulation of its own receptor-subunits (Gürbüz Özgür *et al.*, 2015; Gibney *et al.*, 2014; Levin *et al.*, 2015; Lindholm *et al.*, 2012; Kotermanski *et al.*, 2013; Lai *et al.*, 2012; Miller *et al.*, 2016). These compounds also have actions on neuronal growth modulating substances (e.g. brain-derived neurotrophic factor), second messenger systems as well as various other neurotransmitters within the CNS (First *et al.*, 2011; Prickaerts *et al.*, 2013; Jana *et al.*, 2013; Murck & Harald, 2013; Marvanová *et al.*, 2001) all known to be involved in depressive and cognitive disorders. The therapeutic potential of ketamine and memantine in improving memory and learning has raised much awareness of their use in numerous neurological and psychiatric illnesses, of which depression-induced memory impairment is one. However, allopurinol and sodium benzoate have only recently been comprehensively explored for their therapeutic abilities regarding antidepressant-like and procognitive abilities. Targeting synaptic function, neuroplasticity markers and related systems represent a new therapeutic approach in the treatment of depression-induced cognitive deficits.

Ketamine and memantine have been administered in both animal and human subjects via several routes, such as subcutaneous, intraperitoneal, intravenous, oral and intramuscular in order to assess both their antidepressant and cognitive enhancing activity (Irwin *et al.*, 2013; Machado-Vieira *et al.*, 2009; Kostadinov *et al.*, 2014; Serafini, 2012; Murck & Harald, 2013; Marvanová *et al.*, 2001). Sodium benzoate has been administered via the oral route to

evaluate its plausible procognitive and antidepressant actions within several human studies as well as in rodents (Jana *et al.*, 2013; Lin *et al.*, 2014). Earlier work with the sodium benzoate analogue, methyl parabenzoate, first described the central nervous system effects of the benzoates, demonstrating distinctive effects on cortical second messengers, cyclic adenosine monophosphate (cAMP) and cGMP, as well as altered cyclic nucleotide phosphodiesterases, in rats after chronic exposure (Harvey *et al.*, 1992). Considering the prominent role for both cAMP (Bernabeu *et al.*, 1997; Lynch, 2004) and cGMP (Bernabeu *et al.*, 1996) signalling in memory, these data are provocative for further research into the possible use of benzoate as a procognitive agent. Allopurinol has previously been used in animal studies relating to depressive behaviours as well as several other studies relating the effects of immobilization on rodent liver tryptophan pyrrolase, brain 5-hydroxytrytamine metabolism, tryptophan metabolism and xanthine oxidase activity (Møller & Kirk, 1978; Gibney *et al.*, 2014; Akhondzadeh *et al.*, 2006; Karve *et al.*, 2013; Miller *et al.*, 2006). If these treatment modalities prove effective, they might have a far reaching impact on the future of neuropsychopharmacology, not only as viable and novel treatment options in major depressive disorder and depression-induced deficiencies in cognition, but also in other neuropsychiatric illnesses. Furthermore, current discrepancies in data obtained from the literature may be due to various factors, with differences in routes of administration and dosages, different behavioural assessments applied, differing experimental conditions, as well as differences in the pharmacokinetics of each drug (Kotermanski *et al.*, 2013; Zoladz *et al.*, 2006).

Numerous animal models of depression have been developed (Chapter 2, Table 2-2) and include the Flinders sensitive line (FSL) rodent model. To date, no animal model has been developed that is capable of accurately reproducing the depression-like phenotype as observed in depressed human (Overstreet, 2005). The FSL rat is an ideal animal model of depression based on its sound face (ability to display symptoms relatable to the human depressive-like condition), construct (presents with dysregulation in key neurological systems similar to depressed humans) and predictive validity (responds to antidepressant therapies proven effective in treating human MDD) (Chapter 2, §2.6.1). Though not all depression-related characteristics and behavioural symptoms can be induced or measured in an animal, the FSL rat expresses a great deal of that which is seen in the depressed human, for example serotonergic, glutamatergic and neurotrophic alterations (Overstreet, 2005) have been observed as well as cognitive disturbances (Gómez-Galán *et al.*, 2013).

Thus far, an animal model of depression clearly displaying depression-induced cognitive deficiencies is yet to be generated and the FSL model has only recently been investigated for such abnormalities (Abildgaard *et al.*, 2011; Gómez-Galán *et al.*, 2013; Mokoena *et al.*,

2015). The behavioural analysis employed to do so is the novel object recognition test (NORT) which assesses recognition/declarative memory which depends on recalling/retrieving memories from long-term storage. Another available behavioural analysis is the Morris water maze (MWM) test and has not been utilised for assessing cognitive function in the FSL model. The MWM test not only evaluates spatial memory, but also memory consolidation and acquisition (Chapter 3, §3.2.3.3).

1.1.1 Primary aims and objectives:

- To establish whether the FSL model presented with depression-induced cognitive impairments with regard to learning and memory compared to its healthy control, the Flinders resistant line (FRL) rat, using a modified version of the Morris water maze (MWM) test (Hamlyn *et al.*, 2009).
- To determine by means of a dose-response curve if and at what acute dose two novel treatment options (allopurinol and sodium benzoate), both with activity in the glutamatergic pathway, are able to reduce depressive-like behaviours in the FSL model (with specific reference to immobility) when subjected to the FST.
- To determine if chronic treatment with a fixed dose of allopurinol and sodium benzoate are able to reverse depressive-like behaviours expressed by the FSL rat model using the FST.
- To investigate the effects of chronic treatment with ketamine, memantine and fluoxetine on depressive-like behaviours in the FSL model using the FST.
- To determine if chronic treatment with a fixed dose of allopurinol and sodium benzoate have effects on cognitive behaviour expressed by the FSL rat model using the MWM.
- To investigate the effects of chronic treatment with ketamine, memantine and fluoxetine on cognitive behaviour in the FSL model using the MWM test.
- To investigate the effects of chronic treatment with a fixed dose allopurinol and sodium benzoate on brain monoamine (e.g. noradrenaline, dopamine and serotonin) and brain-derived neurotrophic factor (BDNF) concentrations.
- To investigate the effects of chronic treatment with ketamine, memantine and fluoxetine on brain monoamine and BDNF concentrations.

1.1.2 Secondary objective:

- To reaffirm that the FSL model express depressive-like behaviours comparable to that expressed by human individuals with depression using a modified version of the forced swim test (FST) (Cryan *et al.*, 2002).

1.2 Expected outcomes

Based on current literature surrounding the neurobiological and pathophysiological mechanisms thought to underlie major depressive disorder and its associated cognitive abnormalities; the established compound (fluoxetine), two novel test compounds (allopurinol and sodium benzoate) and two reference compounds (ketamine and memantine) are expected to have the following outcomes on depressive-like behaviours and cognitive functioning as well as brain monoamine and BDNF levels:

- It is expected that the FSL model will present with depression-induced cognitive impairments pertaining to learning and memory compared to the FRL rat when using the MWM test as assessed in previous research (Abildgaard *et al.*, 2011; Erasmus *et al.*, 2015; Mokoena *et al.*, 2015).
- It is expected that the FSL model will express depressive-like behaviours that are phenotypical of the model using the FST as assessed in previous research (Cryan *et al.*, 2002; Overstreet, 1993)
- It is expected that treatment with allopurinol and sodium benzoate using an acute dose-range analysis protocol will produce a dose-response curve illustrating reduced depressive-like behaviours in the FSL model when exposed to the FST.
- It is predicted that chronic treatment with a fixed dose allopurinol and sodium benzoate will reverse depressive-like behaviours displayed by the FSL model using the FST.
- It is predicted that fluoxetine, ketamine and memantine will evoke effects on the depressive-like behaviours displayed by the FSL model when assessed using the FST.
- Furthermore, it is anticipated that chronic treatment with a fixed dose allopurinol and sodium benzoate will bring about changes is cognitive behaviours displayed by the FSL model during MWM testing.
- It is also anticipated that chronic treatment with fluoxetine, ketamine and memantine will bring about changes in cognitive behaviours displayed by the FSL model during MWM testing.
- Second to last, it is expected that chronic treatment with a fixed dose allopurinol and sodium benzoate will alter brain monoamine and BDNF concentrations.
- Finally, it is expected that chronic treatment with fluoxetine, ketamine and memantine will alter brain monoamine and BDNF concentrations.

1.3 Project layout

The study comprised of three phases *viz.*, Phase one: confirmation of the depressive-like phenotype of FSL rat and confirmation of the expression of cognitive insufficiencies within this model. Phase two: acute dose-ranging analysis for allopurinol, sodium benzoate. Phase three: the main experimental study (chronic drug treatment):

Phase 1: This phase was crucial in confirming the depressive-like phenotype of the FSL model compared to its healthy counterpart (FRL rat) using the FST. Furthermore, the FSL model had to be assessed for the expression of cognitive abnormalities compared to the FRL rat and this was done using the MWM test. Only after attaining the results of the aforementioned analyses could the second phase of the study be implemented – establishing a dose-response curve for allopurinol and sodium benzoate in the FSL model using the FST (Chapter 3, §3.2.4.1).

Phase 2: Before any chronic treatment studies could be performed an acute dose-ranging analysis had to be conducted for allopurinol and sodium benzoate. The results were used to generate a dose-response curve to indicate which dose allopurinol and sodium benzoate proved most effective in reducing depressive-like behaviours in the FSL model using the FST (Chapter 3, §3.2.4.2). To the best of our knowledge, no studies were published during the design of this dissertation, exploring the effects of varying doses allopurinol or sodium benzoate on depressive-like behaviours in the FSL rat using the FST.

Phase 3: The main experimental phase of the study investigated the effects of chronic treatment allopurinol, sodium benzoate, fluoxetine, ketamine and memantine on depressive-like behaviours displayed by the FSL model using the FST. Furthermore, administration of the aforementioned drugs according to the same treatment protocol took place in order to investigate their effects on cognitive behaviours in the FSL model using the MWM test. Finally, these compounds were assessed for their effects on brain monoamine and BDNF concentrations in the FSL model. Once again, the same treatment protocol was applied. To the best of our knowledge, no studies were published during the design of this dissertation exploring the effects of chronic allopurinol and sodium benzoate treatment on depressive-like and cognitive behaviours in the FSL model of depression using the FST and MWM test.

1.4 Dissertation layout

This dissertation will be written and submitted according to the standard traditional format guidelines as provided by the North-West University. The outlined format is as follows: Chapter 1: an introduction encompassing the aforementioned points (1.1 -1.4), Chapter 2: an overview of the literature relevant to this study, Chapter 3: project layout and concise description of the methods and materials utilised in this study, Chapter 4: comprising of experimental results, Chapter 5: a discussion surrounding the obtained results and finally, Chapter 6: containing concluding remarks and suggestions applicable to this study for further investigations.

Chapter 2 : Literature Review

Major depressive disorder is a severe neuropsychiatric condition capable of causing incapacitation and detrimental debility in several individuals independent of age, race, gender and ethnicity. MDD may be brought about by various environmental or psychological stressors inducing biological and/or bio-psychological adaptations with associated physical and neurochemical malignancies expressed as depressive symptomatologies and behaviours. Despite the fact that a vast array of therapies are available, therapeutic efficacy relevant to symptom improvement and remission (rate) is limited and several of these options present with numerous unpleasant consequences. Though these inadequacies may seem grim, research has led us to unearth multiple novel biological targeting options that may be proficient in eradicating the pathological causalities thought to underlie MDD hence, the focus of this study. The following literature surrounding MDD will be discussed in this chapter: epidemiology relevant to MDD, its associated signs and symptoms as well as current diagnostic frameworks, the disease aetiology with relevance to verifiable and theorised causations as well as a more thorough explanation on the involvement of the glutamatergic system with relevance to MDD, cognition and novel targeting opportunities, existing therapeutic options for the treatment of MDD, ratified animal models of depression followed by a conclusive précis of the explored literature.

2.1 Epidemiology

Major depressive disorder (MDD) forms part of several affective disorders in existence (NIMH, 2011). Others include dysthymia, minor-, psychotic- and postpartum depression as well seasonal affective disorder (SAD) (NIMH, 2011) – excluding bipolar disorder (Kessler & Bromet, 2013). MDD is one of the most common neuropsychiatric disorders in the world (O'Donnell & Shelton, 2011) being ranked the 4th primary source of global incapacitation (WHO, 2012) with an unpredictable course and low remission rate causing substantial disability and impaired socio-relational interactions on a global scale (DSM-5™, 2013). Approximately 350 million individuals worldwide endure this complex syndrome - an approximated 1 in 20 people (WHO, 2012) – which may well be an underestimation due to inadequate diagnostics and/or deficient case reports. Research conducted both locally and internationally estimates that the lifetime prevalence for major depression in South Africa is nearly 10%, which is lower compared to the projected 19.2% for the United States of America (Kessler & Bromet, 2013; Rumble, 1994; Tomlinson *et al.*, 2009), with the average age of onset being between 22-26 years (Kessler & Bromet, 2013; Tomlinson *et al*., 2009).

2.2 Signs and symptoms

Individuals living with MDD may experience both physical and psychological symptoms, manifesting as feelings of hopelessness, emptiness, guilt and negativity, irritability, psychomotor retardation, middle/terminal/initial insomnia or hypersomnia, diminished or increased appetite, weight gain or loss (a change of >5%), anxiety, continuous depressed mood, suicidal ideation and behaviours, anhedonia, lethargy, tedium and other physical manifestations (viz. muscular aches and pains, headaches and digestive abnormalities) as well as psychomotor agitation or retardation (O'Donnell & Shelton, 2011; NIMH, 2011; DSM-5™, 2013). These symptoms may be chronic or recurrent, affecting the manner in which these individuals go about their daily activities (Kemp *et al*., 2012).

Researchers have found that multiple brain regions involved in mood, appetite, sleep cycle and thought processes are also altered in depressed individuals (NIMH, 2011). Evidence for brain atrophy and neurodegeneration, such as structural brain changes and hippocampal shrinkage, have been documented and are causally related to neurocognitive insufficiencies' such as impaired memory, loss of concentration and indecisiveness (Kemp *et al*., 2012; Solé *et al*., 2015). Additionally, the hippocampus (HPC) and prefrontal cortex (PFC) present with reduced activity and impaired excitatory potentiation (Pittenger & Duman, 2008). Contradictory, the amygdala is enlarged and hyperfunctional with heightened medial PFC projection (Pittenger & Duman, 2008). The VTA, ventral striatum and NAc have also been implicated in MDD (Nestler & Carlezon, 2006). Abnormalities in projections and functions within these areas have been known to induce anhedonia and weakened stress response (Nestler & Carlezon, 2006). These changes and symptoms may also persist well after depressive symptoms have subsided (Solé *et al*., 2015). The HPC is also involved in HPA axis stress response regulation (Pittenger & Duman, 2008). Needless to say, structural or neurochemical changes in the HPC may then lead to impaired stress response coordination (Pittenger & Duman, 2008). Depressed individuals with vulnerability to stress have been found to express stress-induced histological changes in brain regions related to reward perception (e.g. nucleus accumbens – NAc and basolateral amygdala) causing a reduction in the number of spines (Figure 2-1) as well as the number, length and functionality of dendrites (Figure 2-2), contributing to the anhedonic features as seen in MDD and an enhanced risk of developing addictive behaviours (Russo & Nestler, 2013).

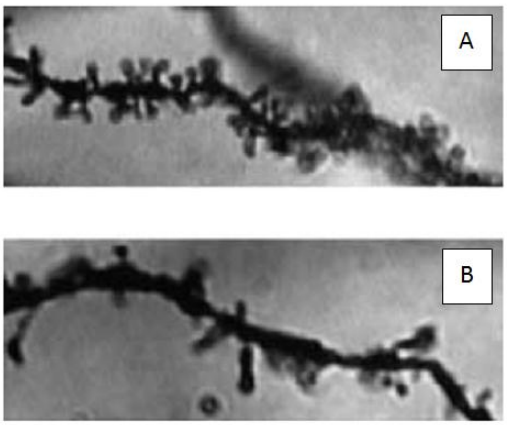

Figure 2-1: Reduced mPFC dendritic spine count in a rodent model subjected to a chronic stress paradigm (B) with relevance to MDD compared to controls (A) (Pittenger & Duman, 2008)

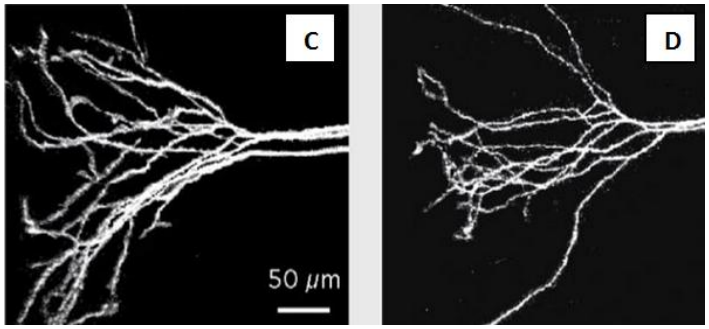

Figure 2-2: Reduction in volume and length of apical dendrites in the PFC of a rodent model subjected to a chronic stress paradigm (D) compared to controls (C) (Duman, 2009)

Other structural changes involve a diminished number of excitatory synapses and related gene expressions in both the HPC and PFC along with reduced cortical width and cell thickness in the PFC (Rajkowska *et al*., 1999).

2.3 Diagnosis

The *Diagnostic and Statistical Manual of Mental Disorders* fifth edition provides us with a well-constructed diagnostic framework for MDD (DSM-5™, 2013). Based on the aforementioned, for an individual to be diagnosed with MDD he/she has to experience five or more of the symptoms (as listed under §2.2) which must include depressed mood and/or anhedonia and may exclude weight change and suicidal ideation (DSM-5™, 2013). The patient has to experience these symptoms each day for the majority of the day and for a period no less than two weeks, significantly impairing or interfering with the individual's ability to function and go about his/her daily life and should not be a subsequent result of any

substance or other underlying medical disorders - between which clear distinction must be made during diagnostic procedures (DSM-5™, 2013).

2.4 Aetiology

MDD is caused by both genetic (40%-50%; e.g. neuroticism) and environmental factors, such as external stressors, traumatic experiences, viral infections, poor lifestyle, unrelated/related comorbidities, drug effects and alterations during brain development (Nestler *et al*., 2002; WHO, 2012; NIMH, 2011; DSM-5™, 2013; Kiyohara & Yoshimasu, 2009). MDD may co-exist or be precipitated by other illnesses for example, anxiety disorders: generalised anxiety disorder (GAD), obsessive compulsive disorder (OCD), post-traumatic stress disorder (PTSD), borderline personality disorder (BPD), social phobias and panic disorder as well as other chronic disease states that includes i.e. cardiovascular and infectious disease (e.g. myocardial infarction and HIV/AIDS), metabolic disorders (e.g. diabetes mellitus), sarcomas and other central nervous system (CNS) disorders (e.g. Parkinson's disease) (NIMH, 2011; Ménard *et a.l*, 2015). Substance and alcohol abuse have also been found to present alongside MDD (NIMH, 2011) and the co-presentation of the disease conditions listed above reduce time to recovery substantially (DSM-5™, 2013).

2.4.1 Genetic causalities

Not all individuals are genetically inclined to develop MDD as it may present itself independent of familial history, however, the amalgamation of several anomalous genes with environmental factors, traumatic experiences and stressful events, relational/social hardships or diseases complexes may exacerbate this disorder (NIMH, 2011; Kiyohara & Yoshimasu, 2009). Over the years various genes that have undergone polymorphism and have been identified and linked to the development of MDD (Table 2-1) (see Kiyohara & Yoshimasu, 2009 for review).

Table 2-1: Genes subject to polymorphic changes along with relevant functions within biological systems (Kiyohara & Yoshimasu, 2009):

Genes subject to polymorphism:	Biological/neurochemical function:
5-HT$_{1A}$	Facilitate cortical and limbic serotonergic activity. Act as auto-receptors on their own synapses → ↓5-HT release in raphe nuclei negative feedback mechanism
BDNF	Essential for synaptic plasticity, neuronal function modulation.
COMT	Enzyme responsible for catecholamine (DA/NA/adrenaline) metabolism
NA transporter	Regulation of pre-synaptic NA re-uptake and physiological noradrenergic effects
5-HT transporter	Modulation of neurotransmission and clearance of 5-HT from extracellular space
Tryptophan hydroxylase 1	Rate-limiting enzyme responsible for 5-HT synthesis (tryptophan (oxygenation) → 5-hydroxytryptophan (decarboxylation) → 5-HT)
Tyrosine hydroxylase	Enzyme responsible for catecholamine (DA) synthesis

5-HT receptor gene variations may affect their pre- and post-synaptic activities within the brain, for instance the release and/or consequent functioning of gamma-aminobutyric acid (GABA), glutamate, 5-HT (which specifically acts post-synaptically) and DA (Kiyohara & Yoshimasu, 2009). Of specific importance is the 5-HT$_{1A}$ receptor located both pre- and post-synaptically with a variety of important functions (Table 2-1) (Kapur & Remigton, 1996). In depressives, all 5-HT receptors are found to be quantitatively increased (Kapur & Remigton, 1996) possibly owing to receptor up-regulation. Polymorphic variants of the 5-HT transporter have been the focus in several MDD studies as they are known to cause reduced presynaptic cellular uptake of 5-HT in the brain of sufferers (Lesch *et al*., 1996).

Women and men experience depressive symptoms (Kiyohara & Yoshimasu, 2009) and respond to antidepressant therapies differently (NIMH, 2011). The same is true for older and younger adults, adolescents and children (NIMH, 2011). Depression is more common in woman than men, with disease burden 50% greater in women (Nestler *et al.*, 2002; NIMH, 2011). The higher frequency of depression occurring in women may be linked to their unique biological (vis-à-vis hormonal cycles) and psychological frameworks (NIMH, 2011). Women are especially susceptible to mood (heightened feelings of guilt, sadness, worthlessness) and behavioural vicissitudes leading to the development of depression consequential of hormone fluctuations as can be seen with post-partum depression (PPD), premenstrual dysphoric disorder (PMDD), the commencement of menopause and its possible progression into osteoporosis (NIMH, 2011). Men, instead, become more ill-tempered, aggressive or even abusive, exasperated, anhedonic, lethargic, irresponsible, regularly suffer from insomnia and often resort to substance use and/or abuse (NIMH, 2011). Though the number of suicide attempts is greater for women, the success in doing so (resulting in death) is

exceeded by men (NIMH, 2011). Women may also exhibit improved response to SSRIs compared to men, who in turn, benefit more greatly from being treated with TCAs (Kornstein *et al*., 2000). Older adults and geriatrics express depressive symptoms and behaviours in a different less obvious manner than seen in young adults, making it difficult to differentiate between feelings of grief and severely depressed mood (melancholia) when diagnosing such persons (NIMH, 2011; DSM-5™, 2013). However, older adults more commonly present with psychomotor disturbances (DSM-5™, 2013). These individuals are also more likely to present with other comorbidities that trigger the development of MDD such as, cancer and cardiovascular disease (NIMH, 2011). The rate of depression driven-suicide in geriatrics is shown to be elevated which is surprising considering that older depressed adults respond well to antidepressant mono- and combination therapy (NIMH, 2011). Likewise, diagnosing children with depressive disorder is challenging due to the ambiguity of their symptoms (DSM-5™, 2013). The prevalence of depression in boys and girls prior to adolescence is equivalent, after which the frequencies start to favour female adolescents (Bernal *et al*., 2007). They often become anxious, experience hypersomnia and hyperphagia, try to avoid school or related environments by feigning illness, and may even fear the harm or death of a parent or express separation anxiety (NIMH, 2011; DSM-5™, 2013). As they approach puberty they start experiencing fluctuated mood, feel misunderstood and often present with co-morbidities that are unearthed during adolescence and include anxiety and eating disorders, substance use and/or abuse as well as suicidal tendencies (NIMH, 2011). Notably, children and adolescents diagnosed with MDD express symptoms of severe irritability and crabbiness rather than depressed mood along with an inability to reach an ideal weight (DSM-5™, 2013). Adolescents with MDD can be effectively treated with combined therapies (NIMH, 2011).

2.4.2 Hypothesised causalities

Numerous hypotheses exist for the neurochemical basis of MDD, with nine theoretical hypotheses/models put forward to explain its underlying pathology. These theories include the following systems: cholinergic, monoaminergic, GABAergic, glutamatergic, neuropeptide Y, the hypothalamic-pituitary-adrenal (HPA) axis, circadian rhythm adaptations, neurotrophic and neuroplasticity alterations along with cytokine related neuro-inflammatory changes.

2.4.2.1 Hypercholinergic hypothesis: Cholinergic model of depression

In 1972, Janowsky *et al.* (1972) proposed that there may be cholinergic-hyperactivity and -super sensitivity with an associated adrenergic under-activity in depressed individuals which led to the birth of the cholinergic model of depression that implicates cholinergic hyperactivity in a depressive brain (Overstreet *et al*., 2005). Unfortunately, this theory could not be

supported as anticholinergics were found to be ineffective in the treatment of depression. Nevertheless, subsequent preclinical and clinical research progressing from their findings supports this theory (reviewed in O'Leary *et al*., 2015). Some decades later, Drevets & Furey (2010) found that an anticholinergic agent is effective in treating treatment-resistant depression (TRD) (Drevets & Furey, 2010). Today, various muscarinic and nicotinic receptor antagonists are under evaluation for antidepressant-like qualities (Drevets *et al*., 2013). Dihydro-beta-erythroidine and mecamylamine (with β_2 and α_7 receptor activity) are two anticholinergics known to possess antidepressant-like effects in rodents. Other drugs include sazetidine-A ($\alpha_4\beta_2$ partial agonist), varenicline and cytisine (partial agonists at nicotinic receptors) showing preclinical antidepressant-like efficacy (Turner *et al*., 2010; Mineur et al, 2011). Augmentation strategies have also been investigated in combining SSRIs and MAOIs with α_7 receptor agonists, exhibiting positive results in various animal models with the latter also causing procognitive effects (Andreasen *et al*., 2011). The muscarinic receptor antagonist, scopolamine, has also proven effective in the rapid reversal of depressive-like behaviours (Drevets & Furey, 2010).

Of special significance is that depression often co-presents with cognitive deficits (central to the DSM-5™ MDD diagnostic criteria) (Solé *et al*., 2015) - such as indecisiveness, disruptive learning, memory and attention processes (Hasselmo, 2006; McGaugh & Cahill, 1997). Brain regions involved in these processes, viz. the HPC and the frontal cortex (FC), are both regulated by the cholinergic system (Hasselmo, 2006; McGaugh & Cahill, 1997). Depressive patients presenting with HPC atrophy and volume reduction, also show traces of reduced neuropil volume and neuron size (Stockmeier *et al*., 2004). Moreover, centrally acting cholinergic agents are widely used for their procognitive effects (Kruk-Slomka *et al*., 2012). The cholinergic theory of depression is also supported by animal work, where the Flinders-Sensitive Line (FSL) rat model of depression presents with increased cholinergic activity/sensitivity in various regions of the brain (Overstreet *et al*., 1984; Pepe *et al*., 1988).

2.4.2.2 Monoamine deficiency hypothesis: Serotonergic, noradrenergic and dopaminergic models of depression

The monoamine deficiency hypothesis specifically involves 5-HT, NA and DA and proposes that monoamine levels and/or activity are dysregulated in the brain of depressives (Overstreet *et al*., 2005). This may result from impaired neurotransmitter-synapse signal transfers that may hamper the essential signalling cascades that follow (see Figure 2-3, 2-4 and 2-5 for neurotransmitter pathways), ultimately preventing receptor activation, gene transcription and target responses leading to the development of depressive symptoms and behaviours (Kiyohara & Yoshimasu, 2009). Irregularities in concentrations of the

aforementioned neurotransmitters may also originate from genetic mutations in key enzymes and/or receptors involved in monoaminergic pathways (Table 2-1) (reviewed in Kiyohara & Yoshimasu, 2009). Upon investigation of various compounds unrelated to affective disorders specifically imipramine (the first TCA and originally developed for the treatment of psychosis), iproniazid (first MAOI) and reserpine (antihypertensive that causes monoamine store depletion and induces depressive symptoms); researchers were able to conjoin 5-HT and NA abnormalities to psychopathologies that underlie depressive disorders (Krishnan & Nestler, 2008). Serotonergic abnormalities have long been investigated in individuals with depression; although both an under-activity (reduced 5-HT reactivity) and over-activity (enhanced 5-HT_{1A} reactivity) has been reported (Lesch, 1990; Overstreet *et al*., 2005). Serotonergic hyperactivity may lead to higher 5-HT catabolism, as is the case in chronic stress, where 5-HT synthesis demands cannot match 5-HT metabolism (Kiyohara & Yoshimasu, 2009). This finding is largely based on the observation that most clinically effective antidepressants modulate either serotonin or noradrenalin, or both. Interestingly, both serotonin reuptake enhancers and inhibitors are effective antidepressants (Harvey, 2008). Overall, the theory is that depression is associated with reduced serotonergic sensitivity (Shayit *et al*., 2003). However, the role of other biogenic amines cannot be ignored, with various antidepressants acting to increase synaptic levels of dopamine or noradrenaline.

Copious amounts of DA are found in the brain where it plays an important role in the mesocorticolimbic modulation of hedonic, motivational and affective behaviours or sensations (Kiyohara & Yoshimasu, 2009). Various pre-clinical and clinical investigations have surfaced DA's involvement in affective disorders where manic symptomatologies have been linked to increased DA concentrations in contrast to the lower concentrations measured in depression (Furlong *et al*., 1999). Decreases in NA concentrations and DA transporter binding molecules have also been documented in depressed individuals (Overstreet *et al*., 2005). Most antidepressants marketed today are aimed at restoring monoamine balance and functionality in the brain by enhancing neurotransmission through reduced neurotransmitter reuptake (e.g. SSRIs and SNRIs) and/or metabolism (e.g. MAOIs) (SAMF, 2010). Indeed, new generation antidepressants, such as agomelatine, act to increase cortical dopamine and noradrenaline levels thereby bolstering frontal cortical activity, especially that of cognitive processing (Harvey & Slabbert, 2014). Other approaches have also been taken in the development of multi-modal/targeting strategies that encompass all of the aforementioned monoamines (O'Leary *et al*., 2015). However, the conclusions drawn from monoamine-based antidepressant research over the years rely more on the mechanistic actions/efficacies of antidepressant therapies than actual tissue or body fluid

measurements of these neurotransmitters leading to the generation of this theory (Krishnan & Nestler, 2008).

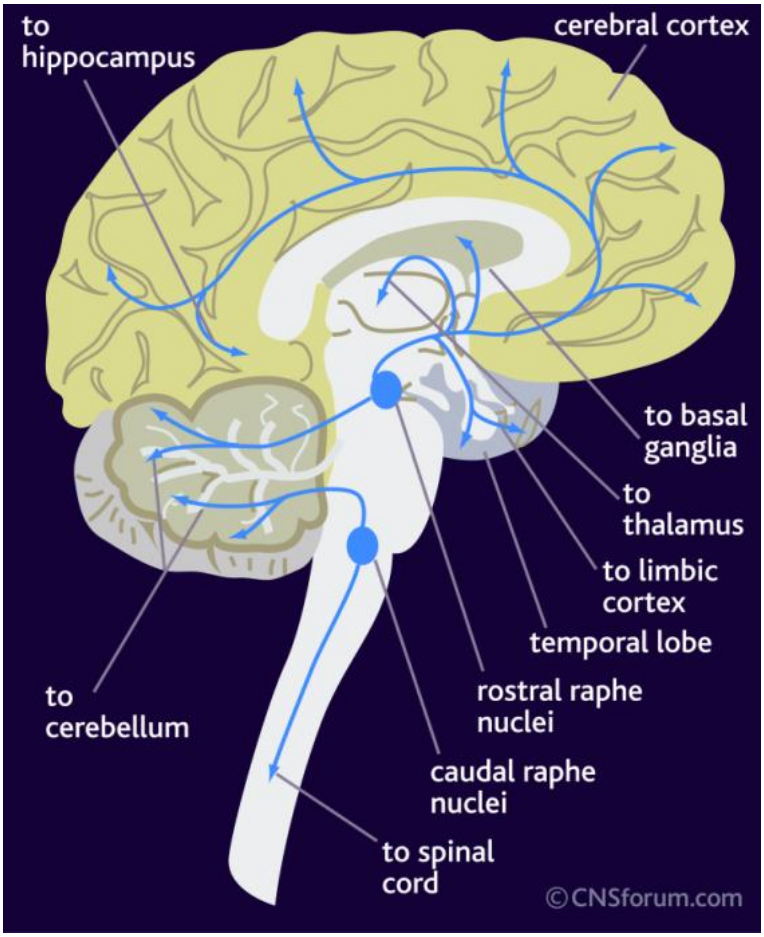

Figure 2-3: Serotonin pathways in a normal human brain (Lundbeck institute, 2014).

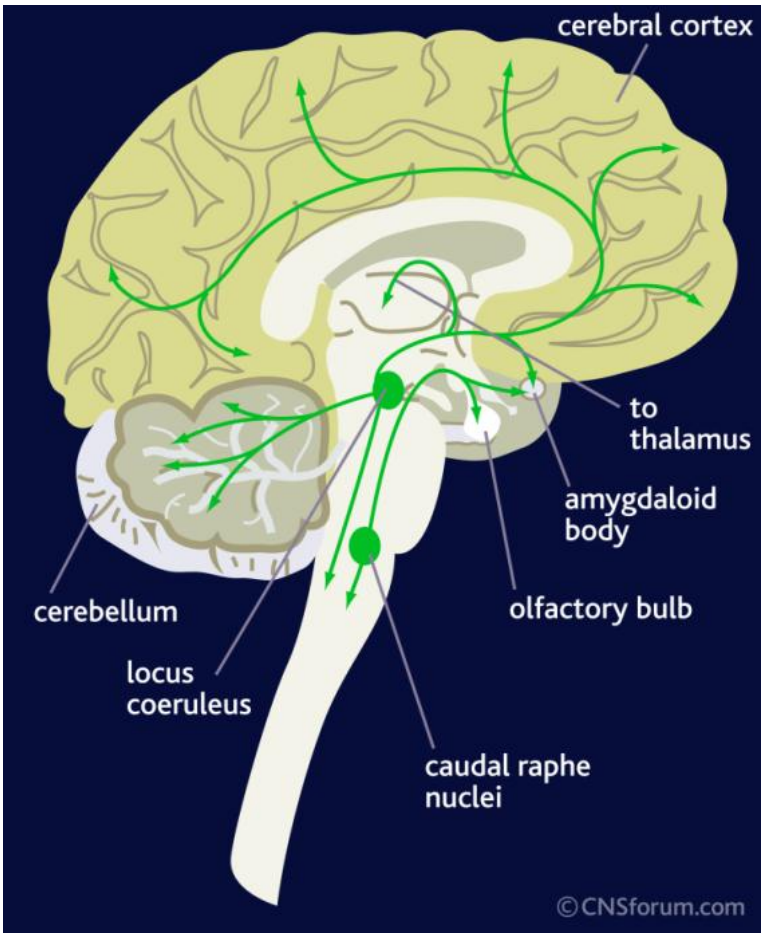

Figure 2-4: Noradrenaline pathways in a normal human brain (Lundbeck institute, 2014).

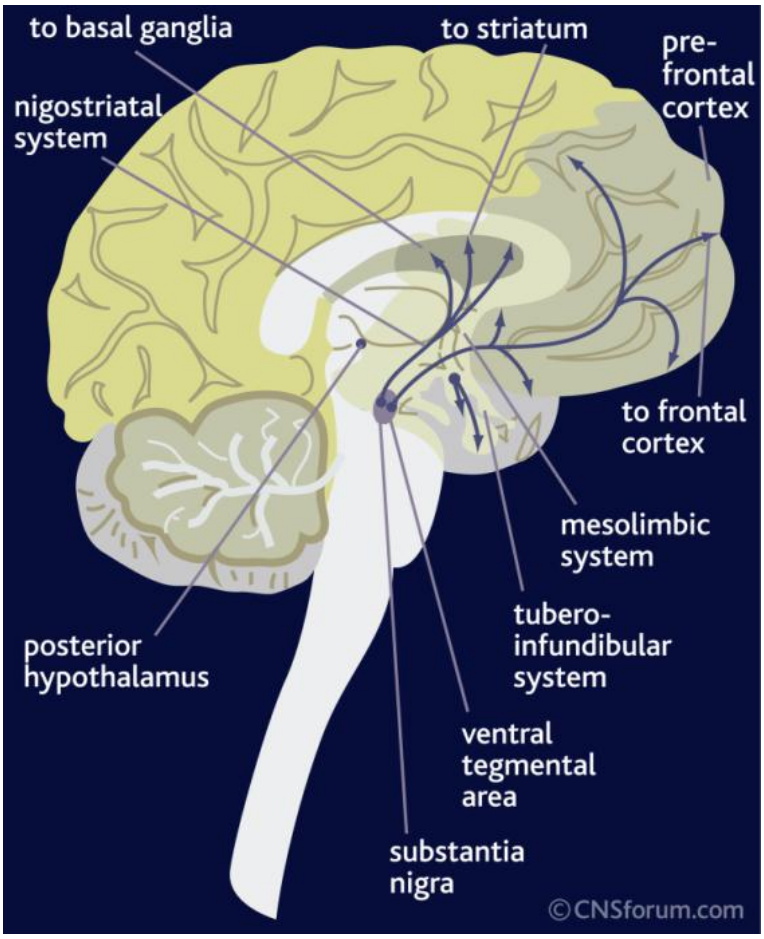

Figure 2-5: Dopamine pathways in a normal human brain (Lundbeck institute, 2014).

2.4.2.3 GABAergic deficiency hypothesis: GABAergic model of depression

A large body of preclinical and clinical research has gone into examining GABA and related receptors in connection to depression treatment and its underlying pathologies (Cryan & Slattery, 2010; Luscher *et al*., 2011). GABA neurotransmitter deficits have been noted in the brain, plasma and cerebrospinal fluid of depressed patients leading to up-regulation of certain GABA-receptor subunits (Petty & Schlesser, 1981). Both $GABA_A$ and $GABA_B$ receptors have been implicated in the pathophysiology of both anxiety- and affective disorders (Luscher *et al*., 2011). GABAergic deficits in rodent models have shown to induce depressive-like behaviours along with related cognitive abnormalities (Crestani *et al*., 1999). Genetic $GABA_A$ receptor polymorphisms have also been associated with the development of affective disorders (Craddock *et al*., 2010). Prior to the year 2010, Klempan *et al*. (2009) had uncovered $GABA_B$ neurophysiological discrepancies in post-mortem brain examinations of depressed individuals (Klempan *et al*., 2009) and more than 15 years ago, researchers were

able to demonstrate the antidepressant-like effects of GABA-receptor blockade with later studies being able to support these findings (Slattery *et al*., 2005). Benzodiazepine ($GABA_A$ receptor modulators) administration to depressives has also been shown to produce antidepressant-like effects (Rudolph *et al*., 1999), further validating the GABAergic hypothesis of depression. It is also worth mentioning that upon investigation of GABAergic mechanisms and pathways (Figure 2-6), distinct connection between GABAergic, monoaminergic and HPA-axis activity have emerged (Luscher *et al*., 2011).

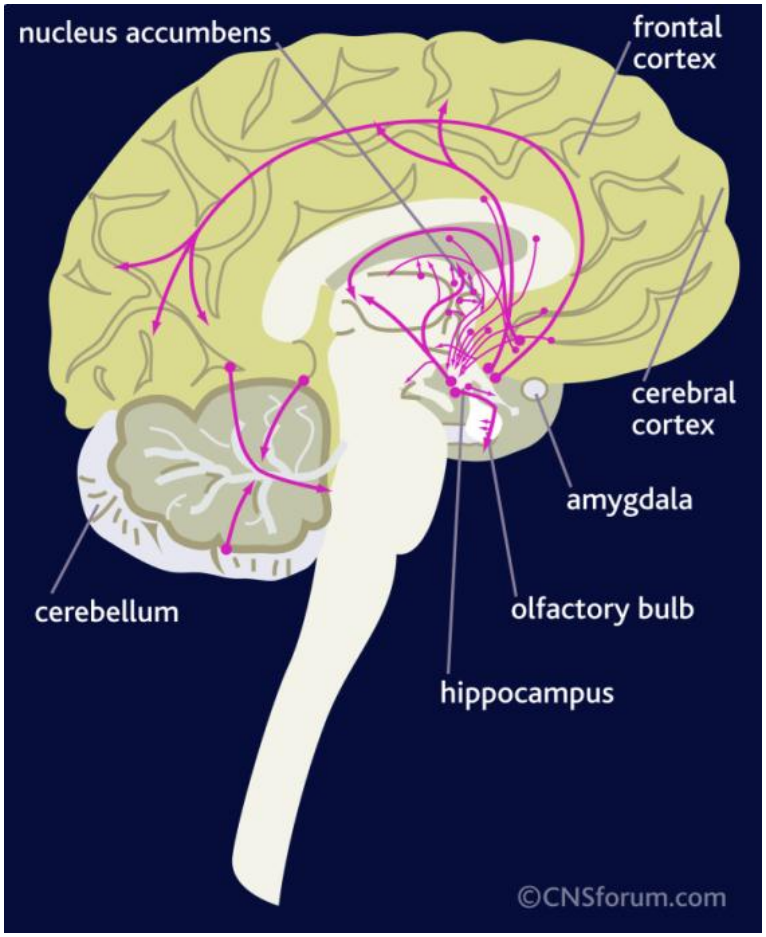

Figure 2-6: GABA pathways in a normal human brain (Lundbeck institute, 2014).

2.4.2.4 Glutamate hyper-/hypo-activity hypothesis

The glutamatergic hypothesis thought to underlie depression is closely associated with that of neuroplasticity with regard to pathophysiological outcomes on neuronal circuitry, neurogenesis and neuroplasticity (Sanacora *et al*., 2012). The basis for this theory had been set upon the discovery that NMDA receptor antagonists cause antidepressant-like effects

(Skolnick & Trullas, 1990). In a review by Sanacora *et al*., (2012) the relationship between glutamate release and functionality and monoamine modulation, stress outcomes and glucocorticoid activity were investigated and clinical evidence supporting glutamatergic abnormalities as an underlying theory for the development of affective disorders in association with cognitive dysfunction was highlighted. Variations in glutamate concentrations measured in the brain, cerebrospinal fluid and plasma of those suffering from affective disorders have prompted further exploration into the causative mechanisms (refer to Figure 2-7 for glutamatergic pathways) (Sanacora *et al*., 2012). Subsequent investigations reported the following conclusions: increased glutamate concentrations, reduced glutamine-to-glutamate plasma ratio in depressed individuals with visible reductions in plasma glutamate measurements after antidepressant therapy, increased platelet glutamate concentrations and an inverse relationship between cerebrospinal fluid glutamine and glutamate levels – the latter being reduced (Hasler *et al*., 2007). Additionally, increased glutamate concentrations were reported upon post-mortem investigations of depressed patient frontal cortices (Hashimoto *et al*., 2007). Surplus glutamate release may result from severe stress stimuli, followed by impaired glutamate catabolism with subsequent reformations in synaptic neurotransmission, dendrite malformation, decreased synaptic spine formation and loss of glial cells followed by volumetric decline (Pittenger & Duman, 2008; McEwen, 2005). However, both increased and decreased glutamate metabolite levels have recently been reported in various brain regions of patients with MDD experiencing a major depressive episode, late-life depression, remissive patients as well as young patients at risk of developing MDD (Hasler *et al*., 2007; Sanacora *et al*., 2004).

It is evident from research that the exact mechanisms thought to underlie the glutamatergic hypothesis require further extensive investigation before any concrete conclusions can be made with regard to its involvement in MDD. In spite of this, there have been studies demonstrating the detrimental effects of excess extra synaptic glutamate on surrounding tissues giving rise to neurodegeneration, neurotoxicity, and decreased neuroplasticity with subsequent regional brain volume reductions as can be seen in patients with affective disorders (Koolschijn *et al*., 2009).

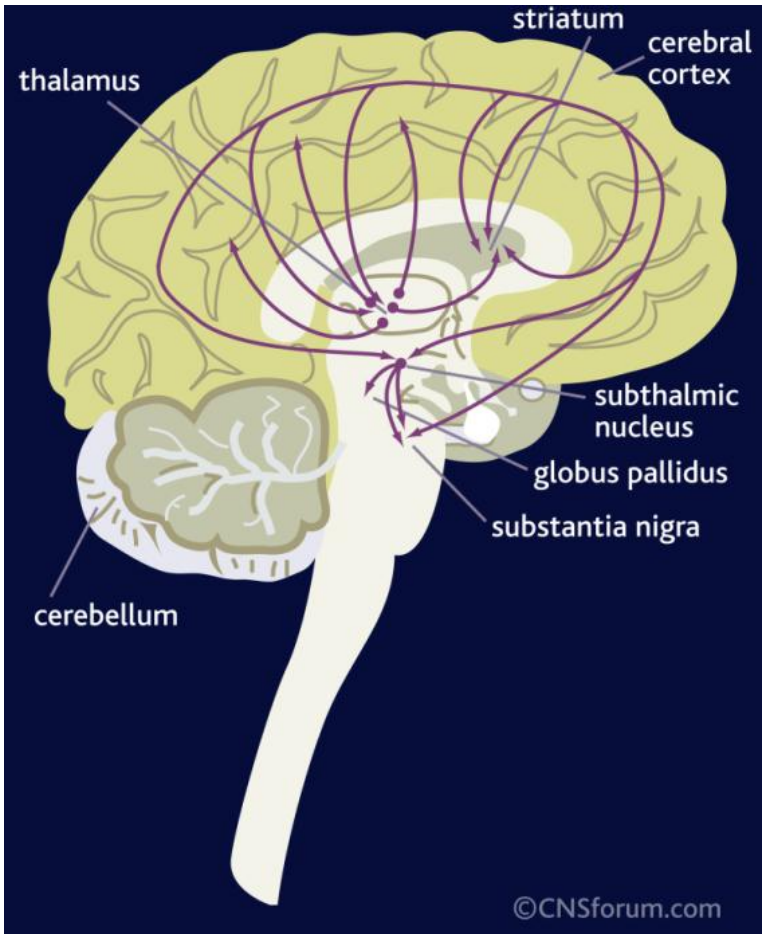

Figure 2-7: Glutamate pathways in a normal human brain (Lundbeck institute, 2014).

2.4.2.5 Neuropeptide hypothesis: Neuropeptide Y (NPY) model of depression

Neuropeptides (e.g. NPY) are implicated in several key functions within the CNS that include the regulation of monoamine release and transmission in addition to HPA axis activity modulation - depending on brain region and/or receptor location (Overstreet *et al*., 2005; O'Leary *et al*., 2015). The aforementioned alongside substantiating evidence of reduced CSF and plasma NPY concentrations measured in depressives, allows a connection to be drawn between MDD and NPY activity (Hashimoto *et al*., 1996). To further support its involvement, data from ECT studies in depressed individuals confirms reversal of NPY deficits leading to the conclusion of pathological NPY hypofunction underlying MDD (Overstreet *et al*., 2005). Additionally, NPY deficits in animal models were also inverted using ECT, antidepressant treatment and lithium administration (Overstreet *et al*., 2005). Preclinical and clinical studies have been conducted to investigate the underlying mechanisms of NPY in both depression and anxiety disorders (Overstreet *et al*., 2005).

Research states that NPY acts both pre- (Y_2) and postsynaptically (Y_1) with the Y_2 receptor being the target involved in antidepressant-like effects pertaining to receptor antagonism (Bacchi *et al*., 2006).

2.4.2.6 Hypothalamic-pituitary-adrenal (HPA) axis hyperactivity hypothesis: HPA axis model of depression

HPA axis anomalies (hyperfunction) have been extensively investigated and substantiated in MDD with the following distinct changes documented: enhanced central corticotropin releasing factor (CRF), enhanced peripheral adrenocorticotropic releasing hormone (ACTH) and glucocorticoid cortisol (Figure 2-9) (in humans)/corticosterone (in rodents) (Overstreet *et al*., 2005; DSM-5™, 2013). These changes have also been associated with symptoms of psychosis, suicidal tendencies and melancholy in MDD sufferers (DSM-5™, 2013). Glucocorticoids play an important role in HPA axis functionality under both baseline and stressful conditions (e.g. early-life traumas) (Pariante & Miller, 2011) with regard to CRF, vasopressin, monoamine and ACTH release modulation (Figure 2-8) (Pariante & Lightman, 2008). Dysfunction within this system has detrimental effects on both central (e.g. emotional and cognitive processing, neurogenesis, neuroplasticity and various brain structures) and peripheral processes (e.g. immunomodulation and metabolic processes) (Raison *et al*., 2006). Abnormal activity, such as impaired glucocorticoid feedback inhibition has been observed in MDD and successfully reversed via antidepressant treatment (Pariante, 2006). The mechanisms underlying the pathological outcomes can be better expressed in view of Figure 2-9: Hypothalamic-pituitary-adrenal axis in a depressed human. Due to the impaired glucocorticoid feedback inhibition, as stated earlier, cortisol oversecretion (hypercortisolaemia) and excessive receptor stimulation occurs with subsequent receptor desensitisation (Pariante, 2006). This in turn leads to dysfunctional monoaminergic neurotransmission in the brain, increased HPA axis activation, hypothalamic CRF hypersecretion and excessive ACTH release from the pituitary gland (Pariante, 2006). The surplus ACTH further stimulates adrenal gland causing adrenal gland enlargement and cortisol release, intensifying the extent of receptor desensitisation and lack of inhibitory feedback (Nemeroff & Vale, 2005). Consequently, the impaired feedback also results in an enhanced immune-inflammatory response that further activates the HPA axis (Raison *et al*., 2006). Increases in circulating cortisol have been proposed to mediate hippocampal shrinkage in MDD, possibly via increasing glutamate release (Holsboer, 2000).

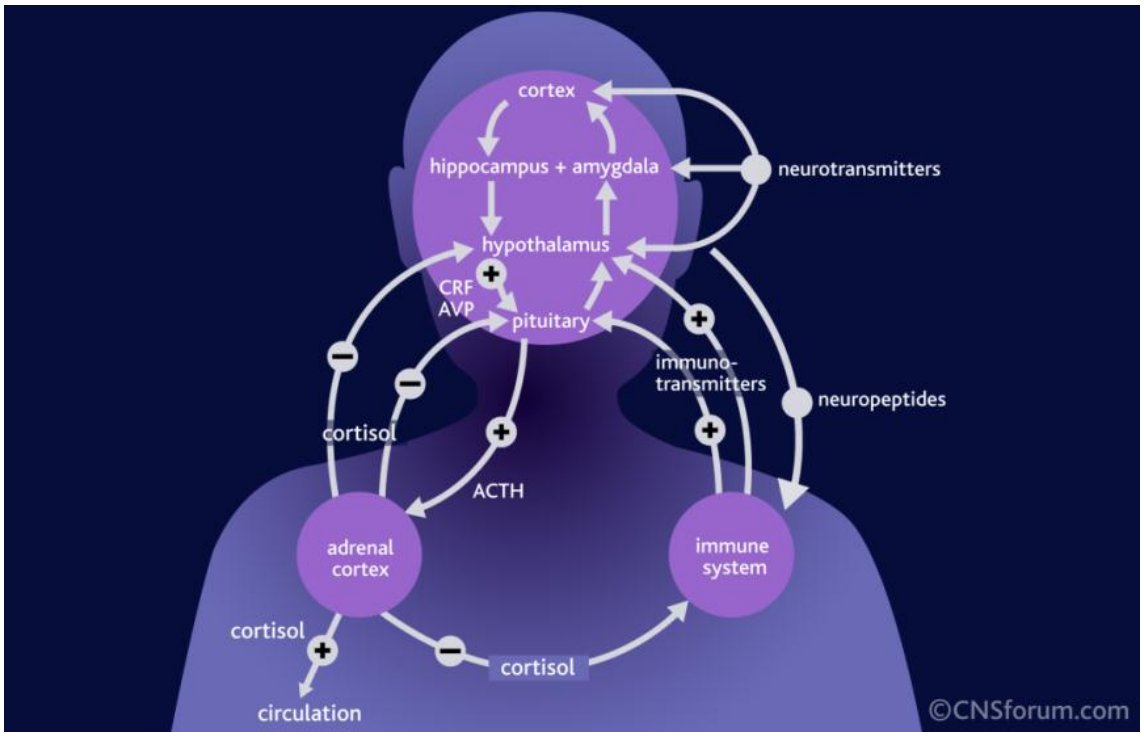

Figure 2-8: Hypothalamic-pituitary-adrenal axis in a normal human (Lundbeck institute, 2014).

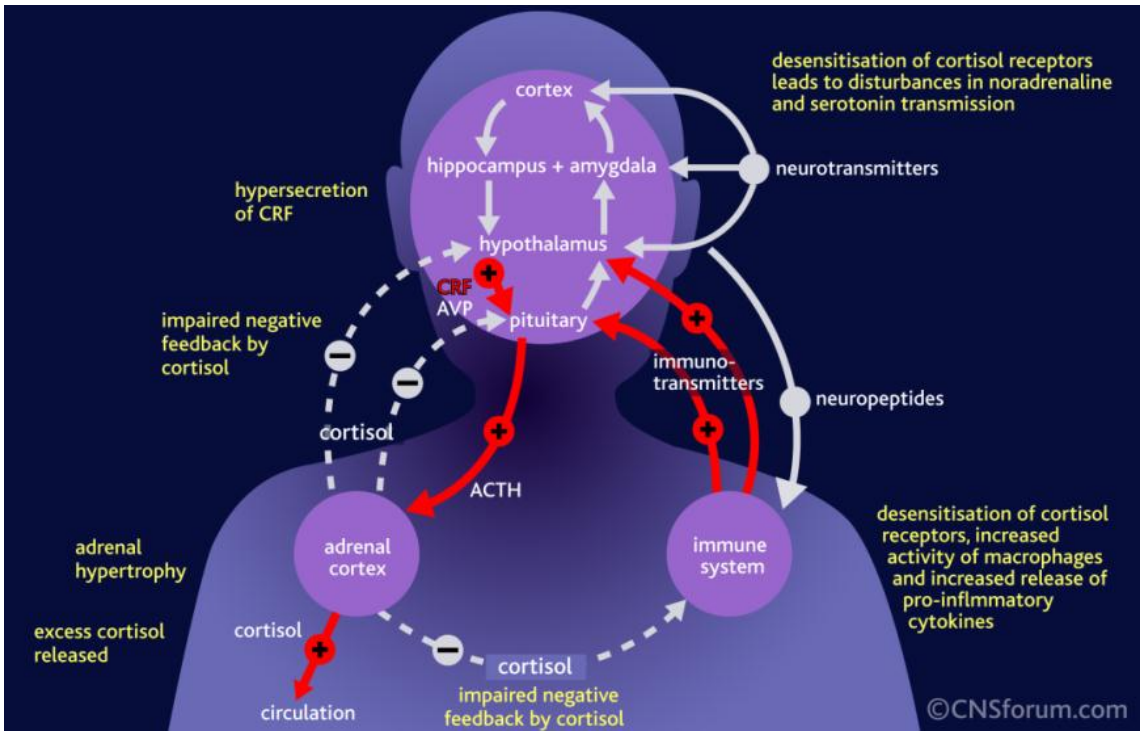

Figure 2-9: Hypothalamic-pituitary-adrenal axis in a depressed human (Lundbeck institute, 2014).

2.4.2.7 Circadian rhythm hypotheses: Phase-shift, reduced latency to REM and social rhythms in depression

Humans are dependent on the sound preservation of internal physiological rhythms that fluctuate over a 24-hour period (Germain & Kupfer, 2008). A review by Germain and Kupfer (2008) provides clear elaboration on how disruptions within these rhythms may lead to physical and psychological manifestations detrimental to individual health, increasing susceptibility to illness development as evinced in depressed patients. They further expound how circadian rhythm modulation encompasses mood, sleep-wake patters, social activity, temperature and various endocrine, neuroendocrine and metabolic processes (Germain & Kupfer, 2008). The circadian rhythm (expanding over a 24-hour dark-light cycle) hypothesis postulated to underlie depression proposes that sufferers experience oscillating glycolysis and gluconeogenesis periods that differ from healthy individuals as well as associated mood improvement closer to the evening (Bunney & Bunney, 2000). Pertaining to the latter, the inverse is true in depressives upon awakening (Germain & Kupfer, 2008). These individuals also present with varying cortisol (rousing hormone) and melatonin (sleep hormone) modulation (Peeters *et al*., 2003). Altered sleeping patterns are very common under depressed individuals with the main complaints being inability to fall asleep, remain asleep and early-morning wakefulness (Tsuno *et al*., 2005). Individuals with MDD also present with rapid REM sleep initiation, prolonged REM sleep, higher number of eye movements during REM and reduced slow-wave sleep (Tsuno *et al*., 2005). Chronic stress has also been found to have deleterious effects on circadian corticosterone and cortisol rhythms in rodents and humans, respectively (Sephton *et al*., 2000). Aside from physiological evidence supporting this hypothesis, pharmacological therapies have also been able to validate these findings. Examples include antidepressants (specifically MAOIs), antipsychotics, mood stabilisers, agomelatine and melatonin therapy which prove to be beneficial in modulating circadian rhythms with specific regard to the sleep-wake cycle (Winokur *et al*., 2001). Agomelatine acts to re-entrain circadian rhythms to effectively treat MDD (Harvey & Slabbert, 2014).

2.4.2.8 Neuroplasticity hypothesis: Neurotrophin model of depression

Neurotrophins are renowned for their ability to modulate growth and activity of neurons while especially BDNF, plays a prominent role in mood disorders (Hasselbalch *et al*., 2012). MDD has been known to present with abnormal modulation of various neurotrophic factors, such as BDNF, vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF) and fibroblast growth factor (Fakhoury, 2015). BDNF is a secretory protein expressed profusely in the adult limbic system (Krishnan & Nestler, 2008), primarily by CNS neurons and to a lesser extent by astrocytes and microglial cells and which plays a crucial role in neuron and

dendrite endurance (and death), dendrite withdrawal and synaptic plasticity (Lessmann & Brigadski, 2009). BDNF levels are decreased in depressed individuals and are normalized after treatment with antidepressants (Hasselbalch *et al*., 2012). Both BDNF and its relevant receptor (TrkB) are reduced in the HPC and serum, but oppositely increased in the NAc and NAc-VTA pathway, of patients with MDD and pathological/chronic stress disorders (Schecterson *et al*., 2012). Microglial BDNF release promotes synaptic plasticity, whereas astrocyte pro-inflammatory cytokines stimulate BDNF expression (Kettenmann *et al*., 2011; Meeuwsen *et al*., 2003) providing us with a connection to neuro-inflammatory pathways and how immunomodulation may impact neurotrophin release and neuroplasticity conduits. Microglial cells are crucial neuro-inflammatory modulators with either neuroprotective and/or neurotoxic effects capable of impacting neuroplasticity (Kettenmann *et al*., 2011; Saha *et al*., 2006). The phenotypical neuroprotective effects of microglial cells relate to their T-cell activation or interaction that increases anti-inflammatory neurotrophin (i.e. BDNF) and cytokine release with subsequent enhanced synaptic plasticity (Kettenmann *et al*., 2011). At physiological concentrations, cytokines (e.g. IL-1β, IL-6 and TNF-α) promote neurogenesis and enhance neuroplasticity (Eyre & Baune, 2012). However, excessive pro-inflammatory cytokine induction (e.g. by means of LPS administration in animals) causes microglial activation that impairs neurogenesis, reduces BDNF release and prevents beneficial neuroplasticity processes – hence, their neurotoxic effects (Guan & Fang, 2006). Further indication for BDNF's important role in neuroplasticity lies within its genetic polymorph, Val66Met, which is responsible for morphological alterations of the HPC (e.g. reduced volume) and cortices, as evidenced by both animal and human studies, leading to reduced hippocampal volume (reviewed in Ménard *et al*., 2015). Alongside neurotrophic factors, monoamines have also been investigated for their role in MDD related neuroplasticity. In a review by Haase and Brown (2015), they provide a diagrammatic overview of how elevated monoamine levels lead to increased gene transcription followed by the release of neurotrophins involved in HPC synaptic plasticity and neurogenesis and how individuals with MDD have been found to present with both functional and structural cell changes within the HPC, ultimately leading to the reduced HPC volume we are all familiar with today. The latter may then suggest a link between 5-HT activity and BDNF gene transcription based on the ability of monoamines to regulate BDNF modulation and associated downstream activities.

Reduced BDNF concentrations measured in depressives may lead to the enhanced susceptibility of depressed individuals to stress (Kang *et al*., 2013) of which the opposite is also true (Duman & Charney, 1999). MDD is known to be induced by chronic psychosocial stress (Harvey, 2008), while stress in particular increases glutamate release and reduces that of BDNF (Harvey *et al*., 2013). Stressful conditions may cause BDNF gene suppression,

impairing its protective capabilities resulting in hippocampal neuron atrophy and even cell death, followed by the development of depressive disorders or even treatment-resistant depression (Duman & Charney, 1999). However, chronic exposure to stress may also give rise to enhanced BDNF concentrations within the mesolimbic DA system (e.g. in the NAc) (Berton *et al*., 2006). Since BDNF plays an important role in regulating the adverse effects of glutamate on neurons, this scenario leads to destructive effects on brain structure and function, culminating in brain atrophy (Harvey *et al*., 2003). Interestingly, direct intraventricular administration of BDNF and VEGF in animals has anti-depressant-like effects (Duman, 2002; Manji *et al*, 2003; Altar, 1999). Of note is that blocking overt glutamatergic activity with an NMDA receptor antagonist is gaining increasing attention for its rapidly acting antidepressant effects (Slutsky *et al.,* 2010). Moreover, multiple classes of present antidepressants have been proven effective in reversing neuroplasticity deficits by way of stimulating neuro- and gliogenesis, dendrite branching, promotion of cellular endurance as well as enhancing synaptic birth (see Serafini, 2012 for review). SSRI antidepressant effects are dependent on BDNF signalling in order to reduce HPC atrophy by promoting neurogenesis (Haase & Brown, 2012). These and related effects may also be induced upon BDNF administration into the HPC (Shirayama *et al*., 2002) or by enhancing TrkB and subsequent BDNF expression, suggesting a relationship between MDD, BDNF and 5-HT (Krishnan & Nestler, 2008). Extensive reviews conducted by Pittenger and Duman (2008) and Serafini (2012) substantiate the aforementioned results of antidepressant-related (e.g. SSRIs, MOAIs, TCAs, ECT and ketamine) reversal of cognitive (i.e. learning and memory) and neuroplasticity deficits in both animal models and human subjects (Pittenger & Duman, 2008, Serafini, 2012).

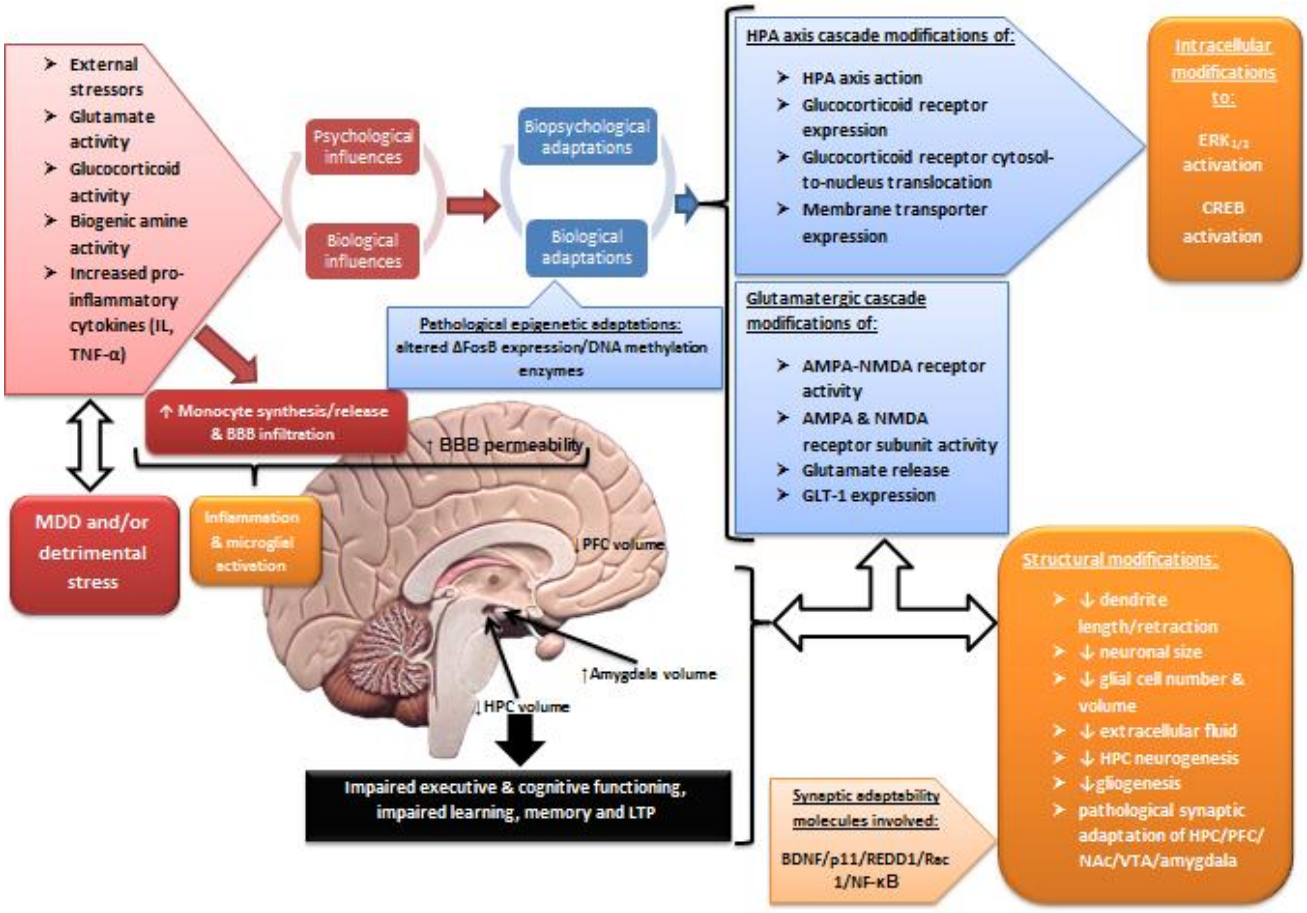

Figure 2-10: Structural and functional adaptations hypthesised to underlie MDD and/or pathological stress conditions with relevance to neuroplasticity modifications (adapted from Serafini, 2012; Ménard *et al.*, 2015).

2.4.2.9 Cytokine hypothesis: Neuro-immunological/inflammatory model

Clear associations between neuroendocrine, neuro-immune, circadian rhythm and HPA axis interactions have been assessed and recorded, incriminating a distinct common denominator – inflammation (Brown *et al.*, 2004). As previously stated, HPA axis hyperfunction leads to hypercortisolaemia which then leads to receptor overstimulation, causing brain tissue damage and structural brain changes. Similar reactions occur in individuals exposed to psychological or physical stress and in some metabolic disorders resulting in expression of depressive-like symptoms and behaviours – depending on the period of stress-induction or disease occurrence (Brown *et al.*, 2004). Continuous stress induction or glucocorticoid activity causes neuroplasticity–related PFC and HPC CA3 regional damage in the form of impaired neurogenesis, cellular degeneration, reduced glial count and dendritic withdrawal with consequential neuropil shrinkage. The aforementioned has been substantiated in depressed individuals and various behavioural models (e.g.

chronic restraint or unpredictable stress test) (Stockmeier *et al*., 2004). With reference to the aforementioned models: these animals also present with reduced glial and endothelial cell proliferation within the medial PFC (Banasr *et al*., 2007). Glial cells form a crucial part of neuronal metabolic support structures and are essentially involved in the synthesis and catabolism of glutamate, indirectly affecting neuroplasticity (Uranova *et al*., 2004). Henceforth, altered glial cell number and functionality will indefinitely cause neuroplasticity changes as seen in MDD and chronic severe stress (Pittenger & Duman, 2008).

In addition to glucocorticoids other molecules are also released in response to stressful stimuli, such as inflammatory cytokines (Haase & Brown, 2015). These inflammatory markers are capable of regulating mood, implicating them in CNS processes (Dantzer *et al*., 2008). CNS cytokine receptors can be stimulated by both central and peripheral cytokines (Dantzer *et al*., 2008). This is demonstrable by low dose lipopolysaccharide (LPS) treatment in rats causing behavioural changes that include reduced exploratory and sexual behaviours along with social withdrawal (Dunn *et al*., 2005). In this instance, LPS administration stimulated pro-inflammatory cytokine (interferon-α, TNF-α, IL-6 and IL-1β) release which consequently caused monoaminergic system and HPA axis activation (Figure 2-10) (Dunn *et al*., 2005). Similar effects are visible after interleukin 1 (IL-1) therapy in rats (Dunn *et al*., 2005). The increase in cytokine activity also caused increased indolamine-2,3-dioxygenase (IDO) followed by increased kynurenine and quinolinic acid (QA) levels in microglia and an associated increase in astrocyte kynurenic acid (KYNA) concentrations (refer to §2.4.5 The kynurenine pathway: Role in depression and cognition). Interestingly, the aforementioned mechanism is linked to the glutamate hypothesis thought to underlie MDD (O'Connor *et al*., 2009). Intensified pro-inflammatory cytokine release, be it peripherally or centrally, leads to altered immune system and inflammatory responses inducing a heightened inflammatory state capable of damaging CNS neurogenesis, neurotransmission and neurotransmitter production, surfacing depressive symptomatologies (e.g. reduced appetite, lethargy and anhedonia), reducing brain derived neurotrophic factor (BDNF) - providing yet again another link to a hypothesis (neuroplasticity hypothesis) thought to underlie depression (Haase & Brown, 2015). These outcomes form the basis for the cytokine hypothesis of depression which suggests that chronic immune system activation via cytokines leads to the development of depression (Smith, 1991). This can be verified by measured increases in IL-6 and TNF-α levels in the plasma of depressed individuals that are effectively reduced to baseline-levels with SSRI treatment (Hannestad *et al*., 2011). This further explains why individuals suffering from autoimmune/metabolic diseases are prone to developing MDD (Loftis *et al*., 2010).

2.4.3 Depression and its association with cognitive abnormalities

Cognitive abnormalities within MDD are not uncommon and have been researched for several years, with memory and attention impairment being regular complaints (Zakzanis *et al*., 1998; Luo *et al*., 2013; Burt *et al*., 1995). Researchers postulate that MDD patients that convey such complaints additionally struggle with memory acquisition and recall (Burt *et al*., 1995). Patients also frequently present with reduced episodic and spatial memory performance (Shelton & Kirwan, 2013; Söderlund *et al*., 2014). It is important to note that not all types of memory are affected to the same extent in depressed individuals. More commonly, patients exhibit impairments within explicit (consciously stored and recalled) memory rather than implicit (previously stored and subconsciously recalled) memory (Shelton & Kirwan, 2013; Zakzanis *et al*., 1998). Unfortunately, the pathological mechanisms that underlie these symptomatologies remain unclear and necessitate further exploration. However, abnormal neuro-inflammatory pathologies have been investigated and implicated in the development of cognitive irregularities (Pittenger & Duman, 2008). For example, both chronic stress and excessive glucocorticoid activity affect neuroplasticity events in brain regions involved in learning and memory (Pittenger & Duman, 2008). Unwarranted glucocorticoid activity causes neuronal damage and degeneration visible in the CA3 hippocampal region and the medial PFC as well as increased CA1 HPC glutamate release, reduced GLT-1 expression followed by excess extracellular glutamate that prevents neuroplasticity and neuronal growth (Zschocke *et al*., 2005). Chronic stress also causes increased HPC (CA3) glutamate release and also inhibits CREB activity, preventing adequate BDNF production (Pittenger & Duman, 2008). Furthermore, chronic stress affects other crucial neuroplasticity-related molecules, such as MAPK, NCAM, PKC and synapsin-I (Figure 2-11) (Muller *et al*., 1996). There is, however, evidence suggesting that the emotional and cognitive disturbances associated with depression are reversible with both antidepressants, such as selective serotonin-reuptake inhibitors (SSRI's), but also by non-antidepressant therapies (e.g. NDMA-receptor antagonists) (Burt *et al*., 1995; Luo *et al*., 2013; Shelton & Kirwan, 2013). Studies that are inconsistent with these findings disprove the existence of a link between depression and memory deficits and state that such deficits are only visible during depressive episodes (Burt *et al*., 1995; Maeshima *et al*., 2013; Mowla *et al*., 2008). The aforementioned clearly indicates that there are discrepancies in explaining a plausible link (or lack thereof) between MDD and cognitive abnormalities and therefore permits room for further investigation. A central brain region involved in memory is the HPC. Confirmed pathologies that may explain why several depressed individuals complain of memory relapse, include that of reduced hippocampal volume observed in many of these patients (Shelton & Kirwan, 2013). Reduced hippocampal volume may contribute to impaired

cognitive activity, not only due to a reduced number of active neurons in that region but also owing to diminished neurotransmitter (e.g. glutamate and glutamine) activity and lower hippocampal activation (Shelton & Kirwan, 2013).

2.4.3.1 Neurobiology of memory

Memories are either declarative (explicit; based on facts and events) or non-declarative (implicit; referring to skills, habits, non-associative memory, simple conditioning and priming) (Squire & Knowlton, 1994). Memory formation processes are dependent on various brain regions, including the HPC, striatum, amygdala, cerebellum, and neocortex as well as the biochemical cascades that take place within them (Squire & Knowlton, 1994). The different cascades comprise glutamate, GABA, AMPA and other receptor modulation, retrograde messengers, several protein kinases and transcription factors – all essential for memory formation and retention (Izquierdo & Medina, 1997). The HPC is essential for learning and memory processes that form part of sustained adult-life neuron synthesis (Bruel-Jungerman *et al*., 2007) and only recently researchers have been able to prove that new neurons can be produced in the adult mammalian brain (specifically in the HPC dentate gyrus) (Kempermann *et al*., 1997). Synaptic plasticity processes in the HPC also play a crucial role in memory-related LTP (Pittenger & Duman, 2008). The combined activity of neurogenesis and electrical potentiation in the HPC is essential for the production and incorporation of new neurons during and after learning-procedures (Pittenger & Duman, 2008).The emotional aspects of memories are encoded and retrieved via the amygdala, but are not stored here (Izquierdo, 1989; Bevilaqua *et al*., 1997).

Animal research regarding acute/mild stress, shows that short bursts of stress induction promotes learning and memory formation, contrary to chronic stress which interrupts HPC-dependent memory processes (Shors, 2001). This type of stress may also induce synaptic plasticity changes within the amygdala-PFC projection (Maroun & Richter-Levin, 2003). Severe/chronic stress animal models, however, show HPC neuron degeneration, reduced pyramidal cell number, increased amygdala synaptic connectivity as well as increased dendrite complexity, length, branching and number of spines (reviewed in Pittenger & Duman, 2008). As a result, neurogenesis is compromised causing impaired LTP and enhanced LTD. These long-term effects are reversible after NMDA receptor antagonist treatment (Zarate *et al*., 2006).

Memory acquisition, formation and retrieval, as well as learning is known to be reliant on hippocampal integrity (refer to the section on *Depression and its association with cognitive abnormalities*) with numerous biochemical cascades taking place in and around

this region (Izquierdo & Medina, 1997; Guzowski & McGaugh, 1997). These cascades are evident within the HPC during both early (memory consolidation) and late-learning (long-term synaptic plasticity) processes and involve many of the same substances (Figure 2-11) (glutamate receptors, PKG, PKC, CaMKII, cAMP, PKA and CREB) as those known to be involved in neuroplasticity processes and long-term potentiation (LTP) (Bliss & Collingridge, 1993; Huang *et al*., 1994; Izquierdo & Medina, 1997). There are also several other substances involved after training processes have taken place including: serotonin, glucocorticoids, adrenaline, noradrenaline, vasopressin, oxytocin, glucose and several others (Izquierdo & Medina, 1997). All of the aforementioned are involved in training procedures, memory consolidation and retrieval, and hippocampal LTP (Izquierdo & Medina, 1997; Izquierdo, 1989). For example, cholinergic agents that stimulate muscarinic receptors lead to the upregulation of NMDA receptors, causing protein kinase C (PKC) activity changes (Jerusalinsky *et al.*, 1997). Additionally, catecholamines increase cAMP levels and subsequently enhance protein kinase A (PKA) activity (Bevilaqua *et al*., 1997). In some instances, low concentrations of these substances may facilitate learning and memory, where the opposite may be true for high concentrations of the same substances (Izquerdo, 1989). BDNF activity also plays a vital role in hippocampal, cortical and amygdala-related LTP, both pre- and post-synaptically (Pittenger & Duman, 2008).

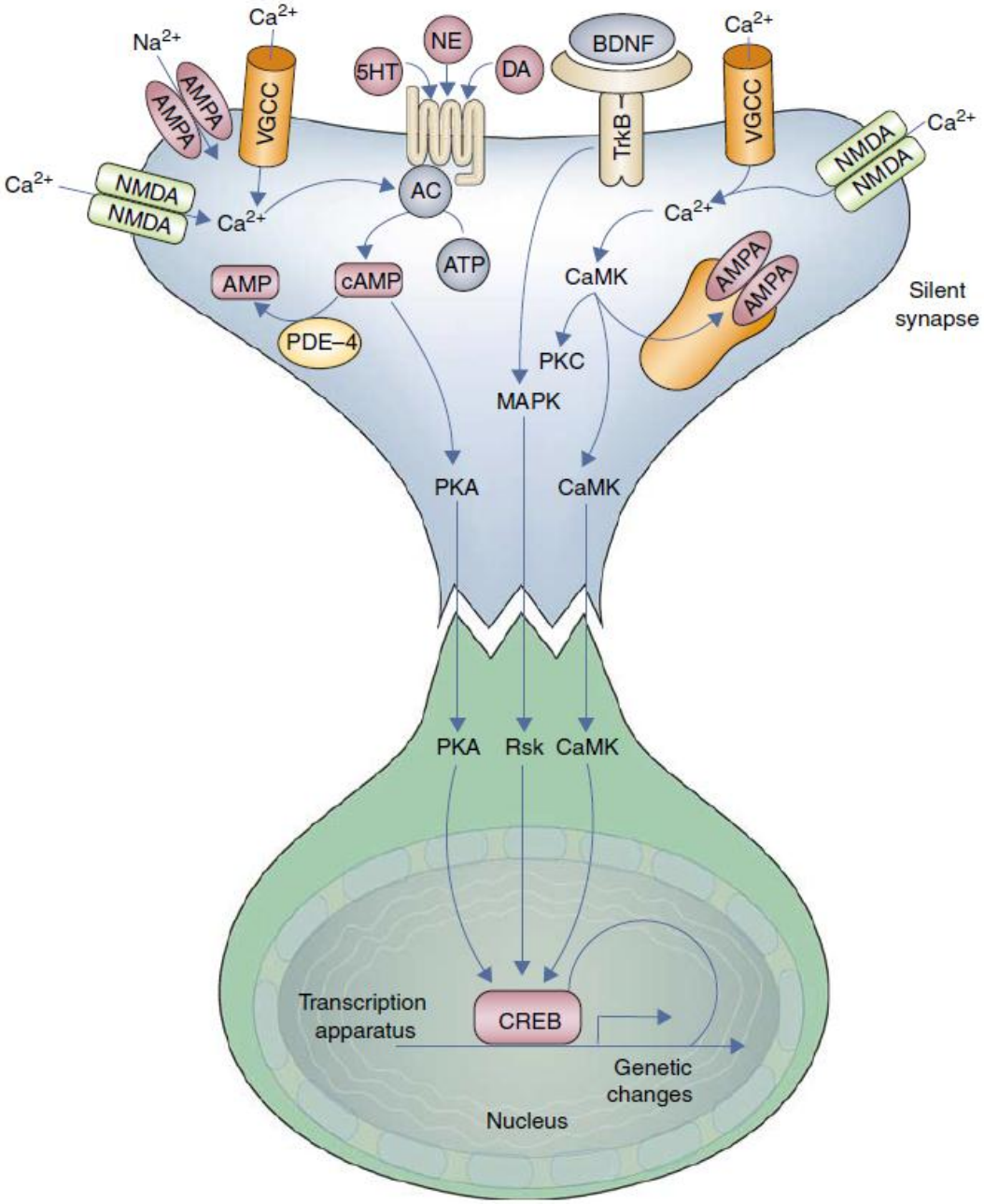

Figure 2-11: Key molecular processes involved in neuroplasticity pathways involving NMDAR (Pittenger & Duman, 2008)

NMDA (NMDAR1), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA; GluR1) and metabotropic glutamate receptor activation has been shown to be involved in different types of learning – especially within the HPC, with much evidence already in existence since the 1980's (Behnisch & Reymann, 1993; Izquierdo & Medina, 1997). NMDA receptor neuroplasticity regulation is dependent on receptor loci (Hardingham *et al*., 2002). NMDA receptors located synaptically positively modulate MAPK, CREB, BDNF and LTP, stimulating

long-lasting beneficial plasticity changes and neuronal endurance; whereas extrasynaptic NMDA receptor activation induces the opposite and when overstimulated may even cause cellular deterioration (Hardingham *et al*., 2002). These receptors have shown increased expression within the CA1-3 hippocampal regions of animals after avoidance-training exposure (a type of learning behavioural analysis) and LTP. As a result, these changes may lead to enhanced neuronal excitability and plasticity with a potential link to Calmodulin-Dependent Protein Kinase II (CaMKII) and PKA phosphorylation involved in the regulation of the above mentioned receptors (Bernabue *et al*., 1997; Izquierdo & Medina, 1997). Modulation of gamma-aminobutyric acid-A ($GABA_A$), β-noradrenergic and cholinergic muscarinic receptors has also been implicated in memory formation (Izquierdo & Medina, 1997).

The CaMKII cascade is well regulated by synaptic or local Ca^{2+} (Bliss & Collingridge, 1993). NDMA receptor activation leads to increased synaptic Ca^{2+} and cAMP concentrations which activate the various signalling cascades responsible for transitory synaptic plasticity (Behnisch & Reymann, 1993). In addition, the higher Ca^{2+} levels are also involved in activating and phosphorylation of CaMKII which then triggers LTP in hippocampal CA1, CA3 and other brain regions as well as additional synaptic alterations necessary for maintaining hippocampal-dependent learning and plasticity (Behnisch & Reymann, 1993). As critical as CaMKII is for LTP, it is also essential for memory processes in the HPC and amygdala in conjunction with PKA (Pittenger & Duman, 2008). All the above have been known to increase after training/learning processes (such as with avoidance training and water maze tests) (Ito *et al*., 1991).

CaMKII is further responsible for the phosphorylation of CREB (cAMP response element-binding protein) to CREB-P and ionotropic (NDMA and AMPA) glutamate receptors followed by subsequent enhancement of LTP, functionality and synaptic strength (Ferrer *et al*., 1996). CREB is an important transcription factor involved in learning-related synaptic plasticity within the HPC (Carlezon *et al*., 2005) and its activity is important for various neuroplasticity processes and alterations related to learning processes within the amygdala, HPC and cortex (Carlezon *et al*., 2005). CREB activation promotes neurogenesis and is capable of producing antidepressant-like effects in the HPC, whereas inhibition reduces this action (Nakagawa *et al*., 2002a).

CREB-P is responsible for many processes (Figure 2-11) involved in plasticity and is activated by PKA (as well as cAMP; cyclic adenylyl monophosphate) (Bernabeu *et al*., 1997). Following its stimulus, it is able to modulate gene activation and protein synthesis (such as c-fos synthesis) (Ferrer *et al*., 1996). CREB-P, c-fos and PKA levels have been

found to be increased after LTP and several training processes, signifying their vital role in memory formation and storage (Ferrer *et al*., 1996; Huang *et al*., 1994).

Evidently, platelet activating factor (PAF), nitrous oxide (NO) and carbon monoxide (CO) have also been implicated in early memory formation within the HPC (CA1). These retrograde messengers are known for their glutamate-regulating capabilities, revealing their association with glutamate receptor activity within brain regions involved in memory processes (Medina & Izquierdo, 1995). CO and NO are both responsible for the activation of the enzyme, guanylyl cyclase. This enzyme is essential for the synthesis of cyclic guanosine monophosphate (cGMP) (Zhuo *et al*., 1994). All three these substances have been hypothesised to underlie prolonged synaptic potentiation induction within the HPC (CA1). Additionally, the involvement of cGMP protein kinase (PKG) is also critical for CA1 LTP and possible plasticity (Zhuo *et al*., 1994). Furthermore, there are also several cell adhesion factors implicated in memory processing and LTP – especially in the HPC (CA1). L1- and neural cell adhesion molecules (L1CAMS and NCAMs) are responsible for regulating hippocampal LTP and levels are found to fluctuate in animal brains after avoidance training procedures (O'Connell *et al*., 1996; Field & Itoh, 1996).

Protein kinase C (PKC) is an additional second messenger essential for spatial and contextual learning memory processes and LTP (Bliss & Collingridge, 1993). After being activated by glutamate and acetylcholine, it stimulates adenylyl cyclase and subsequently cAMP synthesis (Yoshimura & Cooper, 1993). This cascade is vital for activation of PKA as well as the sequence of events responsible for long-term memory storage and plasticity processes, which will follow (Izquierdo & Medina, 1997). The precise role of hippocampal PKC in LTP and memory however, remains unclear (Behnisch & Reymann, 1993).

2.4.4 The glutamate system: Role in depression and cognition

Cognitive domains involved in MDD include memory, attention, executive activity and processing speed (Pehrson *et al*., 2015). Cognitive impairments such as reduced concentration and attention, declarative memory insufficiencies, disrupted thought processes and impaired neurogenesis are phenotypical of MDD (Hasler *et al*., 2007). Concentration and attention depends on dorsolateral PFC functioning whereas declarative memory requires intact HPC, PFC and temporal lobe activity (Harvey *et al*., 2005). An intact HPC is also essential for PFC, NAc and VTA operations of which the former work together to promote concentration and the latter regions in response to novelty (Lisman & Grace, 2005). As noted earlier, the glutamatergic-system appears critical in MDD, cognitive abnormalities and neuroplasticity modulation (Luo *et al*., 2013; Harvey *et al*., 2004). Neuroplasticity modulation may either be adaptive or maladaptive and the majority of these adaptations or

malformations take place in the glutamatergic system (Sanacora *et al*., 2012). The glutamatergic system encompasses the vast majority of neurons and synapses which form part of emotional and cognitive mediation processes (Sanacora *et al*., 2012). Thus far, the excitatory transmission of glutamate has regularly been held accountable for maladaptation as well as emotional and cognitive abnormalities as demonstrated in selected animal models of pathological stress and depressive disorders (see Sanacora *et al*., 2012 for review). These models also present with reduced HPC volumes, suggesting a link between stress, depression and neuroplasticity (reviewed in Pittenger & Duman, 2008). Glutamate plays a crucial role in inter-neuronal communication and its activity is well-regulated by Mg^{2+}, K^{+}, Na^{+} and Ca^{2+} – the ions responsible for regulating the binding of glutamate to the glutamate-NMDA receptors (Morris, 2013). The NMDA receptor is widely distributed in brain regions involved in memory and cognition (Morris, 2013). It is essential for synaptic adaptation and its deliberate distribution within the brain ensures that it utilises several triggering mechanisms involved in cognitive processing (Morris, 2013). Imbalances in the activation of synaptic (beneficial) vs. extrasynaptic (damaging) NMDA receptors have been uncovered in patients with MDD (Pittenger *et al*., 2007). Changes in the glutamatergic-system are well known to underlie the pathology of a number of neuropsychiatric symptoms and disorders (e.g. psychoses, affective disorders and cognitive abnormalities) (Krystal *et al.,* 1999; Harvey *et al*., 2004; Overstreet & Wegener, 2013). Under normal resting conditions, Ca^{2+} enters the synapses via ion-channels and stimulates its own release within the synapses, propagating signal transduction cascades (Morris, 2013).

Of relevance is that Mg^{2+} binds antagonistically to the NMDA receptor during the resting state. This state is known as steady-state baseline NMDA conductance (Murck, 2013; Morris, 2013; Morimoto-Tomita *et al.*, 2009). It then blocks the calcium channels that form part of the NMDA receptor complex thereby temporarily preventing Ca^{2+} flux into and out of the neuron, which only occurs if conditions necessitate it (Eby & Eby, 2010). The enzymatic sodium-potassium-adenosine-triphosphatase (Na^{+}/K^{+}-ATPase) pump present in the synapses maintains pre- and postsynaptic membrane stability by pumping sodium ions out and potassium ions into the synapses (Lackie & Callaghan, 2010). Any changes in the gradient, such as excessive calcium influx into cells, cause irregularities in essential ion concentrations and altered synaptic membrane charges. The resulting over-excitation of the CNS will compromise depletion of cellular energy stores, excessive release of synaptic glutamate leading to NMDA receptor overstimulation, and enhanced activity of oxidative processes (such as increased NOS production) as well as the synthesis and release of the catecholamines (Möller *et al*., 2012; Harvey *et al*., 2004).

Excessive glutamate may also be triggered by stress and glucocorticoids, followed by reduced glutamate clearance by glial cells that are either damaged or abolished (Banasr *et al*., 2011). This in turn generates abnormalities in metabolism and synthesis of multiple neurotransmitters; affecting a variety of vital central processes as well as causing cellular damage and apoptosis (Möller *et al*., 2012). The resulting symptoms are neuromuscular and neurological in nature and may include: anxiety behaviour, migraines, tremors, muscle fatigue and spasms, irritability, depressive behaviour, mood instability and psychosis (Wacker & Parisi, 1968). The most common neuropsychiatric disorders and symptoms to surface from this are several types of depression, anxiety, personality changes and memory deficits (Cardoso *et al*., 2009; Singewald *et al*., 2004; Eby & Eby, 2006; Wang *et al*., 2013).

Reduced hippocampal and amygdala size may also result from amplified inflammation-related processes and changes in CNS which may involve kynurenine metabolism, leading to dendritic atrophy and the development of MDD and other neuropsychiatric conditions (Savitz *et al*., 2014; Miller *et al*., 2010; Banasr *et al*., 2011; Réus *et al*., 2015; Colín-González *et al*., 2013). These inflammatory processes may also be set in motion upon stress-induced activation of stress-related glucocorticoid hormones with succeeding immunosuppressive effects and subsequent CNS damage (Munck *et al*., 1984). Increased concentrations of inflammatory markers prior to antidepressant therapies have been associated with treatment resistance noted in patients with treatment resistant depression (Miller, 2013).

2.4.5 The kynurenine pathway: Role in depression and cognition

Kynurenine is one of two products arising from tryptophan metabolism (Gibney *et al*., 2014) (Figure 2-12). The second being serotonin (5-HT) (Gibney *et al*., 2014). The majority of tryptophan is metabolised by two key enzymes (IDO, indole-amine 2,3 dioxygenase and TDO, tryptophan 2,3 dioxygenase) to form kynurenine (Gibney *et al*., 2014; Réus *et al*., 2015), as illustrated in Figure 2-12. Brain tryptophan is mainly catabolised in astrocytic- and microglial-cells (Heyes *et al*., 1997). IDO is responsible for oxidative metabolism of tryptophan and 5-HT throughout the body and is activated by inflammatory cytokines (Erhardt *et al*., 2013). TDO, that specifically catabolises tryptophan, is found mostly in the liver and is activated by stress-induced corticosteroids (Gibney *et al*., 2014; Miller *et al*., 2010). Kynurenine is further metabolised to produce several other metabolic end-products of which two are key - quinolinic acid (QA, neurotoxic and often found in microglia) and kynurenic acid (KYNA, neuroprotective and often found in astrocytes) (Savitz *et al*., 2014; Miller *et al*., 2010; Réus *et al*., 2015) (Figure 2-12).

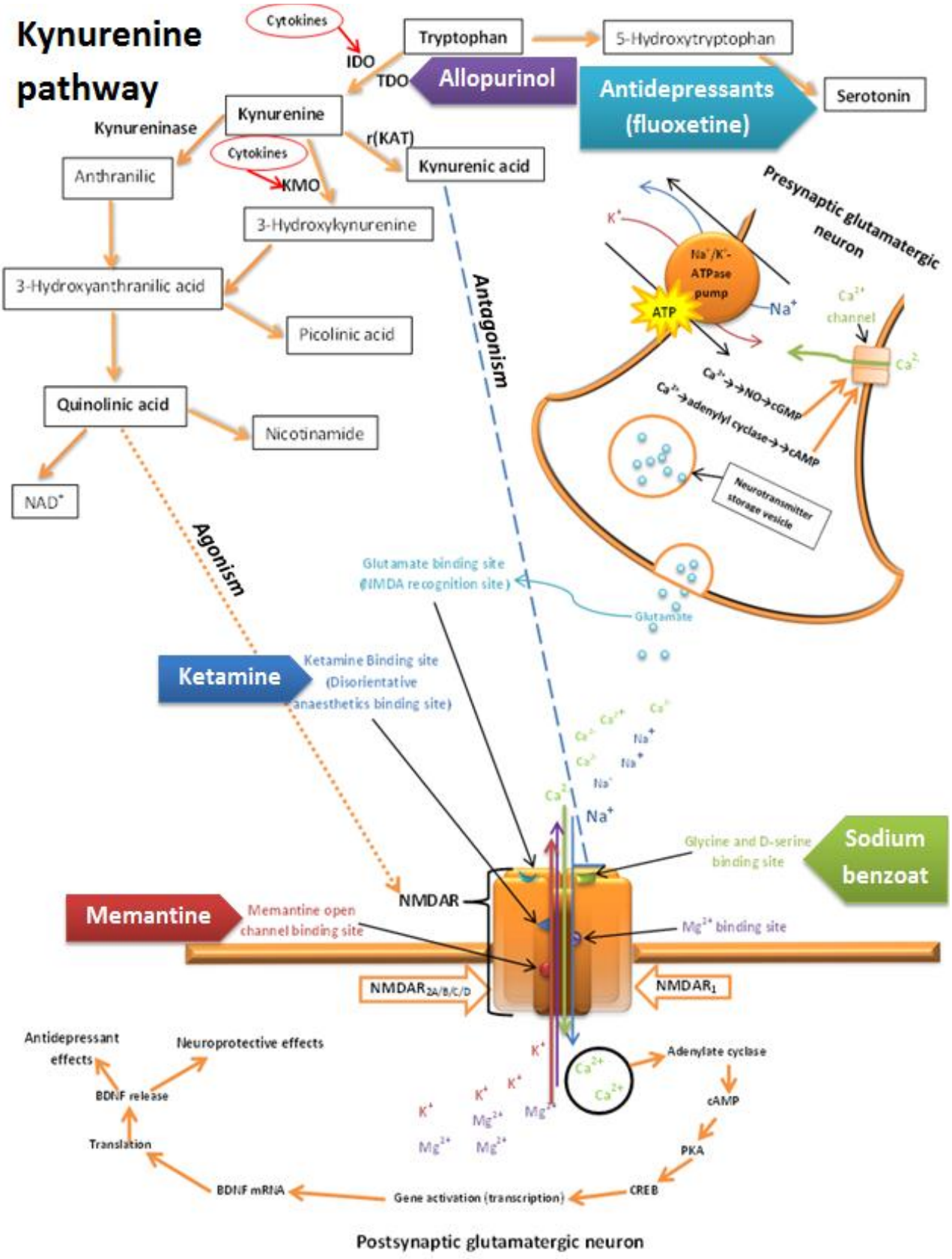

Figure 2-12: Schematic representation of synaptic processes influenced by the kynurenine-pathway and how these may be modulated by NMDA receptor active drugs (Adapted from Steiner *et al*., 2013; Miller, 2013; Lindholm *et al*., 2012; Möller *et al*., 2012).

Continuous stimulation of the kynurenine-pathway leads to reduced 5-HT synthesis, favouring the formation of QA and KYNA. Both metabolites are capable of NMDA receptor modulation and expressing depressive behaviours (Gibney *et al*., 2014; Möller *et al*., 2012). QA activates the NMDA receptor at either the NR1 or NR2A/B subunits. KYNA, however, antagonises the NMDA receptor at the glycine-binding site (Erhardt *et al*., 2013; Réus *et al*., 2015). Therefore, the kynurenine-pathway may be implicated in the glutamatergic-pathway and lately, researchers have found that this relationship may indeed lead to the development of degenerative pathology, depressive symptoms and behaviours (Gibney *et al*., 2014; Möller *et al*., 2012).

Present research supports the link between enhanced stimulation of TDO causing increased production of metabolites in the kynurenine-pathway, leading to enhanced circulatory kynurenine concentrations in rodent models (Gibney *et al*., 2014; Möller *et al*., 2012; Réus *et al*., 2015) as well as in humans (Erhardt *et al*., 2013; Réus *et al*., 2015). Gibney *et al*. (2014) provided evidence of such occurrences in Sprague Dawley rats after subjecting them to both acute and chronic stress paradigms (i.e. short and long periods of restraint). After stress exposure, the animals were accessed for depressive-like behaviours with/without receiving allopurinol (a TDO activity inhibitor) after undergoing stress. They found that the different periods of stress led to enhanced tryptophan levels, circulatory cortisone, increased depression-like behaviour, and amplified TDO expression and activity – all of which were successfully reversed after treatment. Similar altered levels in these endogenous substances were found to be present in post-mortem brains of schizophrenic and bipolar patients (Miller *et al*., 2006).

2.5 Treatment options

The chance origin of antidepressant compounds occurred near 70 years ago with the goal of increasing monoamine concentrations and neurotransmission (Slattery *et al*., 2004). Since then, several treatment options have come to exist for MDD that act on different neurotransmitters, for example: serotonin (5-HT), noradrenaline (NA) and dopamine (DA) and include a vast array of antidepressant medications (NIMH, 2011). Most commonly prescribed classes are: tricyclic antidepressants (TCAs, e.g. imipramine and nortriptyline); selective serotonin reuptake inhibitors (SSRIs, e.g. fluoxetine, paroxetine, sertraline, citalopram and escitalopram), serotonin and noradrenaline reuptake inhibitors (SNRIs; e.g. duloxetine and venlafaxine), monoamine oxidase inhibitors (MAOIs, e.g. phenelzine) and dopaminergic antidepressants (e.g. bupropion) (NIMH, 2011; SAMF, 2010). Other classes include: tetracyclic antidepressants, noradrenergic and specific 5-HT antagonists, 5-HT antagonists and reuptake inhibitors, NA and DA reuptake inhibitors and selective NA

reuptake inhibitors (SAMF, 2010). Today SSRIs and SNRIs form part of first-line treatment of MDD due to their improved side-effect profiles compared to the older TCAs and MAOIs that present with a multitude of unwarranted adverse effects as well as drug and dietary interactions (NIMH, 2011). For instance, TCAs are capable of binding many other receptors (e.g. 5-HT, muscarinic, histaminic and alpha$_1$ receptors) leading to their undesirable side effect profile (NIMH, 2011; O'Leary *et al*., 2015). It is, however, important to note that the first-line therapies do not possess greater degree of efficacy compared to older classes of antidepressants (Millan, 2006). None of the first-line treatment options present with direct procognitive effects, with the exception of SNRIs and SSRIs which have some degree of cognitive activity (Solé *et al*., 2015; McIntyre, 2013). Various classes of antidepressants are able to reduce the effects of depression-related factors on neurogenesis, dendritic and spine densities, and neuron synthesis and growth (Banasr *et al*., 2011). However, only one antidepressant has been found to have clinical pro-cognitive effects, namely vortioxetine. Vortioxetine is a new type of antidepressant with a multimodal character, capable of exerting both antidepressant as well as procognitive effects (McIntyre *et al*., 2014). In the past, the Food and Drug Administration (FDA) broadcasted a warning regarding the combined use of SSRIs/SNRIs and triptan-containing migraine treatments (NIMH, 2011). It is imperative that individuals placed on antidepressant therapies undergo and maintain treatment for a minimum of 6 weeks without discontinuation unless medically specified, are informed of the unpleasant effects and dangers that accompany them and are closely monitored upon initiation of therapy (NIMH, 2011). In 2005, the FDA stipulated that a "black box" warning be issued on all antidepressant medications to inform users of the potential risk of suicidal ideation and behaviours in children and adolescents and in 2007, this warning started including young adults below 24 years of age (NIMH, 2011).

Aside from antidepressant medications, additional therapies are also available and comprise of herbal remedies (such as St. John's wort/ *Hypericum perforatum*), psychotherapy and brain stimulation therapies (SAMF, 2010; NIMH, 2011). St. John's wort has gained great popularity in today's communities for the treatment of mild to moderate depressive disorder (NIMH, 2011). However, this herb presents with its own set of undesirable effects and contraindications, not to mention the controversies regarding its success as a treatment alternative (NIMH, 2011). Psychotherapy or talk-therapy has been found to be effective as add-on therapy or even as monotherapy in some instances with two types being applied, 1) cognitive-behavioural therapy (CBT) and 2) interpersonal therapy (IPT) (NIMH, 2011). Up until now, brain stimulation therapies (BSTs) haven't always been viewed in good light as treatment for mood and other neuropsychiatric disorders based on poor outcome and harmful effects (NIMH, 2011). Current advancements in the field have led to the safe and

effective application of these therapies in individuals that do not respond to treatment or suffer from severe/treatment resistant depression (TRD) with therapeutic side-effects that are generally short-lived (NIMH, 2011). BSTs consist of electroconvulsive therapy (ECT), vagus nerve stimulation (VNS) and repetitive transcranial magnetic stimulation (rTMS) (NIMH, 2011).

2.5.1 The search for new antidepressants

Despite the fact that multiple treatment options exist for MDD, successful remission after first-line antidepressant therapy is accomplished in a meagre 30%-50% of patients (Solé *et al*., 2015) while therapeutic response is only achieved in roughly 60%-70% of patients possibly due to behaviour-alone diagnostic methods and/or the lacking specificity of current drug therapies (Nestler *et al*., 2002; Ménard *et al*., 2015). Of the patients that respond well to therapy, many still suffer from subsyndromal symptomatic depression with accompanying encumbrances (Solé *et al*., 2015). While current antidepressants are effective, they present with numerous safety, efficacy and pharmacokinetic concerns (Machado-Vieira *et al*., 2009; Nestler *et al*., 2002; Drevets *et al*., 2008) as well as other limitations: slow onset of action (6-12 weeks for symptom improvement and up to 6 months for clinical improvement), toxicity, treatment resistance, low response and remission rates (Solé *et al*., 2015; O'Donnell & Shelton, 2011; Sadaghiani *et al*., 2011; Banasr *et al*., 2011; Réus *et al*., 2015). More than 30% of patients no longer respond to conventional therapies due to treatment resistance (Solé *et al*., 2015). This necessitates the need for novel approaches and investigations toward augmentative strategies and/or other plausible targets/mechanisms involved in the pathologies underlying these conditions which follow shortly below and have also been profoundly reviewed by O'Leary and colleagues (2015).

2.5.1.1 Augmentation strategies:

- Lithium (mood stabiliser) or triiodothyroxine combined with TCAs.
- Atypical antipsychotics (e.g. quetiapine/aripiprazole/olanzapine/risperidone) combined with SSRIs.
- Non-pharmacological, such as diet (e.g. L-methylfolate/omega-3 polyunsaturated fatty acids).
- Permeability glycoprotein (P-gp) inhibition combined with imipramine, desimipramine, nortriptyline and escitalopram.

2.5.1.2 Multimodal targeting strategies:

- Novel SSRIs: vortioxetine (a partial 5-HT_{1B} agonist, 5-HT_{1A} agonist, 5-$HT_{3/7}$ antagonist with procognitive effects) and vilazadone (a multipotent SSRI and 5-HT_{1A} partial agonist).
- Triple reuptake inhibitors: 5-HT/NA/DA reuptake blockers (e.g. amitifadine, BMS-820836) and DA transport inhibitors (e.g. bupropion).
- Reversible MAO_A inhibitors: moclobemide and CX157.

2.5.1.3 Novel targeting strategies:

- Neuropeptides with regard to monoaminergic neurotransmission modulation and HPA-axis regulation.
- Neurokinin (NK_2) receptor antagonism.
- Corticotropin-releasing factor receptor 1 (CRF_1) antagonism.
- G-protein-coupled vasopressin 1b receptor antagonism.
- Oxytocin (an adrenocorticotropic hormone modulator) agonism.
- Neuropeptide Y_2 antagonism.
- Melanin-concentrating hormone (MCH) receptor 1 antagonism.
- Galanin (a 5-HT/NA neurotransmission modulator) agonism and/or antagonism.
- Orexin-2 receptor (role in anhedonic behaviour and circadian rhythm modulation) antagonism.
- Ghrelin (role in anhedonia and neurogenesis) modulation.
- Opioid (e.g. kappa) receptor antagonism.
- Sigma-1 receptor agonism.
- $GABA_B$ receptor antagonism.
- Purine receptor antagonism.
- Immunomodulation (e.g. TNF-α antagonism, cyclooxygenase inhibitors, 3-omega polyunsaturated fatty acids and antibiotics).
- Psychobiotics/probiotics

2.5.1.4 Therapeutic onset rate enhancing strategies:

- Glutamate neurotransmission via NMDA and AMPA receptor modulation, metabotropic glutamate receptor modulation and glial-cell glutamate transporter (GLT-1) augmentation.
- Acetylcholine action via nicotinic and muscarinic receptor.
- Chronotherapeutics/ circadian rhythm modulation.

2.5.1.5 Underlying molecular and pathophysiological mechanisms of MDD as targeting strategies:

- Neurotrophic factors (e.g. BDNF, vascular endothelial growth factor/ VEGF, insulin-like growth factor, fibroblast growth factor-2) and associated signalling pathways (mTOR pathway).
- Epigenetic modifications (gene alterations resulting from environmental influences without subsequent DNA sequence modification): e.g. miRNA, histone and DNA epigenetic modifications.
- Glycogen synthase kinase-3 (GSK-3) inhibition.
- FKBP5 (role in modulation of glucocorticoid receptor activation) modulation.
- TREK-1 inhibition.

2.5.2 Target options in the glutamate system

As highlighted, glutamate is one of several neurotransmitters (Machado-Vieira *et al*., 2009) believed to underlie depression while drugs that either selectively or non-selectively block the glutamate-NMDA receptor are widely studied as novel antidepressant compounds. To compensate for reduced NMDA receptor activity, the brain will counterpoise the receptor blockade by increasing NMDA receptor expression in those areas to restore steady-state conductance. The result being, enhanced number of receptors that will promote NMDA receptor signalling, memory, learning and synaptic plasticity (Morris, 2013). An accompanying theory is that of neuroplasticity – a system that is dual in nature. Its duality refers to its ability to be both adaptive (beneficial) and maladaptive (detrimental). Variable neuroplasticity within neuronal networks is characteristic of glutamate modifications, presenting as altered emotional and cognitive processes as seen in MDD with regard to both human and animal models (Drevets *et al*., 2008). As of yet, there are no antidepressant therapies available that directly target the glutamatergic-system. Overall, this system involves several other key neurotransmitters aside from glutamate – all essential in modulating crucial brain functions with several being linked to affective- and thought-processing (Drevets *et al*., 2008). Hence, it is not unexpected that researchers have been investigating NDMA-receptor antagonists for antidepressant activity.

One such a drug is ketamine (Figure 2-12, in dark blue), a well-known NMDA receptor antagonist (Johnson *et al*., 2015) commonly used to induce and maintain anaesthesia (Lindholm *et al*., 2012). Ketamine produces substantial beneficial antidepressant effects in several animal models (Lindholm *et al*., 2012; Dutta *et al*., 2015) of depression as well as in human studies (Sanacora *et al*., 2012; Irwin *et al*., 2013; Koike *et al*., 2011; Zarate *et al*.,

2013; Naughton *et al*., 2014; Dutta *et al*., 2015; Lee *et al*., 2015). An acute dose exhibits rapid (Machado-Vieira *et al*., 2009; Duman & Voleti, 2012) and long-lasting (Miller, 2013) antidepressant activity at both low and therapeutic doses in those suffering from MDD, TRD and several other neuropsychiatric conditions (Dowben *et al*., 2013; Li *et al*., 2011; Duman *et al*., 2012; Johnson *et al*., 2015; Lee *et al*., 2015; Belujon & Grace, 2014). Low dose therapies have also shown to be neurotrophic and neuroprotective (Solé *et al*., 2015). Contrariwise, high ketamine doses lead to cognitive impairment and neurotoxicity (Solé *et al*., 2015; Liu *et al*., 2014). Its antidepressant activity is linked to calcium and sodium channel modulation, cholinergic-, noradrenergic-, serotonergic-, and glutamatergic-transmission, synapse formation, AMPA receptor modulation and BDNF expression (Irwin *et al*., 2013; Duman *et al*., 2012; Dutta *et al*., 2015; Perrine *et al*., 2014; Ballard *et al*., 2014). Ketamine's antidepressant-like effects may result from BDNF-related actions in the HPC (Murrough, 2012). With respect to the kynurenine pathway, ketamine targets QA production by inhibiting kynurenine 3-monooxygenase (KMO) (see Figure 2-12), thereby reducing the frequency of NMDA receptor activation by QA (Steiner *et al*., 2013; Miller, 2013). Ketamine can reverse certain synaptic plasticity deficits (Belujon & Grace, 2014) and augment synaptogenesis by rapidly enhancing spine numbers and mushroom spine density, providing an additional rationale for its rapid antidepressant effects (Li *et al*., 2011; Duman *et al*., 2012). Studies have shown it to amplify BDNF expression and synaptic growth in rat PFCs as well as enhance NR2B expression (Murck, 2013). Ketamine's antidepressant actions in humans are visible within two hours and can persist for roughly seven days after a single dose treatment (Zarate *et al.,* 2006; Duman *et al*., 2012). Ketamine has also been shown to reduce suicidal behaviours in human trials (Erhardt *et al*., 2013). Other investigations include its activity as add-on therapy and theoretical neuroprotective properties during ECT in patients with TRD (Solé *et al*., 2015). In 2014, Murrough *et al* evaluated the effects of IV ketamine on cognitive function in TRD patients where they concluded that the individuals who experienced unfavorable cognitive impairment after infusion were also poorly responsive to its antidepressant-like effects, as opposed to those who presented with reduced depressive symptoms and an associated reduction in memory recollection (Murrough *et al*., 2014; Liu *et al*., 2014). Several other related studies provide corroborative results pertaining to the inverted relationship between the antidepressant–like response and cognitive outcomes resulting from ketamine administration, some of which provide no evidence of neurocognitive regression after multiple infusions (refer to Solé *et al*., 2015). However, its use is limited due to potential toxicity and abuse potential (Li *et al*., 2011). Several studies have shown that ketamine treatment regrettably leads to CNS irregularities such as diminished memory, learning and concentration, hyperlocomotion and impaired social behaviours at varying

doses (Smith *et al*., 2011; Duan *et al*., 2013; Imre *et al*., 2006; Peng *et al*., 2011; Pitsikas *et al*., 2008; Moosavi *et al*., 2011; Krystal *et al*., 2005; Wang *et al*., 2006).

Aside from ketamine, memantine also blocks NMDA receptor activity and causes similar effects (Smith *et al*., 2011; Kotermanski *et al*., 2013) (Figure 2-12, in red). Like ketamine, it is also a noncompetitive, charge-dependent NMDA receptor antagonist but presented with good tolerability with respect to behavioural, cognitive and global functioning (Kotermanski *et al*., 2013). Although not used clinically as an antidepressant, it is registered for the treatment of Alzheimer's disease (Kotermanski *et al*., 2013) and presents with several procognitive effects when tested in different neuropsychiatric conditions and/or models (see Sanie *et al*., 2012 for review; Wilkinson, 2011; Ramaswamy *et al*., 2015; Tarragon *et al*., 2014; Koola *et al*., 2014). However, an investigation into the comparative treatment of depressed patients with comorbid alcohol dependence using memantine vs. the effects of escitalopram in reducing depressive and cognitive symptoms revealed that memantine presented with comparable efficacy to that of escitalopram relative to antidepressant activity (Muhonen *et al*., 2008). Unlike ketamine, at therapeutic doses memantine is without psychomimetic effects (Quan *et al*., 2011). Memantine has proven effective in reducing depressive symptoms and related behaviours as a result of its effects on monoaminergic system pertaining to 5-HT and DA (Johnson & Kotermanski, 2006; Ferguson & Shingleton, 2007), enhancing memory (Wilkinson, 2011) and learning processes along with reducing oxidative damage within the brain of animal and human subjects (Abraham *et al*., 2014: Quan *et al*., 2011; Wesierska *et al*., 2013; Strzelecki *et al*., 2013; Pietá Dias *et al*., 2007; Parsons *et al*., 2007). Several studies have also been conducted on its dose-related effects with regard to neurological behaviours as well as treatment-duration related effects (Réus *et al*., 2010). Unfortunately, despite its clinical use in a disorder of memory, such as Alzheimer's disease, memantine causes impaired memory in animals (Johnson & Kotermanski, 2006; Creeley *et al*., 2006), although this is contradictory to that found in other research (Zoladz *et al*., 2006; Camarasa *et al*., 2010).

In retrospect to the relationship between the NMDA receptor and the kynurenine-pathway, this pathway presents with potential drug targets that indirectly regulate NMDA receptor activity (Figure 2-12). One example is the catabolism of D-serine and D-alanine (D-amino acids) by D-amino acid oxidase (DAAO, a flavoenzyme) (Lin *et al*., 2014; Lane *et al*., 2013; Levin *et al*., 2015). D-amino acids act as neurotransmitters at the NMDA receptor co-agonist-binding site as well as enhancing their circulatory concentrations that in turn will increase NMDA receptor functionality. This can be achieved by compounds that inhibit DAAO. One such compound is sodium benzoate – a well-known preservative used in food

and beverages (Figure 2-12, in green) (Lin *et al*., 2014; Lane *et al*., 2013; Lai *et al*., 2013). As a result, the enhanced activity may lead to improved neurogenesis, neuroplasticity, learning and memory and possibly lessen depressive symptoms in patients with MDD (Kamel & Abd El Razek, 2013; Lin *et al*., 2014; Lane *et al*., 2013; Lai *et al*., 2012; Lai, 2013).

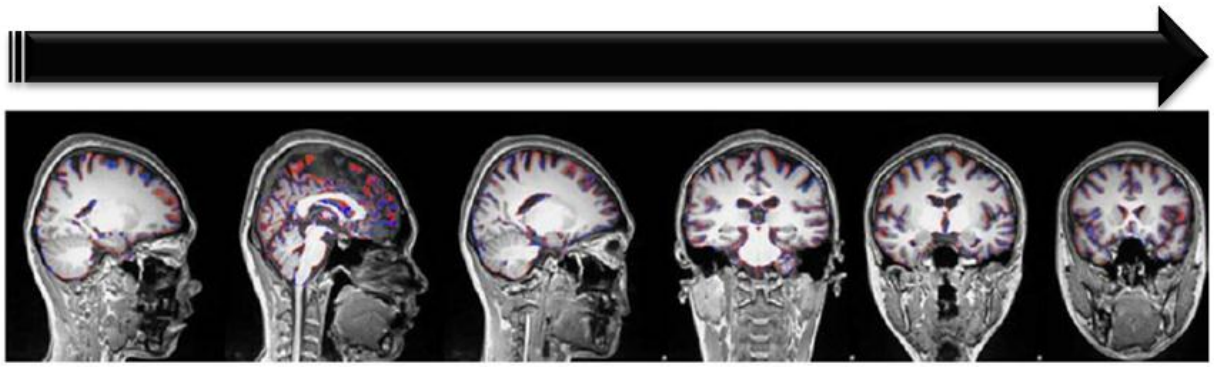

Figure 2-13: Grey matter structural increase subsequent of a 6 week period sodium benzoate (500 mg/day) treatment (Lai *et al*., 2012).

Regrettably, few preclinical trials have been conducted regarding sodium benzoate's procognitive and antidepressant effects in animal models, while limited research with sufficient numbers has been undertaken in human subjects (Figure 2-13) suffering from neuropsychiatric pathologies (Kamel & Abd El Razek, 2013; Lin *et al*., 2014; Lane *et al*., 2013; Lai *et al*., 2012; Lai, 2013).

Considering the NMDA receptor modulating abilities of various metabolites of kynurenine metabolism, relevant compounds that act on the enzymes operating within this pathway may thus be capable of indirectly regulating NMDA receptor activity. One such compound is Allopurinol (Møller & Kirk., 1978; Gibney *et al*., 2014; Curzon & Green, 1969; Julian & Chytil, 1970; Green *et al.*, 1976). The mechanism of action of allopurinol within the kynurenine pathway is well explained by a study conducted by Møller and Kirk in 1978. The aforementioned found that after administration of allopurinol in post-stress-exposure animals, post-mortem analyses indicate that the compound acts as a TDO (tryptophan 2, 3-dioxygenase) activity inhibitor (Figure 2-12, in purple) (Møller & Kirk, 1978). Allopurinol proved capable of reversing enhanced tryptophan levels (by inhibiting TDO, the enzyme responsible for the catabolism of tryptophan), circulatory cortisone, increased depressive-like behaviour as well as amplified TDO expression and activity as induced by stress exposure within the animal subjects (Miller *et al*., 2006). As a result (Figure 2-12), less tryptophan is metabolised leaving more available for the synthesis of serotonin (5-HT, 5-hydroxytryptamine) – an essential monoamine capable of reducing depressive-like behaviours. Additionally, Yonden *et al*. (2010) studied the effects of allopurinol on NMDA

receptor modulation after ammonia toxicity induction in an animal model to establish the extent of CNS damage and whether or not allopurinol administration could reverse these detrimental effects (Yonden *et al*., 2010). Consequently, they observed extensive CNS damage and function abnormalities pertaining to NDMA receptor over excitation followed by enhanced downstream processes contributing to excitotoxicity and neuronal damage (Yonden *et al*., 2010). Treatment with allopurinol proved effective in reducing these pathologies, however, the exact mechanism relevant to its NMDA receptor modulatory capabilities remain unclear (Yonden *et al*., 2010). Allopurinol as adjunctive therapy has also been explored in depression and related neuropsychiatric conditions (Akhondzadeh *et al*., 2006; Karve *et al*., 2013).

Based on the above literature, it is clear that there may in fact be a link between depressive disorders and cognitive abnormalities and within them various correlations between glutamatergic-, kynurenic-, and serotonergic-systems; even though some discrepancies may exist. Recent advances in research have revealed several drug compounds that act on these systems, providing us with near avenues of investigation and possibly novel antidepressant and/or procognitive therapies with specific relevance to MDD. Such compounds include for example ketamine, memantine, sodium benzoate, and allopurinol (Lindholm *et al*., 2012; Smith *et al*., 2011; Kotermanski *et al*., 2013; Lin *et al*., 2014; Lane *et al*., 2013; Forrest *et al*., 2013), which will be the focus of this study. Other compounds that have been studied for similar properties include donepezil, erythropoietin, galantamine, lanicemine, lisdexamphetamine, modafinil, omega-3, oxytocin, S-adenosyl-methionine, scopolamine and vortioxetine (see Solé *et al*., 2015 for review). In addition to these agents, fluoxetine (a standard compound used in the treatment of depression) may well be involved in the kynurenine-pathway. Fluoxetine is a selective serotonin reuptake inhibitor (SSRI) known to increase the production of KYNA and reduce 3-hydroxykynyrenic acid production in studies conducted using astroglial cultures (Yang *et al*., 2012). This clearly provides evidence of its multifunctionality at various target regions in the brain, and not only in enhancing 5-HT levels (Yang *et al*., 2012).

2.6 Animal models of depression

When developing animal models of depression, it is important to note that depression manifests itself through many characteristics and behavioural symptoms and not all them can be induced or measured. As of yet, no animal model capable of precisely replicating the depression-like phenotype as perceived in depressed human individuals has been rendered (Berton & Nestler, 2006; Overstreet *et al*., 2005). Several animal models of depression have been developed over the past ± 50 years and are listed and shortly described in the table below (Table 2-2; see Ménard *et al*., 2015 for review).

Table 2-2: Animal models of depression (Ménard *et al*., 2015):

Animal model of depression:	Short description of model:
Chronic social defeat stress (CSDS)	Based on social defeat in a highly competitive environment or as a result of intimidation (bullying). Rodent depression-like phenotype expressing anhedonia, evasive social behaviours and social stress vulnerability which is reversible with chronic antidepressant therapy.
Chronic unpredictable or mild stress (CUS/CMS)	A model based on chronic mild and unpredictable stressors that lead to development of depression in susceptible human individuals experiencing such events in daily life. Rodent model expresses reduced sucrose preference, motivation, sexual behaviours, olfactory bulb volume and function; increased aggression and anxiety; disrupted sleep cycle and HPA axis activity. Reversible with chronic (not acute) standard antidepressant therapies.
Early-life stress and maternal separation	Model based on early-life traumatisation affecting development, inducing behavioural abnormalities, vulnerability to stress and neurochemical changes resulting depression later in life. Rodent model presents with HPA-axis functional anomalies, stress vulnerability, altered hippocampal glucocorticoid activity, grooming-behaviour changes.
Genetic rodent models	Rodent models/strains with genetic predisposition to stress susceptibility. Wistar Kyoto rat: depression-like phenotype expressing anhedonia, behavioural despair, social evasion reversible with chronic antidepressant therapies, deep brain stimulations, electroconvulsive seizure (ECS) therapy. *Flinders sensitive line rat (FSL): increased immobility in the forced swim test (FST) decreased nurturing behaviours, altered social coping mechanisms. Moderately reversible with chronic antidepressant therapies*. Reports of BDNF polymorphisms in animals affecting stress and antidepressant response. Val66Met (with relevance to BDNF gene) genetically modified mice express depression-like phenotype similar to humans: increased anxiety-like behaviours decreased HPC volume, decreased dendrite density. Reversible with desimipramine, not fluoxetine.

Learned helplessness (LH)	Based on an inability to escape from an extremely unpleasant stressful stimulus (e.g. inescapable foot shock) or a depression-induced failure to cope with avoidable circumstances with relevance to cognitive abnormalities associated with MDD. Rodents express reduced fondness for sucrose (relatable to anhedonia), increased depressive-like behaviours. Reversible with chronic antidepressant therapies.
Olfactory bulbectomy (OBX)	Based on reduced olfactory volume and sensitivity in depressed individuals. Rodent model presents with cortical-hippocampal-amygdala circuitry abnormalities resulting in behavioural anomalies; reduced libido, cognitive functioning, avoidance and foraging behaviours, and exploratory behaviours. Reduced hippocampal plasticity affecting spatial and emotional memory is also evident. Reversible with chronic antidepressant therapies.
Pharmacological rodent models	Chronic corticosterone treatment-induced depression-like behaviours with subsequent increased stress hormone levels relatable to depressed humans. Tryptophan depletion/tryptophan hydroxylase inhibition induced 5-HT deficiency with subsequent development of depression in human subjects and animal models of depression.
Repeated restraint stress	Based on animal's inability to escape from a confined space which it is exposed to on a daily basis for 3 weeks resulting in the development of a depression-like phenotype expressing reduced HPA axis sensitivity, increased immobility in the FST, reduced fondness for sucrose, anxiety-induced reduction in exploratory behaviours. Partially reversible with chronic antidepressant therapies.
Social isolation	Based on a sense of loneliness due to lack of social interaction leading to development of depression-like symptoms and disorders. Rodent model presents with anhedonia and anxiety-like behaviours, enhanced endocrine responsiveness to subject intrusion, and possible altered hippocampal neurogenesis. Reversible with chronic (not acute) antidepressant therapies.
Witness defeat	Model is based on visual exposure to traumatic or stressful episodes leading to development of depression- and anxiety-like behaviours. Model presents with increased serum corticosterone concentrations, altered gene expression in ventral tegmental area (VTA), and memory impairments.

The validity of these models depends on the following criteria: 1) **face validity**, 2) **predictive validity** 3) **construct validity** 4) aetiological validity and 5) population validity (criteria written in bold are most commonly followed) (Overstreet & Wegener, 2013; Schmidt, 2011; Geyer & Markou, 1995). Face validity infers that the model mimics or expresses the same symptomatology as seen in the human disorder (Overstreet & Wegener, 2013; Schmidt, 2011). Predictive validity infers that the animal model responds to antidepressant therapy in the same manner as depressed individuals (Overstreet & Wegener, 2013; Schmidt, 2011). Construct validity infers that the model presents with the same bio-pathologies that underlie

the condition as seen in humans with the same condition (Overstreet & Wegener, 2013; Schmidt, 2011). Aetiological and/or genetic (causal) validity infers a model that presents with a pathological/genetic predisposition to depression as seen in some individuals with MDD, e.g. an increased genetic-environmental relation that increases the risk for development of affective disorders or as evidenced by the enhanced cholinergic sensitivity of the FSL model (Neumann *et al*., 2011; Schmidt, 2011). Finally, population validity infers that the rate of condition incidence in an animal model population (depression-like phenotype) is relatable to that seen in human populations of individuals suffering from the same condition (Schmidt, 2011). Though the specific depression-like phenotype as seen in individuals with MDD is yet to be mirrored in an animal model of depression, there are several human and rodent studies on MDD that validate the disease's underlying pathological mechanisms (Table 2-2).

Table 2-3: Pathological mechanisms underlying major depressive disorder expressed by both humans and animal models (Ménard *et al*., 2015 for review).

Mechanism underlying MDD pathologies:	Alterations in humans and rodent models:
Neuronal or synaptic alteration	*≈ ↓ hippocampal (HPC) neurogenesis; Δ synaptic adaptation in HPC, prefrontal cortex (PFC), NAc, amygdala, ventral tegmental area (VTA), ↓ neurotrophic factors*
BDNF *(brain derived neurotrophic factor)*	↓ levels in brain
p11 *(calcium effector protein modulating 5-HT receptor signal transduction)*	↓ levels in brain
REDD1 *(regulated in development and DNA damage response 1)*	↑ in PFC ≈ ↓ mTOR *(mammalian target of rapamycin)*
Rac1 *(RAS-related C3 botulinum toxin substrate 1)*	↓ in NAc
IKK *(IκB kinase) NF-κB (nuclear factor kappa B)*	↑ NF-κB in FC and peripherally
Transcription or epigenetics	
ΔFosB	↑ in NAc and PFC

β-catenin/microRNA	↓ in PFC (consequential of ↑ glycogen synthase kinase 3 beta/GSK-3β) Δ microRNA ≈ Δ β-catenin
Histone	↓ Histone deacetylase 2 (HDAC2) in NAc, ↑ HDAC2 peripherally Δ in DNA methylation ≈ Δ histone acetylation
Immune system or inflammation	↑ *inflammation*
Pro-inflammatory cytokines	↑ interleukin 1 (IL-1), tumour necrosis factor alpha (TNFα) (central and peripheral)
Microglia	↑ microglial stimulation in cortex
Blood brain barrier (BBB) permeability & monocyte filtration	↓ BBB permeability ≈ ↑ monocyte permeation ↑ monocyte synthesis (bone marrow/spleen)
Astrocytes	↓ *density in PFC, HPC, amygdala*
VEGF *(vascular endothelial growth factor)*	↑ expression ≈ ↑ BBB permeability
Neuronal function regulation	Pathological Δs in astrocytic function ≈ Δ neuronal function and regulation

2.6.1 The Flinders Sensitive Line rat model of depression

In this study, the Flinders sensitive (FSL) and resistant (FRL) line rats are utilised. The reason for this being: FSL rats represent a widely described and validated genetic animal model of depression (Overstreet & Wegener, 2013) with regard to the following:

a) Face validity

The FSL rat exhibits several symptoms relatable to the human depressive-like condition, for e.g. reduced appetite and body weight, increased REM sleep, reduced activity, inert ability to cope with stress in the form of swimming immobility, social anomalies in the form of aggression, moderate anxiety behaviour, anhedonia, altered cognition, increased risk of cardiovascular disease as well as altered ability to detect pain stimuli (Overstreet & Wegener, 2013; Neumann *et al*., 2011; Overstreet *et al*., 2005).

b) Construct validity

Similar to depressed individuals, the FSL rat presents with heightened cholinergic responsiveness, monoamine and HPA axis dysregulation (Overstreet & Wegener, 2013; Neumann *et al*., 2011; Overstreet *et al*., 2005), abnormalities in the GABAergic system (Overstreet *et al*., 2005; O'Leary *et al*., 2015; Luscher *et al*., 2011), deficient neuropeptide Y levels, abnormal circadian rhythms (Overstreet & Wegener, 2013; Neumann *et al*., 2011; Overstreet *et al*., 2005) as well as altered neurotrophin concentrations and regulation (Hasselbalch *et al*., 2012; Haase & Brown, 2015).

c) Predictive validity

Antidepressant therapies (e.g. TCA, SSRI, MAOI, atypical antidepressants, benzodiazepines and ECT) shown to be effective in the treatment of MDD in humans have been tested and substantiated in the FSL model (refer to §2.4.2 Hypothesised causalities; Bunney & Bunney, 2000; Harvey & Slabbert, 2014; Serafini, 2012; Réus *et al*., 2015). However, this criterion has recently been under scrutiny due to poor antidepressant efficacy in several MDD patients (McArthur & Borsini, 2006).

Initially, the Flinders Line rat model had been developed to be genetically resistant to an organophosphate anticholinesterase agent – diisopropyl fluorophosphates (DFP). Contrariwise, the breeding program developed for these rats led to the creation of a rat strain that was in fact more sensitive to DFP and was termed the Flinders Sensitive Line (FSL) rat model. The Flinders Resistant Line (FRL) rat model is in comparison, more resistant to DFP. It initially came to light in 1980 that the FSL rat could be a plausible model for depressive-like behaviour based on reports of increased sensitivity to cholinergic agonists measured in depressed individuals, as compared to healthy individuals (Overstreet & Russell, 1982). This correlates to the findings that FSL rats had a larger distribution and amount of muscarinic receptors (Overstreet *et al*., 1984) as well as the fact that sensitivity for cholinergic agonists were enhanced in these rats (Overstreet & Russell, 1982). These findings led to the conclusion that there are similarities between depressed humans and the FSL rat model when cholinergic supersensitivity is taken into account.

Several depressive symptoms and behaviours as expressed in human subjects with depressive disorders have been found to exist within the FSL rat model. These include reduced appetite, elevated REM (rapid eye-movement) sleep as well as retardation of psychomotor activity. There is also evidence supporting the involvement of the glutamatergic, serotonergic and neurotrophic signalling pathways within the FSL rat model; based on confirmed abnormalities within these mechanisms (Overstreet *et al*., 2005;

Overstreet & Wegener, 2013). In 2010, Elfving *et al* were able to establish differences in BDNF levels between the FSL and FRL rat. They uncovered that the FSL rat displayed increased blood BDNF levels and decreased hippocampal BDNF levels compared to the FRL rat. Deficits in memory and cognition have also been described in FSL rats (Gómez-Galán *et al*., 2013), thus in line with that often described in depression. As in depressed human individuals, these pathways also require chronic antidepressant treatment in order to reduce depressive-like behaviour and symptoms.

Seeing that not all of the behavioural symptoms found in depressed individuals can be modelled in rodents, evaluation of the FSL model cannot rest on behavioural characteristics of depression alone (Overstreet *et al*., 2005). Of late, researchers have conducted more in-depth examinations into cognitive impairments in the FSL rat model of depression to establish which neurological pathways may be involved (Gómez-Galán *et al*., 2013). In 2013, Gómez-Galán *et al.* (2013) conducted a study to investigate whether dysfunctional astrocytic regulation of glutamate transmission in the FSL rat model did indeed relate to cognitive impairments as experienced by many depressed individuals (Millan *et al*., 2012; Femenía *et al*., 2012). They found conclusive evidence that, like depressed individuals, the FSL rats also had reduced hippocampal volume relating to cognitive dysfunction, and that this related to deficits in memory. Together with the aforementioned, they also noticed that the FSL rat had reduced synaptic plasticity and emotional memory (Eriksson *et al*., 2012) that could relate to astrocytic dysfunction. Ultimately, they found that abnormalities within the NMDA/glutamatergic system were undeniably related to cognitive impairments in the FSL model of depression and may in fact be evident in depressed individuals (Gómez-Galán *et al.,* 2013).

2.7 Synopsis

Major depressive disorder (MDD) is one of several neuropsychiatric disorders in the world inducing detrimental physical, psychological, psychosocial and biological abnormalities with subsequent patient incapacity, high economic liability, social responsibility and even irreparable consequences, such as death (WHO, 2012; NIMH, 2011; Ménard *et al*., 2015). MDD affects hundreds of millions of individuals globally and its effects are not restricted to gender, age, ethnicity or genetic transferability (WHO, 2012; NIMH, 2011). The symptomatologies underlying MDD are extensive and burdensome and may be brought about by an array of environmental, genetic and other unwarranted impacts for example underlying or co-occurring morbidities which may persist or reoccur later in life (NIMH, 2011; DSM-5™, 2013; Kiyohara & Yoshimasu, 2009). MDD is treatable with multiple therapies (e.g. drug compounds, natural remedies, talk therapies and brain stimulation therapies)

being available. Drug treatments include various classes of antidepressant (e.g. TCA, SSRI, SNRI, MAOI and many others), benzodiazepine compounds and certain mood stabilisers (NIMH, 2011; DSM-5™, 2013). Unfortunately, a large number of antidepressants present with undesired adverse effects, poor therapeutic efficacy and low remission rates limiting their use as monotherapeutic options at this stage (NIMH, 2011). More and more sufferers are opting for combined approaches, such as SSRIs combined with ECT (NIMH, 2011). To date, science has provided us with concrete evidence substantiating the causality of MDD; however, much of the research is still based on theoretical assumptions and coincidental phenomena. Many of the theories under investigation involve intricate physiological and neurobiological system interactions that are yet to be fully clarified. It is, however, clear that a multimodal approach to this condition is essential. The systems involved comprise of monoaminergic, cholinergic, GABAergic, glutamatergic, neurotrophic, HPA axis-related, neuroplasticity and neuro-inflammatory pathways and have been studied in both animal models and depressed human subjects. Mounting evidence also exists for the relationship between MDD and cognitive dysfunction once again involving several of the aforementioned systems, more specifically glutamate and neuroplasticity and to some extent neuro-inflammation, HPA axis dysregulation and other critical conduits, such as NDMA receptor and kynurenine pathway functioning (Overstreet *et al*., 2005; reviewed in Kiyohara & Yoshimasu, 2009; Krishnan & Nestler, 2008; Luscher *et al*., 2011; Sanacora *et al*., 2012; Pariante & Lightman, 2008; Germain & Kupfer, 2008; Réus *et al*., 2015). This has led researchers to re-evaluate and revolutionise many perceptions surrounding MDD with accompanying cognitive symptomatologies and the relevant treatment methodologies. Biological systems such as those involving glutamate and kynurenine present multiple investigatory and targeting strategies with regard to neuropsychiatric therapies which form the main focus of this study (review in Pittenger & Duman, 2008; Sanacora *et al*., 2012; Krystal *et al.,* 1999; Harvey *et al*., 2004; Overstreet & Wegener, 2013; Morris, 2013; Wacker & Parisi, 1968; Gibney *et al*., 2014; Réus *et al*., 2015; Heyes *et al*., 1997; Erhardt *et al*., 2013; Savitz *et al*., 2014; Miller *et al.*, 2010). Thus far, research regarding the aforementioned has escalated considerably providing more clarifications into underlying pathologies regarding these systems, how they relate to MDD and their potential as pro-cognitive and antidepressant-like targets as well as research in drug development. To date, no antidepressant compounds have been developed that specifically target the glutamatergic system with relevance to its antidepressant-like qualities. However, a number of studies have gone into targeting this system using existing drug compounds from drug classes other than current antidepressant therapies.

In this study I investigated the bio-behavioural effects of novel glutamate active compounds in a known animal model of depression with regard to antidepressant-like and procognitive effects in comparison to an established antidepressant and selected reference compounds. This was accomplished by assessing the effects of allopurinol and sodium benzoate (test compounds) on depressive-like behaviour and cognitive function (with regard to memory) in FSL rats subjected to a forced swim test and Morris water maze test, respectively. These effects were compared to that of a positive control, fluoxetine and two reference compounds, ketamine and memantine. Moreover, the effects of these compounds on monoamine and BDNF levels in specific brain regions were measured post-mortem via HPLC and ELISA analysis, correspondingly.

Chapter 3 : Materials and Methods

In this chapter the experimental methodologies and related materials are discussed. This chapter additionally entails descriptions of the project layout, the animal subjects employed in this study, drugs and drug dosages used as well as a narrative on the behavioural-, neurochemical- and data analyses applied.

Animals were housed, handled, treated and subjected to behavioural analyses at the Vivarium at the North-West University, Potchefstroom campus according to the specified ethical guidelines of the South African National Standards: The care and use of animals for scientific purposes (SANS 10386:2008) and the approved ethics application, **NWU-00208-14-S5**. This application was evaluated and approved by the animal research ethics committee (AnimCare) of the North-West University.

3.1 Overview

The study comprised of three phases *viz.*

- Phase one: confirmation of both the depressive-like phenotype of the FSL rat and the expression of cognitive insufficiencies within this model.
- Phase two: acute dose-ranging analysis for allopurinol and sodium benzoate.
- Phase three: the main experimental study (chronic drug treatment).

3.1.1 Phase 1: Confirmation of expressed depressive-like phenotype along with cognitive insufficiencies within an animal model of depression – FSL vs. FRL

This phase of the study entailed confirming the depressive-like phenotype of the Flinders Sensitive Line (FSL) rat using a modified version of the rat Forced Swim Test (FST) (Cryan *et al.*, 2002). Furthermore, it also included confirming whether the FSL model expresses cognitive deficiencies when subjected to an adapted version of the Morris Water Maze (MWM) test (Hamlyn *et al.,* 2009). These assessments were done in comparison to the Flinders Resistant Line (FRL) rat, a healthy control. The results from this phase were critical for continuation into Phase 2 of the study in determining the most effective drug dose of the two test compounds (allopurinol and sodium benzoate) in reducing depressive-like behaviours. Results for Phase 1 are found in Chapter 4 and discussed in Chapter 5.

3.1.2 Phase 2: Acute dose-ranging analysis - FSL

Establishing the most effective dose for allopurinol and sodium benzoate was an important step in reaching the main focus of this study, viz. whether chronic treatment with the aforementioned compounds could reduce depressive-like behaviours as well as enhance cognitive functioning when exposed to the chosen behavioural analyses. Only FSL rats were utilised in this phase. The rats were injected intraperitoneally (i.p.) with varying doses allopurinol and sodium benzoate at three time intervals 24 hours prior to being subjected to the FST. After analysing the data obtained from the FST, the most adequate dose was chosen and applied in the main experimental phase of this study for analysis of the effects of chronic (12 day) treatment via i.p. injection.

3.1.3 Phase 3: Main experimental study - FSL

The final phase involved the main focus of this study. FSL rats were exclusively used in this section. The rats were once again treated intraperitoneally with the earlier mentioned test compounds for a period of 12 consecutive days. Additionally, groups of rats were also treated with vehicle (0.1 % methylcellulose control), ketamine and memantine (reference compounds) as well as fluoxetine (established antidepressant). The effects of chronic treatment with the aforementioned compounds, allopurinol and sodium benzoate being the main focus, on depressive-like behaviours and cognitive functioning were assessed using the FST and MWM, respectively. These compounds were further evaluated for their effects on monoamines and their associated metabolites as well as related effects on brain-derived neurotrophic factor (BDNF) concentrations in the FSL rat brain, following the chronic treatment period.

3.2 Materials and methods

3.2.1 Subjects

Male FSL and FRL rats weighing approximately 200 g ± 20 g were employed in this study. The rats were randomly allocated to their specified behavioural and/or treatment groups (n=6) either on the first day of behavioural analysis or initiation of treatment. These rats were bred and housed at the Vivarium at the North-West University, Potchefstroom campus. The Vivarium provides an aseptic living and breeding environment for the animals which is maintained by Vivarium protocol that obligates the wearing of specialised laboratory attire (i.e. a laboratory coat, head cover, mask, gloves and boots) and daily cleaning regimens for all housing and non-housing areas that are maintained and strictly adhered to.

All the rats were housed in H-TEMP polysulfone plastic cages as groups of 3 per cage. The different rat strains were housed separately and subsequent treatment groups were also housed apart from each other to ensure minimal interaction which may affect any behavioural assessments. Each cage (395 mm x 346 mm x 213 mm) is tightly sealable; the lid containing a microbiological filter. Furthermore, each cage is connected to air inlets and outlets that are further connected to a HEPA filter ventilation system that ensures a constant supply of sterile air without contaminating the surrounding air in the housing areas. Additionally, each cage is supplied with chipped corncob bedding, free access to Nutroscience® rodent maintenance chow as well as fresh water *ad libitum*. In the housing areas the surrounding environmental conditions were controlled. The ambient temperature was maintained at 22 ºC± 1ºC with a relative humidity of 55 ± 10 %. A positive air pressure making at least 60 complete air changes per hour was ensured for each cage. A full spectrum 12-hour light/dark cycle (06:00 – 18:00) was also used.

3.2.2 Drug preparation, administration and dosages

Fluoxetine, ketamine, allopurinol and sodium benzoate were all purchased from Sigma-Aldrich, South Africa. Memantine was generously donated by H. Lundbeck A/S, Copenhagen-Denmark.

All drug substances, excluding allopurinol and ketamine, were prepared as solutions to be injected via the intraperitoneal route (i.p.). Allopurinol is notoriously insoluble in water and had to be suspended in a 0.1 % methylcellulose suspension in order to ensure accurate dosing when administered via the specified route (Curzon & Green, 1969; Julian & Chytil, 1970). Ketamine was obtained in solution form as 10 ml vials Ketamine-Fresenius (10 mg/ml). Drug solutions and suspensions were newly prepared each day in a sterile laboratory environment according to the specific quantities required for each animal. All equipment and apparatus was sterilised prior to use. All drug powders were kept within their original containers to ensure product stability is maintained. Drug powders were transfered to sterile tubes prior to adding solvent (normal saline solution) or suspending agent (0.1 % methylcellulose). After administration of the calculated drug dose to each animal the remainding solution/suspension was discarded. Drug administration was done using sterile BD microfine insulin injections (1 ml). A single injection was used for each animal and discarded immediately thereafter.

The rats were weighed (kg) every morning in order to calculate the correct drug dose (mg/kg) to be administered. In Phase 2 of this study, each rat received three injections of one of the following – vehicle or varying doses allopurinol or sodium benzoate. The

injections were given at 24 hours, 4 hours and 30 minutes prior to FST exposure (according to the original dosing schedule for AD screening by Castagné *et al.* (2009)). In Phase 3, each rat received a daily i.p. injection of vehicle, fluoxetine, ketamine, memantine, allopurinol or sodium benzoate. The drug dose for each rat was calculated based on the animal's weight and expressed as mg/kg. Subsequently, the volume (ml) injected was determined based on the total drug dose (mg) to be administered and the concentration of the drug solution/suspension (mg/ml). Volumes injected never exceeded 2 ml. Drugs were administered chronically for 12 consecutive days, with the last dose being administered the morning of behavioural analysis (FST or MWM).

3.2.3 Behavioural analysis

3.2.3.1 Open-field test

Prior to exposing the animals to the FST, they were subjected to the open-field test (OFT). The OFT is generally used to assess the extent of activity and exploratory behaviour. For this study, the open-field test is primarily used to assess locomotor activity (i.e. whether treatment has stimulatory or sedative effects, that may contribute to either a false positive or false negative effect in the FST), with focus being placed on distance moved (Gould *et al.*, 2009; Prut & Belzung, 2003; Sherif & Oreland 1994). All OFT analysis procedures took place at 7 pm during the dark cycle of the day (between 6 pm and 6 am) under infrared light (40 lux). Prior to placing the animals in the testing area, the arenas were wiped down using 10 % ethanol to remove any contaminants and lingering odours that may affect the animal's behaviour. The rats were placed in the testing area where they were allowed to habituate for a period of 30 minutes. Following habituation, each rat was then removed from the home cage and placed in an opaque Perspex® container (1 m x 1 m), viz. the open-field (Figure 3-1, left). General locomotor activity was recorded for 6 minutes, of which 5 minutes of footage was assessed using Noldus – EthoVision® XT tracking software to measure the total distance moved (cm) by each rat during that period of time.

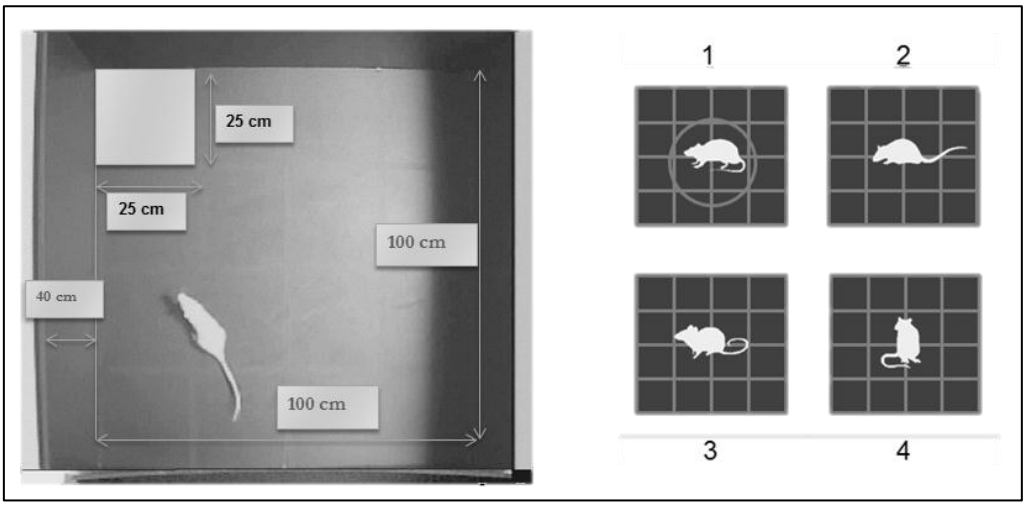

Figure 3-1: Single open-field test arena (left) and placement of the four OFT arenas as presented in testing area of Vivarium at Potchefstroom campus, North-West University (right).

3.2.3.2 Forced swim test

The Forced Swim Test (FST) was initially developed by Porsolt and colleagues (1978) for the preclinical assessment of anti-depressant effects of drugs in rodents. The use of the FST as animal model is based on the observation that rats initially try to escape from a cylinder of water from which it is impossible to escape, after which they adopt a position of immobility – a position thought to indicate behavioural despair (Porsolt *et al.,* 1978; Porsolt *et al.*, 1979, Castagné *et al.*; 2011, Overstreet *et al.*, 2005) and therefore the duration of behavioural despair is measured and used to estimate whether antidepressant effects of drugs are significant.

In this study, the modified forced swim test (Cryan *et al.*, 2002) was employed which includes definitions for swimming (i.e. the (usually horizontal) movement throughout the swim cylinder) climbing (i.e. movements of the front paws directed upward and along the perimeter of the cylinder) and immobility (i.e. when no other activity except that to keep the rat's head above water, is observed) (Figure 3-2, right). These behaviours can be linked to the action of different classes antidepressants based on the monoamines they enhance. For example, selective serotonin reuptake inhibitors (SSRIs) increase serotonin (5-HT) concentrations and consequently lead to increased swimming time in the FST, whereas noradrenaline reuptake inhibitors and other catecholamine enhancers induce climbing behaviours. As a rule, antidepressant treatment reduces immobile behaviour in the FST (Cryan *et al.,* 2005). On the testing day, the rats were allowed to habituate to a well-lit (± 200 lux white light) testing area for a period of 15 minutes. Testing took place at 7 am during the light cycle of the day (between 6 am and 6 pm). Upon test initiation, each individual rat was

placed in a water filled (to 30 cm) transparent Perspex® cylinder for a period of 7 minutes, by which the time of evaluation occurred for 5 minutes and was recorded using a digital camera. The temperature of the water was maintained at 25 °C ± 4 °C. Thereafter, the rats were removed from the cylinders, dried using disposable paper towel and returned to their home cages. The period during which the rats took on a posture of immobility, continued swimming or climbing behaviour was measured (seconds). The total time spent exhibiting all three of these behaviours would amount to a total time of 300 seconds. Each cylinder was wiped clean and refilled with clean water prior to analysing a new set of animals so as to remove any scent cues and drifting waste that may influence their behaviour. All video recordings regarding the FST were scored manually according to the behavioural criteria outlined by Cryan *et al* (2002).

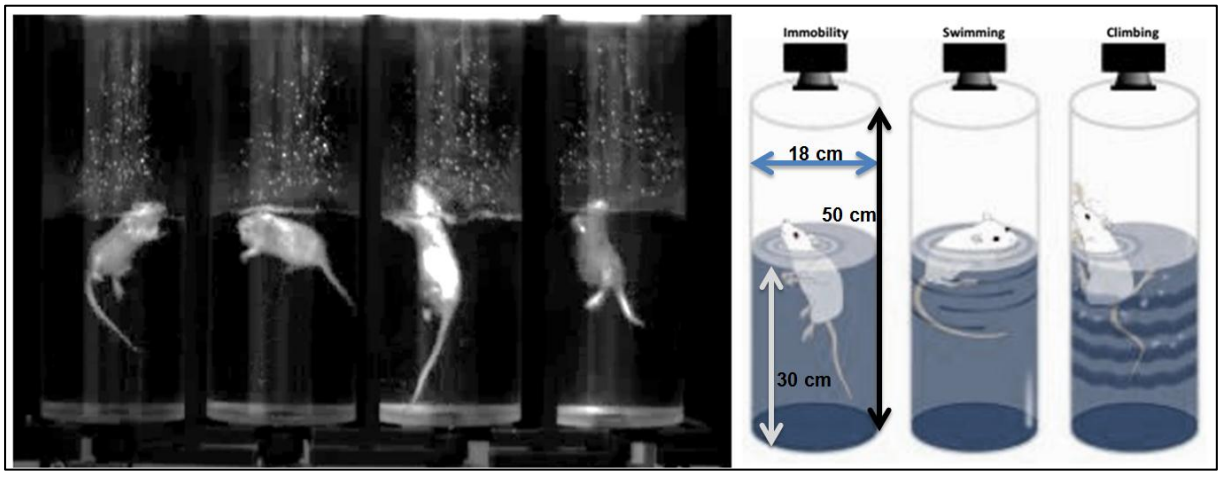

Figure 3-2: Forced swim test as conducted at the Vivarium, Potchefstroom campus of North-West University (left) and swimming behaviours observed during the rat FST (Cryan *et al.*, 2002).

3.2.3.3 Morris water maze

The Morris Water Maze (MWM) is a validated technique (Morris *et al.*, 1986) that tests for spatial learning, reference memory and spatial mapping and provides an understanding of the correlation between NMDA receptor activity, synaptic plasticity and different types of learning (Vorhees & Williams, 2006). It is regularly used to evaluate memory and learning processes with a link to long-term potentiation (LTP) and NMDA receptor function - all key components involved in hippocampal activity (Vorhees & Williams, 2006). The brain regions involved in MWM learning include the hippocampus, cerebellum, striatum, basal forebrain and numerous neurocortical regions (D'Hooge & De Deyn, 2001). The MWM has found application in various test protocols, including: trial-independent learning (such as spatial

acquisition and reversal, spatial double-reversal and repeated learning), spatial working memory, discrimination learning, latent learning and cued learning (Vorhees & Williams, 2006). Spatial learning and MWM-performance are dependent on the coordinated activity of the different brain areas mentioned here (D'Hooge & De Deyn, 2001).

In this study an adjusted version of the MWM was applied to assess spatial memory (Hamlyn *et al.,* 2009). The MWM pool is divided into four quadrants: North-East, South-East, South-West and North-West (Figure 3-3). Each rat was placed in the pool (1.80 m in diameter and 0.65 m in height) filled with water (water depth: 0.275 m; water temperature maintained at: 25 °C ± 4 °C) and the rat had to find the escape platform (Figure 3-3, target quadrant) in order to escape. The hidden platform method was used, with the platform 2 cm below water level. The water maze testing area was well-lit (± 200 lux white light) and pre-set with extra maze cues (viz. visibly stacked large cardboard boxes, computer screen and associated apparatus placed on a high table, a large water reservoir located high against one of the walls and a door that appears as a large black space within a different wall) and a digital camera to collect the swim activities in the four quadrants. After a 7 day drug administration period, the animals were allowed to adjust to the MWM testing area for 30 minutes. Since the MWM requires mental and physical exertion, the animals were also habituated for 60 seconds in the pool 24 hours prior to training so as to limit stress induction caused by performing the training and testing. Learning (acquisition training/memory consolidation) took place in a well-lit testing area and training was performed over 5 days with 4 training sessions per rat per day (Hamlyn *et al.,* 2009). On Day 1 of training (Figure 3-6, day 8 in the entire protocol), the rats had 30 minute intertrial intervals and on days 2-5 (days 9-12 in the entire protocol), 15 min – 20 min intertrial intervals. Training took place during the light cycle of each day at 12 pm (noon). Confirmation that the rat had learned the location of the platform was observed during the probe trial when the platform was removed and the rat searched the location where the platform was previously placed. All swimming activity was recorded and interpreted using Noldus – EthoVision® XT tracking software.

At the start of each training session the rat was placed on the platform for 30 seconds to orientate itself. Hereafter the rat was placed, tail first with its head facing the wall, in a quadrant not containing the platform (South-East, South-West and North-West). Video-recording and timing started when the rat was placed in the water. The rat was allowed to swim for 60 seconds. If it failed in locating the platform in that time, it was assisted onto the platform and left to re-orientate itself for 30 seconds. The time it took to reach the platform was expressed as latency to target zone/quadrant (seconds). The rat was removed from the platform and dried using disposable paper towel before being returned to the home cage. After each training session, the inner walls of the water maze were wiped clean and any

floating waste was collected and disposed of in order to remove any scent cues that my guide the animals to the platform or affect their behaviour and at the end of each training day the water within the pool was drained and the pool refilled with clean water. The training procedure was repeated in other quadrants over the 5 training days (Hamlyn *et al.,* 2009). The extent of memory retrieval (memory storage and recall) was assessed in a probe trial. This trial took place 3 hours after the last training trial on day 5. The platform was removed and the animal was allowed to swim for 60 seconds. The time spent in the target quadrant (expressed as percentage time spent in the target zone) indicated the degree of memory retrieval that took place after learning/acquisition. There was a 30 minute recovery time for the animal between the probe trial and the cued trial in order to correct for visual and locomotor impairment. The cued trial controlled for purely spatial memory deficits and in this trial, a 30 cm long white ruler was fastened lengthwise and at a 90 degree angle to the platform in the original quadrant and the animal allowed to swim for 60 s maximum (Hamlyn *et al.,* 2009).

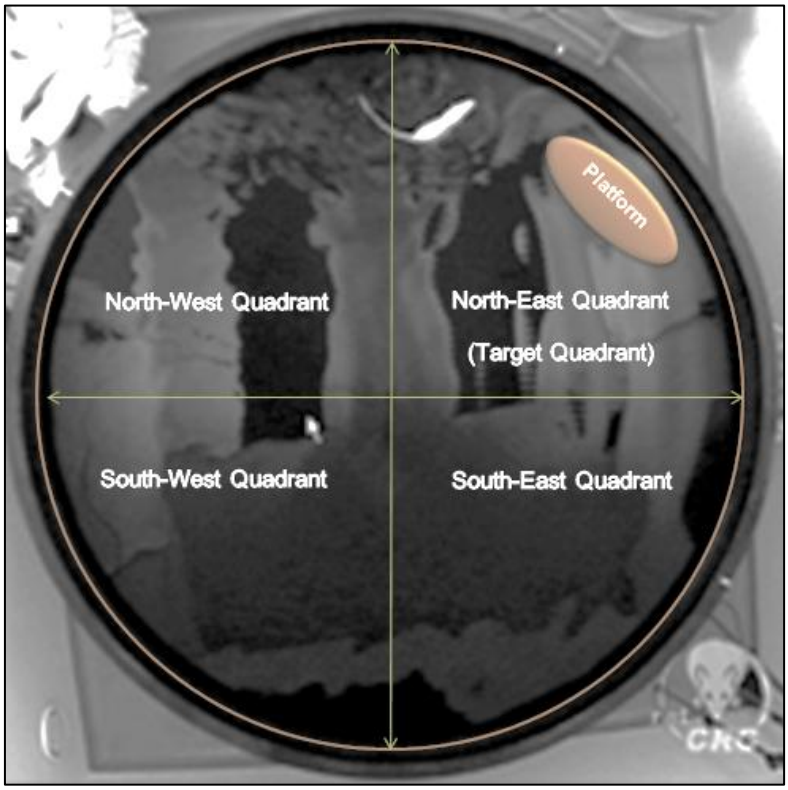

Figure 3-3: Morris water maze as conducted at the Vivarium, Potchefstroom campus, North-West University.

3.2.4 Project layout

3.2.4.1 Phase 1: Confirmation of expressed depressive-like phenotype along with cognitive insufficiencies within an animal model of depression – FSL vs. FRL

Before any drug administration could take place, confirmation of the depressive-like phenotype of the FSL model had to be done using the FST. Furthermore, this model had to be assessed for expression of cognitive deficits upon exposure to MWM testing. The aforementioned is critical in order to corroborate our findings with that of earlier research regarding depressive-like behaviours and cognitive impairments investigated in the FSL model of depression (Overstreet, 1993) prior to assessing any compounds for their effects on the stated behaviours. Chapter 2 of this study further elaborates on this. Depressive disorders have been shown to demonstrate memory deficits within the hippocampal and frontal cortical region (Campbell & MacQueen, 2004; Kemp *et al.*, 2012; Solé *et al.*, 2015; Hasselmo, 2006), and these tests may provide some understanding on how to improve upon such related behaviours. The behavioural analyses used were based on earlier studies (Cryan *et al.*, 2002; Hamlyn *et al.*, 2009) as described in the previous sections of this chapter. Behavioural tests were performed in drug-naïve FSL and FRL rats and the results of the different strains were compared within each test as can be seen in Chapter 4.

Table 3-1: Group layout for the confirmation of the depressive-like phenotype and associated cognitive deficits in the FSL model vs. FRL.

Group		Rat strain	Drug	Dose	n-value
1	FST	FRL	n/a	n/a	6
2		FSL	n/a	n/a	6
3	MWM	FRL	n/a	n/a	6
4		FSL	n/a	n/a	6

3.2.4.2 Phase 2: Acute dose-ranging analysis – FSL

Prior to initiating any chronic treatment studies, an acute dose-ranging analysis was performed to establish appropriate dosages for the two test compounds, allopurinol and sodium benzoate. Performing an acute dose-ranging analysis was necessary to establish whether allopurinol and sodium benzoate presented with antidepressant-like capabilities in the FSL model when evaluated using the FST. The FST has been applied to determine whether acute treatment effects on depressive-like behaviours exist upon treatment, however, not all known antidepressants show response when applied acutely (Overstreet, 1993). This was evidenced by our results obtained in Phase 2 as presented in Chapter 4.

The FSL model does, however, respond positively to chronic antidepressant treatment regimens (Overstreet, 1993).

To our knowledge, there were no studies published during the formulation of this dissertation, investigating the effects of varying doses allopurinol or sodium benzoate on depressive-like behaviours in the FSL rat being subjected to the FST. Furthermore, it was necessary to ensure accurate dosing during the main experimental study. The dosages used were based on previous research (See Table 3-2) and all groups receiving drug treatment were compared to a 0.1 % methylcellulose vehicle-treated group. The dose-ranging analysis was performed in groups of FSL rats according to dosing regimens presented in Table 3-2. All drugs were administered intraperitoneally. The protocol for this phase is illustrated in Figure 3-4 and was based on the dosing-protocol described by Castagné *et al.*, 2009.

Table 3-2: Treatment layout for Phase 2: Acute dose-ranging analysis (i.p.) in Flinders Sensitive Line rats.

Group	Drug	Dose	n-value
1	Vehicle	n/a	6
2	Sodium benzoate[A]	50 mg/kg/d (i.p.)	6
3		100 mg/kg/d (i.p.)	6
4		150 mg/kg/d (i.p.)	6
5		200 mg/kg/d (i.p.)	6
6	Allopurinol[B]	5 mg/kg/d (i.p.)	6
7		10 mg/kg/d (i.p.)	6
8		20 mg/kg/d (i.p.)	6
9		50 mg/kg/d (i.p.)	6
10		100 mg/kg/d (i.p.)	6

The dose range for sodium benzoate and Allopurinol is based on earlier studies:

[A](Lin *et al.*, 2014; Lane *et al.*, 2013, Lai *et al.*, 2012; Lai, 2013)

[B](Møller & Kirk, 1978; Gibney *et al.*, 2014; Curzon & Green, 1969; Julian & Chytil, 1970; Green *et al.*,, 1976)

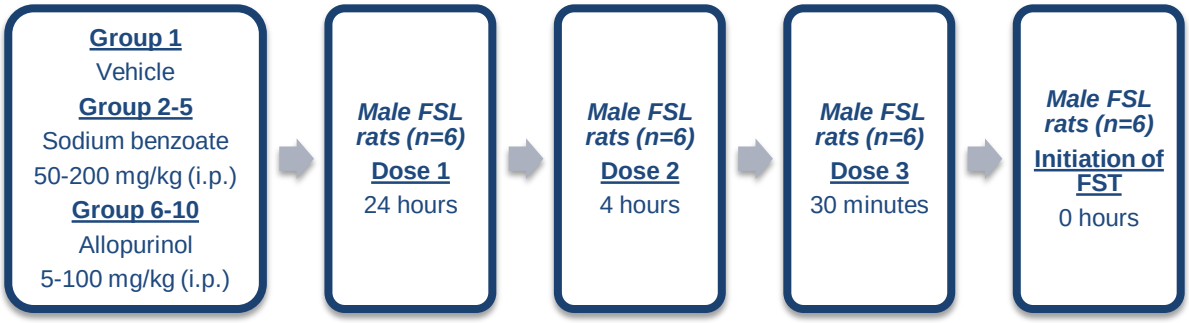

Figure 3-4: Schematic illustration of Phase 2: Acute dose-ranging analysis protocol in the Flinders Sensitive Line rat

- Group 1 served as the control group and only received vehicle (0.1 % methylcellulose solution) via i.p. injection.
- Groups 2-5 received varying doses sodium benzoate dissolved in 0.9 % saline via i.p. injection.
- Groups 6-10 received varying doses allopurinol suspended in the vehicle solution (0.1 % methylcellulose) via i.p. injection.

The dosing intervals ranging from 24 hours to 0 hours depict the various doses administered prior to subjecting the rats to the FST. The drug doses for allopurinol and sodium benzoate in the main experimental study were chosen based on the outcomes of the acute dose-ranging analysis during the FST.

3.2.4.3 Phase 3: Main experimental study – FSL

Evidently, the phenotypical depressive-like behaviours of the FSL rat were confirmed in the FST presenting with enhanced immobility behaviours compared to the FRL control (Chapter 4, § 4.1.1). Unfortunately, no significant differences in spatial memory between the two strains were observed during the MWM (Chapter 4, § 4.1.2). Furthermore, only one dose of allopurinol and sodium benzoate proved effective in reducing immobility time during the acute dose-range analysis phase (Chapter 4, § 4.2.1 – 4.2.2). As stated in Chapter 1 and reviewed in Chapter 2, novel treatment options for MDD have become a necessity. Presently, more researchers have started exploring novel compounds, pathways and strategies – one of which includes the glutamatergic pathway – known to be involved in affective disorders. In this study we investigated two test compounds (allopurinol and sodium benzoate) for their effects on depressive-like behaviours and cognitive functioning based on existing literature implicating their involvement in the glutamatergic pathway and thus, also in the treatment of MDD. Additionally, we also assessed ketamine and memantine for their effects in this regard as both these compounds are involved in the same system. Though all the administered compounds are involved in the glutamatergic pathway, neither one acts via the same mechanism in inducing their effects. Therefore, chronic allopurinol and sodium benzoate treatment was also applied to assess their effects on monoamine and associated metabolite levels as well as BDNF levels.

To our knowledge, no research on chronic allopurinol and sodium benzoate treatment impacts on depressive-like behaviours and cognitive impairments in the FSL model were published during the formulation of this dissertation with relevance to the FST and MWM testing. Therefore, the primary focus of the main experimental study was to investigate

whether allopurinol and sodium benzoate were capable of reducing depressive-like s*ymptoms and behaviours in the FSL rat as well as have an effect on this model's cognition as expressed in the MWM test following chronic treatment. The treatment groups for this study are outlined in Table 3-3 below and graphically illustrated in Figures 3-5 and 3-6. The subsequent results appear in Chapter 4 and are discussed in Chapter 5.

Table 3-3: Treatment layout for Phase 3: Main experimental study (i.p.) in Flinders Sensitive Line rats.

Group	Behavioural analysis	Drug	Dose	n-value
1	FST	Vehicle	n/a	6
2		Fluoxetine[C]	10 mg/kg/d (i.p.)	6
3		Memantine[D]	20 mg/kg/d (i.p.)	6
4		Ketamine[E]	10 mg/kg/d (i.p.)	6
5		Sodium benzoate	100mg/kg/d (i.p.)	6
6		Allopurinol	5 mg/kg/d (i.p.)	6
7	MWM	Vehicle	n/a	6
8		Fluoxetine	10 mg/kg/d (i.p.)	6
9		Memantine[D]	20 mg/kg/d (i.p.)	6
10		Ketamine[C]	10 mg/kg/d (i.p.)	6
11		Sodium benzoate	100 mg/kg/d (i.p.)	6
12		Allopurinol	5 mg/kg/d (i.p.)	6

The dose range for ketamine and memantine is based on earlier studies:

[C](Brand *et al.*, 2011; Karve *et al.*, 2013; Liebenberg *et al.*, 2010)

[D](Parsons *et al.*, 2007; Zoladz *et al.*, 2006; Camarasa *et al.*, 2010)

[E](Perrine *et al.*, 2014; Duan *et al.*, 2013; Duman & Voleti, 2012; Duman *et al.*, 2012)

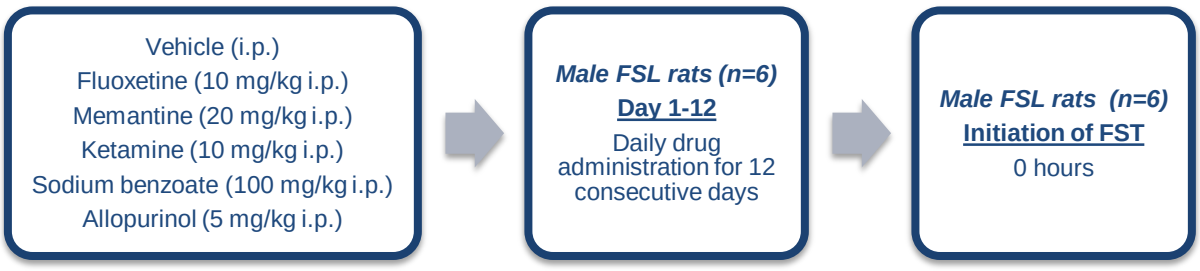

Figure 3-5: Schematic illustration of Phase 3: Main experimental study for chronic treatment in FSL rats subjected to the FST.

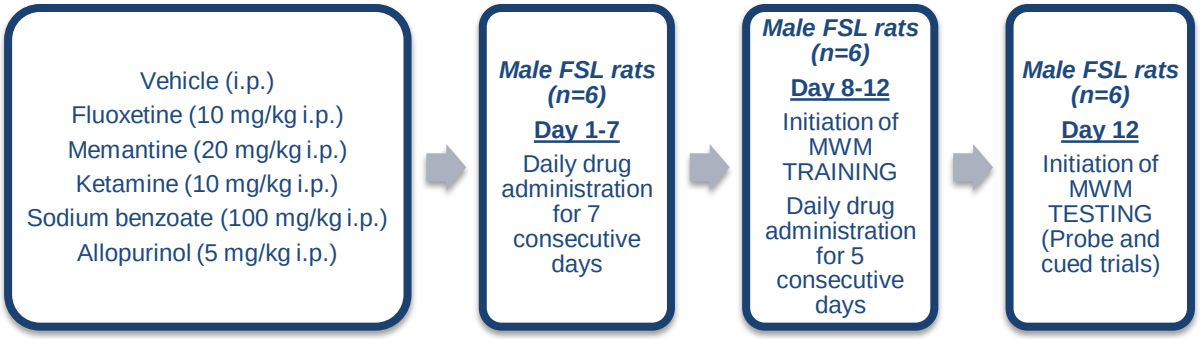

Figure 3-6: Schematic illustration of Phase 3: Main experimental study for chronic treatment in FSL rats subjected to the MWM test.

For the MWM test (Figure 3-6), the animals received daily drug- or vehicle-treatment (i.p.) for 7 days prior to MWM training (Day 8). Treatment continued throughout training for a period of five days (Days 8-12). This was essential in determining whether the drug-treatments had any beneficial effects in terms of spatial memory and learning. A different set of animals were assessed using the FST undergoing the same drug- or vehicle treatment regime (Figure 3-5). These animals were treated for 12 days (the same period as for the MWM) based on the period during which fluoxetine exerts its antidepressant activities (Liebenberg, 2006) in addition to maintaining a constant timeframe for chronic drug treatment.

- Groups 1 and 7 received vehicle treatment via i.p. injection and were later subjected to either the FST or MWM.
- Groups 2-6 received different treatments at the designated doses via i.p. injection and were later subjected to the FST.
- Groups 8-12 received different treatments in the same manner as groups 2-6, but were later subjected to MWM testing.
- Vehicle treated groups served as controls.

3.2.5 Neurochemical analysis

Neurochemical assessments were conducted in order to link the investigated behavioural outcomes to neurobiological outcomes. Separate groups of FSL rats were employed for assessing the effects of chronic treatment (following the same treatment schedule as explained in Table 3-3 with the chosen drug compounds on brain monoamines (noradrenaline/NA, dopamine/DA and serotonin/5-HT), their associated metabolites and BDNF concentrations. On Day 13 the rats were euthanised by means of decapitation, the brains extracted and placed on a cooled slab for dissection and removal of rat hippocampi, prefrontal cortices and striata. The attained brain regions were stored individually in polypropylene tubes and fixed in liquid nitrogen (-198°C) and stored in a -80°C freezer until

assay. These assays would determine whether actions on these pathways are indeed involved in the effects of the drug treatments on depressive behaviours and cognitive function in these animals. Both the left and right brain hemispheres were used.

3.2.5.1 Monoamines

Monoamine deficiencies involving 5-HT, NA and DA have been investigated and are causally related to dysregulation in monoamine activities as established in the brain of depressives (Overstreet *et al.*, 2005). Similar to depressed individuals, the FSL rat too presents with monoamine dysregulation (Overstreet & Wegener, 2013; Neumann *et al.*, 2011).

In this study, DA and its subsequent metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), NA and its end-stage metabolite 3-methoxy-4-hydroxyphenylglycol (MHPG) (Figure 3-7), 5-HT and its end-stage metabolite 5-hydroxyindoleacetic acid (5-HIAA) (Figure 3-8) were quantified in brain samples collected from FSL rats, specifically left hemispheric prefrontal cortices, striata and hippocampi. This was done using a validated method of high-performance liquid chromatography (HPLC) with electrochemical detection (ED) (Harvey *et al.*, 2006; Viljoen, 2012).

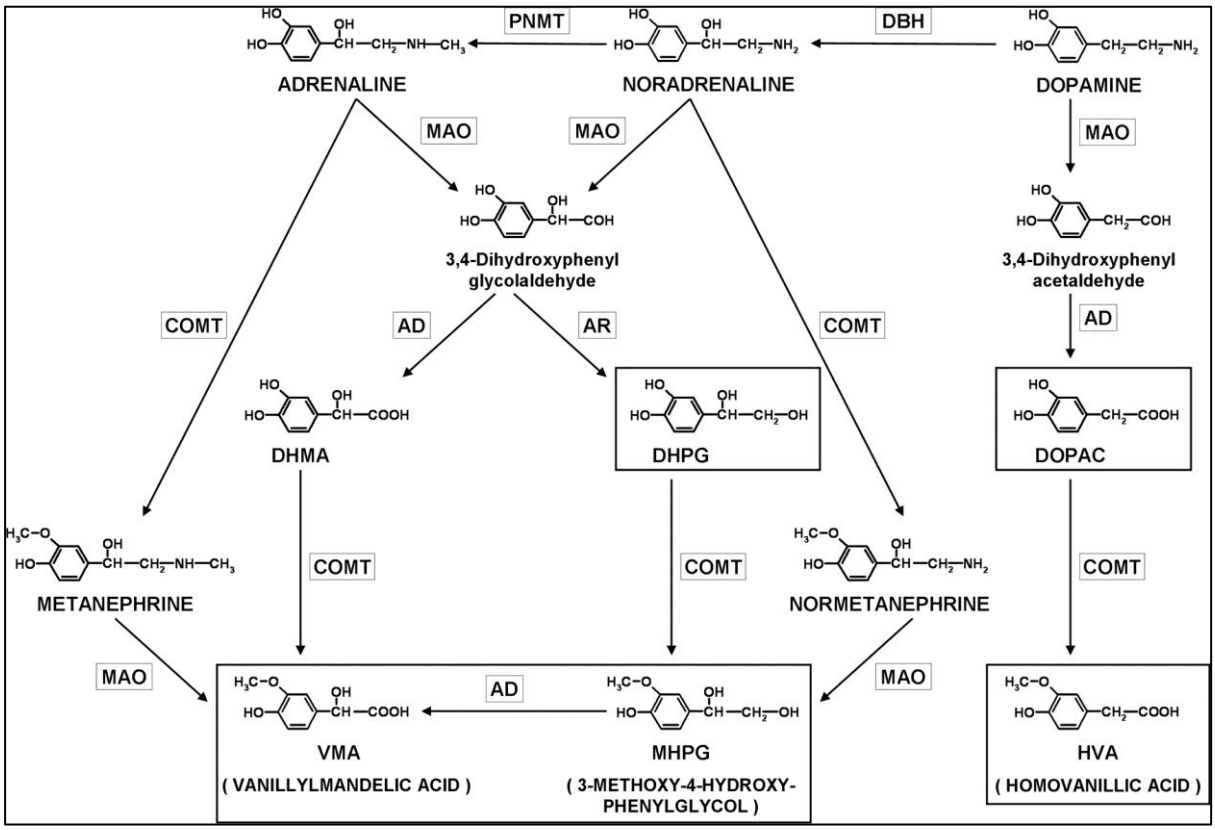

Figure 3-7: Primary catecholamine metabolic pathways (Kvetnansky *et al.*, 2009). AD = aldehyde dehydrogenase; AR = aldehyde reductase; COMT = catechol-O-methyltransferase; DBH = dopamine-β-hydroxylase; DHMA = 3,4- dihydroxymandelic acid; DHPG = 3,4-dihydroxyphenylglycol; DOPAC = dihydroxyphenylacetic acid; HVA = homovanillic acid; MAO = monoamine oxidase; MHPG = 3-methoxy-4-hydroxyphenylglycol; PMNT = phenylethanolamine N-methyltransferase; VMA = vanillylmandelic acid.

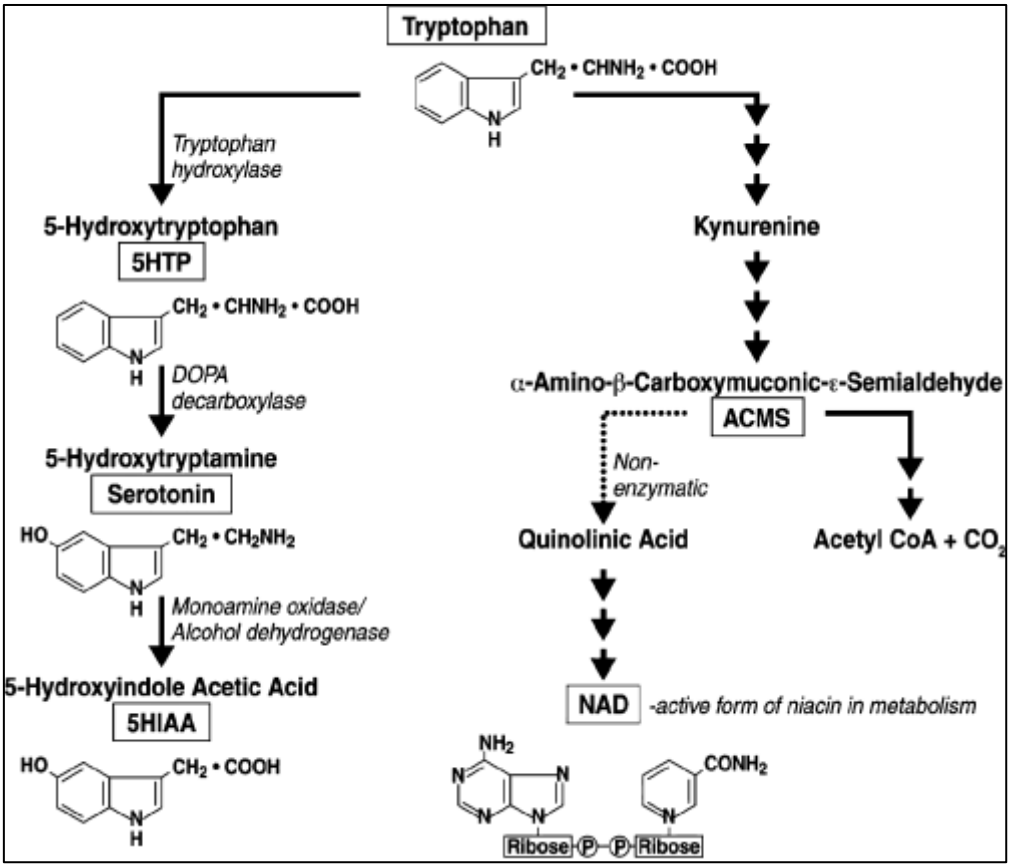

Figure 3-8: The two pathways involved in tryptophan catabolism (Grish *et al.*, 2005).

Table 3-4: Chromatographic apparatus used.

Apparatus:	Specifications:
Stationary phase/Column	Unisol C18, 2.1 x 150mm, 5µm, 100Å
Detector	ESA Coulochem III Electrochemical detector equipped with a Coulometric analytical cell 5011A Settings: Testing electrode 1 – Potential (-150mV), Range (1mA) Testing electrode 2 – Potential (750 mV); Range (500nA) Guard cell potential: 350 mV
HPLC instrument	Agilent 1200 Series HPLC, equipped with isocratic pump and autosampler
Flow rate	0.2 ml per minute
Volume injected	10 µl
Column and room temperature	23-25 °C
Guard column	4.0 x 3.0 mm C18 SecurityGuard™, HPLC Guard Cartridge System, Phenomenex

For this study an Agilent 1200 series HPLC equipped with the apparatus stated in the table below and Chromeleon® Chromatography Management software System (version 6.8) was used.

The biogenic amine and associated end-stage metabolite ***standard stock solutions*** were prepared in the following solution (***Solution A***):

- 0.3 mM Na_2EDTA (0.055836 g) and 0.5 mM sodium metabisulphate (0.047525 g) was dissolved in 400 ml distilled water.
- 0.1 M perchloric acid (5.435 ml) was added to the aforementioned solution and made up to 500 ml with distilled water.

After preparation, Solution A was maintained cool via refrigeration and all working standard solutions were later prepared from this.

Mobile phase preparation:

- 0.1 M sodium formate buffer (6.801 g/L), 5 mM sodium 1-heptanesulfone (1.01125 g/L), 0.17 mM ethylenediaminetetraacetic acid disodium salt (20 mg/L), acetonitrile (4% v/v). The pH was adjusted to between 3.5-4.1 using orthophosphoric acid (85%).
- The final solution was filtered using a membrane Millipore filter (0.22 µm) and then vacuum deaerated before use.

Table 3-5: Preparation methodology for monoamine standards (Harvey *et al.*, 2006).

Monoamine/ metabolite standards:	Raw components:	Preparation methodology:
IS	3,4-dihydroxy-benzylamine (DHBA; 220.1 MW; Sigma-Aldrich)	Dissolved 1 mg in 10 ml solution A. This served as IS stock solution. 30 µl of this stock solution was made up to 2 ml with Solution A, which served as the working internal standard producing a concentration of 1500 ng/ml.
MPHG	3-methoxy-4-hydroxyphenylglycol (184.19 MW; Sigma-Aldrich)	Dissolved 2.47 mg in 10 ml Solution A (100 µg/ml)
NA	L-Noradrenaline hydrochloride (205.6407 MW; Fluka)	Dissolved 1.22 mg in 10 ml Solution A (100 µg/ml)
HVA	Homovanillic acid (182.18 MW; Sigma-Aldrich)	Dissolved 1 mg in 10 ml Solution A (100 µg/ml)
DOPAC	Dihydroxyphenylacetic acid (168.15 MW; Sigma-Aldrich)	Dissolved 1 mg in 10 ml Solution A (100 µg/ml)
DA	3-Hydroxythyramine hydrochloride β-(3,4-dihydroxyphenyl)-aethylamine hydrochloride (189.64 MW; Sigma-Aldrich)	Dissolved 1.24 mg in 10 ml Solution A (100 µg/ml)
5-HT	Serotonin creatinine sulphate (405.43 MW; Merck)	Dissolved 2.30 mg in 10 ml Solution A (100 µg/ml)
5-HIAA	5-hydroxyindole-3- acetic acid (191.19 MW; Sigma-Aldrich)	Dissolved 1 mg in 10 ml Solution A (100 µg/ml)

Sample preparation: The samples were collected from the brain regions mentioned above and stored as described earlier. Prior to starting the analyses, the designated brain regions/samples were allowed to thaw, weighed and placed in newly marked clean tubes. Thereafter, 1 ml solution A was added to each sample-containing tube. These samples were then sonicated (12 second bursts in duplicate at a 14 µ amplitude) so as to disrupt/rupture the tissue cells after which they were placed on ice for 20 minutes and allowed to undergo perchlorate-induced protein precipitation which was necessary for monoamine extraction. After adequate precipitation had taken place over the specified time period, the samples were placed in the centrifuge (Hitachi, Sorvall Discovery ultra-centrifuge, Model 9056) and centrifuged at 4°C, 14 000 revolutions per minute for 30 minutes. The supernatant from each sample after centrifugation was collected and placed in new polypropylene tubes. From here on the samples (supernatant) were prepared for injection into the HPLC column by adding

50 µl/ml potassium acetate (10 M; 98.04 g potassium acetate dissolved in 100 ml distilled water) to each solution to adjust the pH to 5. Finally, 200 µl supernatant was pipetted and placed into an amber eppendorf tube, along with 20 µl DHBA/internal standard (IS). Thereafter, 10 µl of the samples was extracted and injected into the HPLC system. After each sample, chromatograms were generated. Concentration determinations were done the same day as sample injections into the HPLC column. Results were expressed as ng/ml as seen under *Concentration range* and the final results expressed as ng/g wet brain can be viewed in Chapter 4, § 4.3.4 and in Addendum A, § 3.3.

The validity of the HPLC-ED methodology was based on the following:

- *Selectivity and specificity* – Refers to this method as being capable of accurately analysing specific components in the presence of other components with reference to metabolites and other biological substances.

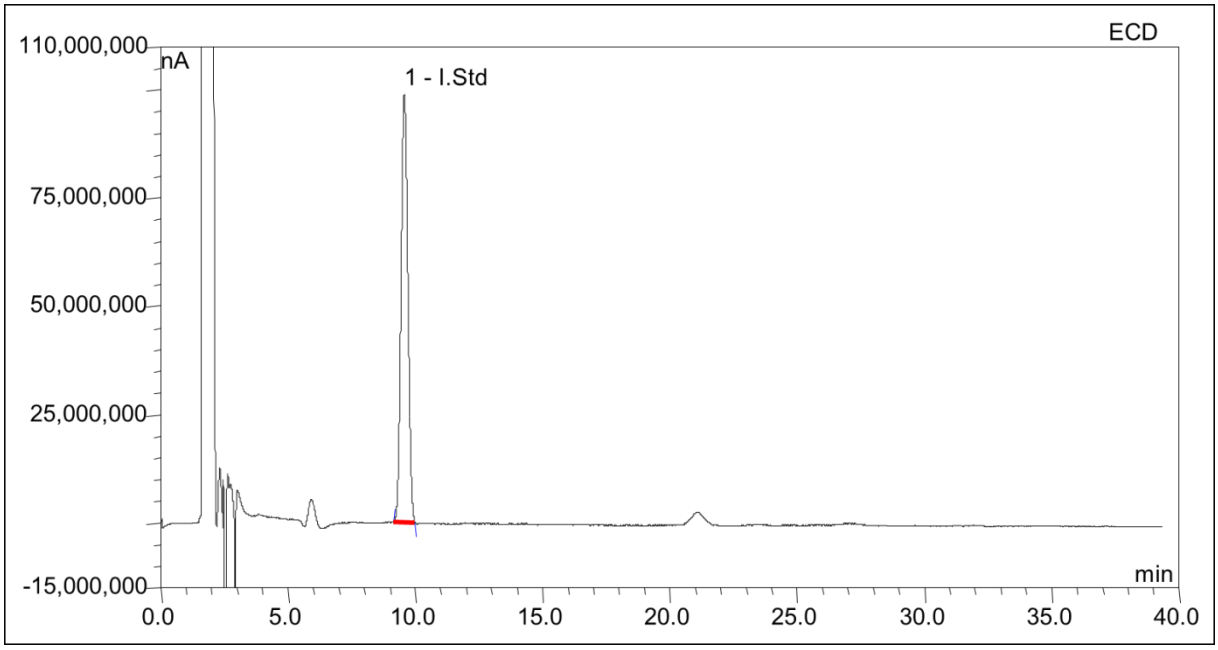

Figure 3-9: HPLC ED chromatograph of a blank sample.

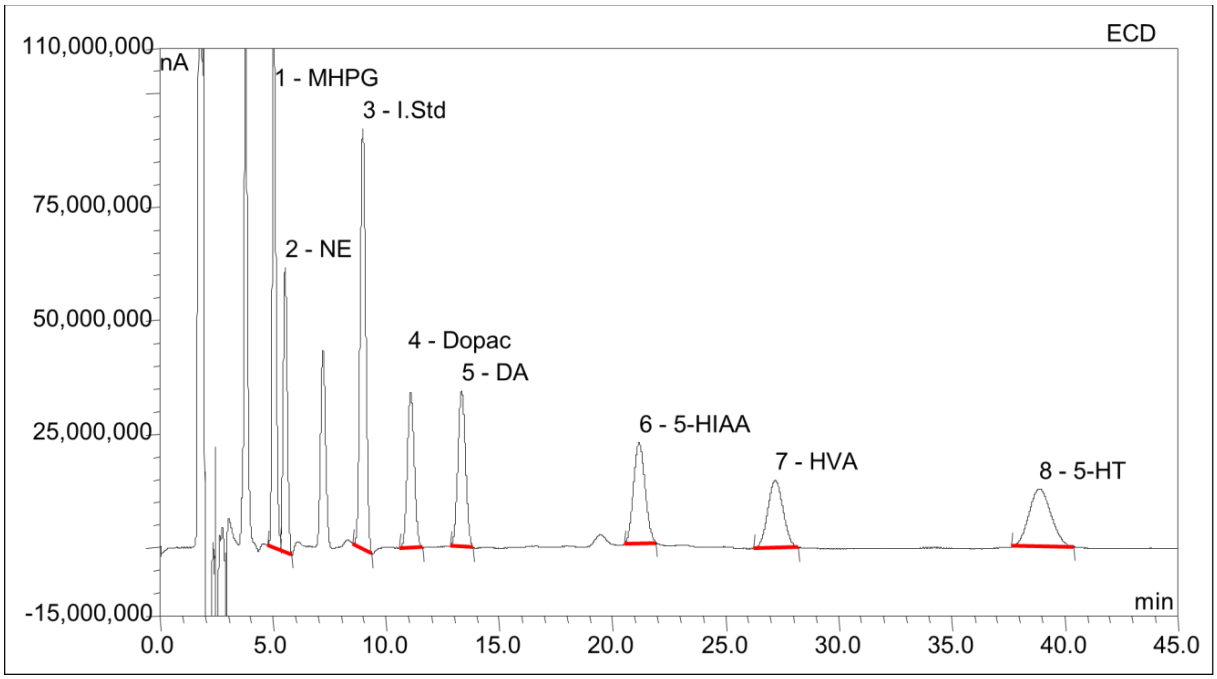

Figure 3-10: HPLC ED chromatograph of the internal standard used in this study (IS).

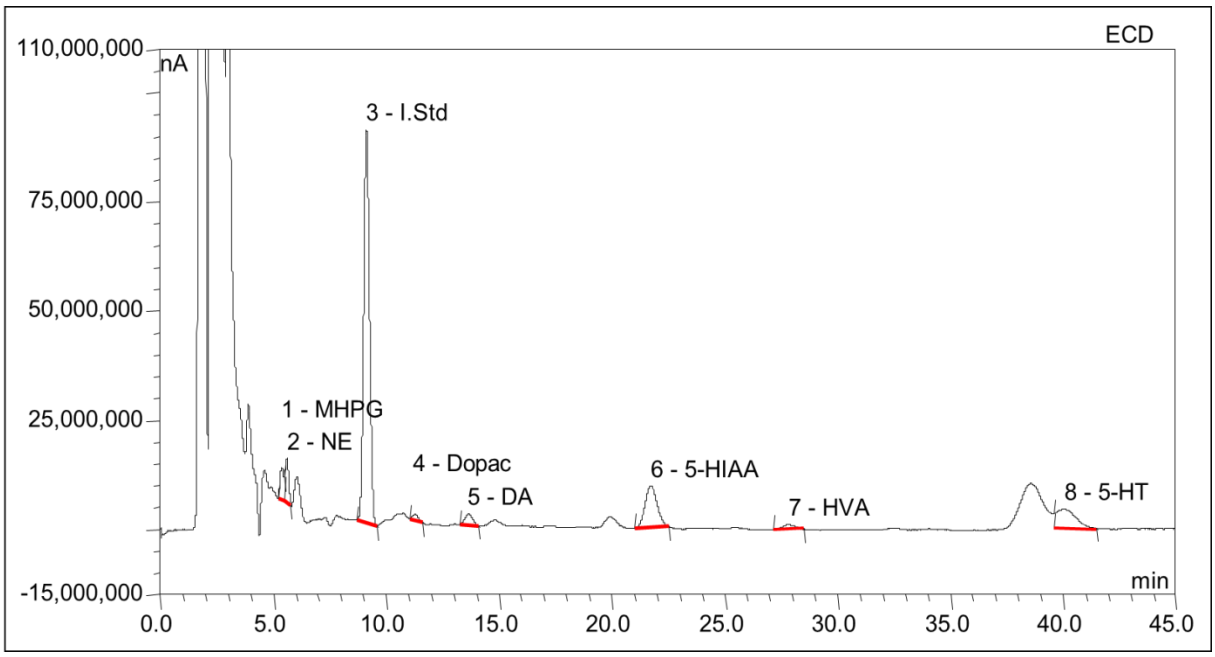

Figure 3-11: HPLC ED chromatograph of a single prefrontocortical sample after chronic allopurinol treatment.

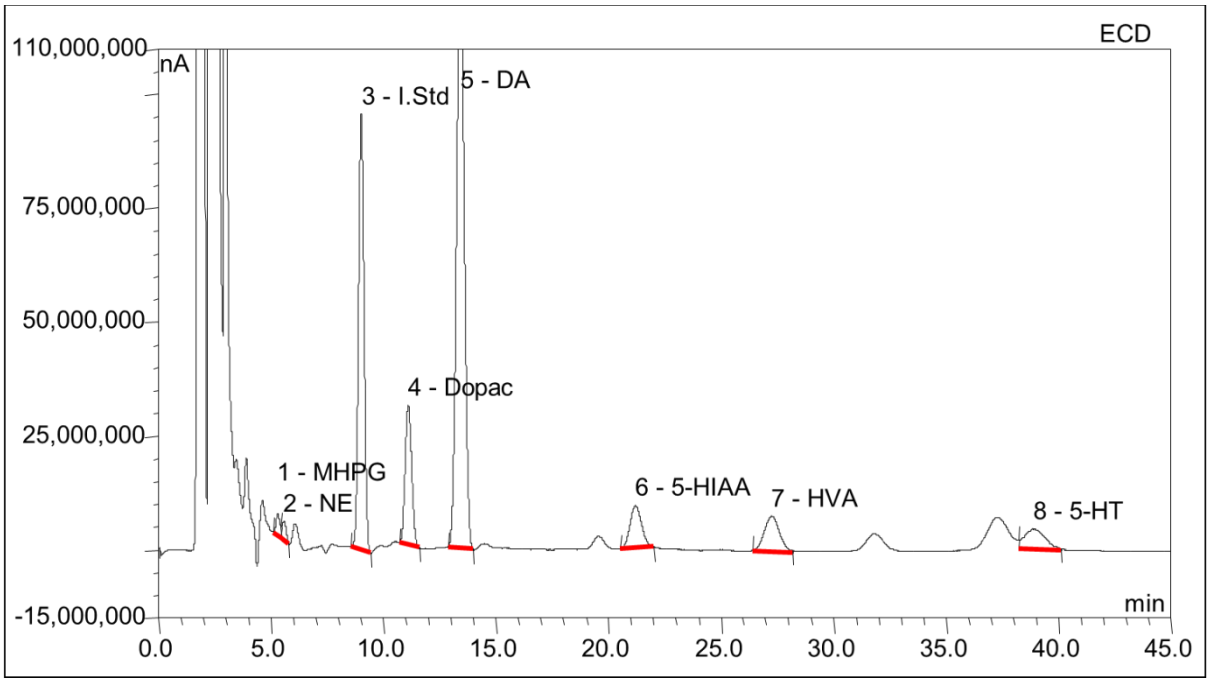

Figure 3-12: HPLC ED chromatograph of a single striatal sample after chronic vehicle treatment.

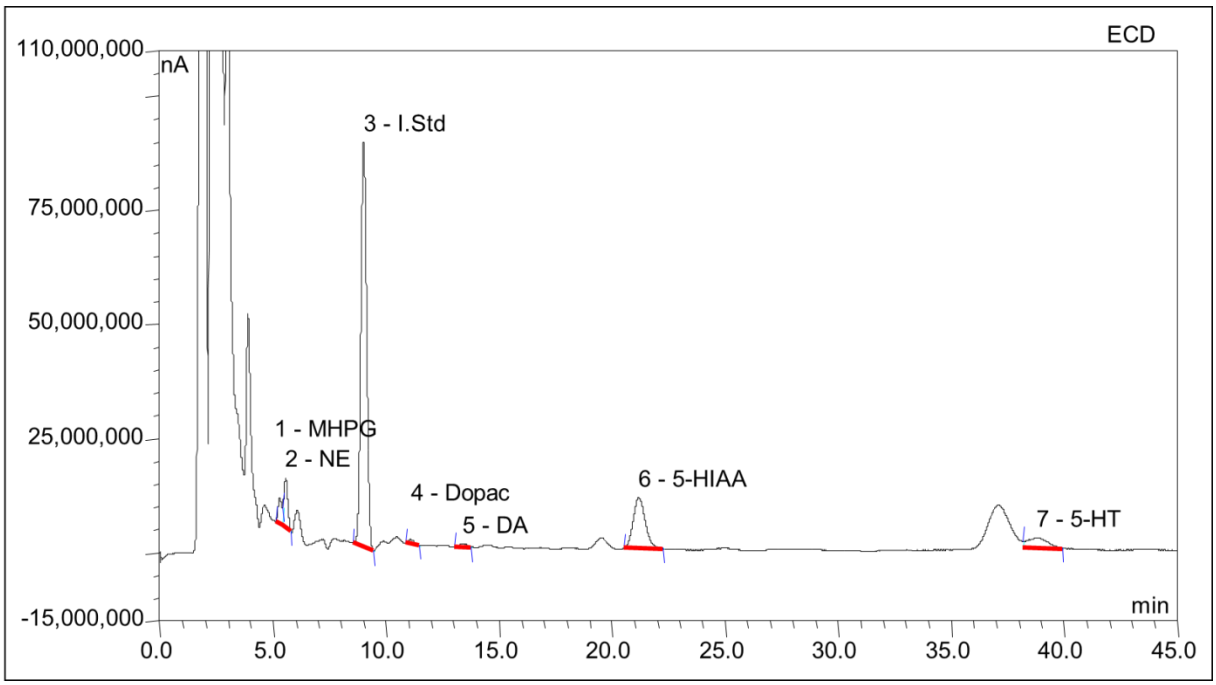

Figure 3-13: HPLC ED chromatograph of a single hippocampal sample after chronic vehicle treatment.

This method proved effective based on its adequate selectivity and sensitivity as evidenced by the lack of overlapping between the different compounds that were analysed in the above chromatographs (Figures: 3-9 to 3-13).

- *Linearity* – Refers to whether there was good linearity between the peak-surface areas across the concentration limits applied upon drafting the standard curves. Therefore, an adequate straight line ($y = mx + c$) could be drawn through the points on a graph of which the peak surface area (y) is plotted against concentration (x [ng/ml]) where m = gradient and c = the y-intercept. The concentration (ng/ml) was converted to concentration ng/g wet brain as expressed in the results chapter.

Table 3-6: Linearity expressed as *y = mx + c* ascertained with HPLC-ED analysis of monoamines and end-stage metabolites along with calculated regression values.

Monoamine or metabolite measured:	Straight line obtained (y = *mx* + *c*):	Regression (R^2) value obtained:
MHPG	*y = 24 144 755.00x*	0.99
NA	*y = 15 144 790.34x*	0.98
HVA	*y = 12 406 947.88x*	0.99
DOPAC	*y = 13 843 772.98x*	0.97
DA	*y = 14 916 503.25x*	0.99
5-HT	*y = 21 621 651.16x*	0.99
5-HIAA	*y = 18 759 302.47x*	0.99

- *Concentration range* – Refers to the sensitivity of its range of detection. In this instance, this method proved effective in detecting standard solution concentrations between 10, 25, 50, 75 and 100 ng/ml.

Table 3-7: Catecholamine and associate end-stage metabolite standard curve concentration ranges.

Standard Number	Concentration (ng/ml)	Dilution (µl)	+	Solution A (µl)	=	Total Volume (ml)
1	**10**	200 (5)	**+**	1800	**=**	2
2	**25**	10 (A)	**+**	1990	**=**	2
3	**50**	20 (A)	**+**	1980	**=**	2
4	**75**	30 (A)	**+**	1970	**=**	2
5	**100**	40 (A)	**+**	1960	**=**	2
A	**5000**	100 (SS)	**+**	1900	**=**	2

- *Repeatability* – Refers to the test-retest reliability of this method, therefore, to what extent the results obtained vary when analysing the same sample several times following the exact procedures. The RSD % for repeated injections (n=6) was < 5% for all the analytes in the measured concentration range and was within acceptable limits (FDA, 2001).

3.2.5.2 BDNF

MDD commonly presents with lower BDNF expression in the brain, which further contributes to neuronal atrophies within the hippocampus and the activation of other apoptotic pathways (Banasr *et al.*, 2011). Neuroplasticity deficits can also be seen in depression-induced cognitive abnormalities and neuronal growth modulating substances, such as BDNF, play a role in mood disorders (Duman, 2002; Manji *et al.*, 2003; Altar, 1999). BDNF levels are found to be decreased in depressed individuals (Campbell & MacQueen, 2004).

In this study, BDNF was quantified in brain samples collected from FSL rats, specifically right hemispheric prefrontal cortices, striata and hippocampi using a biosensis® BDNF *Rapid*™ enzyme-linked immunosorbent assay (ELISA) kit (BEK-2211-2P) and assayed according to the manufacturer's instructions. Samples were collected from the brain regions mentioned above and stored in the same manner as described for monoamine analyses. Prior to quantifying BDNF concentrations, total protein concentrations for each of the samples were measured using a Bradford protein assay method.

Reconstitution of provided materials in BDNF kit:

- The *recombinant BDNF standard* (1000 pg) was reconstituted with 1 ml *acid-extraction sample diluent* to yield a concentration of 1000 pg/ml.
- The *BDNF detection antibody* (110 µl) and *streptavidin-HRP conjugate* (110 µl) were diluted a hundred-fold using *Assay diluent A*.
- The *wash buffer* was diluted ten-fold with ultrapure water (25 ml diluted wash buffer per 96-well plate).

Formulation of solutions required for acid-extraction of BDNF:

- *Acid-extraction buffer*:
  - 0.8204 g sodium acetate (50 mmol/L; 82.0343 MW) dissolved in 200 ml distilled water added to
  - 11.69 g sodium chloride (1.0 mol/L; 58.44 MW) dissolved in 200 ml distilled water added to
  - 200 ml 0.1% Triton X-100
  - pH adjusted to ± 3.95 with acetic acid
  - Prior to use, one Complete Mini protease inhibitors cocktail tablet was added to the buffer (1 tablet per 50 ml buffer) and kept on ice.
- *Incubation/Neutralisation buffer*:
  - 6.8045 g monopotassium phosphate (0.1 mol/L; 136.086 MW)
  - 7.089 g disodium hydrogen phosphate (0.1 mol/L; 141.96 MW)
- *Acid-extraction sample diluent*:
  - Made by mixing 1 part acid-extraction buffer with 3 parts incubation/neutralisation buffer.

Sample preparation: Before starting the Bradford protein assay, the designated brain regions/samples were allowed to thaw, weighed and placed in newly marked clean tubes. Thereafter, the volume ***acid-extraction buffer*** for each sample was calculated and added to the transferred samples in the tubes.

$$Volume\ extraction\ buffer = (Sample\ weight\ in\ mg)(\frac{100\ \mu l\ extraction\ buffer}{10\ mg\ sample})$$

Each tube containing the sample and allotted acid-extraction buffer was then sonicated (bursts of 5-7 seconds done in duplicate) to rupture the tissue cells to allow the release of proteins. After sonication the samples were placed on ice for 30 minutes to allow protein precipitation to take place. This was repeated a second time to ensure complete cell rupture and protein release. The sonicated samples were then centrifuged (Hitachi, Sorvall Discovery ultra-centrifuge, Model 9056) at 4°C, 14 000 revolutions per minute for 30 minutes. After centrifugation, the samples were removed, the clear supernatant extracted from each tube (using a new pipetting tip for each sample) and transferred to a new eppendorf tube and then vortexed. To follow, 100 µl of the supernatant was extracted from each sample (using a new pipetting tip for every sample) and placed in a new clean

eppendorf prior to undergoing Bradford protein analysis. The remainder of supernatant samples were frozen at -80°C for use in the BDNF assay.

Preparation of standards for Bradford's protein assay (Bradford, 1976): 5 mg bovine serum albumin (BSA) was dissolved in 1 ml distilled water (5 mg/ml solution) and vortexed before making a series of 100 µl dilutions as illustrated in Table 3-6. Initially, the different volumes acid-extraction buffer (Table 3-6, last column on right) were added to clean eppendorfs followed by the addition of the respective volumes BSA (Table 3-6, column left of acid-extraction buffer volumes).

Table 3-8: Standards (mg/ml), BSA (µl) and acid-extraction buffer (µl) volumes and concentrations used in Bradford protein assay.

Standard number:	Protein concentration of standard (mg/ml):	Volume (µl) BSA (5 mg/ml) added:	Volume (µl) acid-extraction buffer added:
1	0	0	100
2	0.5	10	90
3	0.8	16	84
4	1.0	20	80
5	1.25	25	75
6	1.5	30	70
7	1.75	35	65
8	2.5	50	50

The refrigerated Bradford's solution was shaken, the volume required for the assay calculated (250 µl per well x 96 wells = 24 000µl) and poured into a 50 ml tube, sealed with foil to protect it from light and allowed to reach ambient temperature prior to being used.

Preparation of 96-well plate: 2 x 5 µl of each standard (Table 3-6, second column on left) and sample (100 µl supernatant extracted earlier) was added to separate wells on a 96-well plate. Thereafter, 250 µl Bradford's solution was added to each plate in three intervals (100 µl + 100 µl + 50 µl) and immediately shaken for 30 seconds on the mixing facility of the plate reader. The plate was then left to incubate for 30 minutes at ambient temperature prior to being read. The absorbance in each well was determined in a 96-well plate reader using a 560 nm filter. The protein concentration of each brain sample (supernatant sample) was determined from the graph produced using net absorbance plotted against standard protein concentrations.

Table 3-9: Linearity expressed as y = mx + c ascertained with Bradford's protein assay of sample preparations along with calculated regression values.

Brain region assayed:	Straight line obtained (y = *mx* + *c*):	Regression (R^2) value obtained:
Prefrontal cortex	*y = 0.1505x − 0.0102*	0.98
Striatum	*y = 0.12x + 0.0017*	0.99
Hippocampus	*y = 0.1397x − 0.018*	0.99

BDNF assay (BDNF Rapid™ ELISA kit):

The brain tissue extracts (supernatant) prepared for the Bradford protein assay were also utilised for the BDNF assay after being thawed and centrifuged.

Preparation of BDNF standards: The supplied recombinant BDNF standard (1000 pg) was reconstituted using 1 ml acid-extraction sample diluent, vortexed and left to stand for 15 minutes. Thereafter, it was further diluted using a serial dilution method as illustrated in Figure 3-9.

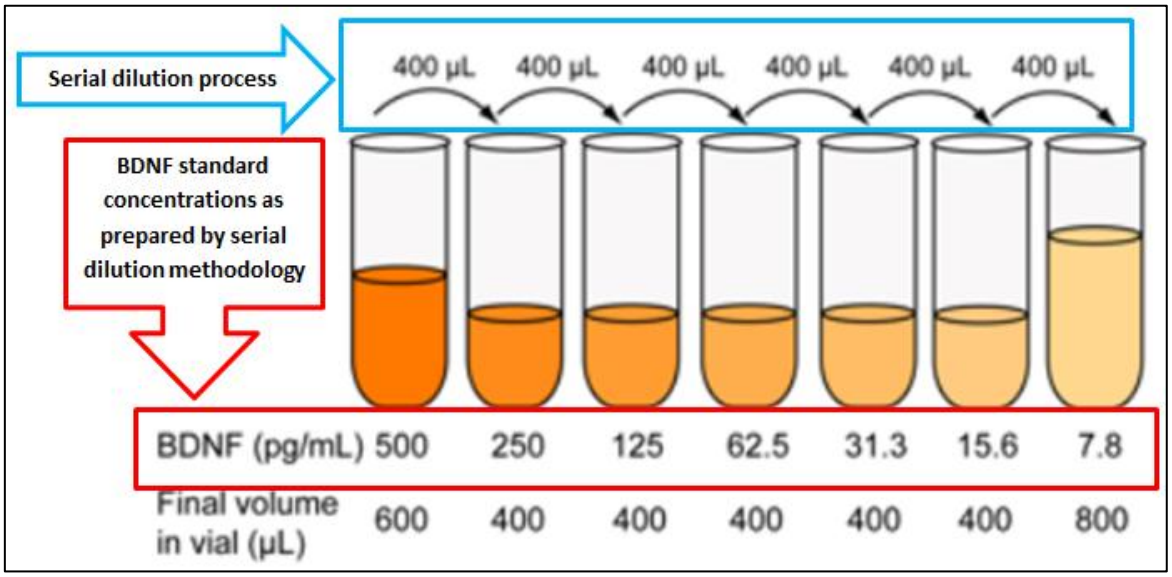

Figure 3-14: Illustration of a 1:2 serial dilution in preparation of BDNF standards for generation of a BDNF standard curve.

Six eppendorf tubes were labelled according to the BDNF standard concentrations as indicated in Figure 3-9. From the 500 pg/ml tube (containing the reconstituted BDNF standard of 1000 pg/ml), 400 µl was extracted and transferred to the 250 pg/ml tube. This process was followed until reaching a concentration of 7.8 pg/ml. All standards were vortexed briefly after formulation, prior to being added to the BDNF antibody coated 96-well microplate.

Preparation of sample dilutions: A stock solution dilution was made for each sample with 1 part tissue extract (supernatant; 100 µl) and 3 parts Incubation/Neutralisation buffer

(300 µl) to a total volume of 400 µl sample dilution and a sample dilution factor of 4. Before analysing all the samples, a test plate was formulated to establish the dilution factors for all the samples in the different brain regions to be analysed. One sample from each brain region from the treatment group vehicle, fluoxetine and allopurinol was chosen to do so. The reason for this group selection was based on the premise that these drug groups would exhibit increased BDNF levels in the specific brain regions. Further sample dilutions from the stock solution dilution were made using the acid-extraction sample diluent (AESD). The dilution factors (DF) chosen for the test plate ranged from 4-512 as illustrated in Figure 3-9. Therefore, 100 µl from then stock solution (DF = 4) was transferred to a new eppendorf tube to which 100 µl AESD was added. The same process was continued for each sample until reaching the final dilution (DF = 512).

BDNF plate preparation: 100 µl of the BDNF standards were added to each pre-coated microplate well in duplicate as illustrated in Figure 3-9. Subsequently, 100 µl of each sample was added to a single pre-coated microplate well as illustrated in the same figure. The plate was then sealed with the provided plate sealer and incubated on a plate shaker (140 rpm) for 45 minutes. The plate was then removed, the sealer taken off and the plate placed in a plate washer programmed to wash each well 5 times using the provided wash buffer (200 µl per well). After washing was completed, 100 µl of the provided detection antibody was added to each well. The plate was sealed again and allowed to incubate in the shaker (140 rpm) for 30 minutes. After incubation the sealer was removed, the solution in the wells discarded and the plate washed as stated earlier. 100 µl of the provided streptavidin-HRP conjugate was added to each well and the plate was once again sealed and incubated in the shaker (140 rpm) for 30 minutes. Thereafter, the plate sealer was removed, the solution discarded and the plate was washed again as stated earlier. Then, 100 µl of the provided TMB substrate was added to each well and allowed to incubate in the dark for 4-15 minutes without shaking until a blue colour started to appear. The reaction was stopped by adding 100 µl of the provided TMB stop solution, changing the colour from blue to yellow. The plate was then placed in the plate reader and read at an absorbance of 450 nm. Reading had to take place within 5-10 minutes to prevent the colour from fading over time.

- Columns 1-2: Standards in duplicate, as prepared in Figure 3-9.
- Columns 3-5: PFC – VEH (3), FLX (4) and ALLOP (5); (DF = 4-512).
- Columns 6-8: STR – VEH (6), FLX (7) and ALLOP (8); (DF = 4-512).
- Columns 9-11: HPC – VEH (9), FLX (10) and ALLOP (11); (DF = 4-512).

	1	2	3	4	5	6	7	8	9	10	11	12	
A	0	0	4	4	4	4	4	4	4	4	4		**A**
B	7.8	7.8	8	8	8	8	8	8	8	8	8		**B**
C	15.6	15.6	16	16	16	16	16	16	16	16	16		**C**
D	31.3	31.3	32	32	32	32	32	32	32	32	32		**D**
E	62.5	62.5	64	64	64	64	64	64	64	64	64		**E**
F	125	125	128	128	128	128	128	128	128	128	128		**F**
G	250	250	256	256	256	256	256	256	256	256	256		**G**
H	500	500	512	512	512	512	512	512	512	512	512		**H**
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	

Figure 3-15: Layout for test plate using a BDNF 96-well plate with varying dilution factors for brain PFC, STR and HPC

From the results of Plate 1's analysis the most effective DF's for the rest of the samples from the various brain regions could be determined. Each subsequent plate was grouped according to brain region, thus: Plate 2 was for PFC samples (DF = 4), Plate 3 for STR samples (DF = 4) and Plate 4 for HPC samples (DF = 16). Samples were allocated to the wells in a random fashion to avoid detection errors that may affect both samples of a duplicate. Each subsequent plate was subjected to the same preparation processes and analyses for Plate 1 as stated under ***BDNF plate preparation***.

Subsequently, BDNF quantities were calculated by dividing the concentration BNDF measured during the BDNF analysis by the concentration protein measured as determined using Bradford's analysis. The succeeding values were expressed as pg BDNF per mg protein (pg BDNF/mg protein) and can be viewed in Chapter 4, § 4.3.5.

The outcomes of the neurochemical analyses (monoamines and BDNF) are illustrated in Chapter 4 and in part in Addendum A, and discussed in Chapter 5 of this study.

3.2.6 Data analysis

The statistical analyses were carried out in association with the Statistical Consultation Services of the NWU, Potchefstroom Campus. All data are expressed as the mean and/or the standard error of the mean (SEM). A p-value of $p<0.05$ was considered statistically significant. GraphPad Prism® version 6.00 for Windows was employed for both statistical analysis and graphical illustrations of behavioural and neurochemical analysis results.

3.2.6.1 Phase 1: Confirmation of expressed depressive-like phenotype along with cognitive insufficiencies within an animal model of depression – FSL vs. FRL

A two-sample t-test (<2 groups compared) was used to compare the FSL groups to FRL groups for the different behavioural studies. This test was used to compare the mean values of two samples, especially since the samples were small.

3.2.6.2 Phase 2: Acute dose-ranging analysis – FSL

Data analysis was done in parallel (multiple comparisons) using one-way analysis of variance (ANOVA) along with a post-hoc Dunnett's multiple comparisons test across all groups (>2 groups compared).

3.2.6.3 Phase 3: Main experimental study – FSL

Data analysis for the FST and its related general locomotor activity assessment was done in parallel (multiple comparisons) using one-way ANOVA along with a post-hoc Dunnett's multiple comparisons test across all groups (>2 groups compared).

Data analysis for the MWM acquisition training over Days 1-5 was done using a two-way ANOVA with repeated measures, followed by a Tukey's multiple comparisons post-hoc test. Acquisition for each day was done using a two-way ANOVA along with a post-hoc Dunnett's multiple comparisons test across all groups. Data analysis for escape latency during the probe trial/memory storage (retrieval) and general locomotor assessment was done using one-way ANOVA along with a multiple comparisons Dunnett's post-hoc test (>2 groups compared).

Furthermore, the results obtained from Phase 3 were subjected to practical significance analysis by calculation of Cohen's d-value. The collective presentation of the quantitative results from both statistical and practical significance analyses, are deemed complementary (Maher *et al.*, 2013). Practical significance testing allowed us to determine the difference in effect sizes between the variables analysed. Consequently, providing a measure of observed variable strength between treatment group responses. As a result, these sizes provide an indication of the practical meaningfulness of the differences measured during statistical analyses.

$$\text{Cohen's } d = \frac{\overline{X}_1 - \overline{X}_2}{\text{SD}_{\text{pooled}}}, \text{ where } \text{SD}_{\text{pooled}} = \sqrt{\frac{\sum (X_A - \overline{X}_A)^2 + \sum (X_B - \overline{X}_B)^2}{n_A + n_B - 2}}$$

Figure 3-16: Formula for calculation of Cohen's d-value (Maher *et al.*, 2013).

All results and graphical illustrations collected from the statistical analyses can be viewed in Chapter 4 and Addendum A and are subsequently discussed in Chapter 5.

Chapter 4 : Results

The purpose of this study entailed the investigation of the potential antidepressant-like and/or procognitive effects of agents with direct/indirect actions on glutamatergic pathways, viz. allopurinol (kynurenine metabolism) and sodium benzoate (N-methyl-D-aspartate (NMDA) receptor modulator). The antidepressant and/or procognitive activity of two reference NMDA active compounds (ketamine and memantine) was also determined. Primarily, we performed a dose-ranging analysis (§4.2) of both allopurinol and sodium benzoate to establish the most effective concentration to be administered as a chronic regime. Thereafter, the effect of chronic treatment with the aforementioned test and reference compounds on depressive-like (FST) and learning-type (MWM) behaviours (§4.3) was investigated as well as the effect of the same treatment regimen on brain monoamine (§4.3.4) and BDNF (§4.3.5) levels with specific reference to the prefrontal cortex, striatum and hippocampus. Our secondary objectives were aimed at confirming the depressive-like phenotype (FST) and possible associated cognitive (learning and memory) abnormalities (MWM) within the chosen animal model, the FSL rat, compared to its healthy counterpart (FRL). Chapter 3 clearly outlines the study approach with special reference to the animals, drug compounds, behavioural-, neurochemical and statistical analyses employed in obtaining the results expressed in Chapter 4.

4.1 Phase 1: Confirmation of expressed depressive-like phenotype along with cognitive insufficiencies within an animal model of depression – FSL vs. FRL

The objective of the initial phase of this study involves confirmation of phenotypical depressive-like behaviours and/or cognitive abnormalities in the form of learning capabilities when subjecting the animal model to specific behavioural analyses (Overstreet, 1993; Overstreet *et al.*, 2005), such as the forced swim test (FST) and the Morris water maze (MWM) (See Chapter 3 for description of methodologies). FSL rat depressive-like behaviour compared to healthy controls (FRL) is assessed during an acute FST (Cryan *et al.*, 2002: Porsolt *et al.,* 1978) whereas learning behaviour in FSL vs. FRL rats is assessed via the MWM test over a 5-day period of acquisition training with the final assessment (memory retrieval) taking place on Day 5 (Hamlyn *et al.*, 2009; Morris *et al.*, 1986). Chapter 3 clearly describes the aforementioned methodologies. During acquisition training, each rat is subjected to 4 training sessions per day (total of 20 sessions over 5 days) each in a different location of the MWM in which they have to find the hidden platform. Memory retrieval is

evaluated using a single probe trial of 60 seconds (per rat) after the platform has been removed. To our knowledge, the Flinders Sensitive Line model has only been validated with regard to recognition memory using the novel object recognition test (NORT) (Abildgaard *et al.*, 2011; Erasmus *et al.*, 2015; Mokoena *et al.*, 2015), but not yet for cognitive impairments (viz. learning and memory disabilities) using the MWM,

4.1.1 Depressive-like phenotype:

4.1.1.1 Open-field test

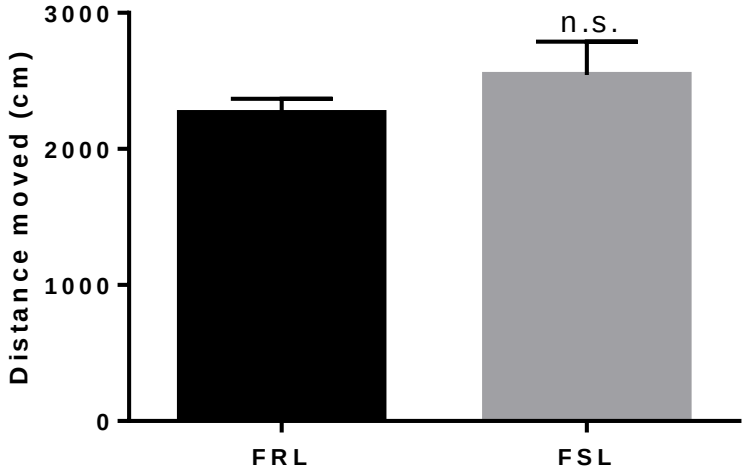

Figure 4-1: Distance moved of untreated FSL vs. FRL rats as measured in the OFT. n.s. p > 0.05 (unpaired t-test) n=6 for all groups.

Figure 4-1 illustrates the general locomotor activity of FSL vs. FRL rats expressed as distance moved (cm) in the open field test. Overall, no significant differences (F = 5.315, p > 0.05) were observed between the two which is in line with earlier research done within our laboratories (Brand, 2011).

4.1.1.2 Forced swim test

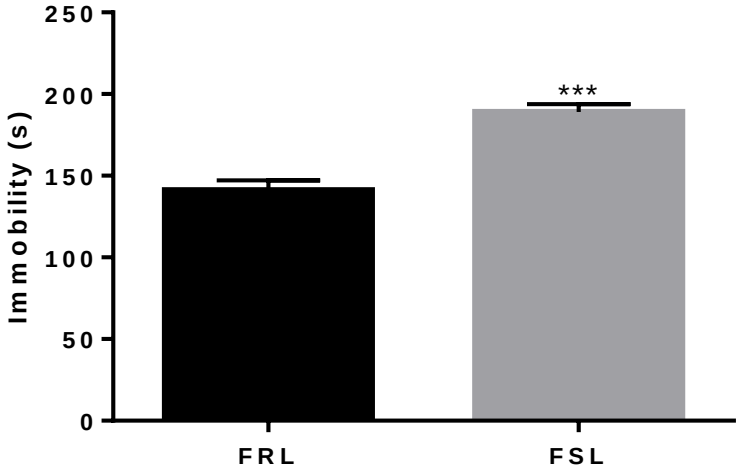

Figure 4-2: Immobility time of untreated FSL vs. FRL rats measured in the FST. ****p < 0.0001 (unpaired t-test). n=6 for all groups.

However, substantial significant differences (F = 1.601, p = 0.0001) in immobility time between FSL (189.1 ± 4.817 seconds) vs. FRL (141.2 ± 6.096 seconds) rats were observed as evidenced in Figure 4-2.

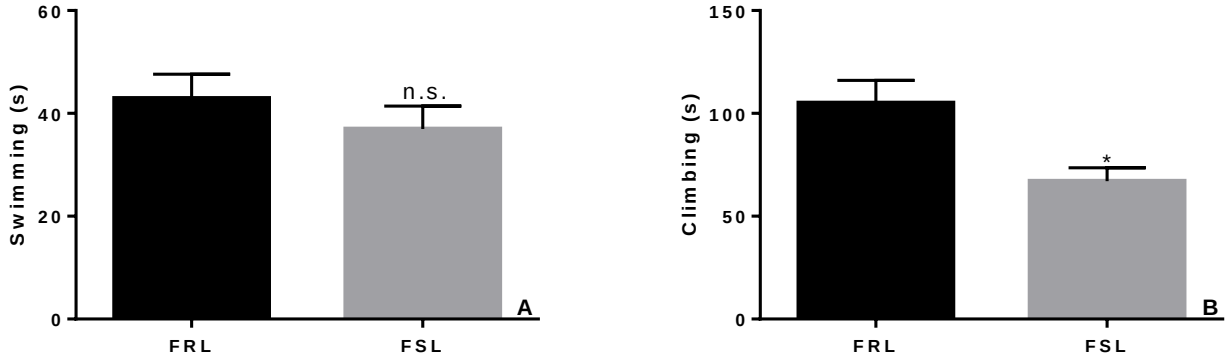

Figure 4-3: Swimming (A) and climbing (B) time of untreated FSL vs. FRL rats measured in the FST. n.s. p > 0.0.5, *p < 0.05 (unpaired t-test). n=6 for all groups.

These results confirm the depressive-like phenotype of the FSL model (as discussed in §5.2.1). No significant differences in swimming behaviour between the FSL and FRL groups were observed (F = 1.131, p > 0.05) (Figure 4-3: A), however the FSL group did present with reduced climbing behaviours compared to the healthy control group (F = 2.961, p = 0.0143) (Figure 4-3: B).

4.1.2 Cognitive function test:

4.1.2.1 Morris water maze

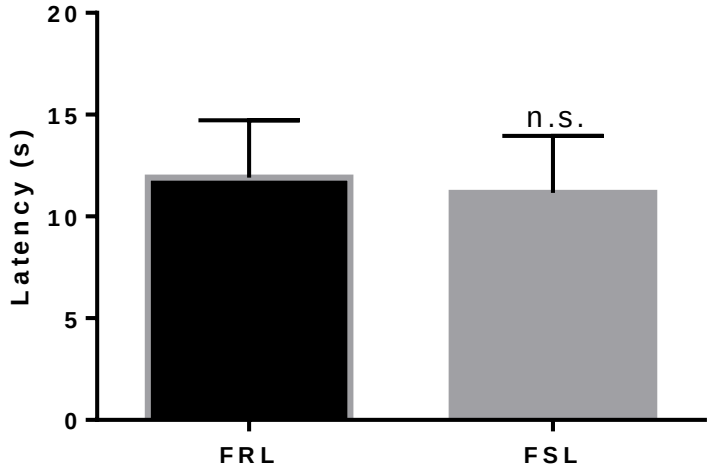

Figure 4-4: Cued trial of untreated FSL vs. FRL rats measured in the MWM. n.s. p > 0.05 (unpaired t-test) n=6 for all groups.

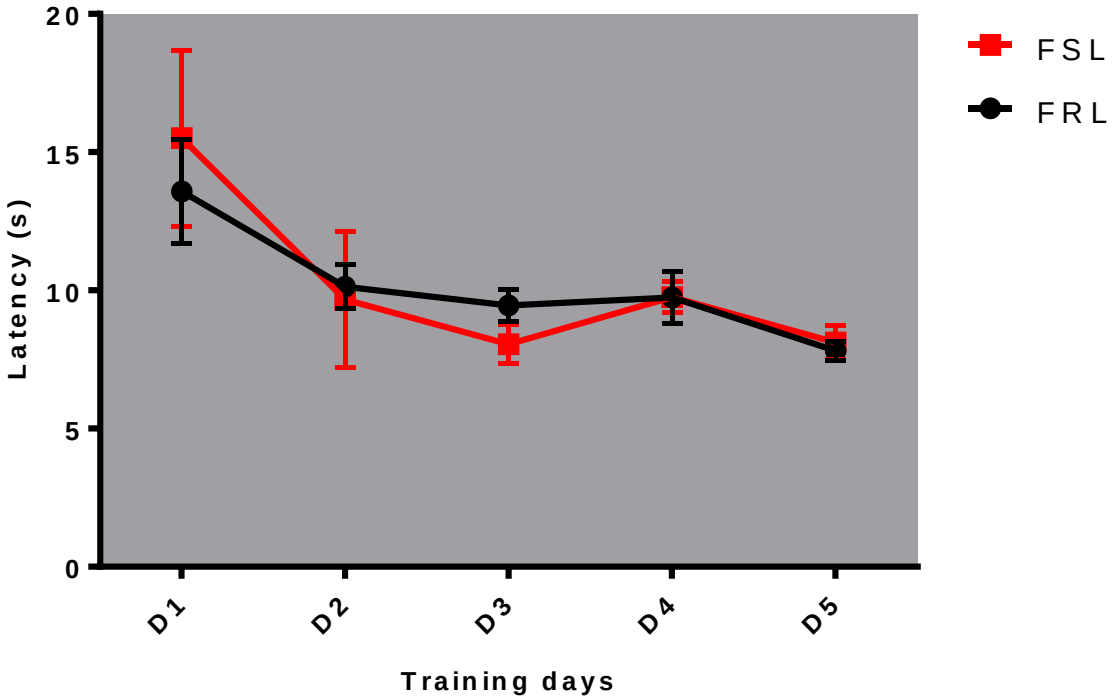

Figure 4-5: Acquisition training Days 1 to 5 of untreated FSL vs. FRL rats measured in the MWM. (Repeated measures two-way ANOVA: Tukey post-hoc) n=6 for all groups.

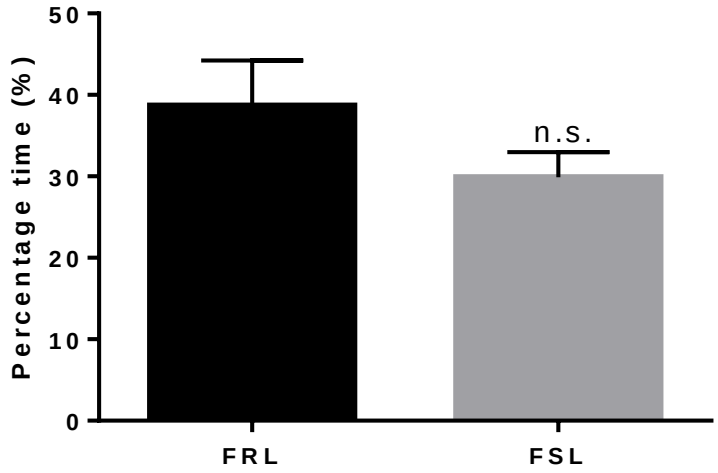

Figure 4-6: Percentage time spent in target zone for untreated FSL vs. FRL rats measured in the MWM.
n.s. p > 0.05 (unpaired t-test) n=6 for all groups.

Locomotor activity with relevance to the MWM (Figure 4-4) was also assessed and is expressed as latency to the hidden platform (seconds). No statistically significant differences (F = 1.013, p > 0.05) in locomotor activity between FSL vs. FRL rats were noted. As illustrated in Figure 4-5, both FSL and FRL rats demonstrate a noteworthy spatial learning curve (F (4, 24) = 7.750, p = 0.0004) over the acquisition training period indicating that memory consolidation had taken place. However, there were no significant differences in learning behaviour (F (1, 6) = 0.003041, p > 0.05) between the two strains. Additionally, there were no noteworthy differences to report on the strain-time interaction (F (4, 24) = 0.4385, p > 0.05) across the 5 days. Furthermore, on a visual level it may seem as though FSL rats present with learning impairments compared to their healthy counterparts during memory retrieval assessment (Figure 4-6), although it cannot be corroborated statistically (F = 3.235, p > 0.05). However, earlier research supports the observation that the FSL model presents with cognitive abnormalities pertaining to learning abilities (Overstreet, 1993; Abildgaard *et al.*, 2011; Mokoena *et al.*, 2015).

4.2 Phase 2: Acute dose-ranging analysis – FSL

The phenotypical depressive-like behaviour (immobility) of the FSL model was established and illustrated in Figure 4-2. Henceforth, the most effective dose for the test compounds (allopurinol and sodium benzoate) could be determined. To our knowledge, no dose-ranging analyses on these two compounds have been published with specific reference to the evaluation of depressive-like behaviours in the FST and cognitive impairments in the MWM in the FSL model.

Groups of FSL rats received either allopurinol (5, 10, 20, 50 and 100 mg/kg) or sodium benzoate (50, 100, 150 and 200 mg/kg) in varying doses via the intraperitoneal (i.p.) route at three different time intervals within 24 hours, prior to being subjected to an acute forced swim test (Chapter 3 for details).

4.2.1 Acute treatment: Allopurinol

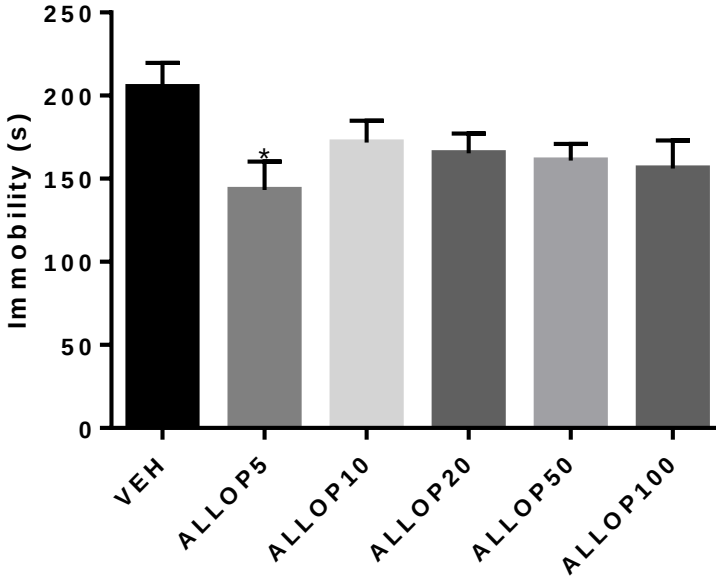

Figure 4-7: Immobility time of FSL rats in the FST after acute treatment with varying doses allopurinol (5, 10, 20, 50 and 100 mg/kg; ALLOP5/10/20/50/100) compared to vehicle (VEH) treated control rats. n.s. p > 0.05 (unpaired t-test), *p < 0.05 (ordinary one-way ANOVA: Dunnett's post-hoc. n=6 for all groups.

The measurable reduction in immobility time for the 5 mg/kg allopurinol treated group (143.3±17.12 seconds) vs. the vehicle treated group (205.1±14.63 seconds) provides marked indication of the most effective dose for allopurinol ($p < 0.05$) after acute FST exposure (Figure 4-7). Similar reductions have been observed at higher doses (> 20 mg/kg) and for longer treatment periods (Gibney *et al.*, 2014; Julian & Chytil, 1970). Though there is a moderate trend in immobility reduction across the different doses (F (6, 34) = 2.684, $p < 0.05$), no other dose induced noteworthy effects on immobility time. Therefore, 5 mg/kg allopurinol was applied in the main experimental study phase that follows in section 4.3.

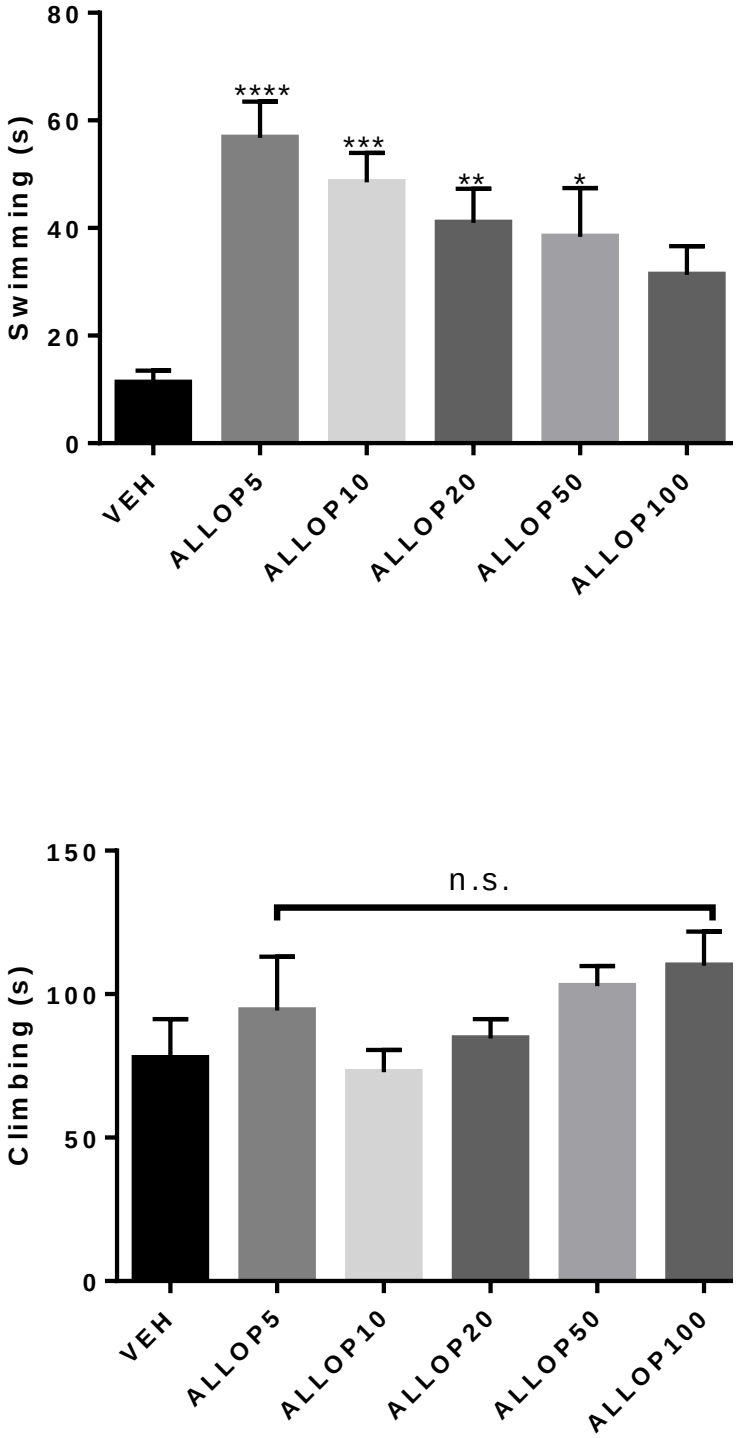

Figure 4-8: Swimming (above) and climbing (below) time of FSL rats in the FST after acute treatment with different doses allopurinol (5, 10, 20, 50 and 100 mg/kg) compared to vehicle treated control rats. n.s. $p > 0.05$, *$p < 0.05$ (50 mg/kg), **$p < 0.01$ (20 mg/kg), ***$p < 0.001$ (10 mg/kg) and ****$p < 0.0001$ (5 mg/kg) (ordinary one-way ANOVA: Dunnett's post-hoc). n=6 for all groups.

Additionally, all doses (ALLOP5 $p < 0.0001$, ALLOP10 $p < 0.001$, ALLOP20 $p < 0.01$ and ALLOP50 $p < 0.05$) excluding 100 mg/kg allopurinol caused a considerable increase in swimming behaviours compared to the vehicle treated group with no significant effect on climbing (n.s $p > 0.05$) (Figure 4-8).

4.2.2 Acute treatment: Sodium benzoate

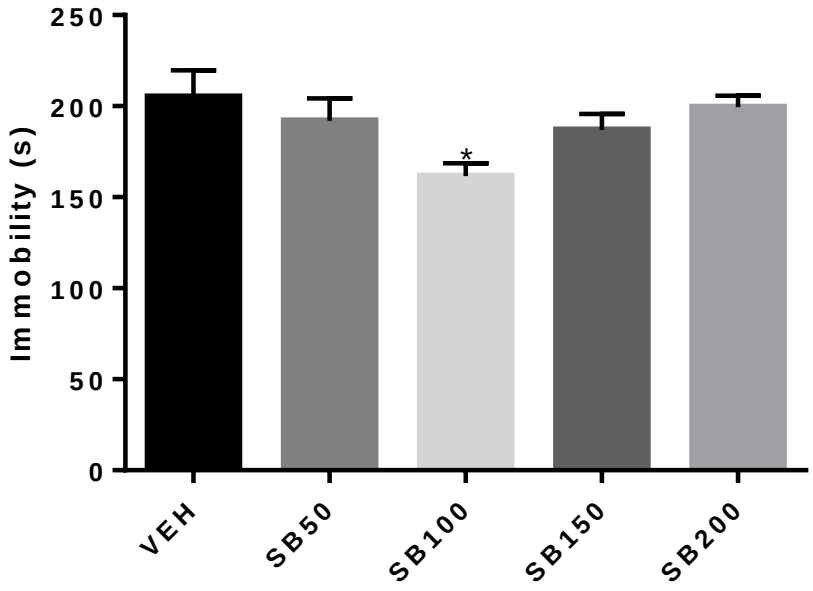

Figure 4-9: Immobility time of FSL rats in the FST after acute treatment with different doses sodium benzoate (50, 100, 150 and 200 mg/kg, SB50/100/150/200) compared to VEH treated control rats. *p < 0.05 (ordinary one-way ANOVA: Dunnett's post-hoc). n=6 for all groups.

As evidenced in Figure 4-9, sodium benzoate 100 mg/kg caused a discernible reduction in immobility ((161.5±7.244 seconds) $p < 0.05$) time compared to the vehicle treated group (205.1±14.63 seconds). None of the other doses proved effective in improving mobility (F (5, 30) = 2.303, $p > 0.05$). Therefore, 100 mg/kg sodium benzoate was applied in the main experimental study phase that follows.

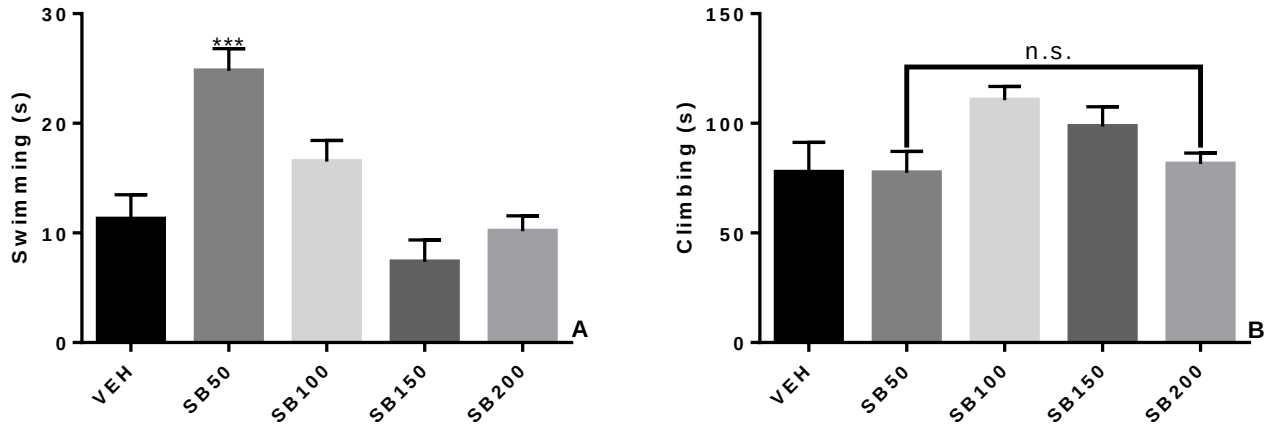

Figure 4-10: Swimming (A) and climbing (B) time of FSL rats in the FST after acute treatment with and different doses sodium benzoate (50, 100, 150 and 200 mg/kg) compared to vehicle treated control rats.
n.s. $p > 0.05$, ***$p < 0.001$ (50 mg/kg) (ordinary one-way ANOVA: Dunnett's post-hoc). n=6 for all groups.

When differentiating immobility time in swimming and climbing behaviour, 50 mg/kg sodium benzoate markedly increased swimming behaviours ($p < 0.001$) compared to the vehicle treated group with no significant effects measured on climbing behaviour (Figure 4-10: A and B).

4.3 Phase 3: Main experimental study – FSL

In the main experimental study phase, it was investigated whether chronic treatment with FLX (10 mg/kg), allopurinol (5 mg/kg), sodium benzoate (100 mg/kg) (test compounds), ketamine (10 mg/kg) or memantine (20 mg/kg) (reference compounds) had any discerning effects on depressive-like behaviours and/or cognitive (learning) function in FSL rats compared to vehicle treated groups. The aforementioned drugs were administered chronically (12 consecutive days) as a single daily dose via the i.p. route. Treatment groups were further subdivided according to type of behavioural analysis, being either the FST or MWM. The FST took place 24 hours after administering the final drug dose. MWM groups received treatment for 7 days prior to, and for the 5 days during acquisition training, after which they were assessed for extent of memory retrieval during the probe trial. Refer to Chapter 3 for more detailed descriptions of the methodologies.

4.3.1 Depressive-like phenotype:

4.3.1.1. Open field test

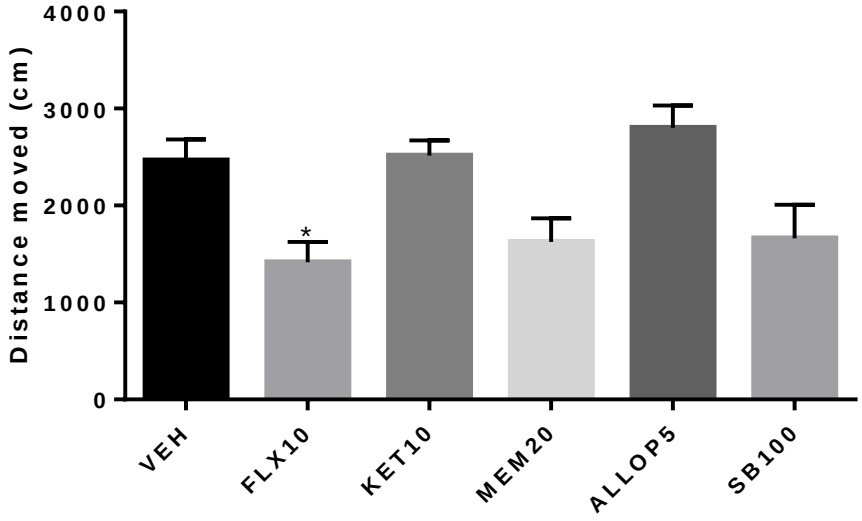

Figure 4-11: Effect of FLX10, ketamine (10 mg/kg, KET10), memantine (20 mg/kg, MEM20), ALLOP5 and SB100 after 12-day treatment on general locomotor activity in the OFT in FSL rats compared to VEH control rats. *p < 0.05 (ordinary one-way ANOVA: Dunnett's post-hoc). n=6 for all groups.

4.3.1.2 Forced swim test

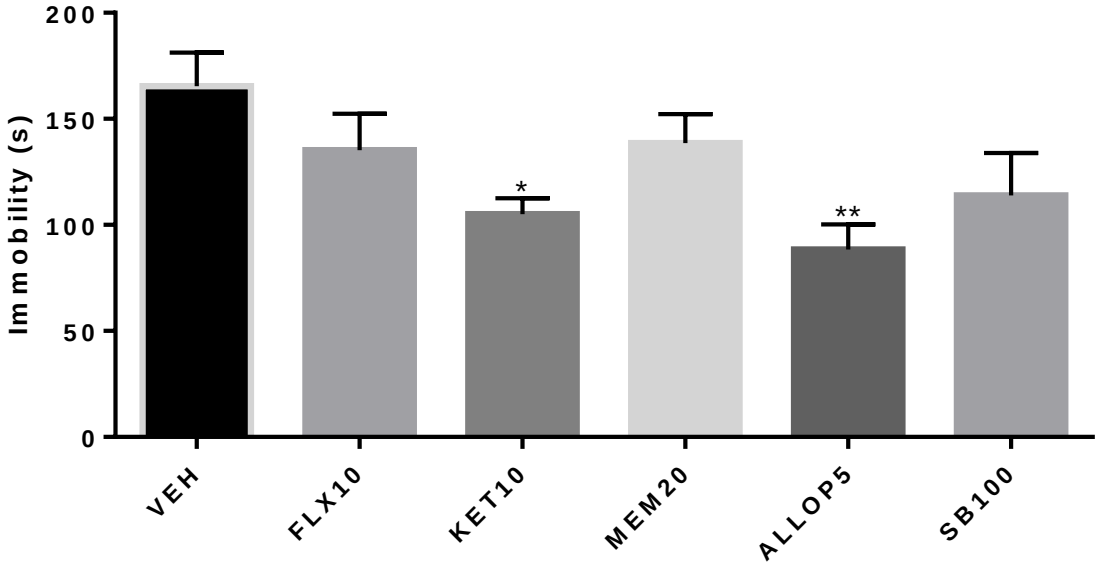

Figure 4-12: Effect of FLX10, KET10, MEM20, ALLOP5 and SB100 after 12-day treatment on immobility in the FST in FSL rats compared to VEH control rats. *p < 0.05 (KET10) and **p < 0.01 (ALLOP5) (ordinary one-way ANOVA: Dunnett's post-hoc). n=6 for all groups.

One-way ANOVA showed a notable inclination toward reduced immobility in the FST across all treatment groups (F (5, 30) = 3.417, p = 0.0146). As depicted in Figure 4-12, allopurinol (88.35±11.89 seconds) significantly enhanced mobility (p < 0.01) in the FST compared to the vehicle treated group (165.4±15.83 seconds) with no significant effect on locomotor activity (p > 0.05) in the OFT (Figure 4-11). This reduction in depressive-like immobility has also

been observed in a study by Gürbüz Özgür *et al* (2015) at higher doses allopurinol (50 mg/kg). Ketamine significantly improved FST mobility time (105.1±7.439 seconds, $p < 0.05$), although not to the same extent as allopurinol which could also not be accounted for by an increase in locomotor activity ($p > 0.05$). Fluoxetine showed an inclination toward reduced immobility, although not in a significant manner ($p > 0.05$). However, fluoxetine markedly caused reduced locomotor activity (1417±208 cm, $p < 0.05$) compared to the vehicle treated group (2466±215.3 cm) (Badenhorst, 2014). Memantine and sodium benzoate caused a reduction in immobile behaviour, although not statistically significant ($p > 0.05$). While fluoxetine, memantine and sodium benzoate had no statistically significant outcomes on immobility behaviour ($p > 0.05$), these compounds exhibited measurable practical significance with fluoxetine and memantine having a medium effect sizes ($d = 0.715$ and $d = 0.689$, respectively) and sodium benzoate a large effect size ($d = 1.049$) compared to the vehicle group (refer to Addendum A for tabulated results). This may imply that both these compounds are capable of antidepressant-like activity and their effects, though not statistically meaningful, should not be discarded but rather undergo further investigation. Both ketamine ($d = 1.554$) and allopurinol ($d = 1.986$) express very large effect sizes which further support the statistical findings as illustrated in Figure 4-12. What's more, allopurinol exhibits an effect size greater than that of fluoxetine ($d = 0.715$). The implication being, that both allopurinol and sodium benzoate possess antidepressant-like capabilities that demand additional exploration.

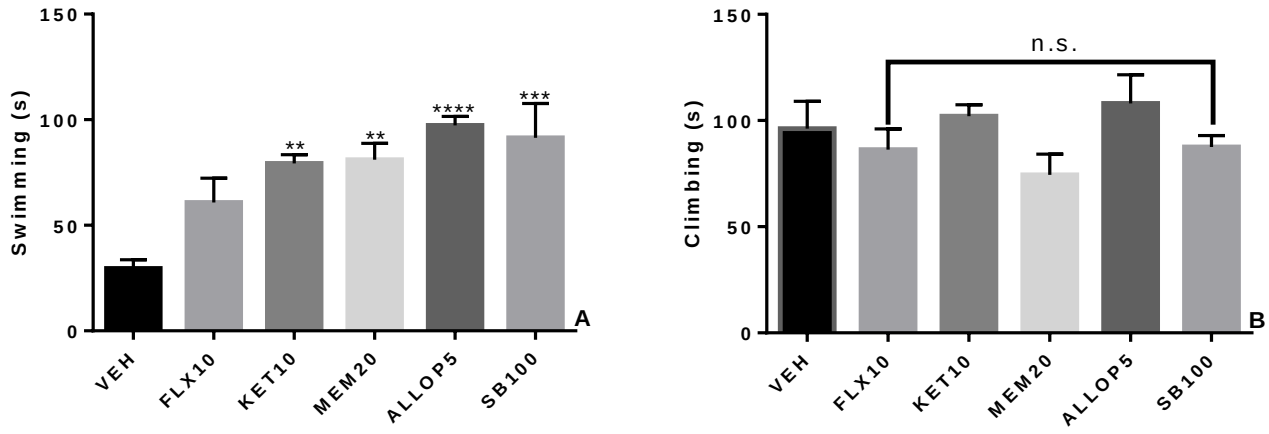

Figure 4-13: Effect of fluoxetine (10 mg/kg), ketamine (10 mg/kg), memantine (20 mg/kg), allopurinol (5 mg/kg) and sodium benzoate (100 mg/kg) after 12-day treatment on swimming (A) and climbing (B) behaviour in the FST in FSL rats compared to control rats. n.s. $p > 0.05$, **$p < 0.01$ (KET10), **$p < 0.01$ (MEM20), ***$p < 0.001$ (SB100) and ****$p < 0.0001$ (ALLOP5) (ordinary one-way ANOVA: Dunnett's post-hoc). n=6 for all groups.

In addition, all treatment groups presented with significantly enhanced swimming time (KET10 and MEM20 $p < 0.01$, SB100 $p < 0.001$, ALLOP5 $p < 0.0001$) compared to the vehicle treated group as evidenced in Figure 4-13: A. No significant effects on locomotor activity (Figure 4-11) or climbing behaviour (Figure 4-13: B) ($p > 0.05$) were observed.

4.3.2 Cognitive function test:

4.3.3.1 Morris water maze

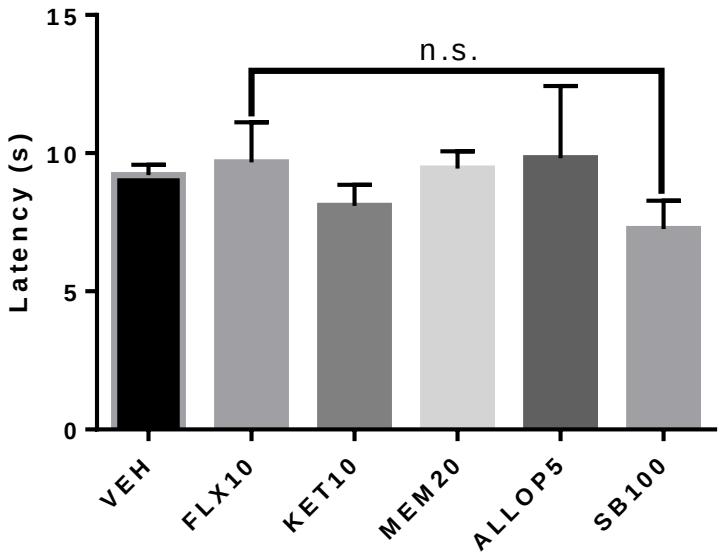

Figure 4-14: Effect of FLX10, KET10, MEM20, ALLOP5 and SB100 after 12-day treatment on general locomotor activity in the MWM cued trial in FSL rats compared to VEH control rats. n.s. p > 0.05 (ordinary one-way ANOVA: Dunnett's post-hoc). n=6 for all groups.

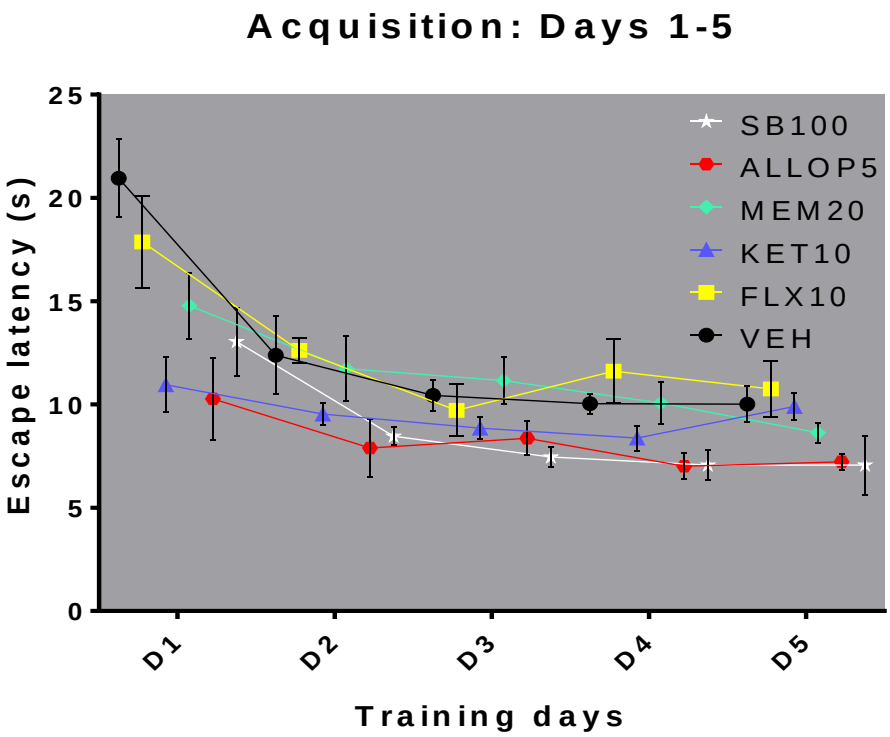

Figure 4-15: Effect of FLX10, KET10, MEM20, ALLOP5 and SB100 after 12-day treatment on memory consolidation (acquisition training) in the MWM over Days 1-5 in FSL rats compared to control rats. (Repeated measures two-way ANOVA: Tukey post-hoc). n=6 for all groups.

Repeated measures two-way ANOVA across Day 1-5 of acquisition training displayed significant improvement in spatial learning capabilities ($F = 21.94$, $p < 0.0001$) in association with drug treatment effects ($F = 3.29$, $p = 0.0278$) on latency to escape compared to VEH treated groups. This is evidenced in Figure 4-15 and provides clear indication of memory consolidation over the 5 day acquisition training period. However, there was no significant treatment-time interaction to report for escape latency ($F = 0.48$, $p = 0.9653$). One-way ANOVA of each of the training days further elaborates on the treatment effects and the extent of variance across the different groups, as depicted in Fig 4.16.

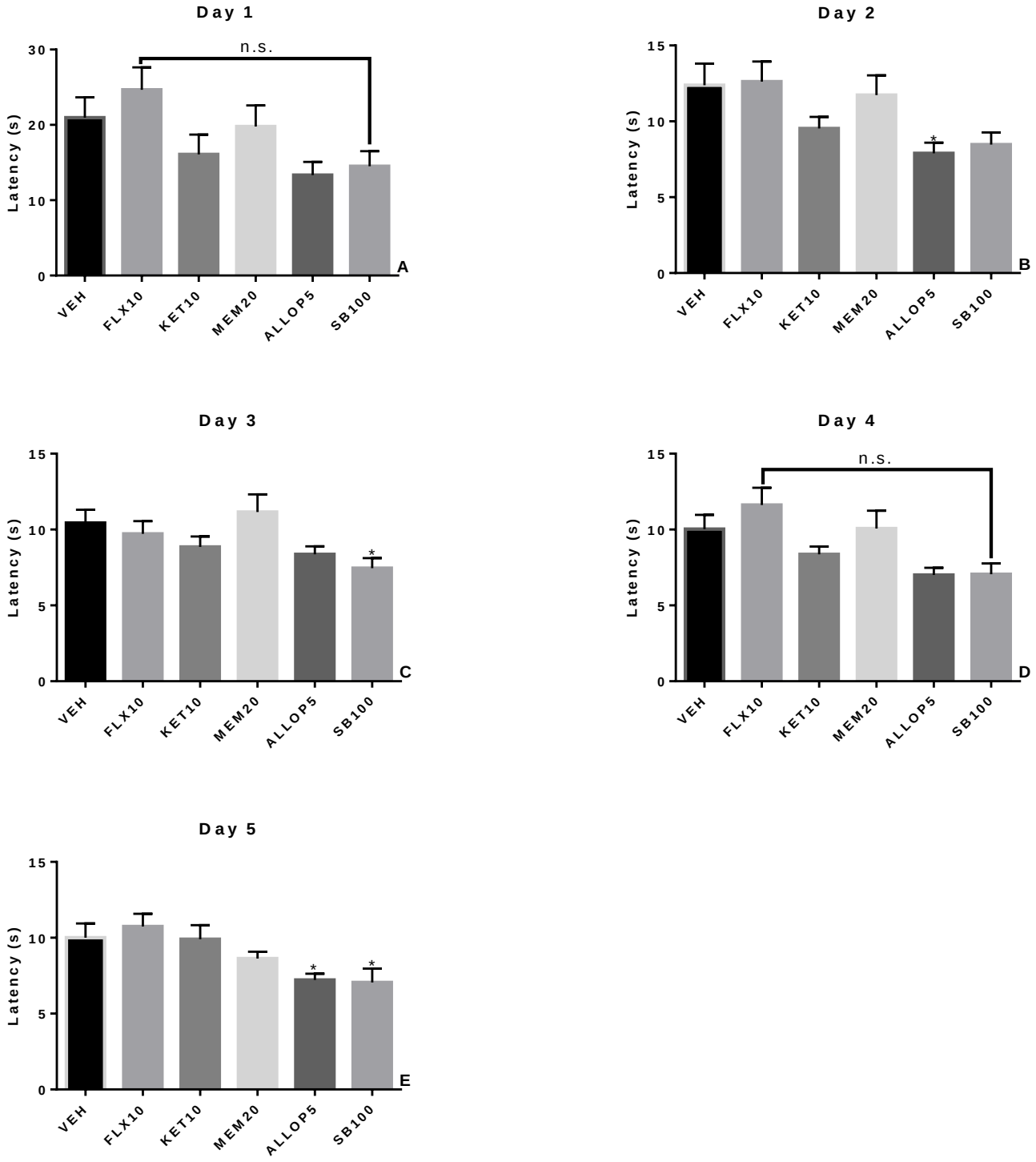

Figure 4-16: Effect of FLX10, KET10, MEM20, ALLOP5 and SB100 after 12-day treatment on Day 1 (A), Day 2 (B), Day 3 (C), Day 4 (D) and Day 5 (E) of acquisition training in the MWM in FSL rats compared to control rats. n.s. $p > 0.05$, *$p < 0.05$ (ALLOP5, SB100) (ordinary one-way ANOVA: Dunnett's post-hoc). n=6 for all groups.

On Day 1 (Figure 4-16: A) there was a visible variance in latency time across the treatment groups (F (5, 138) = 2.979, $p < 0.05$), however, no marked statistical differences were documented between treatments and the vehicle group ($p > 0.05$). Days 2 (F (5, 138) = 3.595, $p = 0.0044$) and 3 (F (5, 138) = 2.783, $p = 0.0199$) (Figure 4-16: B and C) exhibited enhanced consolidation across treatment groups, especially allopurinol (Day 2: 7.888±0.7107 seconds, $p < 0.05$) and sodium benzoate (Day 3: 7.460±0.6568, $p < 0.05$) compared to vehicle (12.39±1.417 and 10.43±0.8814 seconds respectively) treated groups. A substantial significant difference in acquisition latency across treatment groups (F (5, 138) = 4.549, $p = 0.0007$) was documented on Day 4 (Figure 4-16: D). Conversely, no notable differences were observed between the treatment groups and vehicle group ($p > 0.05$). On the final day of training (Figure 4-16: E), one-way ANOVA exhibited sizable significance across treatment groups ($F = 3.959$, $p = 0.0022$) with further measurable reductions in latency time induced by both allopurinol (7.221±0.4157 seconds, $p < 0.05$) and sodium benzoate (7.062±0.9081 seconds, $p < 0.05$) compared to the vehicle group (10.01±0.9261 seconds). The aforementioned results support the discernable effects of the different treatments compared to vehicle administration on acquisition training across Days 1-5 as seen in Figure 4-15, with 5 mg/kg allopurinol (Figure 4-16: B and E) and 100 mg/kg sodium benzoate (Figure 4-16: C and E) having caused more obvious effects on memory consolidation.

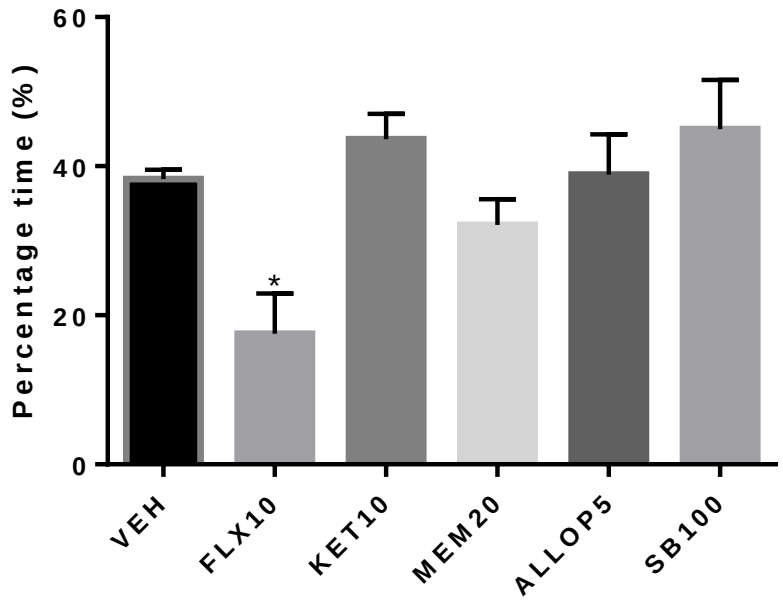

Figure 4-17: Effect of FLX10, KET10, MEM20, ALLOP5 and SB100 after 12-day treatment on memory retrieval in the MWM probe trial in FSL rats compared to control rats. *p<0.05 (FLX10) (ordinary one-way ANOVA: Dunnett's post-hoc). n=6 for all groups.

In addition to 4 final training sessions on day 5 the rats were later subjected to a probe trial to measure whether memory retrieval would take place successfully. (Figure 4-17). A subsequent cued trial followed to assess general locomotor activity (Figure 4-14). One-way ANOVA showed overall significant variance in memory storage/retrieval across all treatment groups ($F = 4.806$, $p = 0.0024$). Unfortunately no striking variances in percentage time spent in the target quadrant were detected for the reference (KET10 and MEM20, $p > 0.05$) or test compounds (ALLOP5 and SB100, $p > 0.05$) when compared to the vehicle treated group. There were no overall effects on general locomotor activity across treatment groups. It is, however, important to note that 10 mg/kg fluoxetine ($p < 0.05$) had a statistically significant impairing effect on memory retrieval compared to the vehicle group (38.29±1.253) as can be seen in Figure 4-17.

Though statistically inconsequential, both ketamine ($d = 0.645$) and memantine ($d = 0.732$) displayed a medium effect size compared to the vehicle treated group (refer to Addendum A for tabulated results of the practical significance). However, ketamine showed a positive effect on memory retrieval contrary to the reduced percentage in the target zone as seen with memantine. Unfortunately, neither one of the test compounds proved considerably practically significant compared to the vehicle ($d = 0.046$ (allopurinol), $d = 0.413$ (sodium benzoate)). However, both the reference compounds demonstrated substantial practical significance (refer to Addendum A for tabulated results for Cohen's d-value).

4.3.3 Monoamine analysis

It has long been hypothesised/proposed that monoamine (NA, DA and 5-HT) levels and/or activity dysregulation in the brains of depressives (Overstreet *et al.*, 2005) and as a result neurotransmitter-synapse communications are affected, subsequently hampering signalling processes that may follow leading to the development of depressive symptoms and behaviours (Kiyohara & Yoshimasu, 2009). So far researchers have been able to link 5-HT and NA abnormalities to psychopathologies that underlie depressive disorders (Krishnan & Nestler, 2008). Chapter 2, section 2.4.2.2 provides a more elaborate literature overview surrounding the involvement of monoamines in MDD. During this part of the study we investigated the effects of ketamine, memantine, allopurinol and sodium benzoate compared to a vehicle treated group on the basis of monoaminergic alterations in specific brain regions. Rats were treated with the above-mentioned compounds for 12 days, after which they were euthanised and their brains removed and dissected for monoamine concentration determination.

Quantification of noradrenaline (NE), dopamine (DA), serotonin (5-HT) and their associated end-stage metabolites was performed using high-performance liquid chromatography with electrochemical detection (HPLC-ED). Rat prefrontal cortices (PFC), striata (STR) and hippocampi (HPC) of the left brain hemisphere were analysed in the final phase of this study. Chapter 3 more clearly describes the methods and materials used in this process. Not all monoamine findings are listed in this chapter, but are recorded in Addendum A for perusal. The results to follow include prefrontocortical noradrenaline, striatal dopamine and prefrontocortical serotonin concentrations as measured using HPLC-ED analysis (ng/g brain tissue).

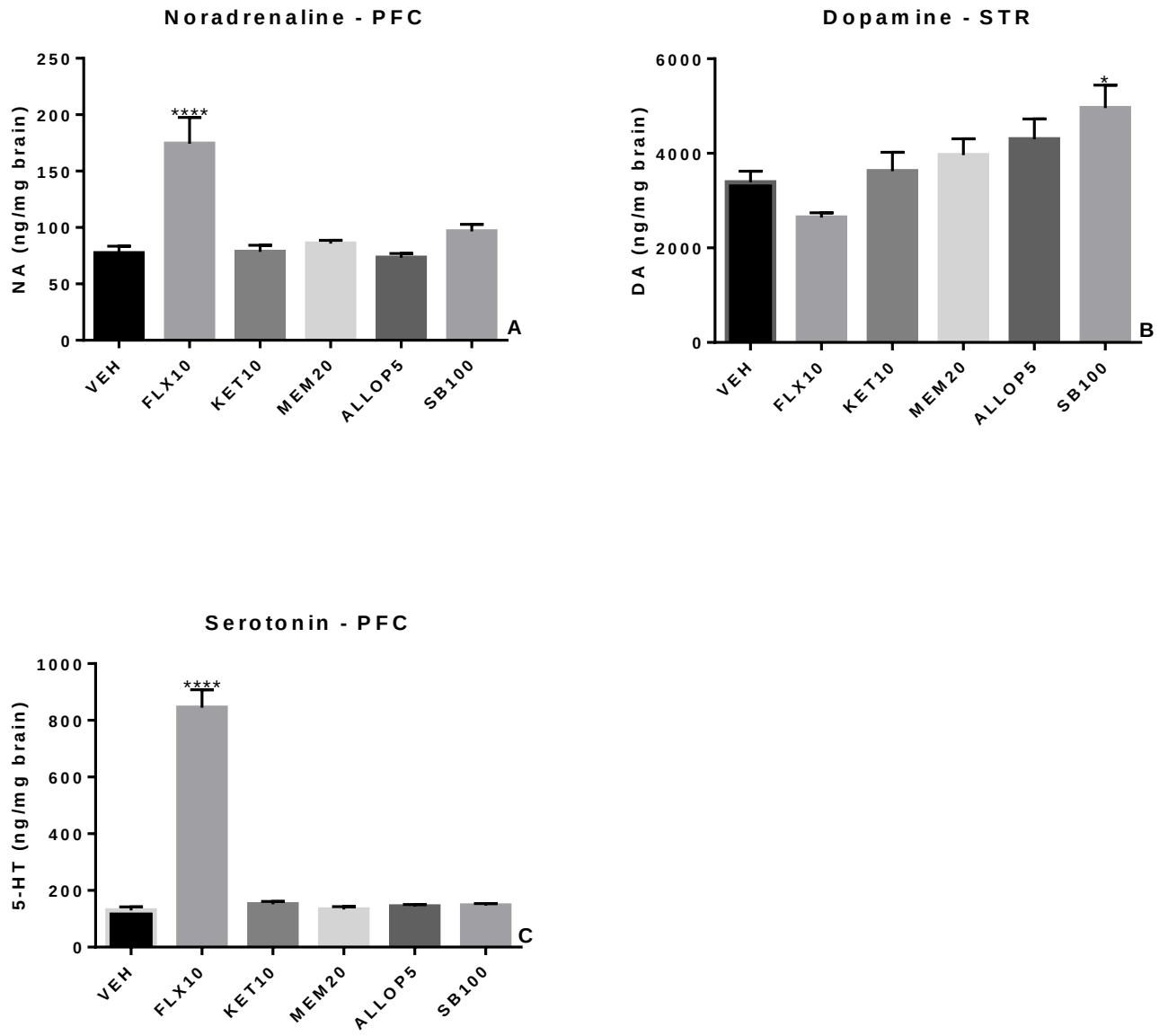

Figure 4-18: Effect of FLX10, KET10, MEM20, ALLOP5 and SB100 after 12-day treatment on prefrontocortical noradrenaline (A), striatal dopamine (B) and prefrontocortical serotonin (C) concentrations in the brain of FSL rats compared to control rats. *p < 0.05 (SB100) and ****p < 0.0001 (FLX10) (ordinary one-way ANOVA: Dunnett's post-hoc). n=6 for all groups.

Due to a lack of data not all HPLC-ED monoamine and relevant metabolite measurements could be presented graphically, however, Table 3 in Addendum A lists all values obtained. One-way ANOVA revealed that chronic treatment with the drug compounds utilised in the main experimental study phase prompted extensive significant variances in monoamine (NA, DA and 5-HT) concentrations (ng/g brain) in the brain regions illustrated in Figure 4-18: A-C (F (5, 30) = 12.91, p < 0.0001 (Fig. 4-18: A); F (5, 30) = 4.935, p = 0.0021 (Fig. 4-18: B); F (5, 30) = 111.3, p < 0.0001 (Fig. 4-18:C)). Fluoxetine demonstrated a sizeable increase in prefrontocortical noradrenaline (174.2±23.51 ng/g brain, p < 0.0001) and serotonin (844.8±63.30, p < 0.0001) levels compared to the vehicle treated group (77.28±6.107 ng/g

brain (NA), 129.9±12.61 ng/g brain (5-HT)). However, fluoxetine had no significant effect on prefrontocortical dopamine levels ($p > 0.05$). Allopurinol (5 mg/kg) caused evident reduction in hippocampal noradrenaline ($p < 0.05$) when compared to the vehicle group (Table 3, Addendum A). A notable enhancement in striatal dopamine was induced upon 100 mg/kg sodium benzoate treatment ($p < 0.05$) vs. the vehicle treated group that appears comparable to memantine's effect (Table 3, Addendum A). No significant differences in striatal serotonin concentrations (n.s. $p > 0.05$) were measured (Table 3, Addendum A). Furthermore, the effects of the different treatment modalities on striatal and prefrontocortical 5-hydroxy-indole acetic acid (5-HIAA) concentrations proved insignificant (Table 3, Addendum A). None of the other treatment groups presented with statistically significant differences in monoamine levels in the specified brain regions, excluding 100 mg/kg sodium benzoate ($p < 0.05$) that appears to have reduced hippocampal 5-HIAA levels practically similar to those of allopurinol (5 mg/kg). As illustrated in Figure 4-18: B, 100 mg/kg sodium benzoate (4957±485.5 ng/g brain, $p < 0.05$) visibly enhanced striatal dopamine levels compared to the vehicle group (3385±283.3 ng/g brain) with the other treatment groups seemingly following the same trend, although not significantly so.

4.3.4 BDNF analysis

Quantification of BDNF was done using a sandwich enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions. BDNF concentrations (BDNF/mg protein (pg)) were analysed in the prefrontal cortex, striatum and hippocampus of the right brain hemisphere of the rat brain.

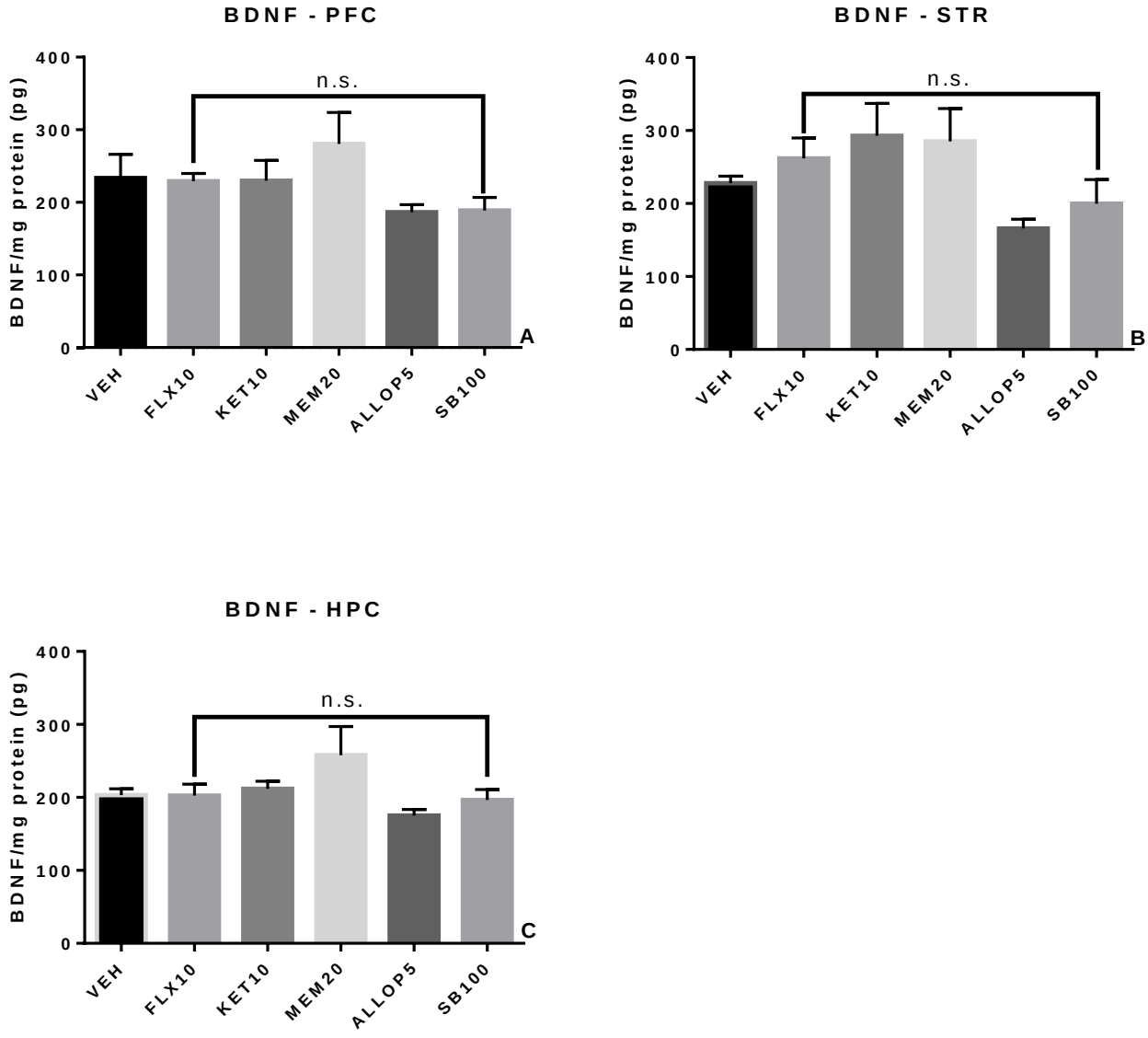

Figure 4-19: Effect of FLX10, KET10, MEM20, ALLOP5 and SB100 after 12-day treatment on prefrontocortical (A), striatal (B) and hippocampal (C) BDNF concentrations in the FSL rat brain compared to control rats. n.s. p>0.05 (ordinary one-way ANOVA: Dunnett's post-hoc). n=6 for all groups.

Unfortunately, none of the analyses displayed statistically significant results across (one-way ANOVA) or between (Dunnett's post-hoc) groups ($p > 0.05$) (Figure 4-19).

Table 4-1: Data summary of expressed general locomotor activity (OFT), depressive-like (FST) and cognitive behaviours (MWM) within the FSL model of depression compared to the FRL rat. (Downward arrows indicate decreases, upward arrows indicate increases, horizontal arrows indicate no significant change and red arrows indicate significant change in movement or behaviour).

Phase 1: Confirmation of expressed depressive-like phenotype along with cognitive insufficiencies within an animal model of depression – FSL vs. FRL					
Open Field Test – Distance moved (cm)					
Flinders Resistant Line		Flinders Sensitive Line			
↔		↑			
Forced Swim Test (seconds)					
Immobility (seconds)		***Swimming (seconds)***		***Climbing (seconds)***	
Flinders Resistant Line	Flinders Sensitive Line	Flinders Resistant Line	Flinders Sensitive Line	Flinders Resistant Line	Flinders Sensitive Line
↔	↑	↔	↓	↔	↓
Morris Water Maze test					
Flinders Resistant Line		Flinders Sensitive Line			
Cued trial - General locomotor activity (Latency to platform - seconds)					
↔		↔			
Probe trial – Percentage time spent in target zone (%)					
↔		↓			

Table 4-2: Data summary of the effects observed with acute administration of varying doses allopurinol and sodium benzoate on depressive-like behaviours in the FSL model using the FST. (Downward arrows indicate decreases, upward arrows indicate, horizontal arrows indicate no significant change and red arrows indicate significant change in the different swimming behaviours).

Phase 2: Acute dose-ranging analysis – FSL					
Acute treatment: Allopurinol					
Vehicle	Allopurinol (5 mg/kg)	Allopurinol (10 mg/kg)	Allopurinol (20 mg/kg)	Allopurinol (50 mg/kg)	Allopurinol (100 mg/kg)
Immobility (seconds)					
↔	↓	↓	↓	↓	↓
Swimming (seconds)					
↔	↑	↑	↑	↑	↑
Climbing (seconds)					
↔	↑	↔	↔	↑	↑
Acute treatment: Sodium benzoate					
Vehicle	Sodium benzoate (50 mg/kg)	Sodium benzoate (100 mg/kg)	Sodium benzoate (150 mg/kg)	Sodium benzoate (200 mg/kg)	
Immobility (seconds)					
↔	↔	↓	↔	↔	
Swimming (seconds)					
↔	↑	↔	↔	↔	
Climbing (seconds)					
↔	↑	↑	↔	↔	

Table 4-3: Data summary of the effects observed with chronic drug treatment on general locomotor activity and depressive-like behaviours in the FSL model using the OFT and FST, respectively. (Downward arrows indicate decreases, upward arrows indicate increases, horizontal arrows indicate no significant change and red arrows indicate significant change).

Phase 3: Main experimental study – FSL					
Open Field Test – Distance moved (cm)					
Vehicle	**Fluoxetine (10 mg/kg)**	**Ketamine (10 mg/kg)**	**Memantine (20 mg/kg)**	**Allopurinol (5 mg/kg)**	**Sodium benzoate (100 mg/kg)**
↔	↓	↔	↓	↔	↓
Forced Swim Test (seconds)					
Vehicle	**Fluoxetine (10 mg/kg)**	**Ketamine (10 mg/kg)**	**Memantine (20 mg/kg)**	**Allopurinol (5 mg/kg)**	**Sodium benzoate (100 mg/kg)**
Immobility (seconds)					
↔	↓	↓	↓	↓	↓
Swimming (seconds)					
↔	↑	↑	↑	↑	↑
Climbing (seconds)					
↔	↔	↔	↓	↔	↔

Table 4-4: Data summary of the effects observed with chronic drug treatment on cognitive behaviours in the FSL model using the MWM test. (Downward arrows indicate decreases; upward arrows indicate increases, horizontal arrows indicate no significant change and red arrows indicate significant change).

Phase 3: Main experimental study – FSL					
Morris Water Maze test					
Vehicle	**Fluoxetine (10 mg/kg)**	**Ketamine (10 mg/kg)**	**Memantine (20 mg/kg)**	**Allopurinol (5 mg/kg)**	**Sodium benzoate (100 mg/kg)**
Cued trial - General locomotor activity (Latency to platform - seconds)					
↔	↔	↔	↔	↔	↓
Training Days 1 to 5 – Memory acquisition (Latency to platform - seconds)					
Training Day 1 (Latency to platform - seconds)					
↔	↑	↓	↔	↓	↓
Training Day 2 (Latency to platform - seconds)					
↔	↔	↓	↔	↓	↓
Training Day 3 (Latency to platform - seconds)					
↔	↔	↓	↔	↓	↓
Training Day 4 (Latency to platform - seconds)					
↔	↑	↓	↔	↓	↓
Training Day 5 (Latency to platform - seconds)					
↔	↔	↔	↔	↓	↓
Probe trial – Percentage time spent in target zone (%)					
↔	↓	↑	↓	↔	↑

Table 4-5: Data summary of the effects observed with chronic drug treatment on brain monoamine and end-stage metabolite LEVELS in the FSL model. (Downward arrows specify decreases; upward arrows specify increases, horizontal arrows specify no significant change, red arrows indicate significant change and x indicates a lack of data).

Phase 3: Main experimental study – FSL					
Monoamines and Associated end-stage metabolites (ng/g wet brain tissue)					
Left prefrontal cortex (PFC)					
Vehicle	**Fluoxetine (10 mg/kg)**	**Ketamine (10 mg/kg)**	**Memantine (20 mg/kg)**	**Allopurinol (5 mg/kg)**	**Sodium benzoate (100 mg/kg)**
Noradrenaline / NA (ng/g wet brain tissue)					
↔	↑	↔	↔	↔	↔
Dopamine / DA (ng/g wet brain tissue)					
X	X	X	X	X	X
Dihydroxyphenylacetic acid / DOPAC (ng/g wet brain tissue)					
X	X	X	X	X	X
Homovanillic acid / HVA (ng/g wet brain tissue)					
X	X	X	X	X	X
Serotonin / 5-HT (ng/g wet brain tissue)					
↔	↑	↔	↔	↔	↔
5-Hydroxyindole Acetic Acid / 5-HIAA (ng/g wet brain tissue)					
↔	↑	↑	↔	↔	↔
Left striatum (STR)					
Noradrenaline / NA (ng/g wet brain tissue)					
↔	↔	↔	↔	↔	↔
Dopamine / DA (ng/g wet brain tissue)					
↔	↔	↑	↑	↑	↑
Dihydroxyphenylacetic acid / DOPAC (ng/g wet brain tissue)					
↔	↔	↔	↑	↔	↑
Homovanillic acid / HVA (ng/g wet brain tissue)					
↔	↔	↔	↔	↔	↔
Serotonin / 5-HT (ng/g wet brain tissue)					
↔	↔	↔	↔	↔	↔
5-Hydroxyindole Acetic Acid / 5-HIAA (ng/g wet brain tissue)					
↔	↔	↑	↔	↔	↑
Left hippocampus (HPC)					
Noradrenaline / NA (ng/g wet brain tissue)					
↔	↔	↔	↔	↓	↔
Dopamine / DA (ng/g wet brain tissue)					
X	X	X	X	X	X
Dihydroxyphenylacetic acid / DOPAC (ng/g wet brain tissue)					
X	X	X	X	X	X
Homovanillic acid / HVA (ng/g wet brain tissue)					
X	X	X	X	X	X
Serotonin / 5-HT (ng/g wet brain tissue)					
↔	X	↑	↔	↑	↔
5-Hydroxyindole Acetic Acid / 5-HIAA (ng/g wet brain tissue)					
↔	↔	↑	↓	↓	↓

Table 4-6: Data summary of the effects observed with chronic drug treatment on brain BDNF concentrations in the FSL model. (Downward arrows indicate decreases; upward arrows indicate increases, horizontal arrows indicate no significant change and red arrows indicate significant change).

Phase 3: Main experimental study – FSL					
Brain-derived neurotrophic factor (BDNF) per mg protein (pg)					
Vehicle	**Fluoxetine (10 mg/kg)**	**Ketamine (10 mg/kg)**	**Memantine (20 mg/kg)**	**Allopurinol (5 mg/kg)**	**Sodium benzoate (100 mg/kg)**
Right prefrontal cortex (PFC)					
↔	↔	↔	↑	↔	↔
Right striatum (STR)					
↔	↑	↑	↑	↔	↔
Right hippocampus (HPC)					
↔	↔	↔	↑	↔	↔

Chapter 5 : Discussion

5.1 Introduction:

Major depressive disorder is a pervasive neuropsychological disorder affecting a multitude of individuals with no precise genetic, cultural, environmental, sociological or psychological predilection. MDD encompasses a variety of debilitating symptoms and pathologies of which several remain under speculation. Systems and/or pathways established and hypothesised to be involved in this disorder include the cholinergic-, monoaminergic-, GABAergic-, glutamatergic pathways, the HPA axis, circadian rhythms as well as neuroplasticity and neuro-immunological pathways. Though over the years much research has gone into establishing the exact cause of disease and course of treatment of MDD, none indefinitely clarify or remit the associated symptomatologies. Furthermore, the current therapeutic options available do not prove extensive in abating the underlying cause(s) of MDD in all patients undergoing treatment. Several sufferers no longer respond to first-line therapies and many others never experience remission. This may be attributable to either treatment resistance or the surfacing of newly undefined causalities. Many physiological and psychological signs and symptoms have proven evident in the course of this disease and have presented as both structural and neuro(bio)logical brain changes that may either be induced by or consequential of each other. As far, researchers have documented structural adaptations in the frontal cortex, hippocampus, striatum, ventral tegmental area, nucleus accumbens and amygdala. These adaptations may present as reduced volume, projection or activity within a specified brain region for example, reduced hippocampal volume with associated neurocognitive impairments. Additionally, these alterations provide evidence for coherent associations between MDD and disruptive learning and memory processes. Unfortunately, these effects may not be eradicated as quickly as the signs and symptoms that arise therefrom.

As stated, though multiple treatment options are available for MDD, therapeutic effects and remission rates are below adequate expectations not to mention the accompanying safety, efficacy, toxicity and pharmacokinetic trepidations they hold. Furthermore, existing antidepressants present with a ‘lag-period’ before any significant positive clinical effects are seen (Frazer & Morilak, 2005). However, these therapeutic shortcomings provide opportunities prompting investigation into novel biological targeting approaches and treatment options that may prove capable of reducing the pathologies believed to be the cause of MDD. The aforementioned statement forms part of the basis of this study.

One of the more recently frequented hypotheses postulated to underlie depression is that of glutamatergic dysregulation. The involvement of glutamate (an NMDA receptor stimulator) in MDD had first been implicated upon discovering that certain drug compounds were able to induce antidepressant-like effects by modulating NMDA receptor activity. Further investigations were later able to associate glutamate release and functioning with monoamine modulation, providing more concrete grounds for its involvement in depression. In addition, glutamate is in close relation to neuroplasticity processes, and thus, involves the neuroplasticity hypothesis. Glutamate excess observed in post-mortem evaluations has been assessed for induction of structural brain changes resulting in dendrite deformities, glial cell number reduction as well as reduced synaptic spine densities; all contributing to volumetric and cognitive changes in the brain (Zakzanis *et al.*, 1998; Luo *et al.*, 2013; Burt *et al.*, 1995). Additionally, depression may also be linked to the complex relationship between the kynurenine and glutamatergic pathways and their NMDA receptor modulating capabilities (Réus *et al.*, 2015). This relationship is illustrated and described in more detail in Chapter 2, Figure 2-12 and under §2.4.5. Two metabolic products are formed from tryptophan: serotonin (a key monoamine capable of antidepressant effects) and kynurenine. Kynurenine is further metabolised to kynurenic acid and quinolinic acid. Both these end-stage metabolites are able to modulate NMDA receptors. Kynurenic acid acts neuroprotectively and has inhibitory effects on NMDA receptors. Conversely, quinolinic acid has neurotoxic effects and stimulates NMDA receptor functioning.

Therefore, by attempting to modulate the glutamatergic pathway and all the associated conduits involved in its functioning using therapeutic drug interventions, it may be possible to induce either antidepressant-like or procognitive effects using certain drug compounds that act on key biological targets in these areas. Unfortunately, the evidence surrounding glutamate's participation in the development of affective disorders associated with cognitive impairments remains vague and speculative. However, these findings should not discourage further investigations and as such, this study examined the effects of specific compounds (viz. allopurinol and sodium benzoate) on depressive-like behaviour and cognitive function in an animal model of depression by targeting significant areas involved in glutamatergic pathway functionality either directly or indirectly.

Allopurinol has long since the 1960's been investigated for its role in the kynurenine pathway. Researchers have found that allopurinol, an antigout preparation, is capable of inhibiting the enzyme responsible for catabolising tryptophan – tryptophan 2,3-dioxygenase (TDO). Consequently, higher levels of tryptophan are available which in turn favours the synthesis of serotonin - a key monoamine involved in antidepressant response which may be the mechanism by which this compound exerts its antidepressant-like actions. Only

recently allopurinol has been applied in studies using animal models of depression to assess antidepressant-like effects using the forced swim test (Gibney *et al.*, 2014; Gürbüz Özgür *et al.*, 2015; Karve *et al.*, 2013). Even more so, as of late allopurinol has been under assessment for its ability to reduce/reverse toxin-induced neuronal damage and enhance cognitive functioning as well as its ability to increase brain-derived neurotrophic factor (BDNF) (Dong *et al.*, 2015; Prickaerts *et al.*, 2013; Yonden *et al.*, 2010). A different target within the glutamatergic pathway involves the indirect modulation of NMDA receptors by targeting D-amino acids. Sodium benzoate, a well-known preservative, has been studied for its effects in association with affective disorders and cognitive functioning (Jana *et al.*, 2013; Kamel & Abd El Razek, 2013; Lai *et al.*, 2012; Lin *et al.*, 2014). Sodium benzoate acts by inhibiting the enzyme (D-amino acid oxidase) responsible for catabolising D-amine acids (D-serine and D-alanine) and as a result, the concentrations of these *neurotransmitters* are higher and are able to stimulate NMDA receptors more frequently. Very few preclinical and clinical studies have been conducted with regard to sodium benzoate's effects on depressive-like behaviours as well as cognition. Though the research is sparse, the results that have been produced prove enthusing, encouraging further investigation into the mechanism relating to its actions.

Chapter 5 will elaborate on the results as displayed in Chapter 4 in the following manner:

- *Phase 1 results on-*
    - The depressive-like phenotype of the FSL model compared to the healthy FRL control
    - The cognitive function deficits of the FSL model compared to the healthy FRL control
- *Phase 2 results on-*
    - The acute dose-ranging analysis of allopurinol and sodium benzoate in the FSL rat
- *Phase 3 results on-*
    - The antidepressant-like effects of chronically administered compounds
    - The effects of chronically administered compounds on cognitive behaviours
    - The effects of chronically administered compounds on brain monoamine and associated end-stage metabolite concentrations
    - The effects of chronically administered compounds on BDNF concentrations

5.2 Phase 1:

5.2.1 The depressive-like phenotype of the FSL model compared to the healthy FRL control

As illustrated in Figure 4-1, no significant differences between the FSL and FRL rat groups in terms of locomotor activity were observed using the OFT under our laboratory conditions. These results correspond with that of earlier research done in our laboratories presenting similar findings (Badenhorst, 2014). Most importantly, there was a statistically significant difference in immobility time between the two strains. As evidenced in Figure 4-2, FSL rats present with longer durations of immobile behaviour compared to the healthy controls. These aforementioned findings are in line with that of earlier studies (Brand, 2011), which additionally confirms the depressive-like phenotype of the FSL and further provides rational for the use of the FST in assessing potential depressive-like behaviours in the FSL model after drug administration in the subsequent phases of this study (Cryan *et al.*, 2002; Overstreet *et al.*, 2005; Porsolt *et al.,* 1978). Additionally, no differences in swimming-time were observed; however, the FRL group did exhibit increased climbing behaviour (Figure 4-3).

5.2.2 The cognitive function deficits of the FSL model compared to the healthy FRL control

No significant differences in general locomotor activity between FSL vs. FRL rats were observed using the MWM cued trial assessment (Figure 4-4). To our knowledge, there is no evidence for assessing general locomotor activity or memory/learning abilities in the FSL model using the MWM test. As far, learning impairments in the FSL model compared to the FRL rat have been measured using behavioural assessments other than the MWM. In 2011, Abildgaard and colleagues were able to confirm memory impairments within the FSL model compared to its healthy counterpart (FRL) after subjecting these animals to the novel object recognition test (NORT). However, NORT only assesses for declarative (long-term) memory whereas the MWM test measures spatial memory by using memory consolidation across multiple training sessions as well as memory retrieval, which is based on long-term memory storage. Further studies subsequently based on their findings were able to reverse these impairments using different treatment modalities (Mokoena *et al.*, 2015; Erasmus *et al.*, 2015). Figure 4-5 provides clear illustration of an evident learning curve between both strains as assessed over 5 days of acquisition training. These findings indicate that memory consolidation had taken place successfully. Unfortunately, no statistically significant variances in memory retrieval during the probe trial were documented between the FSL vs. FRL groups - although differences may seem apparent upon first sight of Figure 4-6

illustrating an extent of memory impairment in the FSL model. The lack of substantial significance may be attributable to the small sample size (n=6) used in this study.

5.3 Phase 2:

5.3.1 The acute dose-ranging analysis of allopurinol and sodium benzoate in the FSL rat

As stated earlier, we have yet to come across publications with regard to an acute dose-ranging analysis using allopurinol or sodium benzoate in the FSL model in order to assess antidepressant-like capabilities using the FST. In this study, groups of FSL rats received varying doses of either allopurinol or sodium benzoate as described in Chapter 3 (Table 3-2). Solutions/suspensions of the drugs were injected intraperitoneally at three time periods 24 hours prior to subjecting the animals to the FST (Figure 3-4). Figure 4-7: B provides clear evidence for the most effective dose allopurinol (5 mg/kg) as demonstrated by its enhanced ability to reduce immobility time compared to the vehicle (control) group in the FST. This dose, however, is lower than that applied in other studies in exploring the effects of chronic allopurinol on depressive-like behaviours in the FST using different animal models of depression. In 2014, Gibney and colleagues were able to establish that administration of allopurinol (20 mg/kg) in combination with TDO to a repeated stress depression-induction animal model proved effective in reducing depressive-like behaviours in the FST by means of the kynurenine pathway. More recently, Gürbüz Özgür *et al.* (2015) suggested that two weeks of allopurinol (50 mg/kg) administration generated antidepressant-like effects comparable to that of fluoxetine (10 mg/kg) given for the same time period. Their results were in line with research done by Karve *et al.* (2013). Although no statistically significant differences in climbing behaviours were documented across the different doses (Figure 4-8: D), all doses (excluding 100 mg/kg) lead to significantly longer swimming times compared to the control group, especially 5 mg/kg allopurinol (Figure 4-8: B). Subsequently, 5 mg/kg allopurinol was applied in the main experimental study.

Sodium benzoate at a dose of 100 mg/kg proved effective in reducing immobility time during the FST when compared to the control group (Figure 4-9). No noteworthy effects were observed with the other doses. Additionally, no statistically significant observations were documented across the different doses regarding climbing behaviours (Figure 4-10: B). However, 50 mg/kg sodium benzoate did substantially enhance swimming behaviours compared to the control (Figure 4-10: A). The 100 mg/kg dose led to increased swimming and climbing behaviours, but not in a significant manner. Although few studies have been conducted assessing sodium benzoate's abilities in treating affective disorders, the

antidepressant-like effects of this compound have been observed in humans receiving a dose of 500 mg/day for a period of 6 weeks (Lai *et al.*, 2012).

5.4 Phase 3:

Finally, after establishing the most effective dose for the two test compounds (allopurinol and sodium benzoate) using the FST, these compounds were assessed in the FSL model using a chronic treatment regime of 12 days. Two reference compounds (ketamine and memantine) and fluoxetine were also employed in this phase of the study (Chapter 3: Table 3-3, Figures 3-5 and 3-6). Although monoamine and associated end-stage metabolite analyses did not produce complete results, an attempt was made to associate the expressed depressive-like behaviours (viz. immobility, swimming and climbing) of the FSL rat in the FST with the measured brain concentrations of the above mentioned substances after a chronic treatment protocol with the stated compounds.

5.4.1 The antidepressant-like effects of chronically administered compounds

Figure 4-12 illustrates the effect of chronic 12 day treatment with vehicle, fluoxetine (10 mg/kg), ketamine (10 mg/kg), memantine (20 mg/kg), allopurinol (5 mg/kg) and sodium benzoate (100 mg/kg) on depressive-like behaviours in the FSL model as measured using the FST.

As evidenced in Chapter 4 (§4.3.1.2), a notable trend in reduced immobility time was observed across all treatment groups. More specifically, allopurinol (5 mg/kg) significantly enhanced mobility in the FST compared to the control group (Figure 4-12). This enhancement could not be attributed to an increase in locomotor activity observed in the OFT (Figure 4-11) but may have resulted from a minor increase in hippocampal noradrenaline concentrations (§5.4.3.1 and Addendum A, Table 3). Furthermore, allopurinol also promoted swimming behaviour which may be subsequent to a slight increase in hippocampal serotonin levels (§5.4.3.3 and Addendum A, Table 3). These findings correlate with other similar studies in animals. Karve and colleagues (2013) found that chronic treatment with allopurinol (39 mg/kg p.o.) had significant antidepressant-like effects that were comparable to that of fluoxetine (10 mg/kg). Gibney *et al.* (2014) were further able to substantiate these findings by administering 20 mg/kg allopurinol intraperitoneally for 9 days. Most recently, Gürbüz Özgür and colleagues (2015) additionally recorded antidepressant-like effects using the FST after a 2 week treatment period with allopurinol (50 mg/kg). Therefore, based on the findings from our study and from the results published from earlier research it is obvious that chronic treatment with allopurinol proves effective in reducing

depressive-like behaviours in a range of animal models when exposed to the FST. However, monoamine data is insufficient in fully supporting these outcomes (§5.4.3).

Sodium benzoate caused a reduction in immobile behaviour (Figure 4-12), although not significantly so and which could not be contributed to any increase in brain noradrenaline concentrations (Figure 4-18: A and Addendum A, Table 3). Nor did it have any significant effect on general locomotor activity (Figure 4-11). Sodium benzoate did, however, significantly enhance swimming behaviour which may have been due to a significant increase in striatal DOPAC and dopamine levels (§5.4.3.2; Addendum A, Table 3). These results weren't as encouraging as expected seeing that earlier research provides more noteworthy evidence for this compound's antidepressant-like capabilities. Furthermore, our study provides evidence of this compound's ability to reduce 5-HIAA levels in the brain (§5.4.3.3; Addendum A, Table 3). A case study by Lai *et al.* (2012) involving sodium benzoate administration to a patient with major depressive disorder was the first attempt in investigating the effects of this compound on depressive behaviours. Their investigation was motivated by earlier research conducted by Heresco-Levy *et al.* (2006) stating that add-on therapy using D-cylcoserine, an antibiotic that acts as a partial agonist at the D-serine binding site on NMDA receptors, visibly reduced depressive symptoms in patients with MDD. Subsequently, they found that after 6 weeks of 500 mg/day sodium benzoate therapy, the patient experienced reduced depressive symptoms and subsequent remission. In addition, structural brain changes were also observed in the form of increased thalamic, amygdalar and brain stem volumes. Then again, Kamel and Abd El Razek (2013) observed that chronic treatment with sodium benzoate induced antisocial as well as anxiety and depressive-like behaviours in albino male Wistar rats. Based on the aforementioned, it may be necessary to further investigate the potential antidepressant-like actions of sodium benzoate to substantiate our findings.

Ketamine (10 mg/kg) also caused a significant improvement in FST mobility time compared to the control (Figure 4-12) as well as an associated increase in swimming time (Figure 4-13: A) which could not be accounted for by an increase in locomotor activity (Figure 4-11). These findings are in line with our findings with regard to monoamine analyses based on ketamine's ability to moderately increase striatal noradrenaline and dopamine (Figure 4-18: B) and hippocampal serotonin (§5.4.3.1-5.4.3.3 and Addendum A, Table 3) as well as a substantial amount of evidence existing for ketamine's antidepressant-like capabilities, making this drug an ideal reference compound when assessing the effects of other (novel) drug compounds (i.e. allopurinol and sodium benzoate) with suggested activity in the glutamatergic pathway. As discussed earlier, ketamine has proven effective in treating MDD and treatment-resistant depression (TRD) as evidenced by both animal and human studies

(Dutta *et al.*, 2015; Sanacora *et al.*, 2012). Ketamine's positive effects on depressive-like behaviour in the FST may result from its unique mechanism with regard to the glutamatergic pathway (Miller *et al.*, 2016). Moreover, previous studies provide evidence of ketamine's effects on monoamines (Tso *et al.*, 2004) and their metabolites (Kari *et al.*, 1978) which may explain its antidepressant-like effects as seen in our results from the FST (Figure 4-12). Initially, Kari *et al.* (1978) observed that ketamine (50 mg/kg) induced increases in 5-HT and its end-stage metabolite, 5-HIAA. They also found that adrenaline concentrations were moderately increased. However, all other monoamines were decreased 12 hours after ketamine administration. Tso *et al.* (2004) found that ketamine induced effects in a stereoselective manner. More specifically, (+)-ketamine increased dopamine outflow and inhibited the dopamine transporter thus reducing dopamine reuptake and increasing extracellular concentrations. Memantine enhanced mobility in the FST (Figure 4-12) and reduced locomotor activity in the OFT (Figure 4-11) without having any statistically significant effect on either behaviours. A sizable increase in swimming behaviour was observed (Figure 4-13: A), however, this could not be attributed to an increase in serotonergic activity (Figure 4-18: C). No positive effects were observed for climbing during the FST as evidenced by the lacking effects of this compound on noradrenaline. However, memantine did cause a minor increase in striatal dopamine (Figure 4-18: B) which is substantiated by a measurable increase in striatal DOPAC (Addendum A, Table 3). The lack of sizable significance may once again be consequent of the small number of animal subjects employed in this study. Other preclinical and clinical studies suggest that memantine is capable of reducing depressive symptoms and behaviours which may assist in validating our findings. Réus and colleagues (2012) observed that both acute and chronic administration of memantine (5, 10 and 20 mg/kg) reduced immobility time of rats in the FST reinforcing their hypothesis that NMDA receptor modulators possess antidepressant potential. Muhonen *et al.* (2008) found that 26 weeks treatment with memantine (20 mg) presented with comparable efficacy to escitalopram (20 mg) in treating depression with co-morbid alcohol abuse. Ferguson and Shingleton (2007) observed that up-titrated doses of memantine (20, 30 and 40 mg/d) reduced depressive symptoms in MDD patients after undergoing 12 weeks of treatment. Unexpectedly, fluoxetine (known antidepressant) did not reduced immobility in the FST although it did express a trend in that direction. These results may be attributed to the increase prefrontocortical noradrenaline and serotonin (Figure 4-18: A and C). However, it did markedly reduce locomotor activity in the OFT compared to the control group which is in line with earlier research conducted in our laboratories (Badenhorst, 2014).

Despite the fact that sodium benzoate did not present with statistical improvement in depressive-like behaviours during the FST, this compound did exhibit measurable practical significance expressed by its large effect size (Addendum A, Table 1) compared to the control group. Allopurinol, more so, presented with a very large effect size compared to the control group suggesting that both the two test compounds are capable of antidepressant-like activity and that their effects should not be rejected, but rather undergo further investigation for future practical implications. Finally, all treatment groups induced enhanced swimming behaviours compared to the control group with allopurinol and sodium benzoate bearing the greatest significance (Figure 4-13: A). No significant effects were recorded for climbing behaviours (Figure 4-13: B).

5.4.2 The procognitive effects of chronically administered compounds

New groups of FSL rats were treated using the same chronic protocol applied for assessing antidepressant-like abilities (§5.4.1) using the FST, now in the MWM testing procedure (Table 3-3, Figure 3-6). To our knowledge, the effects of chronic allopurinol and sodium benzoate treatment on cognitive function in the FSL model have yet to be assessed using the MWM.

As evidenced by Figure 4-15, all treatment groups displayed a significant improvement in spatial learning abilities across Day 1-5 of acquisition training compared to the vehicle (control) group, demonstrating that memory consolidation had taken place effectively. More specifically, allopurinol proved effective in aiding consolidation on Day 2 (Figure 4-16: B), whereas sodium benzoate exhibited similar results on Day 3 (Figure 4-16: C) compared to the control group. Overall, both compounds proved positive in promoting memory consolidation on Day 5 (Figure 4-16: E) and across all training days. However, these results cannot be contributed to locomotor activity enhancement (Figure 4-14). The cognitive enhancing abilities of allopurinol may be explained by its ability to enhance BDNF and neuroplasticity as evidenced by research done in piglets in 2013 (Prickaerts *et al.*, 2013). However, it may seem as though this is the only study to date examining allopurinol's effects on neuroplasticity markers and related effects. This does not include research done by Yonden *et al.* (2010) where allopurinol was investigated for its abilities to reverse ammonia toxicity-induced neuronal damage and excitotoxicity resulting from NMDA receptor overstimulation. On a different note, sodium benzoate has proven capable of promoting neuroplasticity processes suggesting a role in the treatment of neurodegenerative disorders such as those associated with memory impairments (Jana *et al.*, 2013).

After completion of the final 4 training sessions on Day 5, the different groups were subjected to a probe trial to assess the extent of memory retrieval by measuring the time

spent in the target zone were the platform had initially been located. The results were then expressed as percentage time spent in the target zone. As illustrated in Figure 4-17, no prominent variances in percentage time spent in the target zone were observed between the groups to suggest successful memory storage/retrieval when compared to the control group. However, allopurinol has only now entered stages of investigation regarding its potential procognitive effects during Morris water maze (MWM) testing. A study conducted by Dong *et al* (2015) found that carbon monoxide-induced cognitive impairments were successfully reversed via 50 mg/kg allopurinol treatment. They further found evidence for reduced prefrontocortical and hippocampal death along with reduced microglial activation after treatment. Relevantly, fluoxetine did cause memory impairment as evidenced by Figure 4-17. These effects have been observed in past research with regard to fluoxetine-induced learning and/or memory impairment in the MWM as well as other measures of cognitive function (viz. NORT), using different animal models of depression (Ampuero *et al.*, 2013; First *et al.*, 2011; Han *et al.*, 2015; Keith *et al.*, 2007; Song *et al.*, 2006; Wilson & Hamm, 2002). Conversely, several of these studies produced inconsistent results.

Finally, it may also seem as though memantine induces memory impairments to a certain extent which is consistent with findings from earlier studies (Figure 4-17). Despite the fact that memantine is used clinically for the treatment of memory disorders, such as AD, this compound is known to induce memory impairment in animals (Creeley *et al.*, 2006; Johnson & Kotermanski, 2006), although conflicting findings have been published (Camarasa *et al.*, 2010; Zoladz *et al.*, 2006). Conversely, ketamine did not impair memory retrieval which is an unexpected outcome seeing that much research has been published on its debilitating cognitive effects (Smith *et al.*, 2011; Duan *et al.*, 2013; Peng *et al.*, 2011; Moosavi *et al.*, 2011). However, in support of our findings, Solé *et al.* (2015) found that low doses of ketamine may prove beneficial with regard to cognition.

5.4.3 The effects of chronically administered compounds on brain monoamine and associated end-stage metabolite concentrations

It has long been hypothesised/proposed that monoamine (NA, DA and 5-HT) levels and/or activity are dysregulated in the brain of depressives (Overstreet *et al.*, 2005) and as a result neurotransmitter-synapse communications are affected, subsequently hampering signalling processes that may follow leading to the development of depressive symptoms and behaviours (Kiyohara & Yoshimasu, 2009). So far researchers have been able to link 5-HT and NA abnormalities to psychopathologies that underlie depressive disorders (Krishnan & Nestler, 2008). Chapter 2, §2.4.2.2 provides a more elaborate literature overview surrounding the involvement of monoamines in MDD. Due to lacking data from our analyses,

not all results could be presented graphically. This may have resulted from low sample tissue mass or undetected errors during the sample preparation and/or analysis procedure. However, all raw data collected from the HPLC ED monoamine analysis are presented in Table 3 in Addendum A.

5.4.3.1 Noradrenaline

As illustrated in Figure 4-19, few compounds elicited effects on monoamine and associated end-stage metabolite concentrations in the analysed brain regions. Unsurprisingly, fluoxetine did considerably enhance prefrontocortical (PFC) noradrenaline (NA) levels compared to the control group (Figure 4-19: A) which coincides with findings by Bymaster *et al.* (2002). No noteworthy effects on striatal (STR) or hippocampal (HPC) NA were recorded (Addendum A, Table 3). Additionally, no significant changes in NA levels were observed for any of the other treatment groups in the different brain regions (Figure 4-19: A) with the exception of allopurinol in the HPC (Addendum A, Table 3). Allopurinol caused a measurable reduction in hippocampal NA levels compared to the control group. The reason for this, however, is unclear and cannot be explained by literature. However, earlier research provides information suggesting that this compound may be capable of enhancing striatal NA concentrations at a dose (300 mg/kg for three days) considerably higher than ours (Desole *et al.*, 1995). This outcome requires further investigation.

5.4.3.2 Dopamine, dihydroxyphenylacetic acid and homovanillic acid

It may seem as though all treatment groups (excluding fluoxetine) exhibited an inclining increase in STR dopamine compared to the control (Figure 4-19: B). However, sodium benzoate was the only compound that had a significant effect in this regard. Furthermore, sodium benzoate caused an additional increase in striatal dihydroxyphenylacetic acid (DOPAC) (Addendum A, Table 3) which may be consequence of its effects on dopamine. The exact mechanism behind this outcome remains unclear since no sufficient evidence exists for sodium benzoate's effects on monoamines. Crane and Lachance (1985) attempted to do so and came to the unsatisfying conclusion that sodium benzoate had no significant effects on monoamine levels. Sadly, an insufficient amount of data was gathered during the analysis, making it impossible to depict the results from the effects of the different treatment groups on DA levels in the other brain regions. Furthermore, fluoxetine had no statistically significant effects on striatal (STR) dopamine levels (Figure 4-19: B) and its effects on prefrontocortical and hippocampal DOPAC are unconfirmed due to absent data and/or significance. Once more, data regarding fluoxetine's effects on prefrontocortical DA under our study conditions was lacking (Addendum A, Table 3). However, there are animal studies that provide evidence of enhanced prefrontocortical DA (Bymaster *et al.*, 2002). Moreover, it

may seem as though memantine is capable of enhancing striatal DA as it displays a noteworthy increase in striatal DOPAC comparable to that of sodium benzoate (Addendum A, Table 3). Finally, it appears as though allopurinol may potentially increase striatal DA (Johnson & Kotermanski, 2006; Ferguson & Shingleton, 2007). No notable differences in effect on striatal homovanillic acid (HVA) across treatment groups were observed and regrettably, no adequate data was available to assess these effects in the PFC and HPC (Addendum A, Table 3).

5.4.3.3 Serotonin and 5-hydroxyindoleacetic acid

Fluoxetine was the only compound to induce a substantial increase in prefrontocortical serotonin (5-HT) compared to the control group (Figure 4-19: C) which was further supported by an increase in prefrontocortical 5-hydroxyindoleacetic acid (5-HIAA) (Addendum A, Table 3). Fluoxetine had no positive effects on striatal 5-HT and the data pertaining to its effects on hippocampal 5-HT, were insufficient (Addendum A, Table 3). In addition, none of the other compounds enhanced prefrontocortical or striatal serotonin concentrations. However, it appears as though ketamine and allopurinol caused an increase in hippocampal 5-HT levels compared to the control group (Addendum A, Table 3), though not significantly so. Furthermore, ketamine seemingly causes a noticeable increase in 5-HIAA levels in all three brain regions. These results correlate with that found in research. Researchers have established that ketamine acts in a stereoselective manner with regard to monoamine enhancement and/or inhibition (Nishimura & Sato, 1999; Tso *et al.*, 2004). Kari *et al.* (1978) indicated that ketamine initially enhances serotonin (5-HT) and 5-Hydroxyindoleacetic acid (5-HIAA) in the first 30 minutes after administration in rats, whereas noradrenaline (NA) and dopamine (DA) are decreased. The latter two reductions persist up to 12 hours after treatment in association with a notable increase in adrenaline concentrations also being documented (Kari *et al.*, 1978). Nishimura and Sato (1999) found that S-ketamine caused DA-transporter inhibition, but had no significant effects on 5-HT or NA-transporters. Lastly, it appears as though sodium benzoate increases striatal 5-HIAA. However, the data suggests that memantine, allopurinol and sodium benzoate reduce hippocampal 5-HIAA levels. These findings remain unclear.

5.4.4 The effects of chronically administered compounds on BDNF concentrations

BDNF is one of several neurotrophins found in the human body and plays a crucial role in affective disorders and cognitive function (Hasselbalch *et al.*, 2012; Pittenger & Duman, 2008; Serafini, 2012). BDNF activity also plays a vital role in hippocampal, cortical and amygdala-related LTP, both pre- and post-synaptically (Pittenger & Duman, 2008). MDD has been known to present with abnormal modulation of BDNF (i.e. reduced levels) (Fakhoury,

2015) that is reversible with antidepressant therapy (Hasselbalch *et al.*, 2012). Extensive reviews conducted by Pittenger and Duman (2008) and Serafini (2012) substantiate the aforementioned results of antidepressant-related (e.g. SSRIs, MOAIs, TCAs, ECT and ketamine) reversal of cognitive (i.e. learning and memory) and neuroplasticity deficits in both animal models and human subjects (Pittenger & Duman, 2008, Serafini, 2012). Chapter 2, §2.4 elaborates more clearly the involvement of BDNF in both MDD and cognition. Unfortunately, none of the analyses displayed statistically significant results on BDNF across the various treatment modalities which contradict earlier reports by investigators. For example, a study conducted by First *et al.* (2015) on fluoxetine showed increased frontocortical BDNF levels which they suggested may have enhanced cognitive functioning during MWM testing at a lower dose (5 mg/kg fluoxetine) than applied in our study. Other studies also corroborate the aforesaid research in various brain regions (especially the hippocampus) and/or serum (Coppell & Zetterstram, 2000; de Faubert *et al.*, 2004; Liu *et al.*, 2014; Pilar-Cuéllar *et al.*, 2012). This is consistent with earlier statements that antidepressants have procognitive qualities as detailed in Chapter 2.

Ketamine's effects on BDNF are still controversial, but researchers have been able to link its activity to NMDA and AMPA receptor function affecting BDNF expression and release (Murck & Harald, 2013). Ketamine's ability to alter hippocampal BDNF levels has been under speculation to be involved in its antidepressant-like actions (Murrough, 2012). Though ketamine is known for its neurotoxic effects and cognitive impairment, other studies provide evidence for these effects to occur in a dose-dependent manner. That is to say, high doses of ketamine may result in detrimental cognitive effects whereas lower doses promote neuroprotection and neurotrophin expression (Solé *et al.*, 2015) which subsequently leads to synaptic growth and reinforced synaptic connections (Belujon & Grace, 2014; Murck & Harald, 2013). Furthermore, memantine appears to have BDNF enhancing capabilities (Réus *et al.*, 2010) on all three brain regions in our study which is typically related to its procognitive effects as seen in Alzheimer's and Parkinson's disease treatment (Marvanová *et al.*, 2001) (Figures 4-20: A-C). In 2013, Jana *et al* published research results regarding the effects of sodium benzoate (derived from cinnamon) on brain neurotrophic factors. Astonishingly, they observed increased BDNF and neurotrophin (NT)-3 levels and were able to establish the mechanism (PKA-CREB pathway) via these enhancements took place. Yet, this is not the case in our investigation. It may appear as though there is a positive inclination to enhanced BDNF levels in the striatum caused by fluoxetine, ketamine and memantine compared to the vehicle group (Figure 4-20: B). Be that as it may, none of these effects prove substantial and will require further investigation.

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To summarise, the depressive-like phenotype of the FSL model was confirmed using the rat forced swim test (FST) making it suitable for use in this study. Regrettably, substantial cognitive impairments were not observed in this model using the Morris water maze (MWM) test. A suitable dose for both allopurinol and sodium benzoate was established using the rat FST allowing further investigation into the effects of chronic treatment with these compounds on depressive-like behaviours, cognitive function, brain monoamine levels as well as brain-derived neurotrophic factor (BDNF) concentrations.

Chronic 5 mg/kg allopurinol presented with antidepressant-like effects in the FST after a 12 day consecutive treatment protocol which corresponds with results obtained by other studies (Gürbüz Özgür *et al.*, 2015). These outcomes may not necessarily be subsequent to allopurinol's effects on monoamines and their associated end-stage metabolites as assessed in this study. Sodium benzoate (100 mg/kg) similarly exhibited antidepressant-like capabilities using the same protocol, though not to the same extent as allopurinol. These findings were welcomed by a previous study assessing sodium benzoate's effects in a drug-naïve patient diagnosed with MDD (Lai *et al.*, 2012). These results could not be effectively corroborated by changes in monoamine and associated end-stage metabolites even though moderate effects were observed. Disappointingly, neither of the test compounds proved significantly effective in promoting cognitive (learning/memory) function during the MWM test, though it may appear as if sodium benzoate is capable of procognitive actions. This contradicts findings by Prickaerts *et al.* (2015) regarding allopurinol's procognitive effects with reference to neuroplasticity enhancements. Interestingly, fluoxetine visibly impaired memory retrieval during MWM testing which has previously been documented (First *et al.*, 2011; Han *et al.*, 2015). Furthermore, none of the compounds proved substantially effective in enhancing brain BDNF levels. This is in opposition to the findings by Jana *et al.* (2013) providing evidence of allopurinol-induced enhancements of BDNF which related to documented neuroplasticity improvements.

Chapter 6 : Conclusion

Although major depression is a global disability of ambiguous origin entailing multiple psychological and physical vicissitudes of great complexity, it remains treatable. Though the available treatment modalities prove ineffective in some individuals, there remains room for improvement. One of the areas that provide opportunity for investigation is the glutamatergic pathway and associated systems related to its activity viz., the kynurenine pathway and N-methyl-D-aspartate (NMDA) receptor function. To date, much research provides supporting evidence for involvement of this pathway in the pathology and treatment of major depression.

Primarily, this study was aimed at investigating the effects of chronic treatment with allopurinol and sodium benzoate on depressive-like behaviours and possible associated cognitive impairments as well as brain monoamine and brain-derived neurotrophic factor (BDNF) concentrations using a genetic animal model of depression, viz. the Flinders sensitive line (FSL) rat, in order to further elaborate on possible biological targeting areas with the use of novel compounds.

Part of the primary objective of this study entailed confirming whether the FSL rat exhibited cognitive (i.e. learning and memory) impairments compared to its healthy counterpart, the Flinders resistant line (FRL) rat, when subjected to the Morris water maze (MWM) test. Our results did not confirm this expectation based on the lack of substantial and statistically significant differences between the two strains upon assessing memory retrieval. However, a notable difference was seen. Memory processing has previously been assessed in the FSL model using the novel object recognition (NOR) test demonstrating memory impairment. However, the MWM and NOR tests differ in terms of the type of memory assessed. Subsequently, we went on to establish at which doses allopurinol and sodium benzoate proved capable of decreasing depressive-like behaviours in FSL rats in the forced swim test (FST). The results suggested that 5 mg/kg allopurinol and 100 mg/kg sodium benzoate administered via the intraperitoneal route were effective in reducing depressive-like behaviours in the FST using an acute dosing regimen.

Chronic treatment with both these compounds resulted in reduced expression of depressive-like behaviours in the FSL rat compared to the vehicle treated control group. More so, allopurinol exhibited the greater effect between the two compounds. These results are in line with other research employing similar behavioural tests, but different animal models of

depression (Gürbüz Özgür *et al.*, 2015; Karve *et al.*, 2013). Unfortunately, the data obtained during the monoamine analyses were somewhat obscure making it difficult to link possible monoaminergic changes to the observed effects with the exception of dopamine measurements in the striatum.

To our disappointment, chronic treatment with allopurinol and sodium benzoate did not result in substantial procognitive effects with regard to memory retrieval in the MWM test. However, the data gathered over the 5 days of acquisition training (viz. the extent of memory consolidation) did clearly indicate that both these compounds promoted a degree of learning and associated memory consolidation, suggesting that the learnt task was successfully stored in long-term memory. These findings can be supported with findings by Dong *et al* (2015). Even though neither of the two compounds demonstrated beneficial effects on brain BDNF concentrations, there is literature evidence for both allopurinol (Prickaerts *et al.*, 2013) and sodium benzoate's (Jana *et al.*, 2013) BDNF enhancing actions.

In reference to our secondary objectives, we were able to confirm the inherent depressive-like phenotype of the FSL model of depression using the FST. Consequently, we were able to validate the use of this model in assessing the effects of chronic treatment with allopurinol and sodium benzoate on depressive-like behaviours. Furthermore, we measured the effects of two reference compounds (viz. ketamine and memantine) on depressive-like behaviours, cognitive function as well as brain monoamine and BDNF concentrations. Both compounds exhibited antidepressant-like actions, though more so true for ketamine than memantine. These results coincide with that found in literature (Dutta *et al.*, 2015; Lindholm *et al.*, 2012; Muhonen *et al.*, 2008). Their effects on monoamines and associated end-stage metabolites in our study were not substantial enough to support the results obtained during the behavioural analyses, however, much research exists in support of their positive effects on monoamine concentrations (Ferguson & Shingleton, 2007; Duman *et al.*, 2012; Dutta *et al.*, 2015; Johnson & Kotermanski, 2006). Similar to allopurinol and sodium benzoate, neither of the two compounds evoked extensive cognitive enhancing effects when tested in the MWM, even though it may appear as though ketamine may be capable of such action. Concerning brain BDNF concentrations, though no significant increases were documented in this study, ketamine and fluoxetine may have enhanced striatal BDNF levels (Solé *et al.*, 2015), whereas, memantine assuredly increased BDNF levels in all three brain regions.

In summary, the FSL model does not explicitly express cognitive impairments when compared to the FRL counterpart as assessed using the MWM test. However, differences can be seen. Allopurinol, a xanthine-oxidase inhibitor capable of exerting effects on the kynurenine pathway, presents with antidepressant-like actions. The same is true for sodium

benzoate, a D-amino acid oxidase inhibitor. Neither of the compounds enhances monoamine and associated metabolite concentrations in an extensive or predictable manner. Unfortunately, the same is true for BDNF. Despite the fact that the results were not entirely as expected based on the stipulated aims and objectives, there is much room available for exploration. Though the statistical significance of the gathered results may not be substantial in all instances of this study, the practical implications may be of great value for future studies thus necessitating further investigation into this area of research.

6.1 Recommendations for future investigation:

Since this study made use of only six animal subjects per group, which consequently may have led to a lack of substantiating data, future studies should consider increasing subject numbers to ensure accurate results that present significant differences.

It may be useful to investigate a wider dose-range for allopurinol and sodium benzoate in a chronic treatment protocol to assess antidepressant-like effects in the FSL model of depression should it be that these doses prove effective in eliciting a more substantial effect.

Applying a different or even additional behavioural analysis for assessing cognitive function pertaining to memory and learning may prove beneficial in establishing whether a chosen animal model of depression expresses associated cognitive impairments.

Moreover, future investigators could consider using another animal model, e.g. chronic unpredictable or mild stress models of depression for investigating the aforementioned impairments.

It is also important to consider examining the effects of chronic treatment with allopurinol and sodium benzoate on other biological components within the glutamatergic pathway with relevance to depressive-like behaviours and cognitive function, e.g. kynurenine metabolites, NMDA and AMPA receptor expression, glutamate concentrations and tropomyosin receptor kinase B (TrkB).

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Addendum A:

1. Phase 3: Main experimental study – FSL

1.1. Depressive-like phenotype:

Table 7: Calculation, quantification and expression of Cohen's d-value in the form of effect size for immobility time in the forced swim test (Figure 4-12).

Time spent in target zone during probe trial expressed as percentage (%)						
	Vehicle	**Fluoxetine (10 mg/kg)**	**Ketamine (10 mg/kg)**	**Memantine (20 mg/kg)**	**Allopurinol (5 mg/kg)**	**Sodium benzoate (100 mg/kg)**
	155.2	192.4	129.5	102	95	74.2
	137.8	74.3	96.7	178.7	46.3	53.7
	195.4	154.3	81.4	153.3	83.3	158.4
	168.8	109.6	124	147.9	70.7	159.5
	114	160.7	102	155.9	131.6	155.8
	221.1	120.4	97	94.1	103.2	81.9
Calculated average and standard deviation for each treatment group						
	Vehicle	**Fluoxetine (10 mg/kg)**	**Ketamine (10 mg/kg)**	**Memantine (20 mg/kg)**	**Allopurinol (5 mg/kg)**	**Sodium benzoate (100 mg/kg)**
Average	165.3833	135.2833	105.1	138.65	88.35	113.9167
Standard deviation	38.7861	42.11904	18.22109	33.2556	29.12228	49.07005
Calculated Cohen's d-value for expression as effect size: *0.2 ≤ x < 0.5 (small), 0.5 ≤ x < 0.8 (medium), 0.8 ≤ x < 1.30 (large), x ≥ 1.30 (very large)*						
	Vehicle	**Fluoxetine (10 mg/kg)**	**Ketamine (10 mg/kg)**	**Memantine (20 mg/kg)**	**Allopurinol (5 mg/kg)**	**Sodium benzoate (100 mg/kg)**
Vehicle		0.714641	1.554251	0.68925	1.986107	1.048841
Fluoxetine (10 mg/kg)			0.71662	0.079932	1.114302	0.435432
Ketamine (10 mg/kg)				1.008853	0.575161	0.179675
Memantine (20 mg/kg)					1.512527	0.504041
Allopurinol (5 mg/kg)						0.521024

Sodium benzoate (100 mg/kg)	

1.2. Cognitive function test:

Table 8: Calculation, quantification and expression of Cohen's d-value in the form of effect size for memory retrieval in the Morris water maze (Figure 4-17).

Time spent in target zone during probe trial expressed as percentage (%)						
	Vehicle	**Fluoxetine (10 mg/kg)**	**Ketamine (10 mg/kg)**	**Memantine (20 mg/kg)**	**Allopurinol (5 mg/kg)**	**Sodium benzoate (100 mg/kg)**
	34.42267	20.13	54.16417	25.85867	43.88	59.169
	39.4275	8.51	44.59917	47.2685	31.48	58.50167
	37.75917	14.07	39.37183	30.3075	50.99	43.54267
	40.31717	10.07	50.38267	33.42167	25.53	57.50067
	35.31233	9.4	30.75233	23.13383	55.94	27.41567
	42.486	43.1	42.486	32.75433	25.53	23.63433
Calculated average and standard deviation for each treatment group						
	Vehicle	**Fluoxetine (10 mg/kg)**	**Ketamine (10 mg/kg)**	**Memantine (20 mg/kg)**	**Allopurinol (5 mg/kg)**	**Sodium benzoate (100 mg/kg)**
Average	38.28747333	17.54666667	43.62602833	32.12408333	38.89166667	44.96066833
Standard deviation	3.069665865	13.23377296	8.279874612	8.423946016	13.22043784	16.16871949
Calculated Cohen's d-value for expression as effect size: *0.2 ≤ x < 0.5 (small), 0.5 ≤ x < 0.8 (medium), 0.8 ≤ x < 1.30 (large), x ≥ 1.30 (very large)*						
	Vehicle	**Fluoxetine (10 mg/kg)**	**Ketamine (10 mg/kg)**	**Memantine (20 mg/kg)**	**Allopurinol (5 mg/kg)**	**Sodium benzoate (100 mg/kg)**
Vehicle		1.567263299	0.644762783	0.731651175	0.045701462	0.412722542
Fluoxetine (10 mg/kg)			1.970667152	1.101531416	1.61291871	1.695496152
Ketamine (10 mg/kg)				1.365386836	0.358109294	0.08254457
Memantine (20 mg/kg)					0.511903117	0.793914757
Allopurinol (5 mg/kg)						0.375354503
Sodium benzoate (100 mg/kg)						

1.3. Monoamine analysis

Table 9: Results for monoamines and metabolites as measured using HPLC-ED expressed as ng/mg brain.

			Monoamines and metabolites measured (ng/mg wet brain)					
Sample no.	Brain region	Treatment	NA	DOPAC	DA	HVA	5-HT	5-HIAA
1	Prefrontal cortex	Fluoxetine (10 mg/kg)	211.20	29.11	0.00	11.94	1066.88	360.62
2			187.61	37.61	25.61	68.61	883.73	296.35
3			201.09	28.86	7.75	28.23	868.67	279.95
4			225.92	15.81	0.00	12.80	874.07	329.03
5			69.05	25.54	0.00	5.97	591.20	240.39
6			150.20	16.55	0.00	23.62	784.46	293.63
7		Memantine (20 mg/kg)	93.69	22.50	23.85	33.30	141.99	226.35
8			92.58	21.41	11.84	29.44	119.85	208.91
9			76.33	23.51	15.22	22.84	145.75	197.77
10			87.78	18.05	15.61	20.98	101.13	191.54
11			85.31	17.48	15.32	10.49	171.14	144.68
12			78.93	38.19	22.06	34.51	116.14	196.26
13		Ketamine (10 mg/kg)	69.52	8.76	0.00	0.00	97.50	256.09
14			95.56	9.63	6.09	16.01	186.57	273.35
15			71.73	11.22	0.00	10.37	113.83	188.15
16			95.59	7.72	0.00	23.95	136.91	341.50
17			59.01	4.38	0.00	0.00	115.76	147.11
18			72.29	4.38	0.00	0.00	128.63	221.92
19		Vehicle	74.49	0.00	0.00	0.00	143.14	310.70
20			83.63	0.00	0.00	0.00	166.90	227.78
21			75.70	0.00	0.00	0.00	121.76	315.60
22			87.45	0.00	0.00	0.00	190.17	264.78
23			54.18	0.00	0.00	0.00	147.01	244.65
24			95.75	12.38	27.69	23.82	140.44	205.20
25		Sodium benzoate (100 mg/kg)	103.12	19.27	17.16	22.48	154.05	177.82
26			123.52	19.33	15.66	7.46	159.42	254.85
27			90.91	26.76	21.39	29.61	126.28	197.90
28			89.89	28.47	32.71	7.46	162.57	212.56
29			78.78	13.83	16.96	19.51	126.42	178.61
30			92.55	35.98	24.70	14.92	151.86	225.87
31		Allopurinol (5 mg/kg)	85.23	14.58	17.62	24.57	145.68	217.48
32			64.57	14.51	25.85	29.56	149.86	208.65
33			78.80	18.68	36.26	26.64	129.33	180.49
34			70.33	11.82	27.39	32.05	146.27	208.81
35			78.86	13.43	28.21	33.72	169.34	153.81
36			61.28	30.70	29.69	46.73	122.37	176.19
37	Striatum	Fluoxetine (10 mg/kg)	83.32	764.39	2434.42	420.18	253.19	348.90
38			21.98	1084.27	2499.43	614.32	183.41	317.82

39			48.24	540.71	2658.59	396.64	153.54	283.30
40			41.46	791.14	3022.14	490.56	224.00	386.93
41			29.21	824.55	2394.20	464.67	137.21	302.58
42			57.22	604.75	2831.48	486.25	186.48	234.77
43		Memantine (20 mg/kg)	33.83	1319.23	4750.43	503.05	264.67	337.41
44			58.77	1134.05	3893.80	498.86	270.20	367.17
45			24.34	1422.55	4005.93	526.26	194.84	315.51
46			21.91	1429.83	4814.81	584.85	213.34	358.73
47			39.02	518.35	2466.39	244.87	210.01	213.84
48			34.90	1213.27	3843.09	489.82	301.11	319.31
49		Ketamine (10 mg/kg)	26.43	852.52	3129.52	396.55	187.05	326.37
50			44.88	917.23	4030.16	484.64	295.07	354.82
51			57.68	596.96	3192.41	397.04	259.20	285.96
52			72.14	593.51	2447.75	400.51	213.53	361.36
53			56.60	766.06	3630.14	477.20	220.57	303.48
54			53.17	706.60	3881.72	384.41	258.36	307.13
55		Vehicle	57.15	560.44	2504.16	279.48	191.44	311.14
56			48.21	453.69	3258.68	337.38	240.64	281.55
57			67.60	1147.55	5029.77	674.02	295.89	471.60
58			45.30	1160.78	4633.14	496.33	261.81	404.11
59			71.11	588.33	3300.31	276.11	259.25	308.94
60			63.14	532.84	3005.81	345.68	254.45	318.59
61		Allopurinol (5 mg/kg)	33.23	743.49	3832.46	413.07	253.94	261.21
62			37.00	665.08	3680.19	449.97	247.46	265.60
63			48.96	557.77	3087.22	350.75	225.22	249.75
64			41.71	948.21	4290.26	494.83	279.66	315.79
65			68.48	909.96	4824.43	540.49	342.04	366.46
66			38.93	1204.87	6077.25	701.15	263.50	364.67
67		Sodium benzoate (100 mg/kg)	24.79	933.23	4080.21	386.94	232.57	289.91
68			57.07	1070.71	4838.28	516.78	275.63	362.53
69			37.27	1637.56	6362.16	690.61	307.82	436.70
70			79.62	1015.82	4144.52	429.10	300.97	350.51
71			24.41	1621.20	6494.10	697.93	268.15	378.10
72			62.86	798.19	3820.93	451.81	269.84	347.76
73	Hippocampus	Fluoxetine (10 mg/kg)	38.99	0.00	14.15	0.00	0.00	289.26
74			81.87	0.00	0.00	0.00	0.00	295.17
75			45.51	0.00	0.00	0.00	0.00	201.38
76			101.22	0.00	20.82	0.00	0.00	427.58
77			72.25	0.00	0.00	0.00	53.36	215.72
78			100.67	0.00	0.00	0.00	0.00	248.75
79		Memantine (20 mg/kg)	74.22	0.00	0.00	0.00	48.76	170.95
80			89.49	0.00	0.00	0.00	58.47	244.00
81			63.49	0.00	0.00	0.00	40.41	184.22
82			71.32	0.00	0.00	0.00	38.50	123.86
83			109.93	0.00	0.00	0.00	120.00	273.00
84			108.93	0.00	0.00	0.00	81.76	258.71
85		Ketamine	85.73	11.72	19.66	0.00	52.60	314.01

86		(10 mg/kg)	85.50	18.34	9.59	0.00	92.73	306.61
87			93.13	4.06	9.22	0.00	46.25	202.08
88			164.02	28.21	31.10	0.00	95.78	448.66
89			109.16	41.98	47.38	0.00	49.44	209.29
90			66.52	8.11	9.68	0.00	40.18	202.93
91		Vehicle	100.93	7.52	17.77	0.00	79.70	328.15
92			83.16	7.52	8.89	0.00	95.93	291.57
93			123.96	19.16	33.60	0.00	68.26	317.56
94			124.91	15.04	25.21	0.00	101.40	319.90
95			100.60	20.99	42.75	0.00	81.91	300.49
96			80.08	16.26	20.75	0.00	60.74	265.28
97		Allopurinol (5 mg/kg)	70.42	0.00	0.00	0.00	97.92	170.75
98			75.39	0.00	0.00	0.00	96.27	268.58
99			53.68	0.00	0.00	0.00	79.84	167.48
100			52.28	0.00	0.00	0.00	64.32	176.25
101			61.03	0.00	0.00	0.00	76.90	167.56
102			73.48	0.00	0.00	0.00	95.25	199.39
103		Sodium benzoate (100 mg/kg)	65.43	0.00	0.00	0.00	60.69	132.31
104			60.77	0.00	0.00	0.00	41.12	151.28
105			64.16	0.00	0.00	0.00	61.45	195.31
106			109.87	0.00	0.00	0.00	80.08	232.30
107			75.32	0.00	0.00	0.00	72.52	168.59
108			80.50	0.00	0.00	0.00	50.94	172.96