A comparative study of N-acetyl cysteine and an experimental xanthone compound on behavioral, immune-inflammatory and redox biomarkers of depression in the Flinders sensitive line rat

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It all starts here ™

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List of Abbreviations

Numbers

3-HK: 3-hydroxykynurenine

5-HT: serotonin

5-HIAA: 5-hydroxyindoleacetic acid

5-HTTLPR: serotonin-transporter-linked polymorphic region

A

ACTH: adrenocorticotropic hormone

AMPA: alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid

AChE: acetylcholinesterase

ACh: acetylcholine

ANOVA: analyses of variance

A: amygdala

B

BDNF: brain-derived neurotrophic factor

BF: basal forebrain

C

CDC: Centers for Disease Control

CRH: corticotrophin releasing hormone

CNS: Central nervous system

cAMP: cyclic adenosine monophosphate

CREB: cyclic adenosine monophosphate response element binding protein

C: cerebellum

D

DNA: deoxyribonucleic acid

DOPAC: 3, 4-dihydroxyphenylacetic acid

DRN: dorsal raphe nucleus

DA: dopamine

DFP: diisopropylfluorophosphonate

DHA: docosahexaenoic acid

DNA: deoxyribonucleic acid

DSM: diagnostics and statistics manual

DRI: dopamine reuptake inhibitor,

E

EPA: eicosapentaenoic acid

ELISA: enzyme- linked immunosorbent assay

ECT: electroconvulsive therapy

F

FSL: Flinders Sensitive Line

FST: Forced swim test

FRL: Flinders Resistant Line

FC: frontal cortex

G

GABA: gamma aminobutyric acid

GR: glucocorticoid receptor

GPX: glutathione peroxidase

GSH: glutathione

GSSG: glutathione disulphide

GM: *Garcinia mangostana*

H

HPA: hypothalamic-pituitary-adrenal

HVA: homovanillic acid

HPLC: high performance liquid chromatography

Hy: hypothalamus

H_2O_2: hydrogen peroxide

H_2O: water

HIV: human immunodeficiency virus

I

IMI: imipramine

IFN: interferon

IDO: indolamine 2, 3-dioxygenase

IL: interleukin

K

KYNA: kynurenic acid

L

LC: locus coeruleus

LPS: lipopolysaccharide

M

MDD: major depressive disorder

MAOI: monoamine oxidase inhibitor

MR: mineralocorticoid receptor

MDA: malondialdehyde

mGluR: metabotropic glutamate receptor

miRNA: microRNA

N

NAC: N-acetyl cysteine

NORT: Novel object recognition test

NO: nitric oxide

NA: noradrenaline

NMDA: N-methyl-D-aspartic acid

NOS: nitric oxide synthase

NADP: nicotinamide adenine dinucleotide phosphate

NACA: N-acetyl cysteine amide

NRI: noradrenalin reuptake inhibitors

NDRI: noradrenalin and dopamine reuptake inhibitor

O

OFT: Open field test

Q

QA: quinolinic acid

R

ROS: reactive oxygen species

RNS: reactive nitrogen species

REM: rapid eye movement

S

SSRI: selective serotonin reuptake inhibitors

SNRI: serotonin noradrenaline reuptake inhibitors

SCN: suprachiasmatic nucleus

SOD: superoxide dismutase

SNPs: single nucleotide polymorphisms

SERT: serotonin transporter

T

TNF: tumor necrosis factor

TCA: tricyclic antidepressant

TDO: tryptophan 2, 3-dioxygenase

TRI: triple reuptake inhibitor

T: thalamus

TBARS: thiobarbituric acid reactive substance

V

VTA: ventral tegmental area

Abstract

The focus of determining the underlying pathophysiological pathways of major depressive disorder (MDD) has shifted from identifying one single hypothesis to the incorporation of various aspects of the disease. Increasing evidence implicates increased pro- vs. anti-inflammatory activity and oxidative stress as key pathophysiological factors in MDD especially considering its relevance to psychological stress and monoamine mediators. With the realization of the contribution of oxidative stress and inflammation to MDD, treatment with antioxidant and anti-inflammatory compounds has attracted a great deal of attention. One such group of compounds are the xanthones. Of relevance for this particular study, the pericarp of *Garcinia mangostana* Linn. (GM) is an evergreen fruit tree originating from Indonesia that produces approximately 50 bioactive xanthones. Xanthone compounds are known for their antioxidant and anti-inflammatory potential. To establish the antidepressant-like properties of the raw powdered pericarp from this fruit, GM will be compared to N-acetyl cysteine (NAC), a glutathione precursor, antioxidant, and glutamate modulator as well as to imipramine (IMI), a well-known tricyclic antidepressant. This study has set about to address this research question by way of a genetic rodent model of MDD, the Flinders Sensitive Line (FSL) rat. The FSL rat model is a validated genetic animal model of MDD that presents with good face, construct and predictive validity.

The aim of this study is therefore to establish an effective dosage for GM for application in a chronic treatment study with respect to antidepressant effects in the acute forced swim test (FST) in FSL rats. We will also aim to establish whether IMI, NAC and GM have broad psychotropic actions in FSL and Flinders resistant line (FRL) animals using a number of behavioral screening tests of relevance to MDD, and whether a therapeutic distinction with regard to efficacy can be made between these three compounds. Lastly we will establish whether IMI, NAC and GM can reverse redox, immune-inflammatory and monoamine changes related to MDD in FSL and FRL rats, and whether a distinction can be made with respect to the three compounds in this regard.

GM displayed dose-dependent antidepressant-like effects after acute treatment. Translational relevance was established by a similar response after chronic treatment, although a dose of 50 mg/kg may need further characterization for chronic treatment. Behavioral and regional brain monoamine analysis supported early-onset noradrenergic activity following acute administration of GM, as well as a late emerging bolstering of serotonin with long-term administration. GM also displayed antioxidant and anti-inflammatory

properties by reducing lipid peroxidation in brain tissue and increasing plasma IL-10 activity, actions that may underlie the aforementioned behavioral and neurochemical changes. Considering that the data were generated in a genetic animal model of MDD, the antioxidant and anti-inflammatory potential of GM suggest it may be a valuable adjunctive treatment with conventional antidepressants, and warrants further study. These results can be beneficial in the development of a new approach to the treatment of MD.

Keywords

Flinders sensitive line rat; major depressive disorder; *Garcinia mangostana* Linn.; xanthones; oxidative stress; forced swim test.

Opsomming

Die fokus om ‘n onderliggende patofisiologiese weg te vind vir major depressie (MD) het onlangs verskuif vanaf ‘n enkele hipotese na die inkorperasie van verskeie relevante aspekte. Inflammatoriese sitokiene en oksidatiewe stres is ook geidentifiseer as sleutelfaktore wat bydra tot die patofisiologie van hierdie toestand; veral as verwys word na hul verwantskap met psigologiese stres en monoamien merkers. Navorsing ten opsigte van antioksidante en anti-inflammatoriese middels neem ook toe as gevolg van die beduidende rol wat oksidatiewe stres en inflammasie in MD speel.

Die bogenoemde het aanleiding gegee tot die bestudering van xantone en spesifiek tot hierdie studie die perikarp van *Garcinia mangostana* Linn. (GM), ‘n immergroen vrugteboom vanuit Indonesië wat omtrent 50 bioaktiewe xantone bevat. Xantoon samestellings is bekend daarvoor dat hul as antioksidante en anti-inflammatoriese middels optree. Om die effektiwiteit van die rou gepoeierde perikarp met betrekking tot sy antidepressiewe eienskappe te bepaal, sal dit met N-asetielsisteïen (NAC), ‘n glutatioon voorloper, antioksidant en glutamaat moduleerder, asook met imipramine (IMI), ‘n bekende trisikliese antidepressant, vergelyk word. Hierdie navorsing het begin deur die navorsingsvraag te beantwoord met behulp van ‘n geneties depressiewe dieremodel genaamd die Flinders sensitiewe lyn (FSL) rot. Die FSL model is ‘n gevalideerde genetiese dieremodel van MD met goeie gesig-, konstruktiewe- en voorspelbaarheidsgeldigheid.

Die doel van hierdie studie is dus om ‘n geskikte chroniese dosis te bepaal waarby GM as ‘n effektiewe antidepressant optree in FSL rotte deur van die akute geforseerde swem toets (FST) gebruik te maak. Die breër psigotropese effekte van IMI NAC en XC sal met behulp van verskeie gedragstoetse relevant tot MD bepaal word in FSL asook Flinders weerstandbiedende lyn (FRL) rotte en ons sal poog om vas te stel of daar terapeutiese verskille tussen die verskillende middels is. Laastens sal ons, ook met behulp van FSL en FRL rotte, probeer vasstel of IMI, NAC of GM redoks, immuun-inflammatoriese en monoamienergiese veranderinge teweeg bring wat verband hou met MD en of daar onderskei kan word tussen die effektiwiteit van die drie behandelingsgroepe.

Akute behandeling met GM het ‘n dosis-verwante antidepressiewe effek getoon. ‘n Soortgelyke effek is waargeneem na kroniese toediening, alhoewel die 50 mg/kg dosis nog verder ondersoek moet word vir effektiewe kroniese behandeling. Gedrag en neurochemiese analises van monoamiene ondersteun die aanvanklike noradrenergiese aktiwiteit na akute

toediening asook die latere toename in serotonien na kroniese toediening van GM. GM toon antioksidatiewe sowel as anti-inflammatoriese eienskappe deur die verhoogde lipiedperoksidase in breinweefsel te verminder en IL-10 aktiwiteit in die plasma te verhoog wat die grondslag kan wees vir die bogenoemde gedrags - en neurochemiese veranderinge. Indien dit in ag geneem word dat die data verkry is met behulp van ‘n genetiese dieremodel van MD, kan die antioksidant en anti-inflammatoriese potensiaal van GM waardevol wees as bykomende terapie saam met konvensionele antidepressante en verdien dit verdere bestudeering. Hierdie resultate kan van nut wees om ‘n nuwe benadering tot die behandeling van MD te ontwikkel.

Sleutelwoorde

Flinders sensitiewe lyn rot; major depressie; *Garcinia mangostana* Linn.; xantoon; oksidatiewe stres; geforseerde swem toets.

Congress proceedings

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Chapter 1: Introduction

Chapter 1 serves as an introductory chapter to the dissertation, describing its approach and layout.

1.1 Dissertation approach and layout

In this dissertation, the key data will be presented in an article format to be submitted for possible publication in an accredited journal *(Chapter 3).*
Any supplementary data will be presented in various Addenda.
The following outline serves to assist the reader in finding key elements of the dissertation, which can be outlined as follows:

Chapter 1 (*Introduction*)

- Problem statement, study objectives and study layout

Chapter 2 *(Literature review)*

Chapter 3 *(Research article)*

Chapter 4 *(Conclusion and recommendations for future studies)*

References

Addendum A

- Additional methods
- Additional results

1.2. Problem statement

Major depressive disorder (MDD) is a foremost cause of morbidity worldwide (Nestler *et al.*, 2002:13) and is among the most debilitating of diseases (Williams *et al.*, 2007:305). MDD is often misdiagnosed while current treatments have significant shortfalls with respect to efficacy, side effects and onset of action (Domenici *et al.*, 2010:9166), leading to increased healthcare costs (US Preventive Services Task Force, 2009:1223). Chronic, psychosocial and environmental stressors, together with genetic susceptibility, play a major role in the development of MDD (Rao *et al.*, 2008:521). According to the World Health Organization, MDD is the fourth leading cause of disability worldwide (Hasler, 2010:155; Snow *et al.*, 2000:738) and is a major contributory factor to the increase of suicide (US Preventive Services Task Force, 2009:1223). MDD is a clinical syndrome that lasts more than two weeks (Snow *et al.*, 2000:738) with severe symptoms that persist indefinitely (Hasler, 2010:155).

The pathology of MDD is explained by a number of theories, some with varying degrees of success. MDD is a common illness (Hasler, 2010:155) with a lifetime prevalence of up to 20% (Williams *et al.*, 2007:305). As noted earlier, prior and/or ongoing stress plays a significant role in the etiology of MDD, as do genetic and epigenetic factors. The subsequent modification of the hypothalamic-pituitary-adrenal (HPA) axis together with the disruption of inhibitory-excitatory gamma aminobutyric acid (GABA)-glutamate signaling and subsequent disruption of monoamine function, plays a decisive role in its pathology (Harvey *et al.*, 2003:1105; Krishnan & Nestler, 2008:894).

According to the original biogenic amine hypothesis of MDD a deficiency of the neurotransmitters, serotonin (5-HT), noradrenaline (NA) and dopamine (DA) in the central nervous system is the underlying pathology of the disorder. The depletion of tryptophan, a precursor of central 5-HT, leads to the development of MDD symptoms in susceptible patients (Neumeister *et al.*, 2004:765), while the role of NA in MDD is based upon previous evidence of a decrease in NA metabolism and a decrease in the NA transporter in the locus coeruleus in depressed individuals. With regard to DA in MDD, studies showed a reduction in DA metabolites in patients with MD. Moreover the anhedonic symptoms of MDD are linked to reduced dopaminergic transmission in the nucleus accumbens (Charney & Manji, 2004:5). Although there is good support for this theory, antidepressants targeting these neurotransmitters showed a delayed onset of action which suggests the prerequisite involvement of other downstream events, other primary abnormalities or confounding variables introduced by comorbid disorders (Hasler, 2010:155).

One of these contributing factors is the presence of oxidative stress as well as immune-inflammatory mediators (Maes *et al.*, 2011:676). The activation of the immune response, especially the activation of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, has been observed in MDD (Connor & Leonard, 1998:583; Maes *et al.*, 2011:676), while interferon (IFN)-α, used to treat certain cancers, is well-known to cause MDD as a side effect (Capuron *et al.*, 2000:2143). These markers induce "sickness behavior" which shares various symptoms with MDD, including fatigue, anhedonia, psychomotor retardation and cognitive impairment. Sickness response is mediated by the induction of the inflammatory cascade (Hasler, 2010:155) while this response is accompanied by the induction of oxidative and nitrosative stress pathways (Anderson & Maes, 2014:3812; Maes *et al.*, 2012:66). This process significantly depletes plasma concentrations of key antioxidants, such as vitamin E, zinc and coenzyme Q10 and lowers antioxidant enzyme activity, e.g. glutathione peroxidase, superoxide dismutase and catalase. This impairs the ability of the body to protect itself against reactive oxygen and nitrogen species responsible for damaging fatty acids, proteins and deoxyribonucleic acid (DNA) (Maes *et al.*, 2011:676).

Stress, HPA axis modification, disrupted GABA-glutamate signaling and the depletion of neurotransmitters, together with factors such as inflammation, redox imbalance and deficits in neurotrophin release culminate in structural changes in critical brain regions (Hasler, 2010:155; Maes *et al.*, 2011:676). This not only leads to failure to regulate the stress response, but also dysfunction in neuroprotective vs. neurodegenerative processes in the brain. The latter are central to neuroanatomical changes in the brain, particularly hippocampal shrinkage, often described in patients with MDD (Harvey *et al.*, 2003:1105; Krishnan & Nestler, 2008:894).

There is a multitude of pharmacological options with which to treat MDD, including the "older" drugs such as the first- and second-generation tricyclic antidepressants (TCAs), heterocyclics such as mirtazepine, and monoamine oxidase inhibitors, and the "newer" classes of antidepressants such as the selective serotonin reuptake inhibitors (SSRIs), serotonin noradrenaline reuptake inhibitors (SNRIs) and atypical compounds such as bupropion and agomelatine (Snow *et al.*, 2000:738). The selection of an antidepressant is based on the anticipated tolerance of the patient and the risk of adverse effects. Patients using TCAs complain of urinary retention, blurred vision, sinus tachycardia, and cognitive dysfunction (Linder & Keck, 1998:1073) while diarrhea, headache, insomnia, and nausea significantly affects patients being treated with SSRIs. Sedation and sexual dysfunction is also often reported with the use of antidepressants (Snow *et al.*, 2000:738). Issues such as side effects compromise compliance that, together with concerns regarding delayed onset of

action and partial response, contributes to inefficient treatment of the disorder that can have devastating consequences for the patient (Domenici *et al.*, 2010:9166). Inadequate treatment of MDD remains a serious concern (Hasler, 2010:155), along with non-adherence to pharmacotherapy (US Preventive Services Task Force, 2009:1223). Despite substantial progress in new drug development, pharmacological treatments for MDD are at best 50-55% effective (Hasler, 2010:155). Furthermore, less than 60% of patients achieve full remission after antidepressant treatment (Warden *et al.*, 2007:449), while currently available antidepressants do not always show a significant benefit compared to placebo (Maes *et al.*, 2009:27). This emphasizes the importance of identifying new biological targets in MDD and developing new antidepressant treatments. To enable this requires a better understanding of the neurobiological basis of MDD and its treatment.

With the realization of the contributory role of oxidative stress in MDD, treatment with antioxidant compounds has attracted a great deal of attention. One such compound is N-acetyl cysteine (NAC), a glutathione precursor, antioxidant (Kerksick & Willoughby, 2005:38) and glutamate modulator (Bauzo *et al.*, 2012:288; Grant *et al.*, 2007:652). Indeed, a number of clinical (Berk *et al.*, 2008b:346; Berk *et al.*, 2014:628), as well as animal studies (Ferreira *et al.*, 2008:747) have provided preliminary evidence in support of this approach. Studies in our laboratory have also confirmed its ability to reverse oxidative damage *in vivo* (Harvey *et al.*, 2008:508). Another class of compounds worth studying for their putative psychotropic and antidepressant-like effects are the xanthones and of relevance for this particular study *Garcinia mangostana* (GM). The raw powdered pericarp from this fruit will be studied with respect to its antidepressant-like properties and compared to NAC, as well as to imipramine (IMI), a well-known TCA (Maubach *et al.*, 2002:609). Despite the fact that there are more clinically relevant antidepressants, IMI has demonstrated robust results in our laboratories with this specific animal model and is therefore our first choice for a positive control (Mokoena *et al.*, 2010:125; Brand, 2011:1).

This study has set about to address these issues by way of a genetic rodent model of MDD, the Flinders Sensitive Line (FSL) rat. The FSL rat model is a validated genetic animal model of MDD (Overstreet *et al.*, 2005:739). These animals present with exaggerated immobility in the forced swim test (FST), the prototypical screening procedure for antidepressant action (Cryan *et al.*, 2005:547; Slattery & Cryan, 2012:1009). Furthermore, FSL animals present with several characteristic features of 'clinical' MDD, as well as with increased stress sensitivity vs. Flinders Resistant Line (FRL) controls (Overstreet *et al.*, 2005:739). At the neurobiological level, the FSL rat displays multiple abnormalities consistent with proposed theories of MDD, in particular altered monoaminergic, cholinergic and glutamatergic function

(Jiménez-Vasquez *et al.*, 2007:298). Moreover, FSL rats present with evidence of a pro-inflammatory state compared to FRL rats (Blaveri *et al.*, 2010:e12596; Mokoena *et al.*, 2015:1) as well as structural brain changes akin to that observed in MDD (Sierakowiak *et al.*, 2014:1).

1.3. Project hypothesis, aims and objectives

1.3.1. Hypothesis

We postulated that the treatment of a translational animal model of MDD with GM, NAC and IMI (positive controls) would demonstrate the following:

- Dose-dependent antidepressant-like activity for GM in FSL rats after acute treatment using the open field test (OFT) and the FST.
- Pro-cognitive and antidepressant-like activity for all three compounds, as tested in the novel object recognition test (NORT), the OFT and the FST, in FSL rats, after 14 days of chronic treatment.
- FSL animals would present with elevated markers of oxidative stress and immune-inflammation vs. FRL animals and that it would be reversed following sub-chronic treatment with the three compounds.
- Therapeutic distinction could be made between GM, NAC and IMI with regard to their ability to reverse the above-mentioned behavioral and neurochemical changes in FSL rats.
- A differential response with regard to behavior and neurochemistry would be evident in FSL vs. FRL animals for the three test compounds.

1.3.2. Research objectives

Primary objectives:

- To have established a dose response relationship for GM with respect to its antidepressant effects in the acute FST in FSL rats, using NAC and IMI as reference antidepressants.
- To have established an effective dose for GM in the acute study for application in the chronic treatment study.
- To have established whether IMI, NAC and GM have broad psychotropic actions in a translational animal model of MDD using a number of behavioral screening tests of

relevance to MDD, and whether a therapeutic distinction with regard to efficacy could be made between these three compounds.

- To have established whether GM, NAC and IMI could reverse redox, immune-inflammatory and monoamine changes related to MDD in FSL rats, and whether a distinction could be made with respect to the three compounds in this regard.

Secondary objectives:

- To have determined if GM presented with antidepressant effect in the "non-depressed" FRL animals based on the fact that antidepressants should have no mood uplifting effects in healthy individuals (Kanemaru *et al.*, 2009:363).
- To have accessed differences in treatment response as a factor of presenting pathology (i.e. monoamine changes, altered redox, inflammation) and also to establish a genetic basis for the bio-behavioral changes observed.

1.3.3. Conceptual Framework and aims:

- The acute FST in FSL rats was used to identify the most effective dose for GM. Close consideration was made with regard to minimizing locomotor effects that could have adversely affected interpretation of the FST data.
- Once the above dose for GM was established, the full psychotropic actions of NAC (150 mg/kg) and GM were investigated in FSL and FRL rats using a fixed dose, chronic treatment regime over 14 days, compared to the reference antidepressant, IMI (20 mg/kg). At the end of the treatment period, the following was determined, and in this order: Cognitive effects in the NORT, general locomotor activity in the OFT and antidepressant-like effects in the FST.
- Using the doses and chronic treatment regime described in the point above, the following was determined:
    - The ability of GM, IMI and NAC to reverse FSL-associated changes in plasma levels of a pro- and anti-inflammatory cytokine (peripheral markers of immune-inflammation)
    - The ability of GM, IMI and NAC to reverse FSL-associated cortical, hippocampal and striatal changes in markers of oxidative stress in the brain tissue (lipid peroxidation).
    - The ability of GM, IMI and NAC to reverse FSL-associated changes in cortical, hippocampal and striatal levels of 5-HT, 5-hydroxyindoleacetic acid (5-HIAA), DA, 3, 4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and NA.

- In order to consider how presenting pathology (i.e. monoamine changes, altered redox, inflammation) as well as genetic predisposition in an animal predetermined drug response, both behavioral and biological responses following treatment were considered in FSL (depressive-like) and FRL (healthy) animals.

1.4. Project design

Since no treatment-related data on the use of GM for MDD in rats were available at the time of designing the study, an acute dose response analysis was initially performed in FSL and FRL rats (Fig. 1). This firstly allowed proof of concept to be established, i.e. if GM has antidepressant-like effects, and thereafter a suitable dose was established for GM to be applied in the chronic treatment regimen. Studies conducted on GM suggest a dosage range of 100-200 mg/kg (Devi Sampath & Vijayaraghavan, 2007:336; Tangpong *et al.*, 2011:292; Phyu & Tangpong, 2014:151).
In the acute study, GM was compared to NAC, an antioxidant that has demonstrated antidepressant-like effects in humans and animals (Berk *et al.*, 2008a:468; Möller *et al.*, 2013a:156; Smaga *et al.*, 2012:280) and IMI, a well-known reference antidepressant (Maubach *et al.*, 2002:609). Six treatment groups were set up, with each having 6 rats per group. One group consisted of 6 FRL and 6 FSL rats that received water, acting as drug naïve controls. The rest of the groups consisted of FSL rats and received 150 kg NAC (Möller *et al.*, 2013b:687; Smaga *et al.*, 2012:280), 20 mg/kg IMI (Brand, 2011:1; Gigliucci *et al.*, 2014:1349) and 50, 150 or 200 mg/kg GM (Fig. 1). Each group was treated three times – 24 h, 6 h and 1 h before the behavioral tests commenced. These tests included the OFT, the locomotor test and the FST. All drugs were administered by oral gavage.

The chronic study consisted of a behavioral (Fig. 2) and a biology cohort (Fig. 3), performed in both FRL and FSL rats. Six treatment groups were set up, with one group receiving 150 mg/kg NAC (n = 12 FSL, n = 12 FRL), another 20 mg/kg IMI (n = 12 FSL, n = 12 FRL) and another receiving GM at the dose decided upon in the earlier acute study, i.e. 50, 150 or 200 mg/kg GM (n = 12 FSL, n = 12 FRL). In order to control for the vehicle groups used, one treatment group received xanthan gum (vehicle for GM; n = 6 FSL, n = 6 FRL) and another water (vehicle for NAC and IMI; n = 6 FSL, n = 6 FRL). Treatment lasted for 14 days. On day 15 the rats that formed part of the behavioral cohort (Fig. 2) were then subjected to sequential behavioral testing, taking place 12 hours after the last treatment and at the start of the dark cycle (Liebenberg *et al.*, 2010:137). The behavioral tests included the NORT (for the measurement of cognition), the OFT (for the measurement of locomotor activity) and the

FST (for the measurement of the antidepressant-like effects). The FST was conducted at 08:00 the next morning. Behavioral testing was performed according to a sequential protocol validated previously (Mokoena *et al.*, 2015:1). Observations and analysis of data using rats subjected to the FST indicated that exposure to the OFT does not affect behavior in the FST (Overstreet *et al.*, 2005:739).

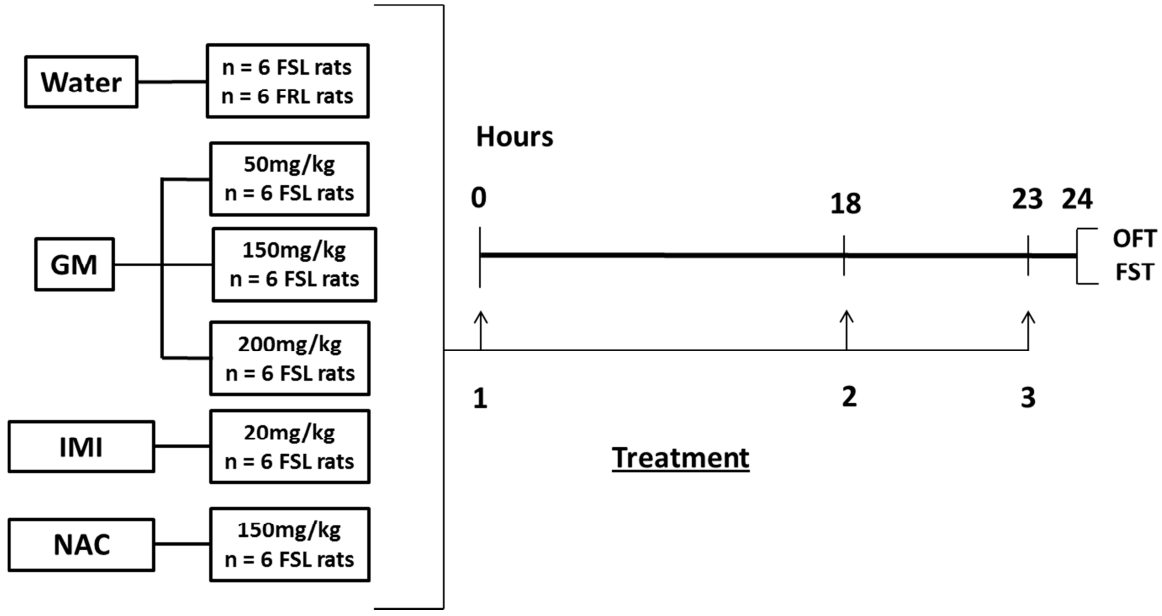


Figure 1. The acute dose-response behavioral study in FSL rats, with FRL rats acting as the drug naïve healthy control. FSL or FRL rats received three dosages of water, GM, IMI or NAC after which the OFT and FST commenced.

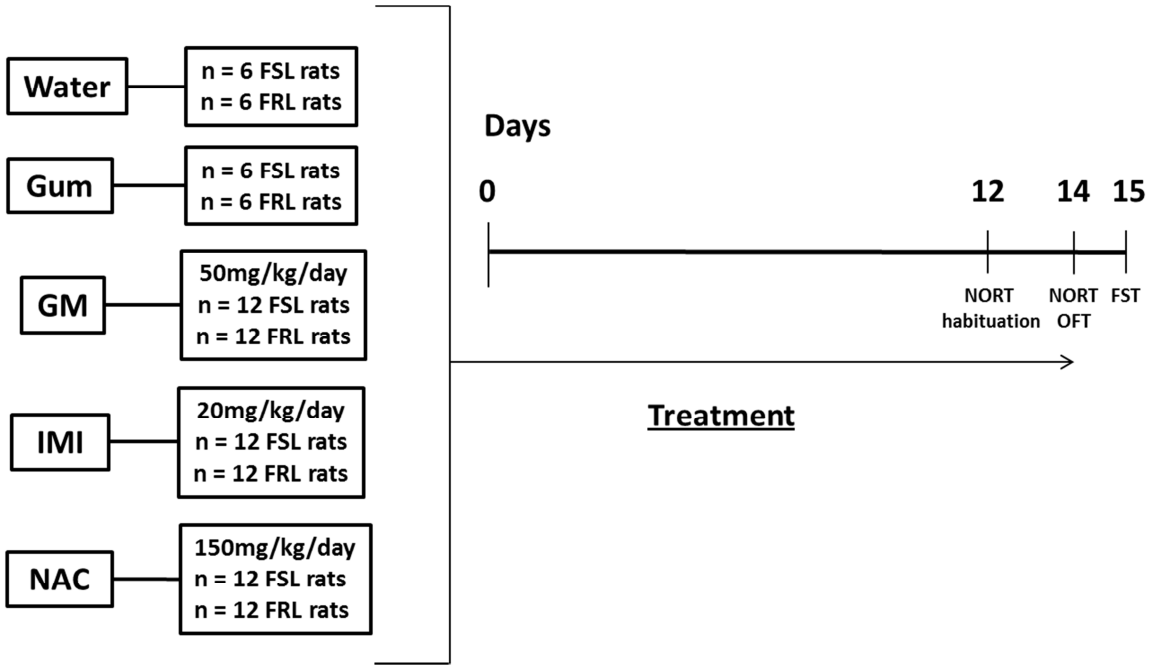


Figure 2. The behavioral cohort of the chronic study in FSL and FRL rats treated with either water, xanthan gum, GM, IMI or NAC. All treatments were administered daily for 14 consecutive days. The behavioral studies commenced on day 14 with the NORT and the OFT followed by the FST the following morning.

The rats that formed part of the biology cohort (Fig. 3) were randomly divided into the same treatment groups as the behavioral study. The dosages obtained from the acute dose-response study (Fig. 1) for GM, and NAC and IMI were administered orally once a day for 14 consecutive days. The biology studies commenced on day 15 following sacrifice. Trunk blood was collected and the frontal cortex, hippocampus and striatum harvested for the various redox, inflammatory and monoamine measurements. As for the behavioral cohort, the complete study was performed in FSL and FRL animals.

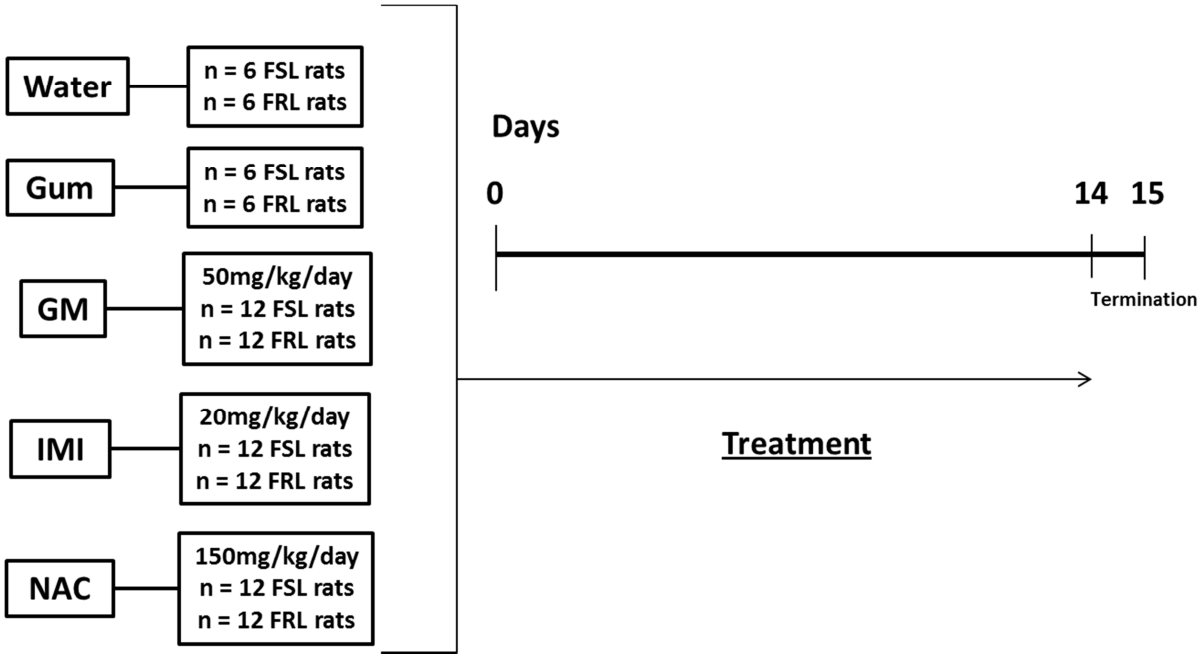


Figure 3. The neurobiological cohort of the chronic study in FSL and FRL rats treated with either water, xanthan gum, GM, IMI or NAC. All treatments were administered daily for 14 consecutive days. The neurobiological studies commenced on the morning of day 15.

1.5. General points

This dissertation was written and submitted in the article format for dissertation submission, as approved by the North-West University. This format includes an introductory chapter, a chapter covering the relevant literature overview, a chapter containing methodologies and experimental results in the form of a concept article for submission to a peer review journal, and a discussion chapter containing concluding remarks and suggestions for future studies. Additional data not included in the article are presented in addenda at the end of the dissertation.

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Chapter 2: Literature Review

2.1 Major depressive disorder (MDD)

MDD is defined as a deviation from a person's normal mood as well as from the perception they have of themselves and all that surround them (Gotlib & Joormann, 2010:285). The symptoms of MDD must persist for more than two weeks and must include persistent sadness and a lack of interest in activities normally enjoyed (Paykel & Priest, 1992:1198). MDD is projected by the World Health Organization to be the second leading cause of disability worldwide by 2020 (Bromet *et al.*, 2011:90) as well as one of the most common causes of premature death (Murray *et al.*, 2014:1005). It is a major cause of morbidity worldwide (Nestler *et al.*, 2002:13) with a lifetime risk of 15% (Richards, 2011:1117) and a 20 times greater risk of suicide than a non-depressed individual (Korte *et al.*, 2015:88). MDD is a major public health issue with surveys showing a similar prevalence around the world (Richards, 2011:1117); with 10% of South Africans falling under this burden (Tomlinson *et al.*, 2009:368).

In the last twenty years our understanding of MDD has transformed from an acute state to a chronic persistent illness (Richards, 2011:1117). According to the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders, MDD can be chronic or episodic and recurrence is frequent (American Psychiatric Association, 2013:970). Although well-established treatment options exist, is there a significant shortfall in efficacy (Domenici *et al.*, 2010:9166), while the relapse rate after remission remains very high (De Raedt & Koster, 2010:50). After the first depressive episode, 75% of patients relapse within two years after recovery (Boland *et al.*, 2002:23; Gotlib & Joormann, 2010:285).

MDD is associated with severe symptoms which are often misdiagnosed (Hasler, 2010:155), and shows a great impact on a personal, interpersonal and societal level (Richards, 2011:1117). MDD is also economically detrimental, leading to a considerable increase in healthcare costs due to psychiatric monitoring, hospitalization and drug treatment (US Preventive Services Task Force, 2009:1223). MDD patients often show a resistance to treatment that together with comorbidity further add to healthcare costs. The indirect costs associated with functional and occupational impairment further contributes to the economic burden patients face (Centers for Disease Control and Prevention (CDC), 2010:1229; Richards, 2011:1117). MDD is often comorbid with substance abuse or other mental and physical health conditions (Gotlib & Joormann, 2010:285), with up to 50% of depressed

patients also experiencing anxiety (Warden *et al.*, 2007:449). It also often affects common chronic conditions such as cardiovascular disease, cancer, diabetes, arthritis, asthma, human immunodeficiency virus (HIV) infection and obesity (Centers for Disease Control and Prevention (CDC), 2010:1229; Richards, 2011:1117). Patients with comorbid illnesses show more severe MDD symptoms and tend to have a poorer treatment response (Richards, 2011:1117). Moreover, many chronic illnesses can exacerbate MDD symptoms (Centers for Disease Control and Prevention (CDC), 2010:1229).

2.1.1 Incidence and demographics of MDD

Globally, MDD represents an enormous health challenge, affecting over 120 million people with neither age, ethnic or social status exempting one from this disorder (Kessler & Bromet, 2013:119). For most countries there are little or no statistics available for the prevalence of MDD, although existing data shows a high prevalence all around the world (Snow *et al.*, 2000:738). In South Africa, MDD was classified as the most prevalent mental disorder, affecting 5-10% of the population (Bromet *et al.*, 2011:90; Williams *et al.*, 2007:305).

Most studies regarding the epidemiology of MDD conclude that gender, age, level of education and marital status are correlated to MDD (Kessler & Bromet, 2013:119), while the prevalence is higher in women (10% to 25%) than in men (5% to 12%) (Snow *et al.*, 2000:738). The onset of MDD reaches a peak between the ages of 15-29 years (Hart *et al.*, 2001:633) with the mean age in South Africa being 22 years old (Bromet *et al.*, 2011:90). Earlier onset can have greater implications, including not finding a spouse, greater comorbidity, more depressive episodes with more severe symptoms and an increased suicide risk (Richards, 2011:1117; Zisook *et al.*, 2007:1539). MDD is the third major contributory factor in the increase in suicide in children between 15 and 24 (US Preventive Services Task Force, 2009:1223) with a reported 1 million lives being claimed worldwide by MDD associated suicide each year (Goldsmith *et al.*, 2002:496).

2.1.2 Symptomatology and diagnosis of MDD

Despite research that is aimed at understanding MDD, the diagnosis and evaluation of treatment is based on subjective evaluation of the symptoms (Domenici *et al.*, 2010:9166). MDD is defined by the Diagnostic and Statistical Manual of Mental Disorders (5^{th} edition) as a clinical syndrome that can be diagnosed if depressed mood, anhedonia as well as at least four of the following symptoms occur for more than two weeks for most of the day, nearly every day:

- Depressed mood
- A diminished interest or pleasure in activities
- Significant weight loss or weight gain or decrease or increase in appetite
- Insomnia or hypersomnia
- Psychomotor agitation (e.g., the inability to sit still, pacing; handwringing, or pulling or rubbing of the skin, clothing, or other objects) or retardation (e.g., slowed speech, thinking, and body movements; increased pauses before answering; speech that is decreased in volume, inflection, amount, or variety of content, or muteness)
- Fatigue
- Feelings of worthlessness or excessive inappropriate guilty preoccupations or ruminations over minor past failings
- A lack of concentration or indecisiveness
- Recurring thoughts of death or suicidal ideation or a suicide attempt (American Psychiatric Association, 2013:970).

Clinically significant distress can be observed as well as impairment in social, occupational, or other important areas, which could lead to the diagnosis of MDD (American Psychiatric Association, 2013:970). During milder episodes, the person may function normally but it will require increased effort to do so. Furthermore, failure to inquire about accompanying depressive symptoms often results in under diagnosis (American Psychiatric Association, 2013:970). Fatigue and insomnia are present in most of the cases while psychomotor disturbances and delusional guilt are less common, but indicate greater severity (American Psychiatric Association, 2013:970). Sometimes there are somatic complaints (e.g., body aches) or increased irritability, while many individuals report impaired ability to think, concentrate, or to make even minor decisions (American Psychiatric Association, 2013:970). MDD patients also appear easily distracted and complain of memory difficulties. Family members often notice the social withdrawal and sometimes there is a significant reduction in sexual interest or desire. To be acknowledged as a symptom of MDD, it must be newly present or have worsened when compared to the previous state (American Psychiatric Association, 2013:970).

If the above symptoms from the diagnostics procedure are considered, it is clear that opposed to most diseases, the diagnosis of MDD is based on relatively subjective criteria. Therefore it is considered a syndrome including various distinct symptoms (Beck, 1967:364).

Although there is extensive literature that describes the neuroanatomical, neuro-endocrinological, and neurophysiological correlates of MDD, there are no laboratory tests of sufficient sensitivity and specificity to be used as a diagnostic tool for this disorder (Brand *et al.*, 2015:324). The evaluation of the symptoms of a MDD episode is especially difficult when they present comorbid to another medical condition (e.g., cancer, stroke, myocardial infarction, diabetes, pregnancy). Seeing that some of the criterion signs and symptoms of a MDD episode are identical to those observed in these conditions (e.g., weight loss with untreated diabetes; fatigue with cancer; hypersomnia early in pregnancy; insomnia later in pregnancy or postpartum), these symptoms count towards the diagnosis of MDD except when they are clearly and fully attributable to a general medical condition. Non-vegetative symptoms of dysphoria, anhedonia, guilt or worthlessness, impaired concentration or indecision, and suicidal thoughts should be assessed with particular care in such cases. The diagnosis of MDD could therefore only be made if the episode is not attributable to the physiological effects of a substance or to another medical condition and there has never been a manic episode (American Psychiatric Association, 2013:970).

2.1.3 Etiology of MDD

A consensus on the etiology of MDD is still elusive. Rather, a multi-factorial cause is suspected to underlie the illness (Hankin, 2006:102). The course of various biological and non-biological processes together with risk factors and environmental aspects may increase the vulnerability for MDD in any given individual (Hankin, 2006:102; Hansson *et al.*, 2010:54).

There is a strong correlation between MDD and prior stressful life events as well as genetic factors (Fava & Kendler, 2000:335). Glucocorticoids (described in section 2.1.5.3) as well as monoamines (described in section 2.1.5.1) are the primary mediators of hormones and an essential aspect for adaptation and maintaining homeostasis (allostasis) (McEwen, 2000:108). The long-term effects of the response to stress activation result in accumulated physiological wear and tear on the body (allostatic load) resulting in the dysregulation of multiple systems (McEwen, 2000:108). In MDD chemical imbalances, alterations in circadian rhythms and often atrophy of brain structures form the allostatic load (McEwen, 2000:108). Stressful conditions, including abuse, neglect and the loss of parents at a younger age (Bedi, 1999:225) add on to the allostatic load later in life. Chronic psychosocial and environmental stressors play a major role in the development of MDD (Disner *et al.*, 2011:467) and can also lead to the development of psychiatric disorders, increased stress sensitivity and negative behavioral traits later in life. Although substantial evidence shows MDD to be

associated with stressful events, stress alone is not the sole cause of MDD. MDD is a highly heritable disorder with a 40-50% risk for MDD if the person is genetically predisposed (Nestler *et al.*, 2002:13). According to animal studies (Mann & Currier, 2010:268), early life adversity during development can cause genotype interactions that in turn may alter serotonin (5-HT) levels as well as modulate the hypothalamic-pituitary-adrenal (HPA) axis that can lay the foundation for a mood disorder (Mann & Currier, 2010:268).

An interaction between emotion and cognition in MDD has been established. According to Beck's cognitive model of MDD, environmental events can influence how an individual interprets future experiences (Disner *et al.*, 2011:467). Negative events early in life can form an idea that can be reactivated by other stressors later in life. This latent dysfunctional outlook of an individual can have an impact on attention, interpretation and memory later in life (Disner *et al.*, 2011:467). Negative ideas about oneself and one's surroundings, together with the biases in cognitive processes, can make an individual more vulnerable to the onset of MDD (Gotlib & Joormann, 2010:285). Side-effects to certain medication (Nestler *et al.*, 2002:13) or a secondary illness such as diabetes, cardiovascular disorders and obesity can also underlie the etiology of the illness (Patten *et al.*, 2005:195), while the unhealthy, less active lifestyle of modern living can further add to the burden of MDD (Beydoun & Wang, 2010:52; Thomson *et al.*, 2010:1812).

2.1.4 Neuroanatomy of MDD

Numerous studies have shown structural modifications in distinct areas in the brain to be prominent in the pathophysiology of MDD (Minkwitz & Himmerich, 2011:199). These areas include the limbic system (hippocampus, basal ganglia, amygdala) as well as in the cortical brain regions, such as the frontal cortex (Brand *et al.*, 2015:324; Bremner *et al.*, 2000:115; Drevets, 2001:240; Nestler *et al.*, 2002:13). The reduction in the volume of the hippocampus may have a genetic determinant but it is also dependent on the duration of MDD together with poor compliance to antidepressant treatment (Brand *et al.*, 2015:324), as will be discussed presently.

Volume reduction in the frontal cortex and hippocampus (Hasler, 2010:155; Sheline, 2003:338) as well as a decrease in blood flow can be observed in patients suffering from MDD, both of which can be reversed by treatment with antidepressants (Drevets & Raichle, 1998:353). The hippocampus interacts with the cerebral cortex which includes the frontal cortex and amygdala (Phelps, 2004:198). These regions affect the cognitive symptoms associated with the illness and are involved in regulation of mood, learning and memory

(Krishnan & Nestler, 2008:894) while the hippocampus is known to control emotional behavior (Dalgleish, 2004:583) (illustrated in Fig. 4). Structural brain imaging studies have associated a smaller hippocampus with an increase in the lifetime duration of MDD (Sheline *et al.*, 1999:5034), suggesting that recurrent MDD episodes may lead to permanent structural damage (Harvey *et al.*, 2003:1105). A few genetic variants seem to modulate the effects of stress on hippocampal volumes as well as the development of the brain (Frodl *et al.*, 2008:363).

Genetic variants seen in MDD patients appear to modulate the effects of stress on the volume of the hippocampus (Frodl *et al.*, 2008:363). In the promotor region of the 5-HT transporter (5-HTTLPR), polymorphism has shown to affect the hippocampus volume of depressed patients possibly by a reduction in serotonergic transmission (Eker *et al.*, 2011:22). Moreover, carriers of the brain-derived neurotrophic factor (BDNF) polymorphism also had smaller hippocampal volumes in both MDD patients and healthy controls (Frodl *et al.*, 2008:363). Considering the FSL genetic animal model of MDD, reductions in hippocampal volume have also been observed when compared to the control Flinders resistant line (FRL) rat. This reduction can be due to the reduction in hippocampal neurons (Chen *et al.*, 2010:1376).

The frontal cortex plays a role in decision making, motivation and mood regulation (Fig. 3) and, importantly, serves as a target for monoaminergic projections from the midbrain (Korte *et al.*, 2015:88). These pathways originate from cell bodies in the ventral tegmental area (VTA; Fig. 3), LC (Fig. 1) and dorsal raphe nucleus (DRN; Fig. 3) from where the respective dopamine (DA), noradrenaline (NA) and 5-HT neurons are projected to virtually all brain regions (Gobert *et al.*, 1998:413; De Raedt & Koster, 2010:50; Hasler, 2010:155). Therefore the effect of monoamine signaling is felt on a broad range of brain functions associated with these areas, including cognition, memory, appetite, sleep, reward related behavior, attention and mood (Hasler, 2010:155). Their close anatomical association means that the monoamines can interact with one another within the locality of their cell bodies in the brain stem at the end of synapses in their distal projection areas (Roos & Burns, 2015 (in press):528). These interactions can occur at synaptic level, via heteroreceptors, or via sub-cellular second messenger networks (Harvey, 1997:540; Roos & Burns, 2015 (in press):528).

Most of the NA cell bodies are located in the LC and lateral tegmental nuclei and innervate large areas of the central nervous system (CNS) with moderate densities (Fig. 1) (Fuchs & Flugge, 2004:171). NA receptors belong to the excitatory postsynaptic ß-adrenergic, α_1- and

inhibitory pre- and postsynaptic α_2 -adrenergic categories (Fig. 2.1a & b) (Falkai *et al.*, 2013:978). Up regulation of α_2 -adrenergic receptors where observed in post-mortem brain tissue of patients with MDD, suggesting noradrenergic deficits (Mann, 1999:99; Meana *et al.*, 1992:471).

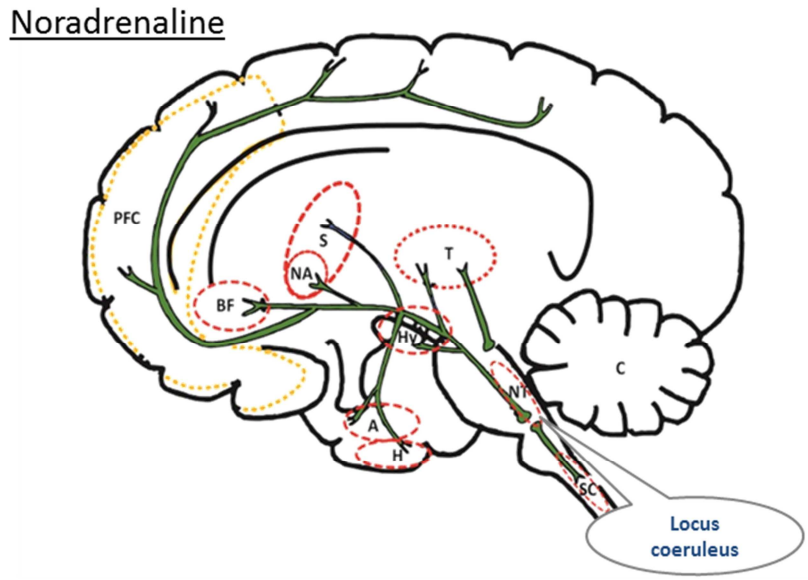


Figure 1. An anatomical map of brain regions innervated by NA neurotransmission (Stahl, 2008:279). PFC – prefrontal cortex, BF: basal forebrain, S: striatum, NA: nucleus accumbens, T: thalamus, Hy: hypothalamus, A: amygdala, H: hippocampus, C: cerebellum.

There is also evidence of a decrease in NA metabolism and density of transporters in the LC in MDD patients (Hasler, 2010:155). In the hippocampus formation of NA may serve as facilitation for 5-HT signalling through a negative feedback mechanism (Falkai *et al.*, 2013:978). NA may inhibit 5-HT as well as DA release via inhibitory presynaptic α_2 heteroreceptors located on 5-HT and DA nerve terminals (Fig. 2.2 & 7) (Falkai *et al.*, 2013:978). An animal study demonstrated 5-HT release in the ventral hippocampus can be modulated by both heteroreceptors on the terminals of 5-HT neurons (Fig. 2.3) and by α_2 -adrenoceptor sites situated pre- and/or postsynaptic to the NA terminals (Fig. 2.1a & b) (Tao & Hjorth, 1992:137). 5-HT may alter NA levels through a tonic input from the DRN (Fig. 2.5) (Gobert *et al.*, 1998:413).

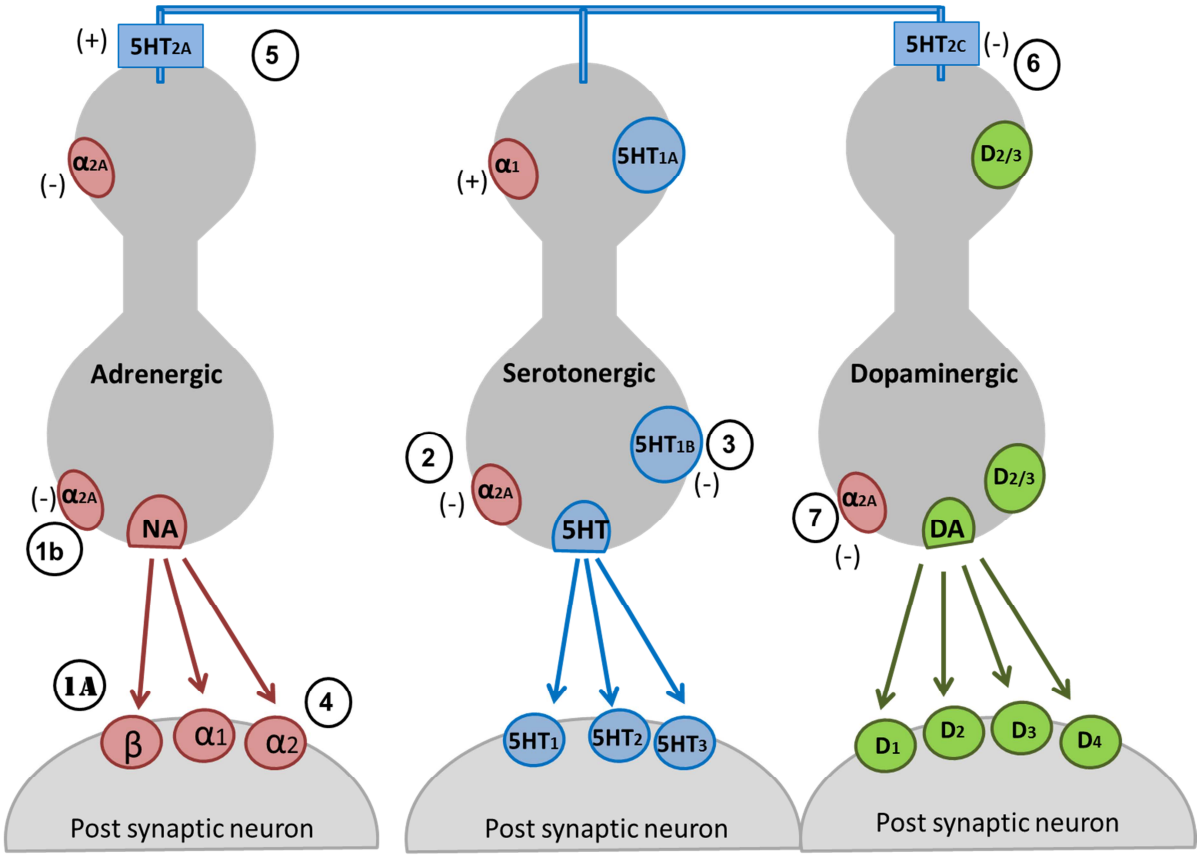


Figure 2. The interaction between the NA, 5-HT and DA nerve terminals and the tonic 5-HT input they receive at cell body level from the DRN (De Boer, 1996:19; Gobert *et al.*, 1998:413).

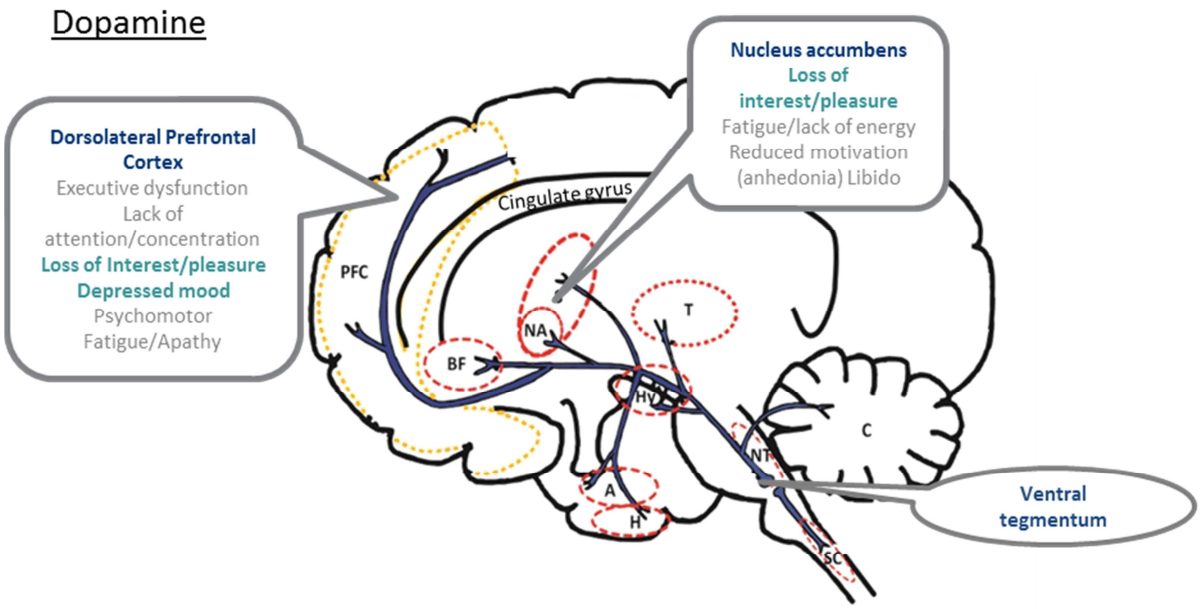


Figure 3. A symptom - anatomical map of DA related brain regions linked to the Diagnostics and Statistics Manual (DSM) criteria for MDD (Stahl, 2008:279). PFC: prefrontal cortex, BF: basal forebrain, A: amygdala, Hy: hypothalamus, T: thalamus, C: cerebellum**.**

The majority of DA cell bodies are situated in the ventral midbrain and give rise to dense innervations in the nucleus accumbens and dorso-lateral frontal cortex, as illustrated in Fig. 3 (Fuchs & Flugge, 2004:171). Dopaminergic neurons bear D_2/D_3 autoreceptors located on the cell body and terminal as well as α_2 heteroreceptors. DA neurons also receive tonic 5-HT input at a cell body level from the DRN (Fig. 2.6). The involvement of DA in MDD can be attributed to the dysfunctions in the mesolimbic DA reward circuit which originates in the VTA (Fig. 3) (Nestler & Carlezon, 2006:1151). The DA reward circuit is linked to the rewarding effects of sex, drugs and food (Nestler & Carlezon, 2006:1151). The levels of DA are also lowered in the striatum contributing to symptoms such as anhedonia together with reduced energy levels and motivation (Brand *et al.*, 2015:324). In Parkinson's disease the decrease in DA projections to the striatum may be associated with MDD (Hasler, 2010:155).

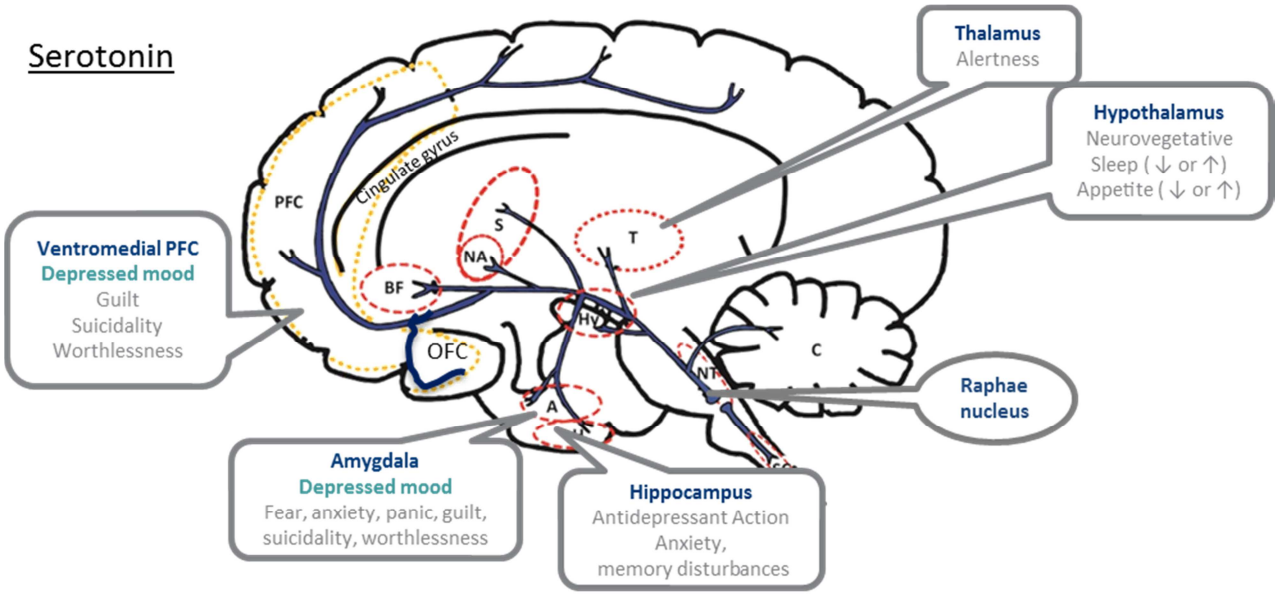


Figure 4. A symptom - anatomical map of 5-HT related brain regions linked to the DSM criteria for MDD (Stahl, 2008:279). PFC: prefrontal cortex, BF: Basal forebrain, OFC: Orbitofrontal cortex, S: striatum, NA: nucleus accumbens, C: cerebellum

The majority of 5-HT nerve cells originate in the raphe nuclei region in the brainstem with the highest concentration located in the DRN, as illustrated in Fig. 4 (Falkai *et al.*, 2013:978; Fuxe *et al.*, 2010:82). The 5-HT nerve cells innervate various limbic structures, which include the hippocampus, the ventromedial frontal cortex, the hypothalamus and the amygdala (Fig. 4) (Falkai *et al.*, 2013:978). Chronic treatment with antidepressants leads to an increase in 5-HT release from the raphe nuclei (Mizoguchi *et al.*, 2001:443), although excessive 5-HT

release, as with a SSRIs, will also reduce 5-HT and NA release in the frontal cortex, giving rise to iatrogenic symptoms of emotional blunting and reduced response with respect to anhedonic symptoms (Harvey & Slabbert, 2014:503). Moreover, reduced synthesis of 5-HT induces MDD symptoms in healthy patients exposed to stress (Falkai *et al.*, 2013:978). An increase in the release of 5-HT in the hippocampus can mediate adaption to stress by inhibiting the consolidation of stressful memories (Joca *et al.*, 2007:227). SSRI antidepressants have also been shown to enhance the gene expression of BDNF that in turn promotes the survival of 5-HT neurons (Martinowich & Lu, 2008:73). Furthermore, 5-HT receptors can be down regulated in the hippocampus by corticosteroid dependant stressors linking 5-HT to the activation of the HPA axis (Falkai *et al.*, 2013:978).

Given that neurovegetative symptoms are prominent in MDD, the involvement of the hypothalamus has been implicated (Nestler *et al.*, 2002:13). The hypothalamus has been extensively studied in MDD, although most of this work has focused on the HPA axis (section 2.1.5.3.) or other neuroendocrine functions (Nestler *et al.*, 2002:13). Hypothalamic related neurotransmitters and nuclei are crucial for appetite, sleep, circadian rhythms and interest in sex, all of which are abnormal in many MDD patients (Nestler *et al.*, 2002:13). Indeed, recent work, and the introduction of agomelatine, has emphasized the relevance of targeting disorganized circadian rhythms in MDD (Mairesse *et al.*, 2013:323).

2.1.5 Pathophysiology

MDD is considered a heterogeneous illness, with no single hypothesis being found adequate to fully explain the illness, especially when considering the relatively low response rate to current antidepressants (Hasler, 2010:155). Nevertheless, the pathophysiology of MDD can be explained by a number of theories, some with varying degrees of success. All theories apply to certain MDD patients with the pathophysiology varying with the course of the illness.

2.1.5.1 The biogenic amine hypothesis

In 1954 tuberculosis patients were treated with isoniazid which exerted a beneficial effect on the well-being of many of the patients (Selikoff *et al.*, 1952:973). Isoniazid was later identified as the first antidepressant and the first monoamine oxidase inhibitor (MAOI) (Wrobel, 2007:3404). During this period the antidepressant efficacy of imipramine (IMI), the first monoamine reuptake inhibitor, was established (Kuhn, 1958:459). During the 1960s, 5-HT and NA where identified as neurotransmitters and it was subsequently hypothesized that deficits in 5-HT in the brain can be corrected by antidepressant treatment. During that time it

was found as well that patients treated with reserpine displayed symptoms of MDD, its action later being ascribed to depleting monoamine stores (Harro & Oreland, 2001:79). This lead to Schildkraut proposing the classic monoamine hypothesis of MDD that concluded that MDD is caused by a deficiency of monoaminergic activity and that it can be treated with drugs that increase this activity (Berton & Nestler, 2006:137; Schildkraut, 1965:509).

Subsequently, the original hypothesis was reformulated as new knowledge was obtained (Healy, 1999:174) and a number of inconsistencies came to light, including:

- Lowered levels of 5-HT in the CNS of healthy controls had only modest effect on mood (Healy, 1999:174).

Meta–analysis indicated that antidepressants are only modestly more effective than placebo in reducing depressive symptoms (Andrews *et al.*, 2015:164; Fournier *et al.*, 2013:392; Khan *et al.*, 2002:40) and that patients respond differently to treatment (Posternak & Zimmerman, 2005:148).

Some drugs that block 5-HT reuptake (e.g., cocaine and amphetamine) are not effective in treating MDD (Charney *et al.*, 1981:1334).

The above mentioned inconsistencies lead to the reformulation of the monoamine theory to state that the rise in monoamine levels is only one part of a complex series of events underlying the effect of antidepressants (Pineyro & Blier, 1999:533). The pathophysiology of MDD has therefore moved away from a condition caused by an absolute deficit in neurotransmitters, in attempt to gain better understanding of their receptors and the downstream actions that they trigger (Stahl & Felker, 2008:855). The formulation of the permissive hypothesis (Harvey, 1997:540) linking 5-HT circuits to deficits in NA signaling (Nemeroff, 1998:42), raised awareness of the complex role that 5-HT plays in the regulation of mood. This hypothesis states that a decrease in central 5-HT levels allows an affective state that can be regulated by NA, therefore MDD will arise from a deficit in 5-HT and NA (Harvey, 1997:540). Serotonergic pathways project to various regions in the CNS including the areas involved in NA and DA release (Gobert *et al.*, 1998:413). Mirtazapine is an antidepressant that enhances noradrenergic and 5-HT_{1A}-mediated serotonergic neurotransmission via the antagonism of α_2 auto- and hetero-adrenoreceptors (Schreiber *et al.*, 2002:143). Contradictory to the monoamine hypothesis, there is some doubt as to whether an increase in 5-HT is necessary for antidepressant response (Brand *et al.*, 2015:324). In fact, the antidepressant effect of SSRIs seems to be independent of sustained

5-HT release since direct 5-HT agonists are ineffective as antidepressants (Brand *et al.*, 2015:324). Moreover, increasing 5-HT can actually worsen certain symptoms by decreasing DA and NA release in the frontal cortex that in turn can complicate treatment (Harvey & Slabbert, 2014:503).

Often different antidepressant mechanisms are combined to allow greater efficacy but consequently resulting in an increase in adverse effects (Garnock-Jones, 2014:855). This has led to the formulation of antidepressants with multi-modal activity combining different pharmacological mechanisms of action. Vortioxetine, for example, has multi-receptor effects that lead to alteration of 5-HT neurotransmission as well as the modulation of NA, DA, histamine, acetylcholine, gamma aminobutyric acid (GABA) and glutamate systems in the forebrain (see section 2.1.6.) (Davis, 2015:34). All of these transmitters are known to have regulatory effects on mood (Brand *et al.*, 2015:324).

2.1.5.2 The GABA & glutamate dysregulation hypothesis

GABA and glutamate are respectively the principle inhibitory and excitatory neurotransmitters in the CNS and contribute to at least 50% of synapses (Falkai *et al.*, 2013:978). Glutamate serves as the precursor of GABA with elevation in glutamate levels resulting in a reactive increase in GABA synthesis thereby restoring homeostasis between inhibitory and excitatory circuits (Fig. 5A) (Brand *et al.*, 2015:324). Their ubiquitous presence allows GABA and glutamate to play a profound role in regulating most neurotransmitter systems in the brain (Brand *et al.*, 2015:324). Behavioral effects of glutamate include modulation of cognition and mood while exerting an influence on neurogenesis and neuroplasticity on a cellular level (Harvey & Shahid, 2012:775).

An overactive glutamate and hypoactive GABA system, together with dysfunction of peripheral glutamate receptors, has been suggested to underlie MDD (Falkai *et al.*, 2013:978; Nunes *et al.*, 2013:1336). A series of magnetic resonance spectroscopy studies have repeatedly shown a reduction in GABA levels in the frontal cortex, amygdala and hippocampus of MDD patients (Sanacora *et al.*, 2012:63; Block *et al.*, 2009:415; Michael *et al.*, 2003:720; Krystal *et al.*, 2002:71). Increased glutamate activity leads to an influx of Ca^{2+} into the neuron and the subsequent accumulation of toxic reactive oxygen and nitrogen species (ROS/RNS; Fig. 5A) (Brand *et al.*, 2015:324). The release of nitric oxide (NO), a potent cell toxin, is another glutamate dependent way of inducing cell damage (Wegener *et al.*, 2010:461). NO can modulate neurotransmission as well as neural toxicity as will be discussed later on (see section 2.1.5.5) (Wegener *et al.*, 2010:461).

Glutamate is also implicated in cell death following glutathione depletion (Berk *et al.*, 2008b:346). Glutathione serves as the primary endogenous antioxidant by neutralizing ROS/RNS through free radical scavenging and thus effectively maintains oxidative balance in the cells (Fig. 5A) (Berk *et al.*, 2008b:346). Specifically the N-methyl-D-aspartic acid (NMDA) receptor mediates the cytotoxic effects of glutamate (McNally *et al.*, 2008:501; Niciu *et al.*, 2014:907). Glutamate induced glutathione depletion via cysteine-glutamate antiporter deactivation, results in an increase of extra-synaptic glutamate, the subsequent over-activation of NMDA receptors and ultimately an increase in ROS/RNS production and cell damage (Fig. 5A) (Berk *et al.*, 2008b:346; Kritis *et al.*, 2015:1).

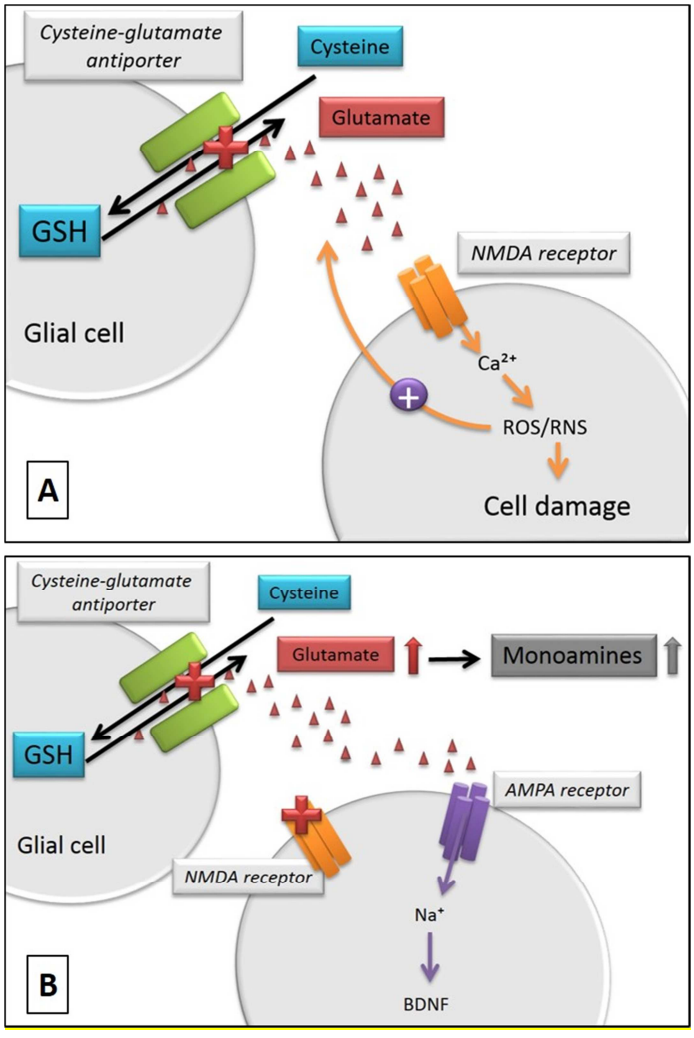


Figure 5. A: Glutamate-induced cell death via glutathione depletion and oxidative damage. B: Synaptic and intracellular events stimulated by the NMDA-antagonistic antidepressant ketamine, adapted from (Hashimoto, 2009:105). GSH: glutathione, NMDA: N-Methyl-D-aspartic acid, ROS/RNS: reactive oxygen and nitrogen species.

NMDA receptor antagonists present with antidepressant effects (Harvey & Shahid, 2012:775), while post-mortem brain tissue of MDD patients shows abnormalities in NMDA

signaling (Hasler, 2010:155). Furthermore, the NMDA antagonist, ketamine displays rapid antidepressant effects when administered as a single dose to patients with treatment resistant MDD (Kerksick & Willoughby, 2005:38). Another study indicated that ketamine and amantadine (another NMDA receptor antagonist) have antidepressant effects in humans (Müller & Schwarz, 2007:988). Antagonism of the post-synaptic NMDA receptor, together with an increase in synaptic glutamate release, resulting in the stimulation of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors suggested to mediate antidepressant properties (Fig. 5B) (Falkai *et al.*, 2013:978). AMPA receptors induce the release of BDNF that is associated with protection against excytotoxic brain damage, a reduction in neurotoxicity and improved neurogenesis (Fig. 5B) (Brunoni *et al.*, 2008:1169; Lynch, 2006:82).

Moreover, the glutamate system can have a direct or indirect effect on the release of monoamines implicated in the pathophysiology of MDD (Brand *et al.*, 2015:324; Möller *et al.*, 2013b:687). Monoaminergic neurons are affected directly by the release of excitatory glutamate (Fig. 5B) or inhibitory GABA from interneurons (Carlsson *et al.*, 2001:237). The antagonism of NMDA receptors has been established to increase central 5-HT levels possibly by an increase in synaptic glutamate (Fig. 5B) (Iversen, 2006:82). It has also been hypothesized that GABA interneurons contain excitatory 5-HT receptors that alter glutamate levels (Fig. 6a) (Aghajanian & Marek, 1999:122; Carlsson, 1998:525). This action also allows 5-HT to indirectly modulate glutamate release via actions on GABA interneurons (Carlsson, 1998:525). In the hippocampus GABA neurons expressing 5-HT_3 receptors in turn suggest serotonergic control over inhibitory GABA interneurons (Fig. 6a) (Bétry *et al.*, 2011:603).

GABA also has an inhibitory effect on DA cell bodies via the $GABA_A$ receptor (Fig. 6d) (Rudolph & Knoflach, 2011:685). Low GABA levels, as seen in MDD, can therefore result in the disinhibition of DA neurons resulting in increased DA release (Rudolph & Knoflach, 2011:685). DA can also modulate post-synaptic GABA neuron activity as well as presynaptic glutamate release via the expression of DA receptors on these neurons (Fig. 6c & e) (Rudolph & Knoflach, 2011:685).

Interestingly a neurodegenerative metabolite of the kynurenine pathway (discussed in section 2.1.5.4), quinolinic acid (QA), is a NMDA receptor agonist (Möller *et al.*, 2013a:156). Studies in animal models have showed that QA increases the release of glutamate in the striatum and cortex (Müller & Schwarz, 2007:988). The neurotoxic effects of QA and cortisone are implicated in the neurodegenerative effects observed in MDD patients, such as hippocampal atrophy discussed in section 2.1.5.8 (Müller & Schwarz, 2007:988). Another

important link between the production of QA and the neurodegeneration observed in MDD is inflammation, as discussed in section 2.1.5.6.

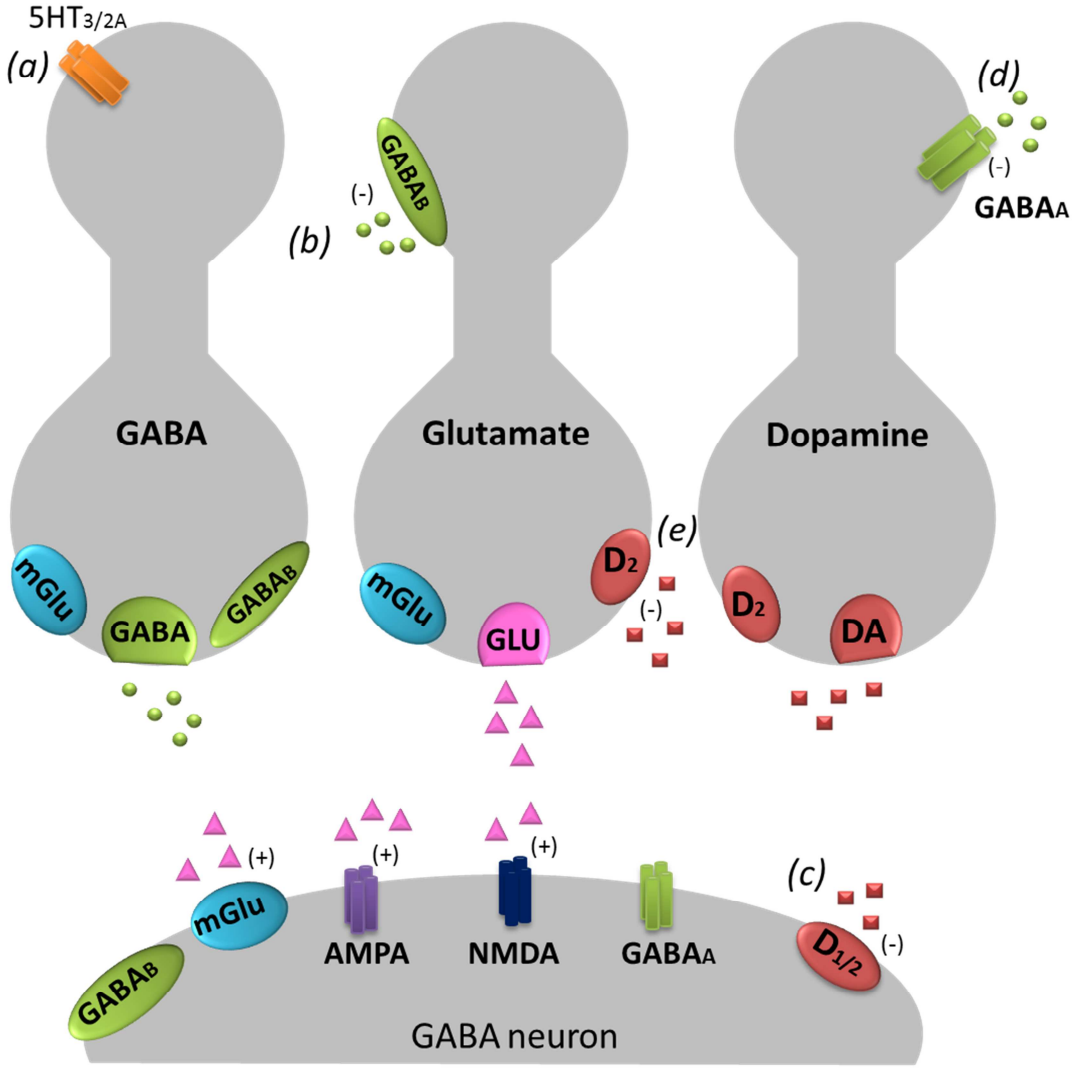


Figure 6. Cross-talk between glutamate, GABA and DA signaling with various receptors (Rudolph & Knoflach, 2011:685; Bétry *et al.*, 2011:603; Carlsson *et al.*, 2001:237). 5HT – serotonin, GABA – Gamma aminobutyric acid, mGlu - metabotropic glutamate, D – dopamine receptor, NMDA - N-methyl-D-aspartic acid, AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid.

2.1.5.3 The hypothalamic-pituitary-adrenal axis hypothesis

The HPA axis is a neuroendocrine system and a major regulator of stress in the human body (Falkai *et al.*, 2013:978). HPA axis hyperactivity is a predisposing factor in the development of MDD in 50% of patients (De Raedt & Koster, 2010:50). It can be activated by stressful life events, as well as by genetic vulnerability to stress (De Raedt & Koster, 2010:50; Nunes *et al.*, 2013:1336). MDD patients often are unable to cope with stress (Müller & Schwarz,

2007:988), where a process of allostasis is gradually moved to allostatic load, which is when psychopathology presents (Korte *et al.*, 2005:3). Numerous studies show that after some time, stressors can induce lasting detrimental changes to hormonal and neurochemical levels that often present, for example as hypercortisolemia or other endocrine disturbances (De Raedt & Koster, 2010:50; Thase, 2009:187).

Psychological stress activates the HPA axis when the hypothalamus releases corticotrophin releasing hormone (CRH) following the action of the perceived stressor on the cortical regions of the brain (Hasler, 2010:155). The effect is the secretion of adrenocorticotropic hormone (ACTH) in the pituitary. ACTH is released into the bloodstream and activates the adrenal cortex to release cortisol in humans or corticosterone in rats (Fig. 7) (De Raedt & Koster, 2010:50; Hasler, 2010:155). The HPA axis can be inhibited by a negative feedback loop whereby excessive cortisol inhibits the release of CRH and ACTH, as seen in Fig. 7 (Falkai *et al.*, 2013:978).

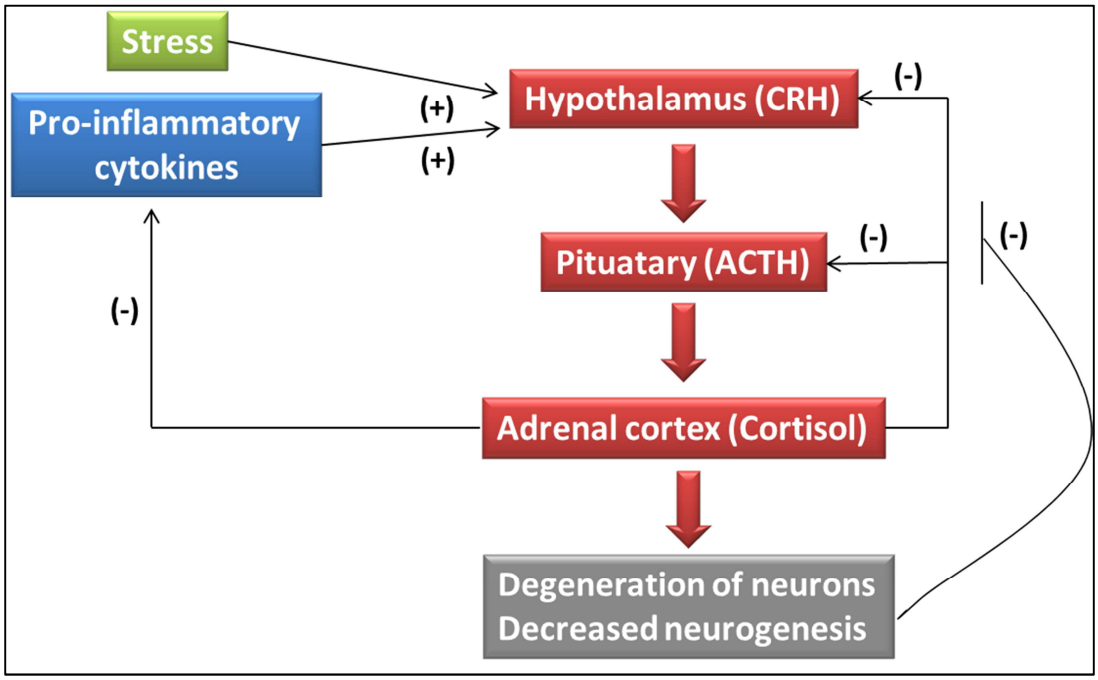


Figure 7. Regulation of the HPA axis under the influence of chronic stress. Cortisol stimulates the negative feedback loop regulating CRH as well as ACTH levels. Degeneration of neurons and decreased neurogenesis modifies the efficacy of the feedback loop of cortisol (Maclaughlin *et al.*, 2011:950461; Maletic *et al.*, 2007:2030).

MDD patients show elevated levels of cortisol, decreased corticosteroid receptor function, enhanced adrenal response to ACTH, decreased sensitivity of the pituitary to CRH and pituitary enlargement (Swaab *et al.*, 2005:141). Corticosteroids bind to mineralocorticoid receptors (MR) and glucocorticoid receptors (GR). GR are found in various brain regions that

involve cognition and memory (e.g. hippocampus, amygdala and frontal cortex – see Fig. 4). These areas, together with the hypothalamus, are involved in the negative feedback loop that regulates the function of the HPA axis (Swaab *et al.*, 2005:141). Damage to the hippocampus, as can be observed in MDD, causes modifications in the efficacy of the negative feedback loop leading to further activation of the HPA axis (Fig. 7) and a subsequent rise in glucocorticoid levels resulting in additional damage to the hippocampus. This is known as the ‘‘glucocorticoid cascade hypothesis’’, a potential mechanism in neurodegenerative diseases associated with HPA axis modification (Swaab *et al.*, 2005:141).

A prominent factor in the negative feedback loop is CRH. It is known for the regulation of various mechanisms in the CNS including cardiovascular function, appetite control, stress-related behaviour and cerebral blood flow (Swaab *et al.*, 2005:141). CRH pathway hyperactivity is evident in many MDD patients despite increased cortisol levels that usually would be an inhibitory factor (Swaab *et al.*, 2005:141). Studies of post-mortem patients that have received glucocorticoid therapy showed a reduction in the expression of CRH in neurons situated in the hypothalamus (Raison & Miller, 2003:1554). The rise in CRH levels can also be attributed to pro-inflammatory cytokines (Fig. 7) with particular immuno-reactivity in the hypothalamus (Swaab *et al.*, 2005:141).

Although stress directly activates the HPA axis, it is indirectly stimulated by pro-inflammatory cytokines (Müller & Schwarz, 2007:988; Swaab *et al.*, 2005:141) that in turn lead to degeneration of neurons and decreased neurogenesis implicated in the pathophysiology of MDD as seen in Fig. 7 (Nunes *et al.*, 2013:1336). Glucocorticoids are potent anti-inflammatory hormones that suppress the activity and the activation of pro-inflammatory cytokines during normal stressful reactions (Raison & Miller, 2003:1554). *In vivo* findings have demonstrated that immune cells in MDD patients show a decrease in the sensitivity to the immunosuppressive effects of glucocorticoids (Raison & Miller, 2003:1554). This may be due to diminishing of glucocorticoid receptor function by cytokines (Raison & Miller, 2003:1554). So although MDD is associated with hypercortisolemia, the inflammatory cascade is not quenched by the anti-inflammatory action of cortisol, thereby perpetuating a pro-inflammatory state.

Moreover, cortisol also has detrimental effects in the CNS by increasing extracellular glutamate levels and causing overstimulation of NMDA receptors and subsequent neuronal damage. (Müller & Schwarz, 2007:988). Animal studies have also proved an association between the dysregulation of the HPA axis and 5-HT neurotransmission. Rats that were

placed under chronic stress show a dysregulation in the HPA axis together with a reduction in 5-HT neurotransmission (Mizoguchi *et al.*, 2001:443). Cortisol is also involved in the stimulation of tryptophan metabolism (see the following section) and therefore directly decreases 5-HT levels in the CNS (Müller & Schwarz, 2007:988).

Hyperactivity of the HPA axis as well as dysfunctions regarding the CRH system is a continuous biological finding in MDD studies (Van Praag *et al.*, 2004:296). Evidence also showed that even when MDD patients are in remission they still display altered HPA axis function (Bhagwagar *et al.*, 2014:1890). Dysregulation of the HPA axis is shown to be associated with the possibility of relapse; therefore the functioning of the HPA axis needs to be normalized following antidepressant treatment, to ensure remission (Mizoguchi *et al.*, 2008:170). When the HPA axis is stabilized with the use of antidepressants it reflects improved mood scores and in the end relieves MDD symptoms (Wainwright & Galea, 2013:805497). There are several types of anti-cortisol agents that have been investigated in MDD including cortisol synthesis inhibitors like ketoconazole, CRH antagonists as well as glucocorticoid receptor antagonists such as mifepristone (DeBattista & Belanoff, 2006:117). Ketoconazole unfortunately has significant side effects at doses that suppress cortisol synthesis. CRH antagonists appear to have antidepressant and anxiolytic properties when evaluated in animal models although clinical studies show a varying degree of success with worsening of affective symptoms when the drug was withdrawn (DeBattista & Belanoff, 2006:117).

2.1.5.4 The kynurenine pathway

The neurodegeneration hypothesis proposes that an imbalance between neuroprotective and neurodegenerative metabolites of the kynurenine pathway can cause MDD (Myint *et al.*, 2007:143) (Fig. 8). Active immunotherapy or immune activation is associated with a decrease in plasma levels of tryptophan, brought about by the activation of tryptophan 2, 3-dioxygenase (TDO) and indolamine 2, 3-dioxygenase (IDO) that metabolize tryptophan (Dantzer *et al.*, 2008:46) (Fig. 8). IDO catabolizes tryptophan to kynurenine (Dunn *et al.*, 2005:891). Importantly, pro-inflammatory cytokines, including IFN-γ, IL-6 and TNF-α, increase the expression of IDO (Dowlati *et al.*, 2010:446; Dantzer *et al.*, 2008:46; Dunn *et al.*, 2005:891). The resulting activation of IDO may play a central role in MDD by decreasing plasma tryptophan, which is an essential amino acid in 5-HT and protein synthesis (Dantzer *et al.*, 2008:46; Wichers & Maes, 2004:11). Lower plasma concentrations of tryptophan have been associated with MDD (Coppen & Wood, 1978:49), while tryptophan depletion in rats causes an increase in anxiety and depressive-like behavior (Blokland *et al.*, 2002:39).

Another characteristic of MDD is its association with prior or on-going stress (Nestler *et al.*, 2002:13). In fact, MDD is associated with hypercortisolemia while TDO is activated by cortisol (Dantzer *et al.*, 2008:46) (Fig. 8), thus consolidating a major role for the kynurenine pathway in the biology of MDD.

A rise in kynurenine, a major metabolite of tryptophan, leads to the formation of 3-hydroxykynurenine (3-HK), QA or kynurenic acid (KYNA), (Fig. 8). Studies found an increased concentration of kynurenine and its metabolites in patients with MDD (Myint *et al.*, 2007:143). An increase in QA, an endogenous NMDA agonist may lead to hippocampal neuron damage and apoptosis (Wichers & Maes, 2004:11). Kynurenine administered alone to mice induced depressive-like behavior (O'Connor *et al.*, 2009:511). KYNA, on the other hand, is a NMDA receptor antagonist that has been speculated to be neuroprotective (Möller *et al.*, 2012:2499). Wu *et al* 2007, found that intra striatal administration of KYNA dramatically reduces extracellular DA in the rat striatum (Wu *et al.*, 2007:33), possibly as a result of modulating glutamatergic activity (Miller *et al.*, 2009:732). Moreover, 3-HK is a potent generator of free-radical species that can cause oxidative stress and lipid peroxidation (Dantzer *et al.*, 2008:46).

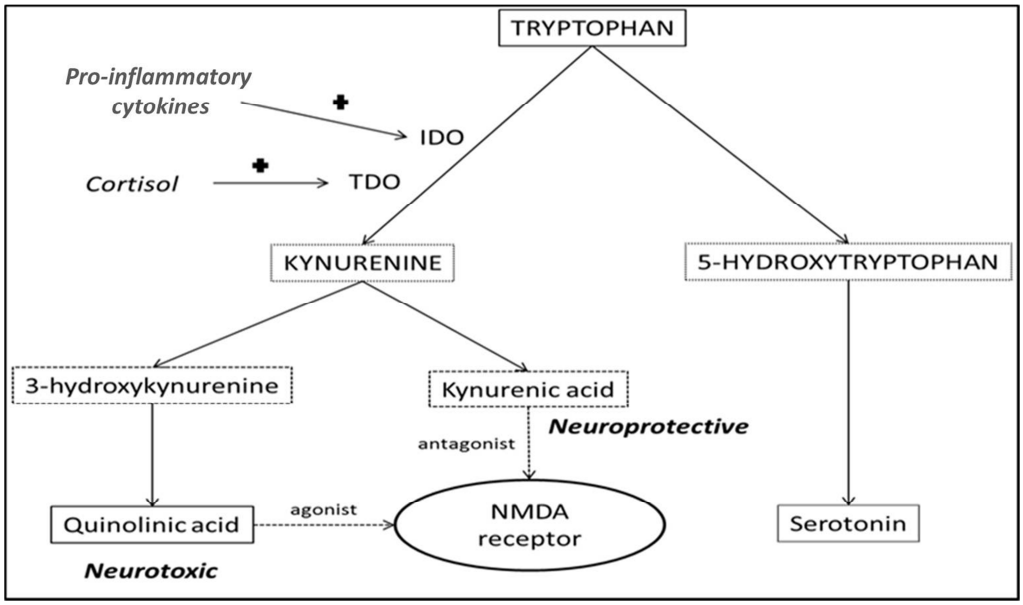


Figure 8. The kynurenine pathway for the formation of 5-HT and the influence of inflammatory mediators and cortisol. End-products result in neuroprotective or neurodegenerative effects via NMDA receptors (Dantzer *et al.*, 2008:46).

2.1.5.5 Oxidative stress

There is abundant evidence for disorganized redox balance and oxidative stress in patients suffering from MDD and in animal models of MDD (Harvey, 2008:371; Khanzode *et al.*, 2003:365; Maes *et al.*, 2011:676). Maes et al 2009 formulated a new hypothesis stating that the activation of inflammatory, oxidative and nitrosative stress pathways is a key pathophysiological factor in MDD (Maes *et al.*, 2009:27). Inflammatory responses are known to be accompanied by the induction of oxidative and nitrosative stress pathways. Animal studies found that experimental MDD is associated with elevated oxidative stress in the rat cortex (Eren *et al.*, 2007:1188). Moreover, they observed that various psychotropics, including antipsychotics and antidepressants such as lamotrigine, aripiprazole and escitalopram, reversed the depressive symptoms as well as manifestations of cortical oxidative stress (Eren *et al.*, 2007:1188).

The brain contains high levels of polyunsaturated fatty acids and low concentrations of endogenous antioxidant enzymes (Sarandol *et al.*, 2007:67). Approximately 20% of the oxygen that is consumed by the body is used by the brain, making this organ more vulnerable to the damaging effects of oxidative free radicals than other tissue (Siegel *et al.*, 1999:531). Due to the aforementioned properties and its high metabolic rate, the brain is especially vulnerable to oxidative stress (Eren *et al.*, 2007:1188; Maes *et al.*, 2011:676), which can be a primary or secondary contributor to MDD and other neurological and psychiatric diseases (Harvey *et al.*, 2008:508; Sarandol *et al.*, 2007:67; Khanzode *et al.*, 2003:365).

A decrease in antioxidant enzymes e.g. glutathione peroxidase (GPX) (Eren *et al.*, 2007:1188; Maes *et al.*, 2011:676) and superoxide dismutase (SOD; Fig. 9) (Maes *et al.*, 2011:676) impair the body's ability to protect itself against ROS and RNS such as superoxide, NO, peroxynitrite and hydrogen peroxide (Fig. 9) (Harvey *et al.*, 2008:508; Maes *et al.*, 2011:676). Various preclinical studies have confirmed the excessive activation of nitric oxide synthase (NOS) and subsequent NO release in the cortex and hippocampus following a stressful event (Harvey *et al.*, 2004:494). These factors contribute to pathology by damaging the cell wall, mitochondria, DNA, functional proteins and fatty acid membrane lipids (Maes *et al.*, 2011:676). This damage will eventually result in apoptosis and cell death (Mokoena *et al.*, 2010:125). Despite the tissue damage and its consequences, damage by ROS & RNS may cause an autoimmune response, thus emphasizing how closely connected the two phenomena are when considering the causal pathology of a particular illness (Maes *et al.*, 2011:676).

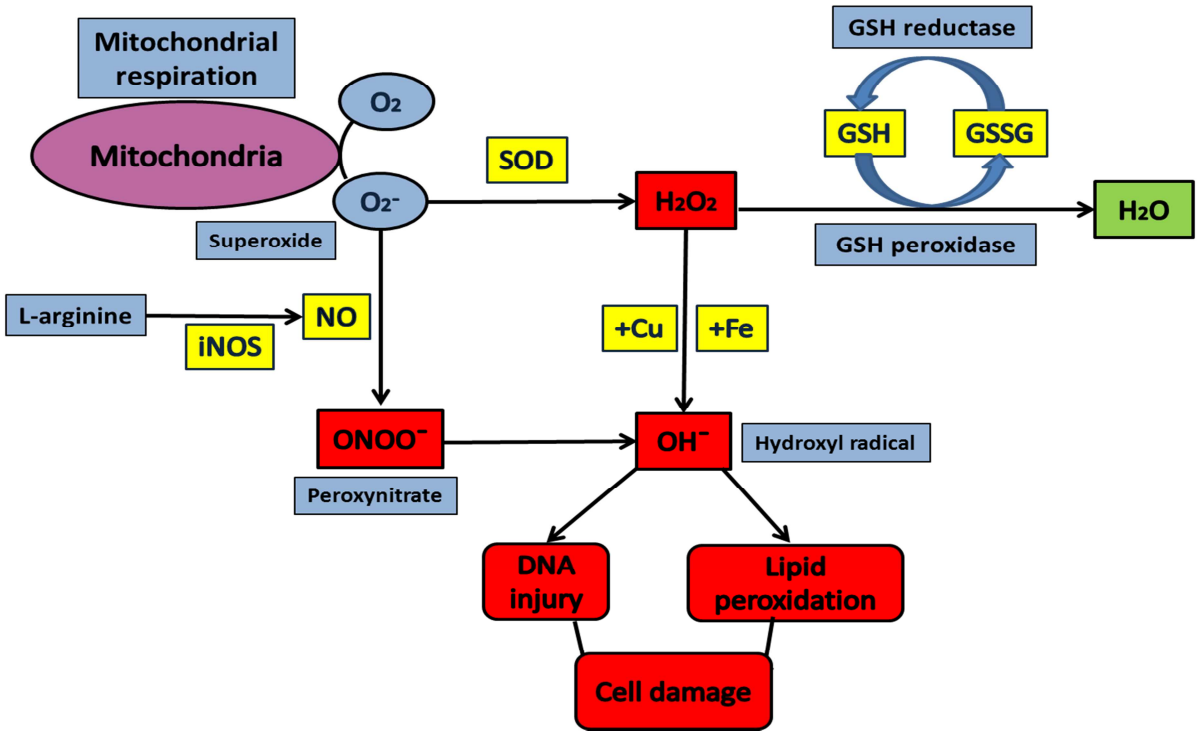


Figure 9. The cellular redox pathway. During MDD oxygen is converted to cell damaging ROS/RNS indicated in red. When there is an overproduction of ROS the activity of antioxidant enzymes including superoxide dismutase SOD and glutathione (GSH) peroxidase are insufficient (Eren *et al.*, 2007:1188; Harvey *et al.*, 2008:508). H_2O_2 - hydrogen peroxide, H_2O – Water, GSSG - glutathione disulphide.

2.1.5.6 Inflammatory and neurodegenerative hypotheses

Evidence implicates increased pro- vs. anti-inflammatory activity and oxidative stress in MDD (Maes *et al.*, 2009:27) and that pro-inflammatory cytokines may be involved in the pathophysiology of the disorder (Maes *et al.*, 2009:27). Cytokines can either be pro-or anti-inflammatory. Pro-inflammatory cytokines include tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-8, IL-1 (Anderson & Maes, 2014:3812; Connor & Leonard, 1998:583) and interferon (IFN) gamma (Dunn *et al.*, 2005:891) that inhibit neurogenesis, induce apoptosis and reduce synaptic and neuronal plasticity (Brand *et al.*, 2015:324). Anti-inflammatory cytokines such as IL-10 and IL-4 (Dantzer, 2004:399; Schiepers *et al.*, 2005:201) dampen the immune response as well as inhibit inflammation (Brand *et al.*, 2015:324).

The association between MDD and inflammation is not new (Bufalino *et al.*, 2013:31; Dantzer *et al.*, 2008:46; Raison *et al.*, 2006:24). In fact, even before the publication of the monoamine hypothesis some prominent antidepressants including MAOIs were found to

have anti-inflammatory activity (Schildkraut, 1965:509). In a clinical study, Xia *et al* in 1996 also demonstrated the anti-inflammatory potential of the TCA, imipramine, by observing its ability to inhibit the release of IL-1 and TNF-α (Xia *et al.*, 1996:27). In 1991 Smith first proposed a link between inflammation and MDD, with his original cytokine hypothesis of MDD, suggesting that MDD is associated with an increase in the secretion of cytokines from macrophages (Smith, 1991:298). Smith also noted the comorbidity between MDD and various other autoimmune and heart diseases and concluded that MDD must be an inflammation related disorder (Berk *et al.*, 2013:200). Since then, there have been consistent findings of increased levels of pro-inflammatory cytokines in patients with MDD (Maes *et al.*, 1995:301; Maes *et al.*, 2009:27). In 1992 Kent et al first noted that pro-inflammatory cytokines are responsible for producing "sickness behavior" (Kent *et al.*, 1992:24), with Yirmiya later connecting this phenomena to MDD (Yirmiya, 1996:163).

Studies in animals showed that MDD-like symptoms can be induced by administering cytokines and by blocking pro-inflammatory cytokine-mediated signaling, antidepressant-like effects can be observed (Krishnan & Nestler, 2008:894). These studies also provided compelling evidence that inflammatory cytokines not only induce "sickness behavior", such as anorexia, but also reduces locomotor activity, exploration, anhedonia and cognitive disturbances, all bearing a strong similarity to the pathophysiology of MDD (Dantzer *et al.*, 2008:46; Maes *et al.*, 2009:27). More recent human research showed elevation of TNF-α and IL-6 in patients suffering from MDD (Dowlati *et al.*, 2010:446; Liu *et al.*, 2012:230) suggesting an exaggerated inflammatory response (Leonard & Maes, 2012:764). Studies conducted on patients treated for cancer or hepatitis C demonstrated that immunotherapy with IFN-α induced full scale MDD in up to 70% of the patients (Maes *et al.*, 2009:27; Amodio *et al.*, 2005:93; Bonaccorso *et al.*, 2002:237; Maes *et al.*, 2001:130; Bonaccorso *et al.*, 2001:45). It is therefore clear that administration of cytokines can precipitate MDD in test subjects (Dowlati *et al.*, 2010:446). After all the above mentioned findings, researchers formed the inflammatory and neuroprogressive hypothesis (Maes *et al.*, 2009:27) suggesting that inflammation is linked to neurotoxicity and neurodegeneration that in turn contribute to the symptoms of MDD. More evidence in support of this hypothesis is the high comorbidity of MDD with inflammatory disorders such as coronary-heart disorder, multiple sclerosis, HIV-infection, inflammatory bowel disease and rheumatoid arthritis (Maes *et al.*, 2009:27).

Investigation into the genetics of the immune system and its relation to MDD has shown that certain polymorphisms of genes can increase the susceptibility of the individual to MDD

(Müller & Schwarz, 2007:988). While the previous research was conducted on peripheral inflammation, Shelton *et al* 2011 reported that at mRNA levels in the central pro-inflammatory network are up regulated in post mortem studies on MDD patients (Shelton *et al.*, 2011:751). Pro-inflammatory cytokines also contribute towards alterations in neurotransmitters as well as neuroendocrine function, synaptic plasticity and behavioral characteristic of MDD (Brand *et al.*, 2015:324). There is also an association between psychological stress and immune activation; studies in experimental animals have demonstrated that psychological stressors increase cytokine levels in the plasma and in various brain regions (Ishikawa *et al.*, 2001:19; Maes *et al.*, 2009:27). Similarly, human studies have also positively linked psychological stress to an inflammatory response with an increased production of pro-inflammatory cytokines (Maes *et al.*, 2009:27; Shapira-Lichter *et al.*, 2008:388).

2.1.5.7 The cholinergic-adrenergic hypothesis

This hypothesis was first described in the 1970s and proposed a central imbalance between cholinergic and adrenergic neurotransmission (Janowsky *et al.*, 1972:632). A cholinergic dominance can be observed during a MDD episode while a manic episode displays a hypo-cholinergic state (Janowsky *et al.*, 1972:632). The hyper- and hypo-cholinergic states cause a decrease and an increase in noradrenergic neurotransmission in MDD and mania, respectively (Dilsaver, 1986:285). The formulation of the hypothesis originated from MDD symptoms caused by organophosphate poisoning (Mineur & Picciotto, 2010:580). Organophosphate is an acetyl cholinesterase (AChE) inhibitor and therefore leads to an increase in central acetylcholine (ACh) (Holmstedt, 1967:433). The centrally acting irreversible AChE inhibitor diisopropylfluorophosphonate (DFP) as well as physostigmine induce MDD symptoms in humans while suppressing symptoms in manic patients (Janowsky *et al.*, 1974:248). However, the cholinergic hypothesis showed a lack in evidence linking it to the etiology of MDD which has complicated its validity, especially that pertaining to predictive validity (drug treatment). However the hypothesis still portrays a major role in certain parts of the disease (Janowsky *et al.*, 1972:632). Despite varying outcomes of older studies more recent literature provide physiological evidence implicating an overactive or hyper-responsive cholinergic system in MDD (Drevets *et al.*, 2013:1156). Moreover variation in genes that code for receptors in the cholinergic system is associated with an increased risk for MDD (Drevets *et al.*, 2013:1156).

Cholinergic receptors are found in various parts of the brain including the hippocampus, hypothalamus, nucleus accumbens and frontal cortex (Alkondon & Albuquerque, 2004:109).

From the basal forebrain cholinergic projections modulate the function of various brain regions associated with MDD (Dagytė *et al.*, 2011:574) to set the appropriate dynamics for neural activity and for the occurrence of learning and memory within the extended hippocampal system (Roland *et al.*, 2008:116). Moreover, preclinical and clinical studies showed that cholinergic dysfunction resulting in a decrease of hippocampal neurogenesis, can contribute to the cognitive symptoms associated with MDD (Duman, 2004a:140). This is more evident when the disease is persistent or resistant to treatment (Jerusalinsky *et al.*, 1997:507). There is also a connection between hippocampal neurogenesis and various learning and memory deficits, including poor attention and concentrations as well as an impairment in memory and information processing that can be seen in MDD (Dagytė *et al.*, 2011:574). Depressive symptoms become evident in healthy individuals treated with cholinomimetics (Janowsky & Risch, 1984:125).

Recent clinical studies (Drevets *et al.*, 2013:1156) have emphasized the role of the cholinergic system in treatment resistant MDD. Pharmacological and gene expression data indicate a different mechanism of action beyond that of direct muscarinic cholinergic antagonism by induction of changes in gene expression and synaptic plasticity (Drevets *et al.*, 2013:1156). Scopolamine, by means inhibiting muscarinic cholinergic receptors, delivers rapid and robust antidepressant effects in randomized, double-blind, placebo-controlled studies involving subjects with unipolar or bipolar depression (Drevets *et al.*, 2013:1156). A preclinical study using a validated animal model of MDD, the Flinders Sensitive Line (FSL) rat, also presented with increased cholinergic activity (Overstreet *et al.*, 2005:739). Chronic stress in rats has also been reported to cause cholinergic dysfunction where a decrease in acetylcholinesterase neurons was observed (Overstreet, 1993:51).

With regards to antidepressant treatment, it has been observed that citalopram, a classic serotonergic selective antidepressant, in part, targets cholinergic neurotransmission to enhance the release of acetylcholine that in return improves memory impairment in patients suffering from MDD (Egashira *et al.*, 2006:161). Furthermore, TCAs have pronounced anti-cholinergic activity that may contribute towards their antidepressant efficacy (Rafaelsen *et al.*, 1981:364). More recently, other preclinical work (Brink *et al.*, 2004:51; Brink *et al.*, 2008:117) has emphasized the cross-talk between the serotonergic, cholinergic and nitrergic systems that may be important in mood regulation.

2.1.5.8 Neuroplasticity

Neuroplasticity includes various mechanisms that allow central neurons to adapt to structural or functional changes in the brain caused by various stimuli (Ganguly *et al.*, 2013:373). These mechanisms encompass the birth, survival, migration and integration of new neurons as well as synaptogenesis and the modulation of synapses (Wainwright & Galea, 2013:805497). For the neurons to mediate and respond to activity, neurons and their synapses must be plastic which in the end is crucial for processing and producing information and ultimately results in a change of behaviour (Wainwright & Galea, 2013:805497). Every aspect of neuroplasticity can independently and additively cause or contribute to the state of MDD (Wainwright & Galea, 2013:805497). Malfunction regarding these alterations in the brain can lead to cellular changes including atrophy, cell death and neurogenesis (Duman, 2002:306; Reay, 2010:1). This hypothesis examines the role of neuroplasticity in MDD (Manji & Duman, 2001:5) where it may provide the bridge between most of the prevailing theories of MDD (Wainwright & Galea, 2013:805497), including the involvement of risk factors for MDD and their origin. The first episode is believed to be caused by external triggers including psychosocial stress, while the triggers for the relapse episodes include endogenous triggers and therefore seem to emerge spontaneously (Hasler, 2010:155), and/or may be brought about by the impact of poor treatment management and non-compliance on neuronal function (Harvey *et al.*, 2003:1105; Harvey & Slabbert, 2014:503).

A decrease in the density of neuronal dendrites and axons, as can be seen in the hippocampus of MDD patients, results in disturbances in connectivity that most likely leads to the well described hippocampal volume loss (Falkai *et al.*, 2013:978). Evidence indicates that new hippocampus neurons are selectively involved in learning and memory (Wainwright & Galea, 2013:805497), thus explaining many of the cognitive deficits evident in the disorder. In the frontal cortex of MDD patients, shrinkage in the cortical thickness along with lower densities of neurons and glia cells can be observed (Falkai *et al.*, 2013:978). Evidence also suggests a correlation between a loss of volume in certain brain areas like the hippocampus and the duration of MDD (Sheline *et al.*, 2003:1516). It is interesting that this volume loss can be the effect of untreated MDD and can present with increased sensitivity to stress and thus increase the likeliness of relapse. Therefore MDD should be treated as soon and as effectively as possible as treatment can possibly normalize hippocampus volume and thereby provide a better long-term outcome (Minkwitz & Himmerich, 2011:199). It has also been suggested that toxicity from corticosteroids and glutamate, together with a decrease in

neurotrophic factors and neurogenesis could be the mechanisms by which brain volume loss is caused (Hasler, 2010:155). The hippocampus is especially vulnerable to stress because of its richness in MR and GR (Wainwright & Galea, 2013:805497). These receptors are responsible for maintaining the basal HPA tone and for the regulation of the negative feedback loop during a stress response (Wainwright & Galea, 2013:805497). The presence of these receptors in the hippocampus, and the latter's role in regulating the HPA axis, also explains why hippocampus atrophy in patients with MDD will promote increased stress-sensitivity, failed coping mechanisms, and in the end predispose these patients to develop subsequent episodes (due to increased allostatic load).

Animal studies have shown that chronic stress or administration of glucocorticoids causes degeneration of the neurons in the hippocampus along with atrophy of dendrites (Sapolsky *et al.*, 1990:2897) while post mortem studies show that stress influences the neuroplasticity of the frontal cortex (Rajkowska, 2000:766; Wainwright & Galea, 2013:805497). These observations led to the formulation of the neurotrophic hypothesis of MDD stating that a decrease in neurotrophic factors may lead to hippocampus pathology when the patient suffers from long term MDD (Castrén *et al.*, 2007:18). On the other hand chronic treatment with antidepressants increases the regeneration of cells as well as neurons (Duman, 2004b:11; Malberg & Schechter, 2005:145). Although neurotrophic factors regulate the growth and differentiation of developing neurons (Cohen-Cory *et al.*, 2010:271), more recent work shows that their regulation of plasticity is primarily responsible for the survival of neurons (Sairanen *et al.*, 2005:1089).

There are various molecular factors involved in the stress response, such as BDNF, cyclic adenosine monophosphate (cAMP) and the transcription factor, cyclic adenosine monophosphate response element binding protein (CREB) (Duman *et al.*, 2000:732; Nibuya *et al.*, 1996:2365). All of these factors are modified by stress with cAMP and CREB being decreased in patients suffering from MDD (Nibuya *et al.*, 1996:2365). BDNF in particular is expressed in the hippocampus and cortex and is involved in the survival and differentiation of axons and dendrites (Falkai *et al.*, 2013:978). In animal models, acute and chronic stress lead to a reduction in BDNF levels in the hippocampus along with a decrease in branching of the dendrites (Falkai *et al.*, 2013:978). Reduced expressions of hippocampal BDNF particularly show a correlation to treatment resistance and recurrence of MDD episodes (Leonard & Maes, 2012:764). Importantly, studies have also showed that treatment with antidepressants, including MAOIs, SSRIs and the SNRIs, increase BDNF (Duman, 2004b:11) as well as CREB levels (Nibuya *et al.*, 1996:2365), while remission of MDD

following antidepressant treatment is associated with reversal of hippocampus volume changes (Chen *et al.*, 2010:1376; Fisar & Hroudová, 2010:1).

2.1.5.9 The circadian rhythm hypothesis

Since the 1950s, abnormal circadian rhythms have been described in patients with mood disorders which include rhythms in sleep, activity, blood pressure, hormone secretion, and monoamines (McClung, 2013:242). In the 1980s the Social Zeitgeber Theory of mood disorders postulated that in a vulnerable individual, changes in the sleep/wake cycle attributes to stressful life events and leads to altered molecular and cellular rhythms and mood-related episodes (Ehlers *et al.*, 1988:948). Impaired sleep regulation and day-time fatigue can be observed in MDD patients and form part of the diagnostic criteria for this disorder (American Psychiatric Association, 2013:970; Hasler, 2010:155). An altered sleeping pattern has effects on mood while manipulation of circadian rhythms in turn has antidepressant effects (Hasler, 2010:155).

Altered cortisol secretion and shortened rapid eye movement (REM) latency in patients suffering from MDD, as well as the effect of antidepressant treatment on circadian rhythms, strongly support this hypothesis (Steiger, 2002:125; Wulff *et al.*, 2010:589). The circadian pacemaker in the brain, the suprachiasmatic nucleus (SCN) of the hypothalamus, coordinates the scheduled release of various peptides and hormones which include melatonin, implicated in the induction of the onset of sleep (McClung, 2013:242).

The pineal gland, located behind the hypothalamus, synthesizes melatonin, which is a derivative of the amino acid tryptophan (Becker, 2001:2283). Unlike other antioxidants, melatonin can be taken up by mitochondrial membranes, thus making it a potential therapeutic tool for treating neurodegenerative disorders (Becker, 2001:2283). Various *in vitro* and *in vivo* experiments have shown that melatonin prevents oxidative stress induced mitochondrial dysfunction (Hardeland *et al.*, 1995:104; Reiter *et al.*, 2001:237). The phase and amplitude of a particular circadian rhythm is determined by the balance between melatonin and 5-HT (at 5-HT_{2c} receptors), with MDD being characterised by a relative loss of melatonin surge in the dark cycle that results in the loss of SCN regulation via the pineal gland (Hickie & Rogers, 2011:621). The misalignment between the circadian pacemaker and the timing of sleep can cause depressive symptoms in vulnerable individuals; moreover the degree of misalignment correlates with the severity of MDD symptoms (Emens *et al.*, 2009:259). The SCN receives mutual inhibitory glutamatergic and serotonergic innervation, with both being able to inhibit circadian rhythm phase changes that is induced by the other

(Germain & Kupfer, 2008:571). A decrease in the activity of the SCN as is seen in MDD can be caused by the increase in plasma cortisol levels that are known to inhibit SCN function (Swaab *et al.*, 2005:141). BDNF, known for its neuroprotective effects, shows a decrease in its proliferation rate when there is a disturbance in the daily cortisol rhythm (McClung, 2013:242). Chronic disruption of circadian rhythm may also inhibit hippocampus neurogenesis depending on the direction and duration of the shift (McClung, 2013:242). Human genetic studies have identified several polymorphisms of circadian genes that are associated with psychiatric diseases (McClung, 2013:242). These genetic factors suggest that abnormal circadian rhythm may cause mood disruptions rather than being an effect of it (McClung, 2013:242).

With the arrival of agomelatine, this hypothesis has grown in stature. Agomelatine portrays both melatonergic and serotonergic receptor activities and has shown to effectively advance circadian rhythms in animal models as well as MDD patients (Leproult *et al.*, 2005:298; Mairesse *et al.*, 2013:323; Taylor *et al.*, 2014:1888). Other non-pharmacological interventions include electroconvulsive therapy (ECT) and transcranial magnetic stimulation that also act on sleep and other circadian rhythms (Germain & Kupfer, 2008:571).

2.1.5.10 Genetic aspects

The heritability of MDD seems meagre when it is compared to the 80% heritability rate of bipolar disorder and schizophrenia (Falkai *et al.*, 2013:978). Studies that implicate twins and families show that MDD is a moderate heritable illness (Levinson, 2006:84), however, recently gene and genome association studies have linked common DNA polymorphisms to MDD (Levinson, 2006:84), and MDD retains a complex polygenetic origin (Minkwitz & Himmerich, 2011:199) with single nucleotide polymorphisms (SNPs) only marginally affecting its pathophysiology (Falkai *et al.*, 2013:978).

Not only is polymorphisms in genes directly associated with monoaminergic synaptic concentration seen in patient with MDD, but it is also associated with affecting cell growth, axon function and the circadian rhythm (Minkwitz & Himmerich, 2011:199). The most consistent gene study results are for gene expression differences associated with glutamate and GABA (Minkwitz & Himmerich, 2011:199). Various studies have examined the interaction of stress and genetic predisposition (Minkwitz & Himmerich, 2011:199). Genetic variation in CRH has been more accurately associated with a decrease in HPA axis response to CRH (Ressler *et al.*, 2010:812). Polymorphism in the GR on hippocampus level is found to be associated with an increased vulnerability to MDD by impairing CRH secretion

in the negative feedback loop (Falkai *et al.*, 2013:978). Certain polymorphisms may also predispose a smaller hippocampus volume that together with a 5-HT transporter polymorphism is associated with stress-induced MDD (Minkwitz & Himmerich, 2011:199). More genes implicated in MDD development are the monoamine oxidase A gene as well as a group of glutamate receptors (Falkai *et al.*, 2013:978).

The various theories of MDD argue against a unified hypothesis of MDD and suggest that it is a clinically and aetiologically heterogeneous disorder (Hasler, 2010:155). Stress response with an added genetic susceptibility lead to the subsequent modification of the HPA axis together with the disruption of GABA-glutamate signalling regulating the stress response as well as monoamine function (Hasler, 2010:155). Inflammatory mediators, cytokines, ROS and QA can result in the release of glutamate within the brain. A decrease in the reuptake as well as the activation of NMDA receptors disrupts neural plasticity. Deficits in neurotrophin release, such as BDNF together with the before mentioned factors culminate in structural changes in critical brain regions (Harvey *et al.*, 2003:1105; Krishnan & Nestler, 2008:894).

2.1.6 Treatment options

2.1.6.1 Conventional options for treating MDD

There is a multitude of pharmacological options with which to treat MDD. These include the older drugs such as the first- and second-generation TCAs, heterocyclics such as mirtazepine, and MAOIs (Snow *et al.*, 2000:738). More recently newer classes of antidepressants have emerged such as the SSRIs, SNRIs (Fig. 10) as well as atypical compounds such as bupropion and agomelatine. Lesser known new treatments include reversible MAOIs, 5-HT_2 antagonists, 5-HT_{1a} agonist, GABA mimetic analogues, DA reuptake inhibitors and DA antagonists (Snow *et al.*, 2000:738). For most patients' first line treatment consist of SSRIs (e.g. fluvoxamine, fluoxetine, sertraline, escitalopram) or SNRIs (venlafaxine, duloxetine), mirtazapine, or bupropion (Gelenberg *et al.*, 2010:1; Korte *et al.*, 2015:88). If there is at least a moderate improvement in symptoms within 4–8 weeks of initiation of the treatment, the diagnosis should be re-evaluated and the side effects together with treatment adherence assessed (Gelenberg *et al.*, 2010:1).

After an additional 4–8 weeks of treatment, if the patient still shows minimal or no improvement in symptoms, contributory factors should be assessed and changes in the treatment plan considered (Gelenberg *et al.*, 2010:1). The first option is to optimize the dosage of the current therapy if side effects are tolerable (Gelenberg *et al.*, 2010:1).

Alternatively another non-MAOI antidepressant or augmenting current therapy with a non-MAOI antidepressant from another class of compounds can be considered (Gelenberg *et al.*, 2010:1). Patients not responding to treatment with SSRIs may find SNRIs more effective whereas non-selective MAOI can be considered if the patient can adhere to dietary restrictions (Gelenberg *et al.*, 2010:1; Korte *et al.*, 2015:88).

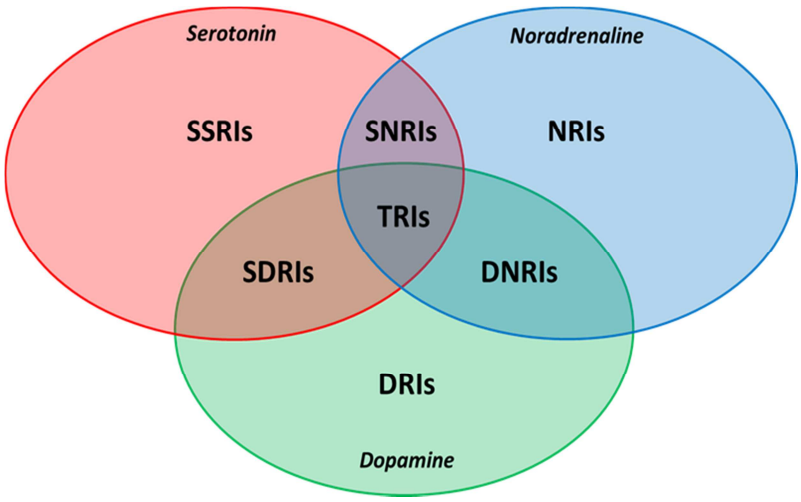


Figure 10. Monoaminergic drugs that block the 5-HT, NA and/or DA transporters (Korte *et al.*, 2015:88). SSRIs – selective serotonin reuptake inhibitors, SNRIs – serotonin and noradrenalin reuptake inhibitors, NRIs - noradrenalin reuptake inhibitors, NDRIs - noradrenalin and dopamine reuptake inhibitors, DRIs - dopamine reuptake inhibitors, TRIs - triple reuptake inhibitors

Some patients experience symptoms including impaired motivation and pleasure which creates a role for DA in their treatment regime. Additional treatment that focuses on the dopaminergic system greatly contributes to the treatment of MDD (Korte *et al.*, 2015:88). This knowledge has led to the formulation of triple reuptake inhibitors (TRIs) that simultaneously block 5-HT, NA and the DA transporters (Fig. 10) (Korte *et al.*, 2015:88), as well as agomelatine that promotes frontal cortical DA release (Harvey & Slabbert, 2014:503). These formulations have led to greater awareness in treating anhedonia by focusing on long-lasting enhancement in the brain reward system (Korte *et al.*, 2015:88).

2.1.6.2 Novel options for treating MDD

Considering the advancements in our understanding of MDD, a number of novel therapeutic strategies may be put forward

Considering MDD as a pro-oxidative state (Leonard & Maes, 2012:764) has led to the investigation of antioxidant compounds as a treatment option. NAC replenishes endogenous antioxidant GSH and increases glutathione peroxidase activity, as well as directly scavenges damaging oxidants (Dean *et al.*, 2011:78). NAC is a safe and affordable antioxidant and has proved effective in clinical and preclinical studies (Berk *et al.*, 2014:628; Bavarsad Shahripour *et al.*, 2014:108; Dean *et al.*, 2011:78).

Another well documented antioxidant compound is polyunsaturated fatty acids (Pandya *et al.*, 2013:214). Omega-3 fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are crucial for development of the CNS (Pandya *et al.*, 2013:214), and are known for their antioxidant as well as anti-inflammatory potential, and possibly increasing BDNF expression (Pandya *et al.*, 2013:214). A meta-analysis study has demonstrated that supplementation with EPA in excess of DHA were effective against primary MDD (Pandya *et al.*, 2013:214).

Considering the important role for glutamate in MDD, another novel compound includes ketamine, a non-competitive NMDA receptor antagonist. This anesthetic agent has demonstrated antidepressant effects in animal models of MDD (Hashimoto, 2009:105). Zarate *et al.* 2006 also demonstrated robust and rapid antidepressant effects in treatment-resistant MDD following a single intravenous dose (Hashimoto, 2009:105).

Minocycline, a second-generation tetracycline, may be a potential new treatment for MDD. Emerging findings in animal and human studies revealed that it has antidepressant-like neuroprotective and anti-inflammatory properties (Investigators NN-P., 2006:664; Lai & Todd, 2006:809; Traynor *et al.*, 2006:20; Zhang *et al.*, 2006:1).

Considering the role of NO in MDD, methylene blue, a vital non-toxic dye structurally related to TCAs (Harvey *et al.*, 2010:1580), has displayed antidepressant-like effects in the forced swim test (FST) (Eroglu & Caglayan, 1997:381).

MicroRNAs (miRNAs) are a class of gene-expression regulators that may be a novel class of therapeutic targets to treat MDD (Mouillet-Richard *et al.*, 2012:272). miRNAs are profoundly involved in the regulation of various physiological processes central to the functioning of the CNS (O'connor *et al.*, 2012:359). MiRNA-expression levels were found to be altered in preclinical models of psychological stress. Furthermore, research suggests that antidepressants utilize miRNAs as downstream effectors (Mouillet-Richard *et al.*, 2012:272).

The alteration of miRNAs levels has been shown to affect depressive behavior in preclinical models (O'connor *et al.*, 2012:359).

Stress, a significant contributing factor in MDD, is known to alter the gut micro biome that has been suggested to contribute to the development of MDD (Logan & Katzman, 2005:533). Probiotics are known to improve nutritional status, correct bacterial overgrowth and to reduce systemic inflammatory cytokines as well as oxidative stress ultimately leading to increased BDNF. The latter underlies how altered gut micro biome contributes to depressive symptomology in humans and in animals (Logan & Katzman, 2005:533).

The essential metal zinc has proved to be significantly lowered in MDD patients (Pandya *et al.*, 2013:214). Zinc crosses the blood-brain barrier and concentrates in the hippocampus and amygdala where it modulates NMDA, AMPA and GABA receptors and improves hippocampus neurogenesis through an increase in BDNF expression (Pandya *et al.*, 2013:214). A review of clinical trials suggests potential benefits of using zinc supplementation as a stand-alone or as an adjunctive therapy to conventional antidepressant treatment (Pandya *et al.*, 2013:214).

Taking into account the kynurenine pathway (section 2.1.5.4), research has been conducted on various IDO inhibitors. The most researched compound, 1-Methyltryptophan, has been found to reduce depressive-like symptoms in a lipopolysaccharide (LPS) induced neuro-inflammatory animal model (Dobos *et al.*, 2012:905). Recently Young *et al.* 2013 suggested the combination of both TDO and IDO inhibitors as possible antidepressant therapies (Young, 2014:168). Existing TDO inhibitors include certain antidepressants, aspirin and nicotinamide (Badawy, 2014:169).

2.1.7 Treatment problems/challenges

Patients using TCAs complain of blurred vision, constipation, dizziness, dry mouth, and tremors while diarrhea, headache, insomnia, and nausea significantly affects patients being treated with SSRIs (Snow *et al.*, 2000:738). Furthermore, patients treated with SSRIs also report sedation as well as sexual dysfunction (Snow *et al.*, 2000:738). Emotional blunting induced by SSRI treatment is a two-edged sword, and can be regarded as a benefit or complication depending on the patient's response and needs (Sansone & Sansone, 2010:14). SSRIs can interact with other drugs that enhance 5-HT release, and can induce a life threatening serotonin syndrome with symptoms including disorientation, confusion, agitation, restlessness, fever, shivering, diaphoresis, diarrhea, as well as ataxia,

hyperreflexia, and myoclonus activity (Lane & Baldwin, 1997:208). SNRIs can cause the same side effects as SSRIs but may also include the effects caused by NA reuptake inhibition, especially over-activation, anxiety and elevated blood pressure (Korte *et al.*, 2015:88). Importantly, side effects compromise compliance to the treatment regime and can result in devastating long-term consequences for the patient (Domenici *et al.*, 2010:9166).

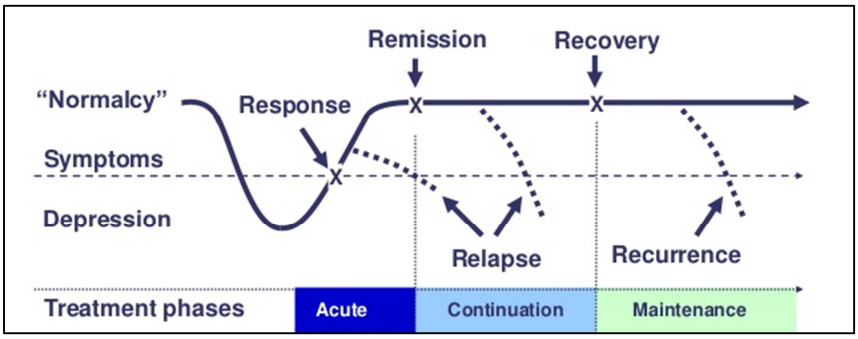


Figure 11. The acute, continuation and maintenance treatment phases in MDD and the associated relapse or recurrence of symptoms after response, remission or recovery (Frank *et al.*, 1991:851).

Following an acute MDD episode, two subsequent phases of MDD follow, viz. the continuation phase where the patient receives antidepressant treatment for up to six months followed by a prophylaxis phase (Fig. 11) (Geddes *et al.*, 2003:653). During the continuation phase MDD episodes often recur, in which case it is termed relapse (Keller *et al.*, 1982:911). If inadequate treatment is received during this phase the relapse rate can be as high as 50% when compared to 20% if treatment is maintained adequately (Keller *et al.*, 1982:911). Relapse occurs during the maintenance phase and more often in patients with previous depressive episodes. The latter often presents in patients with residual symptoms after acute treatment, and in those that lack social support or have social difficulties (Keller *et al.*, 1982:911). The amount of previous episodes is one of the strongest predictors of relapse or recurrence (De Raedt & Koster, 2010:50). If an episode of MDD occurs during the prophylactic phase it is termed recurrence (Fig. 11) (Paykel & Priest, 1992:1198). For an episode to be defined as recurrent there must be a two month interval between episodes during which the patient must not meet the criteria for MDD (American Psychiatric Association, 2013:970). The possibility of future episodes is 30% up to 75%, which increases with subsequent episodes (Andrade *et al.*, 2003:3). After two years the recurrence rate was found to be 25-40% increasing to 60% after five years, 75% after ten and 85% after fifteen years (Keller, 1999:41). Finally, the risk of occurrence increases if the patient is female (43%), is not married (55%) and has a history of MDD (11 %) (Richards, 2011:1117).

Preference for a SSRI as a first line treatment option is generally considered due to their improved tolerability as well as being safer in overdose (Barbey & Roose, 1998:42; Hansen, 2004:93; Isbister *et al.*, 2004:277) and having less cardiovascular toxicity (Sarko, 2000:637). Other studies have found the difference in side effects between TCAs and MOAIs vs. SSRIs to be relatively minor (Disner *et al.*, 2011:467). The TRIs, a more recent antidepressant approach includes the modification of 5-HT, NA and DA neurotransmitters. It has however raised increased awareness regarding its potential for abuse since cocaine is a TRI as well (Korte *et al.*, 2015:88). Hypothetically TRIs should cover a broader range of MDD symptoms but potentially it will increase the side-effects (Korte *et al.*, 2015:88).

The interruption of antidepressant treatment may lead to a complex set of physiological and neuropsychiatric symptoms referred to as antidepressant discontinuation syndrome (Warner *et al.*, 2006:449). This syndrome is primarily associated with the discontinuation of TCA, SSRIs, and SNRIs and lead to symptoms including dizziness, nausea, headache, lethargy, low mood, insomnia, tinnitus and imbalance (Harvey & Slabbert, 2014:503). The syndrome is also more prominent after prolonged antidepressant use or if the patient was treated with high dosages. Antidepressant discontinuation syndrome has significant clinical implication for the management of MDD. The outcome can be detrimental in an already compromised individual resulting in undesirable effects on the long-term outcome of treatment response (Harvey & Slabbert, 2014:503).

2.2 Treatments in this study

2.2.1 N-acetyl cysteine

As discussed in the previously mentioned section 2.1.5.2 and 2.1.5.5 it is clear that glutamate dysfunction together with oxidative stress play a definite role in the pathophysiology of MDD, thus suggesting that an antioxidant and glutamate modulator may offer clinical utility in treating this illness. In this regard N-acetyl cysteine (NAC), a glutathione precursor, antioxidant (Kerksick & Willoughby, 2005:38) and glutamate modulator (Bauzo *et al.*, 2012:288; Grant *et al.*, 2007:652), is a promising therapeutic agent (Dean *et al.*, 2011:78). NAC is conventionally used for the treatment of paracetamol toxicity by acting as an antioxidant precursor to glutathione (Dean *et al.*, 2011:78). It is also used as a mucolytic and in the treatment of chronic obstructive pulmonary disease (Dean *et al.*, 2011:78).

Alterations in cysteine levels modulate neurotransmitter pathways, including DA and glutamate (Fig. 12A) (Dean *et al.*, 2011:78). Cysteine regulates the intra- and extracellular

exchange of glutamate by increasing the activity of the cysteine-glutamate antiporter. This results in stimulation of inhibitory metabotropic glutamate receptors situated on glutamatergic nerve terminals that is responsible for reducing the synaptic release of glutamate (Afshar *et al.*, 2012:797) as well as increasing the synaptic release of DA (Dean *et al.*, 2011:78). NAC has been found to reverse cortico-striatal DA and 5-HT alterations as well as striatal NA alterations in the social isolation reared rat, a neurodevelopmental animal model of relevance for MDD and schizophrenia (Möller *et al.*, 2013b:687). Targeting redox-inflammatory pathways, with subsequent glutamate alterations and effects on cortico-limbic monoaminergic transmission may underlie its therapeutic benefits in the treatment of MDD and schizophrenia (Möller *et al.*, 2013a:156).

Another potential mechanism of action of NAC is its anti-inflammatory properties (Fig. 12B). Animal studies have shown NAC to reduce TNF-α and IL-1 levels following both traumatic brain injury and focal cerebral ischemia. The anti-inflammatory effect of NAC may be associated with inflammatory pathways or work through oxidative processes (Dean *et al.*, 2011:78). Animal studies have confirmed the ability of NAC to reverse striatal oxidative stress *in vivo* (Harvey *et al.*, 2008:508) (Fig. 12C) and to induce antidepressant-like effects in animals (Ferreira *et al.*, 2008:747). Pretreatment with NAC prevented oxidative stress in rats following exposure to prenatal inflammation (Lanté *et al.*, 2008:602), while another study proved the antidepressant effects of NAC in rats exposed to chronic mild stress was related to its ability to reduce oxidative damage (Arent *et al.*, 2012:1072). Recent clinical studies have also highlighted its efficacy as a stand-alone treatment for the depressive symptoms in bipolar disorder (Berk *et al.*, 2008a:468; Magalhães *et al.*, 2011:374), with some benefit as adjunctive treatment observed in MDD (Berk *et al.*, 2014:628). Since the current treatment of MDD remains focused on correcting monoaminergic transmission (e.g. SSRIs and SNRIs) while oxidative stress in itself may mediate altered monoaminergic activity (Möller *et al.*, 2013b:687), drugs targeting redox status may have distinct benefits in treating the illness.

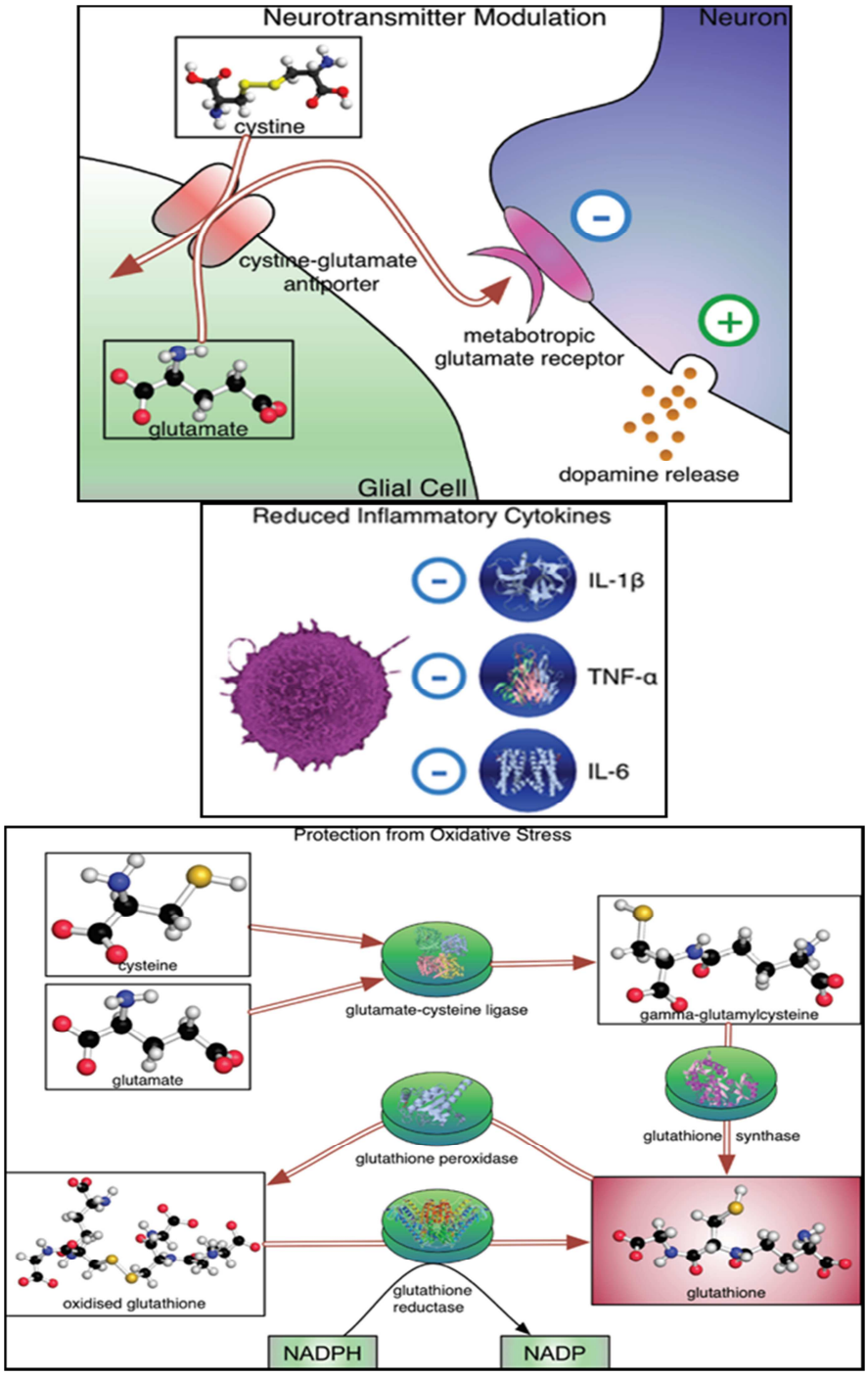


Figure 12. The mechanisms of action of NAC (Dean *et al.*, 2011:78).

A: NAC increases the activity of the cystine–glutamate antiporter. This results in an increase in the activation of glutamate receptors on inhibitory neurons which facilitates DA release. B: NAC is associated with reduced levels of inflammatory cytokines. C: NAC is a substrate for glutathione synthesis in the oxidative stress pathway. All of the above actions are believed to be mechanisms that promote cell survival and growth factor synthesis which leads to increased neurogenesis.
BDNF = brain-derived neurotrophic factor; IL = interleukin; NADP = nicotinamide adenine dinucleotide phosphate; NADPH = reduced form of NADP; TNF = tumor necrosis factor.

NAC seems to hold promise in the treatment of psychiatric disorders and provides a treatment opportunity where current therapies are restricted or suboptimal. A precise dosage for NAC in MDD remains elusive as dose range studies showed equal efficacy at high and low dosages. Some controversy regarding using antioxidants in the *in vivo* (and clinical) scenario, and the doses needed, has attracted a great deal of controversy (Emsley *et al.*, 2014:224; Seybolt, 2014:222). Nevertheless, Emsley and colleagues have emphasized that predicting the therapeutic effects of an antioxidant is complex. In fact, antioxidants may act either as an antioxidant or as a pro-oxidant depending on the redox status of the organism (Flora, 2009:191; Tylicki *et al.*, 2003:303), which in turn relates to illness state (or severity). This already complex cellular environment will also predetermine how the applied dose of the antioxidant will perform, thus, the present study will attempt to address these burning issues by studying chronic NAC treatment and its response in both "depressed" and healthy animals. No serious side effects have been noted for NAC, although chronic use has not been extensively studied (Dean *et al.*, 2011:78).

However, recent work has suggested that the N-amide derivative of NAC (NACA) may offer better efficacy due to improved pharmacokinetics and brain penetration (Ates *et al.*, 2008:372). NACA crosses the blood-brain barrier more effectively and shows greater radical scavenging ability at higher concentrations. Attaching the amide to NAC enhances the antioxidant potential, while NACA's improved membrane penetration allows it to be more effectively hydrolyzed to cysteine, thereby boosting the endogenous glutathione pool (Ates *et al.*, 2008:372). Indeed animal studies have found NACA to be effective in reducing haloperidol-induced oxidative stress (elevated lipid peroxidases and protein peroxidases) and vacuous chewing in an experimental model of tardive dyskinesia in rats (Sadan *et al.*, 2005:285). Another study showed that NACA prevents and suppresses experimental autoimmune encephalomyelitis, in an animal model of multiple sclerosis (Offen *et al.*, 2004:1241), while the compound also prevents neuronal damage caused by DA in an animal model of Parkinson's disease (Imam *et al.*, 2011:157). These data confirm it to be an effective radical scavenger that lowers ROS with potential in certain animal models. However, no head-to-head studies in humans or animals have been carried out to date to confirm the assumption that it is therapeutically superior to NAC.

2.2.2 Imipramine

The parent compound of IMI, iminodibenzyl, was first identified in 1899 but it was only in 1948 when it was first synthesized as an antihistaminic agent (Hafliger & Burckhardt, 1964:35). IMI was the prototype in the iminodibenzyl class of derivatives and the first to

display clinical efficacy as an antidepressant (Klerman & Cole, 1965:101). The TCAs are the most commonly used treatment for MDD and were the first line treatment for almost 30 years before the discovery of SSRIs (Tamminga *et al.*, 2002:589).

The original animal studies showed an effect of IMI on motor retardation, to prolong sleep after barbiturate use, to slow waves in the cortical electroencephalogram and to have atropine-like effects (Klerman & Cole, 1965:101). It was also found to potentiate central and peripheral adrenergic mechanisms (Klerman & Cole, 1965:101). TCAs work by inhibiting NA and 5-HT reuptake. However, although studies showed this action to take place within minutes of dosing, its antidepressant effects only became evident more than three weeks later (Klerman & Cole, 1965:101). IMI blocks the uptake of NA in peripheral as well as brain tissue (Klerman & Cole, 1965:101). Studies also found that it modulates glutamate and nitrergic signaling as well (Dean *et al.*, 2011:78). TCAs are divided into two classes: the tertiary amines, which include IMI, have a high burden of anticholinergic side-effects, and the secondary amines, including desimipramine, which have a reduced side-effect profile (Tamminga *et al.*, 2002:589). Recent work has also highlighted the antioxidant properties of IMI. If one considers MDD to be a pro-oxidative state, this constitutes an additional mechanism of action, and hence of relevance for this study. An increase in SOD (Fig. 9), together with a decrease in lipid and protein damage was observed in the frontal cortex and hippocampus of animals receiving acute or chronic treatment with IMI (Reus *et al.*, 2010:325; Zafir *et al.*, 2009:220). Furthermore, IMI has been found to reduce markers of nitrosative stress in the rat brain (Krass *et al.*, 2011:57).

2.2.3 *Garcinia mangostana* raw pericarp powder

A class of compounds worth studying for putative psychotropic and antidepressant-like effects is the xanthones. Xanthones are found in higher plant families, fungi and lichen as secondary metabolites (Negi *et al.*, 2013:1). Of relevance for this particular study, the pericarp of *Garcinia mangostana* Linn. (GM) was used. GM is an evergreen fruit tree originating from Indonesia and produces approximately 50 bioactive xanthones (Pedraza-Chaverri *et al.*, 2008:3227). The people of Indonesia have for many years used the pericarp (peel, rind, hull or ripe) of GM or mangosteen as traditional medicine for the treatment of trauma, skin infection, abdominal pain, diarrhea, dysentery, infected wounds, suppuration, and chronic ulcers (Negi *et al.*, 2013:1).

A xanthone of GM, α-mangosteen, was the first to be investigated for pharmacological activity and it was found to have monoamine oxidase inhibitor activity (Negi *et al.*, 2013:1).

According to Negi and co-workers, xanthones show a broad spectrum of biological activities that lends it for testing as a treatment for MDD, e.g. immunomodulation, anti-inflammatory and anticholinergic activity (Negi *et al.*, 2013:1). The xanthones of GM also display strong antioxidant effects in the brain, possibly as a result of their potent scavenging abilities of ROS or by chelating metals (Phyu & Tangpong, 2014:151; Negi *et al.*, 2013:1; Rao *et al.*, 2012:848) as well as increasing the activity of glutathione peroxidase (Pedraza-Chaverri *et al.*, 2008:3227). The xanthone, α–mangosteen also shows antioxidant and neuroprotective properties (Márquez-Valadez *et al.*, 2009:35; Pedraza-Chaverrí *et al.*, 2009:491) and was able to scavenge O_2^-, $ONOO^-$, and singlet oxygen species and prevented 3-nitropropionic acid induced neurotoxicity and ROS production (Pedraza-Chaverrí *et al.*, 2009:491).

Evidence for possible application in neurodegenerative and inflammatory disorders comes from animal work where GM has been found to prevent the accumulation of pro-inflammatory cytokines in the frontal cortex and resulting oxidative damage (Márquez *et al.*, 2012:729; Phyu & Tangpong, 2014:151). The above described effects have all been suspected of contributing to the neuroprotective actions of xanthones (Phyu & Tangpong, 2014:151; Rao *et al.*, 2012:848). GM xanthones caused a reduction in lipid peroxidation in a dose dependent manner (Pardo Andreu *et al.*, 2010:124) together with prominent anti-inflammatory activity by inhibiting the production of IL-6 (Yiemwattana & Kaomongkolgit, 2014:1).

Considering the monoamine (Hasler, 2010:155), redox (Myint *et al.*, 2012:245), immune-inflammatory (Maes *et al.*, 2009:27) and cholinergic hypotheses (Mineur & Picciotto, 2010:580) of MDD, GM therefore offer great potential as novel antidepressant compounds (Amani & Nabilah, 2010:1). Although the biological half-life of GM ranges between 3 and 7 hours depending on the administration route (Pardo Andreu *et al.*, 2010:124), not much is known about its pharmacokinetic properties. Looking at the broader group of polyphenols, they tend to be absorbed in the gut where after various metabolites appear in the plasma that retain some degree of antioxidant capacity (Amani & Nabilah, 2010:1).

Thus, while GM shows putative psychotropic effects, the antidepressant activity has not been studied in animals. The overall behavioral pharmacology of GM with respect to the key symptoms of MDD, viz. despair, learned helplessness, anxiety, anhedonia and cognitive deficits, compared to NAC and a reference antidepressant has not been undertaken before. Moreover, their psychotropic actions in a translational animal model, as well as neurobiological testing with regards to the immune-inflammatory hypothesis, would consolidate their claim to being novel antidepressant compounds.

2.3 Animal models of MDD

Animal models serve to study the neurobiology of disease as well as to predict the effects of treatment *in vivo* (Borsini & Meli, 1988:147). As can be seen from the multitude of hypotheses for MDD described earlier, MDD is a heterogeneous disease with an unclear etiology (Hasler, 2010:155). It is thus a great challenge to develop an animal model that can accurately imitate the symptomatology, neurobiology and treatment response evident in MDD.

An animal model is required to be valid with respect to three criteria, viz. face, construct, and predictive validity (Overstreet & Wegener, 2013:143). These are defined as follows:

- Face validity: the extent that the animal model mirrors the behavior and the symptoms of the human condition (Overstreet & Wegener, 2013:143).
- Construct validity: the extent to which the theoretical rationale behind the animal model correlates with that of the human disorder, such as corresponding abnormalities in neurological biomarkers or neurotransmission (Overstreet & Wegener, 2013:143).
- Predictive validity: the ability of the animal model to predict the theorized outcome. The model must therefore respond to the same drug treatment as the human condition (Overstreet & Wegener, 2013:143).

An animal model can be created by means of the etiology of MDD by introducing either pathological or induced mechanisms that underlie the illness in humans (Willner & Mitchell, 2002:701). Creation of a pathological model will result in a “sick” animal representative of the human illness (Willner & Mitchell, 2002:701). These models can be developmental models (early life stress), lesion models (Olfactory bulbectomy), genetic models (FSL) or genomic models (HPA transgenic, serotonin transporter knockout). None of these models fully recreate the human disorder. However, if its limitations are noted, a well-validated animal model may provide useful insight on MDD and its treatment (Overstreet & Wegener, 2013:143).

As discussed in section 2.1.5 MDD is believed to result from the combination of genetic susceptibility and environmental factors. Therefore an animal model with a genetic predisposition for MDD may better represent the human condition (Malkesman & Weller, 2009:153; Overstreet *et al.*, 2005:739). The FSL rat is an example of selective breeding to produce a stress-sensitive animal that to date has been well-described and validated for MDD (Overstreet & Wegener, 2013:143). There is abundant data supporting the face,

predictive and construct validity of the FSL rat model (Malkesman & Weller, 2009:153; Yadid *et al.*, 2000:353). Adding to its value is the FRL rat that presents with an absence of genetic susceptibility to stress that can be used as a corresponding negative control (Overstreet *et al.*, 2005:739). The stress-sensitivity of the FSL rat and its response to antidepressants that are effective in human MDD has been described by numerous laboratories (Malkesman & Weller, 2009:153; Overstreet *et al.*, 2005:739) and will be used in this study as a translational model of MDD.

2.3.1 Flinders sensitive line rat animal model of MDD

The FSL and FRL rats where developed from Sprague Dawley rats by means of selective breeding (Overstreet *et al.*, 1979:15) by choosing lines that displayed high and low sensitivity to the anticholinesterase agent, DFP (Hasegawa *et al.*, 2006:358; Overstreet *et al.*, 1979:15).

Later on it was established that they have other depressive-like physiological and behavioral characteristics that resemble the human depressive state (Overstreet *et al.*, 2005:739). The FSL rat has thus been proposed as a genetic animal model for some aspects of human MDD (Lavi-Avnon *et al.*, 2005:607). When compared to the FSL rats, the FRL control counterpart does not show depressive-like behavior or its characteristic neurochemical abnormalities (Hasegawa *et al.*, 2006:358).

With regards to face validity, the FSL rats resembles depressed humans in that they demonstrate reduced locomotor activity and an associated increase in passive behavior following stress as displayed by greater immobility during the FST (Overstreet & Wegener, 2013:143), resembling behavioral despair evident in MDD (Porsolt, 2000:53). Psychomotor retardation is also evident in FSL rats that have been suggested to be associated with a reduction in locomotor activity as well as reward-seeking behavior (related to anhedonia in MDD) (Overstreet, 1993:51). They also demonstrate an increase in total rapid eye movement (Malkesman *et al.*, 2006:17), alterations in sleep patterns (Overstreet *et al.*, 2005:739) as well as cognitive difficulties (Overstreet *et al.*, 2005:739). Patients with MDD usually experience alterations in appetite (American Psychiatric Association, 2013:970) which is reflected in the FSL rat as reduced body weight when compared to the FRL control (Malkesman *et al.*, 2006:17). The increase in the behavioral vulnerability of the FSL rat to acute or chronic stress, as displayed by its tendency to display anhedonic behavior, is shown by the sucrose consumption test (Kanemaru *et al.*, 2009:529). Comorbidity of cardio-

metabolic illness, including heart disease and diabetes, is evident in MDD. Evaluations of the cardiac function in FSL rats showed a significantly larger myocardial infarct size following ischemia as well as hyperinsulinemia (Overstreet & Wegener, 2013:143). The FSL rat thus has a substantial degree of face validity and therefore has value for studying the underlying neurobiological mechanisms of the illness (Overstreet *et al.*, 2005:739).

On a neurobiological level, the construct validity of the FSL rat is based on multiple abnormalities consistent with various proposed theories of MDD, in particular altered serotonergic (Zangen *et al.*, 1997:2477), cholinergic (Janowsky & Risch, 1984:125), α_2 - adrenergic (Landau *et al.*, 2015:97) and glutamatergic-nitrergic function (Wegener *et al.*, 2010:461) as well as neurotrophic signaling (Elfving *et al.*, 2010:563). Decreased BDNF levels in the hippocampus have been documented in FSL but not in FRL rats (Elfving *et al.*, 2010:563). Moreover a decrease in hippocampal neurogenesis is also documented, thereby providing support for the neuroplasticity hypothesis of MDD (see section 2.1.5.8.) (Neumann *et al.*, 2011:1357).

Contradictory to the monoamine hypothesis, FSL rats exhibit *higher* levels of 5-HT, NA and DA and their metabolites in some mesolimbic regions compared to FRL controls (Malkesman *et al.*, 2006:17; Zangen *et al.*, 1999:243; Zangen *et al.*, 1997:2477). However, there is currently uncertainty as to how this can be translated to human pathology (Neumann *et al.*, 2011:1357). Recent work has revealed a decrease in the density of α_2 - adrenoreceptors in these animals (Landau *et al.*, 2015:97). Considering the important role of these receptors in regulating the release of many monoamine transmitters, any altered expression or functional deficits in these receptors may translate into paradoxical changes and possibly even elevations in monoamine levels (Landau *et al.*, 2015:97). Furthermore, FSL rats are more sensitive to serotonergic drugs, with experiments reporting a positive correlation between the increase in behavioral sensitivity and increased 5-HT receptor number in these animals (Overstreet & Steiner, 1998:261). This observation correlates with MDD, where SSRIs are known to cause hyper excitation and anxiety in depressed individuals due to the up regulated state of 5-HT receptors, which follows from a chronic state of serotonergic insufficiency. FSL rats also present with evidence of immune alterations (Carboni *et al.*, 2010:1037) as well as phase advanced circadian rhythms compared to FRL rats (Overstreet *et al.*, 2005:739). Further characteristics of the FSL rat that emphasizes its stress-vulnerability are differences in the HPA axis, including a heightened CRH response when compared to FRL controls (Yadid *et al.*, 2000:353), as well as heightened NOS activation following stress exposure (Wegener *et al.*, 2010:461).

Considering predictive validity, exaggerated immobility is a key behavioral symptom exhibited by the FSL rat when exposed to the FST. This behavioral abnormality is normalized by a number of well-recognized antidepressant drugs including the classical TCAs and SSRIs (Hasegawa *et al.*, 2006:358; Overstreet & Wegener, 2013:143), as well as atypical antidepressants e.g. agomelatine (Overstreet *et al.*, 2005:739).

Thus the FSL rat model represents a genetic rodent model of MDD that fulfills the criteria of face, construct, and predictive validity (Yadid *et al.*, 2000:353).

2.4 Conclusion

MDD is a heterogeneous disease with many overlapping pathological contributors. Current treatment is mainly based on the amine hypothesis (Healy, 1999:174), although there are significant shortfalls in efficacy with these compounds (Domenici *et al.*, 2010:9166). Much of these deficits in efficacy are due to an inherent misunderstanding of the underlying pathology of the disorder. As a result, attention has shifted from looking at monoamines to considering the involvement of neutrophins, neurogenesis, neuroplasticity, redox and inflammation to aid in the search for novel antidepressant compounds (Duman *et al.*, 2000:732). This study will be specifically considering the redox-inflammatory hypothesis. Various animal models are available to screen new treatment options, with the FSL genetic animal model displaying robust face, construct and predictive validity for this purpose (Yadid *et al.*, 2000:353).

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Chapter 3: Research article

Article to be submitted for publication in *Progress Neuropsychopharmacology and Biological Psychiatry,* entitled:

"Characterisation of the antidepressant-like effects of a commercial extract of *Garcinia mangostana* in a genetic animal model of depression."

Introduction

This chapter presents a full-length article to be submitted for publication in *Progress Neuropsychopharmacology and Biological Psychiatry*, published by Elsevier. The manuscript is presented in the required format prescribed by the Instructions to the Authors, as outlined on the journal website.

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The manuscript will begin with the title, contributing authors and affiliations on a separate page, followed by an Abstract, Graphical abstract, Highlights, Keywords and Abbreviations. Thereafter will follow the main body of the manuscript, including: Introduction, Materials and Methods, Results, Discussion, Conclusion, Author disclosures, Acknowledgements, References. Legends to Figures and Figures are required to be embedded in the article. Supplement 1 presents the quality control data of *Garcinia mangostana* Linn. as provided by the manufacturer.

Authors' contributions

- *I Oberholzer* designed the study in consultation with *BH Harvey and M Möller-Wolmarans* and undertook all the behavioral, analytical and statistical analyses, wrote the first draft of the manuscript, and edited the manuscript after receiving comments from the co-authors.
- *W Dreyer* supervised and assisted in all aspects of the lipid peroxidation analysis.
- *F Viljoen* supervised and assisted in all aspects of the monoamine analysis.
- *M Möller-Wolmarans* co-supervised the study and assisted in the interpretation of the study data.
- *BH Harvey* supervised the study and assisted in the interpretation of the study data, as well as finalized the manuscript for publication.

All co-authors granted permission for the article to be submitted for the purpose of the MSc.

Title page

Title

Characterisation of the antidepressant-like effects of a commercial extract of *Garcinia mangostana* in a genetic animal model of depression.

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Abstract

Background: There is abundant evidence for disorganized redox balance, immune activation and related monoamine changes in major depressive disorder (MDD). As *Garcinia mangostana* Linn (GM) impacts many of these pathways, we investigated whether the powdered pericarp of (GM) affects the above pathophysiological manifestations in a genetic animal model of MDD.

Hypothesis: GM will display antidepressant-like activity in the forced swim test (FST) after acute as well as chronic treatment and will reverse altered monoamines, oxidative stress as well as immune-inflammatory markers in the Flinders Sensitive Line (FSL) rat.

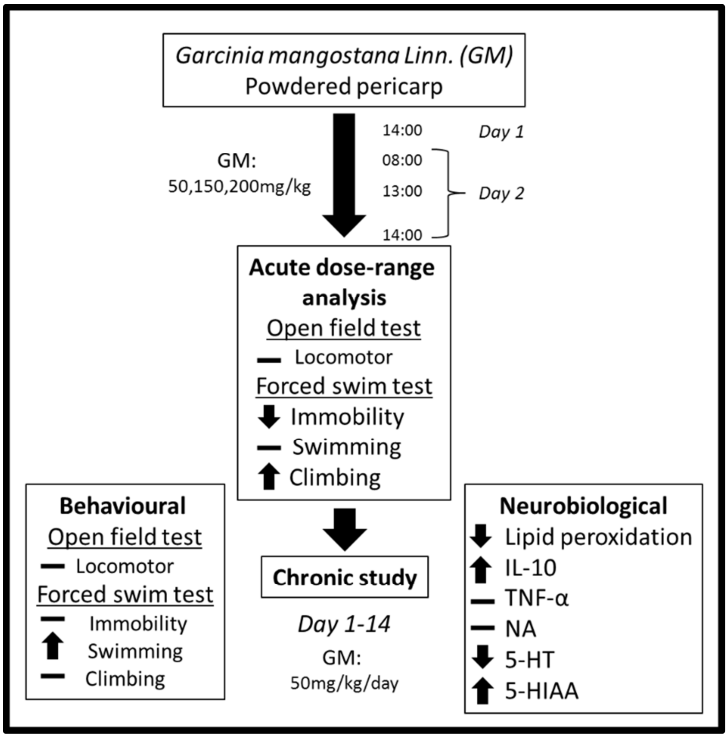
Study Design: The acute dose-dependent effects of GM (50, 150 and 200 mg/kg), imipramine (IMI) (20 mg/kg) versus vehicle were determined in the FST in FSL rats, using Flinders Resistant Line (FRL) rats as controls. Locomotor testing was conducted using the open field test (OFT). Using the most effective dose above and the same behavioral tests, a fixed dose chronic treatment study of GM (50 mg/kg/day x 14 days) was performed and compared to IMI (20 mg/kg/day x 14 days) and vehicle-treated animals. A separate group of animals, receiving the same treatments, were evaluated for regional brain monoamine, lipid peroxidation and immune-inflammatory alterations, and their response to the various drug treatments.

Methods: Behavioral tests included the OFT and the FST. Biological analyses included the HPLC quantification of noradrenaline (NA), serotonin (5-HT) and 5-hydroxyindole-acetic acid (5-HIAA) in the frontal cortex and hippocampus. Lipid peroxidation was analyzed in the hippocampus and pro- and anti-inflammatory cytokines (tumor necrosis factor (TNF)-α and interleukin (IL)-10, respectively) were analyzed in plasma.

Results: FSL rats showed depressive-like behavior with hippocampal lipid peroxidation, although TNF-α, IL-10 and monoamines were unaltered. Acute and chronic treatment with IMI showed antidepressant-like effects by bolstering noradrenergic activity, with chronic treatment lowering limbic 5-HT and NA levels. Chronic IMI treatment also increased IL-10 with a tendency to reverse lipid peroxidation. Acute GM shows noradrenergic antidepressant activity at 50 mg/kg, but a serotonergic action following chronic treatment. GM reduced cortical 5-HT, increased cortico-hippocampal 5-HIAA without affecting NA, reduced hippocampal lipid peroxidation (though not significantly) and elevated plasma IL-10 without affecting TNF-α.

Conclusion: FSL rats present with bio-behavioral changes akin to MDD with evidence of a pro-oxidant state. GM displays antidepressant, antioxidant and anti-inflammatory properties, and offers significant potential as a novel antidepressant compound.

Graphical abstract



Highlights

- The FSL rats showed depressive-like behavior with increased hippocampal lipid peroxidation
- Acute treatment with the pericarp of *Garcinia mangostana* Linn. demontrated antidepressive-like effects in the FST with an underlying noradrenergic mechanism
- Chronic *Garcinia mangostana* Linn. pericarp administration increased serotonergic related swimming behavior
- Potential antioxidant and anti-inflammatory properties were identified

Keywords

Depression; antidepressant; serotonergic; noradrenergic; mangosteen; oxidative stress; treatment, translational animal model.

Abbreviations

MDD: major depressive disorder, GM: *Garcinia mangostana* Linn., 5-HT: serotonin, NA: noradrenaline, DA: dopamine, 5-HIAA: 5-hydroxyindoleacetic acid, SSRI: selective serotonin reuptake inhibitor, ROS: reactive oxygen species, RNS: reactive nitrogen species, FSL: Flinders Sensitive Line, FRL: Flinders Resistant Line, FST: forced swim test, TCA: tricyclic antidepressant, IMI: imipramine, OFT: open field test, ANOVA: analyses of variance, MDA:

malondialdehyde, TBARS: thiobarbituric acid reactive substance, ELISA: enzyme-linked immunosorbent assay

3.1. Introduction

Major depressive disorder (MDD) is a global health challenge, affecting over 120 million people worldwide (Kessler & Bromet, 2013:119), impacting the patient as well as society (Richards, 2011:1117). Genetic predisposition, neurotransmitter abnormalities and chronic stress are but a few factors evident in its pathogenesis (Nestler *et al.*, 2002:13). Although the biogenic amine hypothesis of MDD is the most consistent theory thus far, dysregulated monoamine levels are only one part of a complex set of events underlying the pathophysiology and the response to antidepressants (Pineyro & Blier, 1999:533). Considering the low response rate (50-55%) to antidepressant treatments (Hasler, 2010:155), the identification of new biological targets and novel treatments are crucial.

According to the monoamine hypothesis of MDD, the deficiency of the neurotransmitters, serotonin (5-HT), noradrenaline (NA) and dopamine (DA) in the central nervous system reflects the underlying pathology of the disorder (Neumeister *et al.*, 2004:765). Post-mortem studies have shown a decrease in the levels of 5-hydroxyindoleacetic acid (5-HIAA), the metabolite of 5-HT, in MDD patients together with increased 5-HT_{2A} receptor binding sites in prefrontal cortical sites (Lidberg *et al.*, 2000:395). Regarding NA, a decrease in NA metabolism and NA transporter density is evident in the locus coeruleus of depressed individuals (Klimek *et al.*, 1997:8451), while α_{2A} adrenoceptors are increased in the prefrontal cortex in MDD patients (Valdizán *et al.*, 2010:869). Complicating the picture even more is that increasing monoamine levels, such as 5-HT with a selective serotonin reuptake inhibitor (SSRI), is associated with *reduced* release of frontal cortical NA and DA that is not immediately beneficial on mood (Harvey & Slabbert, 2014:503). Furthermore, antidepressants targeting monoamines show a delayed onset of action which suggests the prerequisite involvement of other downstream events that need to be considered (Hasler, 2010:155).

There is abundant evidence for disorganized redox balance and oxidative stress in both MDD and in animal models of MDD (Maes *et al.*, 2011:676; Mokoena *et al.*, 2015:1). Moreover, the activation of inflammatory, oxidative and nitrosative stress pathways are seen as key pathophysiological factors in MDD (Maes *et al.*, 2009:27). Indeed, the antioxidant and glutathione precursor, N-acetyl cysteine (NAC), has antidepressant effects in animals and has recently demonstrated antidepressant effects in MDD (Berk *et al.*, 2014:628).

A class of compounds that may address disordered immune-inflammatory-redox processes in MDD are the xanthones, abundant secondary metabolites in higher plant families, fungi

and lichen (Negi *et al.*, 2013:1). *Garcinia mangostana* Linn. (GM) is an evergreen fruit tree originating from Indonesia that contain over 85 components including polyphenols, such as xanthones, prodelophinidins anthocyanins, procyanins, tannins and epicatechins including α and γ-mangostin (Pedraza-Chaverri *et al.*, 2008:3227). Xanthones have been found to prevent the accumulation of pro-inflammatory cytokines as well as reverse oxidative damage in the prefrontal cortex of rats exposed to stress (Márquez *et al.*, 2012:729). These findings provide support for their possible application in neurodegenerative and inflammatory disorders. The most abundant xanthone present in the mangosteen fruit, α-mangostin, also shows antioxidant and neuroprotective properties (Pedraza-Chaverrí *et al.*, 2009:491), possibly by virtue of its potent scavenging abilities of cell damaging reactive oxygen species (ROS), by chelating metals (Phyu & Tangpong, 2014:151), or by enhancing the antioxidant effect of endogenous glutathione peroxidase (Pedraza-Chaverri *et al.*, 2008:3227). α-mangostin also demonstrates dose-dependent reduction in lipid peroxidation with neuroprotective actions as a consequence (Pardo Andreu *et al.*, 2010:124). However, while xanthone compounds are putative psychotropic compounds, to the best of our knowledge their ability to correct behavioral and biochemical alterations of relevance for MDD has not been studied in animals or in humans. Clinical trials in patients with schizophrenia using GM as adjunctive treatment to second generation antipsychotics are underway. Moreover, evaluating the effects of GM on the bio-behavioral alterations in an animal model of MDD would clarify its antidepressant potential, as well as shed light on its mechanism of action.

The Flinders Sensitive Line (FSL) rat presents with robust face, construct and predictive validity for MDD (Overstreet & Wegener, 2013:143). FSL rats demonstrate reduced locomotor activity and an associated increase in passive behavior following stress as displayed by greater immobility in the forced swim test (FST) (Overstreet & Wegener, 2013:143), while these behavioral abnormalities can be normalized by chronic treatment with various classes of antidepressants (Hasegawa *et al.*, 2006:358; Overstreet & Wegener, 2013:143). On a neurobiological level, these animals display multiple abnormalities consistent with proposed theories of MDD, such as serotonergic, cholinergic, glutamate-nitric oxide, metabolic and immune dysfunction (Overstreet & Wegener, 2013:143).

In this study we hypothesized that raw freeze-dried powder of GM pericarp (see certificate of analysis in *supplement 1*) will show antidepressant properties in the FSL model of MDD, with reference to selected symptoms of MDD as assessed in the FST, viz. despair and learned helplessness (Cryan *et al.*, 2005:547). Moreover, the involvement of altered monoamines, redox and inflammatory alterations in the FSL rat, and their reversal by GM, will also be implicated. In all instances, the bio-behavioral effects of GM in FSL rats will be compared to the reference antidepressant, imipramine (IMI).

3.2 Materials and methods

3.2.1 Animals

Male FSL rats (n = 66) and a corresponding behavioral control line, viz. FRL rats, were used for the study. All animals were housed under conditions of constant temperature (22 ± 1 ℃) and humidity (50%) with a 12:12-hour light/dark cycle (lights on 06:00 to 18:00). All animals were housed in groups of 3-4 rats/cage in identical polypropylene cages (380 mm x 380 mm x 230 mm) with positive air pressure with an air change rate of 18 times per hour. Food (standard rat chow) and water were provided ad libitum.

The study and all animal procedures conformed to the guidelines of the South African National Standards (SANS 10386, 2008:232) and were approved by the animal research ethics committee (AnimCare) of the North-West University (approval number: NWU-00149-14-S5).

3.2.2 Drug treatment

Imipramine hydrochloride (Sigma Aldrich, Kempton Park, South Africa) was dissolved in double distilled water and administered orally at a fixed dose of 20 mg/kg by oral gavage (Gigliucci *et al.*, 2014:1349). GM powder (Industrial Analytical, Kyalami, South Africa) is insoluble in water. A suspension in water is impractical due to rapid precipitation before being administered to the animal. Therefore, in order to ensure accurate oral dosing, the drug was carefully weighed using a Sartorius® BP211D balance and mixed in a 0.1% xanthan gum solution to aid suspension. GM was administered by oral gavage.

3.2.3 Study design

Since no treatment-related data on the use of GM in rats was available, an acute dose response analysis was initially performed in FSL rats (with FRL rats as control) to assess possible toxicity and to establish a usable dose for chronic treatment. This would also serve as a proof of concept study, i.e. that GM has antidepressant potential. Based on studies conducted on GM in rats using a dosage range of 100-200 mg/kg/day (Devi Sampath & Vijayaraghavan, 2007:336; Phyu & Tangpong, 2014:151), the acute dose range analysis with GM was tested over a dosage range of 50, 150 and 200 mg/kg.

3.2.3.1 Acute study

In the acute study, GM was compared to IMI, a reference antidepressant (Maubach *et al.*, 2002:609). Five treatment groups were set up in FSL rats, with each group having 6 rats. One group received water, acting as drug naïve controls. The rest of the groups received 20

mg/kg IMI or 50, 150 or 200 mg/kg GM, respectively. Each group was dosed three times – 24 hours, 6 hours and 1hour before commencement of the behavioral tests. The latter tests included the open field test (OFT) to determine general locomotor performance followed by the FST.

3.2.3.2 Chronic study

The chronic study consisted of a behavioral *and* a neurochemical cohort, performed in FSL rats with a vehicle-FRL group as treatment-strain control. Six treatment groups were set up for each cohort, with one group receiving 20 mg/kg IMI (n = 12 FSL) and another receiving an appropriate dose of GM dictated by the above acute study (n = 12 FSL). In order to control the vehicle groups used, one treatment group received 0.1% xanthan gum (vehicle for GM; n = 6 FSL, n = 6 FRL) and another received water (vehicle for IMI; n = 6 FSL, n = 6 FRL). A comparative behavioral analysis on these two vehicle groups returned no significant differences (data not shown), whereupon the water and gum groups were pooled and hereafter referred to jointly as the “vehicle” group. The groups received the indicated drug treatment for 14 days. On day 15 the rats in the behavioral cohort were subjected to behavioral testing in the OFT taking place 12 hours after the last treatment and at the start of the dark cycle and the FST conducted the next morning.

Rats used in the neurochemical cohort were randomly divided into the same treatment groups as the behavioral study. On day 15 the animals were sacrificed by decapitation with trunk blood and brain (frontal cortex and hippocampus) harvested for the various neurochemical tests, as described below.

3.2.4 Behavioral tests

Following the vehicle/drug treatment for the acute dose-range analysis and the behavioral cohort of the chronic treatment study, the OFT and the FST were performed, as described below.

3.2.4.1 Open field test

The OFT measures general locomotive behavior of the animal and is used to ensure that any change observed in the mobility of the animals in the FST is not due to general alterations in locomotor activity but has a psychogenic origin (Overstreet *et al.*, 2005:739). Locomotor activity was measured over a 5 minute period in the open field arena. Rats were placed individually into a square arena (100 cm × 100 cm × 50 cm), illuminated with red light (40 lux), and their movement recorded on video. The total distance moved (cm) was scored using EthoVision XT software (Noldus Information Technology, Wageningen, Netherlands).

3.2.4.2 Forced Swim Test

The FST is a validated method to screen for antidepressant properties (Porsolt, 2000:53). When placed in an inescapable water cylinder, rats initially attempt to escape, but once all efforts fail, they adopt an immobile posture that bears a resemblance to behavioral despair observed in MDD (Lucki, 1997:523). Antidepressant treatment evokes persistent escape-directed behavior for a prolonged period (Porsolt, 2000:53). The FST was performed as previously described (Liebenberg *et al.*, 2010:540) directly after the OFT in the acute dose range analysis and the next morning after the OFT in the chronic study.

After 30 minutes of habituation in the testing room, with a light intensity of 200 lux, the rats were placed into Perspex® cylinders (diameter 18 cm, height 60 cm), containing 30 cm of water maintained at a temperature of 25 ℃ (Porsolt , 2000:53). The behavior was recorded on video for 7 minutes with the first and last minute omitted from the final results. Rats were deemed immobile when they only conducted the necessary movements to keep their heads above the water. Swimming was defined as horizontal movements on the water surface while climbing was defined as upward-directed actions at the cylinder wall (Liebenberg *et al.*, 2010:540). Immobility, swimming and climbing behaviors were scored manually by a researcher blinded to treatment and expressed in the amount of time (seconds) spent performing each behavior. In this instance, EthoVision XT software was not used to analyze the FST as it was found unsuitable to accurately distinguish between the different behaviors.

3.2.5 Neurobiological studies

3.2.5.1 Preparation of brain tissue

The animals were decapitated and the brain immediately dissected on ice into the right and left cerebral hemispheres. The olfactory bulb was removed and the frontal cortex was dissected with the corpus callosum anterior tip being the external limit (Toua *et al.*, 2010:492). The remainder of the brain was placed on its ventral side and the hippocampus removed from the remainder of the cortex (Spijker, 2011:13). The brain areas were immediately fixed in liquid nitrogen and stored at −80 ℃. On the day of the analysis the tissue was thawed on ice, weighed and a 10% (weight/volume) tissue homogenate prepared using phosphate buffered saline.

3.2.5.2 Monoamine analysis

Quantification of NA, 3-methoxy-4-hydroxyphenylglycol (MHPG), 5-HT and 5-HIAA were performed using a high performance liquid chromatography (HPLC) system with electrochemical detection, as described previously (Möller *et al.*, 2013:687). Briefly,

monoamine concentrations in the samples were determined by relating the area under the peak of each monoamine to that of the internal standard, isoprenaline (range 5–50 ng/ml). An Agilent 1200 series HPLC, equipped with an isocratic pump, autosampler, and coupled to an ESA Coulochem Electrochemical detector was used, together with Chromeleon® Chromatography Management System version 6.8. Linear standard curves, with a regression coefficient greater than 0.99, were set up in this range. Monoamine concentrations were expressed as ng/mg wet weight of frontal cortical or hippocampal tissue (mean ± SEM).

3.2.5.3 Lipid peroxidation analysis

Lipid peroxidation was measured through quantitative determination of thiobarbituric acid reactive substance (TBARS) in hippocampal brain tissue using an enzyme-linked immunosorbent assay (ELISA) kit (R&D systems, Minneapolis, U.S.A). The assay was performed according to manufacturer's instructions. Tetramethoxypropane was used as standard. Absorbance was measured using a Spectronic 20 spectrophotometer at 532 nm. Data are expressed as pmol MDA formed/mg wet brain tissue.

3.2.5.4 Preparation of plasma

After decapitation, trunk blood was collected in 4 ml vacutainer tubes (SGVac) coated with K_2EDTA anticoagulant solution and kept on ice. The samples were centrifuged at 14,000 rpm at 4 ºC for 15 min, and the plasma stored at -80ºC. On the day of analysis the plasma samples were thawed on ice, centrifuged again and the plasma used for the determination of immune-inflammatory markers.

3.2.5.5 Immune-inflammatory analysis

The plasma levels of tumor necrosis factor (TNF)-α (pro-inflammatory) and interleukin (IL)-10 (anti-inflammatory) were measured in duplicate using a sandwich ELISA kit according to the manufacturer's protocol (Biolegend, San Diego, U.S.A and Sigma Aldrich, Kempton Park, South Africa). Briefly, 100 µl of the plasma sample were incubated in specific capture antibody coated wells. The plates were analyzed using a microplate reader at 450 nm. The final concentration of each cytokine was expressed as pg/mL of plasma used.

3.2.6 Statistical analysis

Multiple comparisons relative to the FSL control rats were analyzed by a one-way analyses of variance (ANOVA) followed by the Bonferroni post-hoc test. Grubbs' test was used to identify outliers. GraphPad Prism® (version 5.00, San Diego, U.S.A) was used for all

statistical analyses and graphical representation. Data are presented as mean ± SEM with a value of $p < 0.05$ regarded as statistically significant.

3.3 Results

3.3.1 Acute dose-range analysis

3.3.1.1 Open field test

A one-way ANOVA of the data showed no significant treatment*distance moved interaction ($F (4, 25) = 0.51$, $P = 0.73$, data not shown). Consequently, no effect on locomotor performance was evident for IMI as well as the 50, 150 and 200 mg/kg doses of GM, compared to the FSL vehicle group.

3.3.1.2 Forced swim test

A one-way ANOVA of the data revealed a significant treatment*immobility ($F (5.35) = 8.69$, $p < 0.0001$); treatment*climbing ($F (4.30) = 6.99$, $p = 0.0001$); treatment*swimming interaction $F (5.35) = 6.63$, $p = 0.0002$).

The Bonferroni post-hoc tests indicated a significant increase in immobility in the FSL vehicle ($p < 0.0001$) vs. the FRL vehicle group (Fig. 1A). Immobility was significantly decreased in the FSL IMI ($p = 0.004$) and the FSL GM (50 mg/kg) groups ($p = 0.02$) vs. the FSL vehicle group, with GM 150 mg/kg and 200 mg/kg ineffective (Fig. 1A).

The FSL vehicle group demonstrated significantly reduced climbing behavior vs. FRL vehicle (Fig. 1B; $p = 0.0004$). FSL IMI ($p = 0.001$) and FSL GM (50 mg/kg, $p = 0.03$) significantly increased climbing vs. the FSL vehicle group, with GM 150 mg/kg and 200 mg/kg ineffective (Fig. 1B).

FSL rats showed significantly reduced swimming behavior vs. FRL vehicle controls (Fig. 1C; $p = 0.003$). However, no significant differences were observed between any of the drug treatment groups with respect to swimming behavior vs. the FSL vehicle group (Fig. 1C).

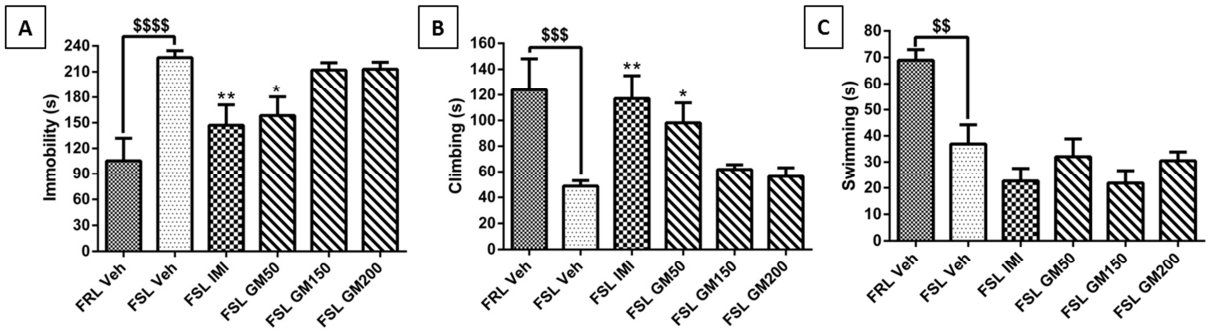


Figure 1. The effect of acute administration of IMI (20 mg/kg) and GM (50, 150 and 200 mg/kg respectively) vs. water (vehicle control) in FSL rats, as well as the strain difference between the FRL and FSL water control groups on A: Immobility, B: Climbing and C: Swimming behavior.

($^{\$\$}p < 0.01$ vs. FRL Veh, $^{\$\$\$}p < 0.001$ vs. FRL Veh, $^{\$\$\$\$}p < 0.0001$ vs. FRL Veh, *p < 0.05 vs. FSL Veh; **p < 0.01 vs. FSL Veh; one-way ANOVA, the Bonferroni post-hoc test)

3.3.2 Chronic treatment study

3.3.2.1 Open field test

A one-way ANOVA of the data showed no significant treatment*distance moved interaction ($F(2, 33) = 1.21$, $p = 0.31$, data not shown). Consequently no effect on locomotor performance was noted in the IMI as well as the GM groups vs. FSL vehicle control.

3.3.2.2 Forced swim test

A one-way ANOVA of the data revealed a significant treatment*immobility ($F(3.44) = 14.93$, $p < 0.0001$); treatment*climbing ($F(3.44) = 9.80$, $p < 0.0001$); as well as a treatment*swimming interaction ($F(3.44) = 14.46$, $p < 0.0001$).

The Bonferroni post- hoc tests indicated a significant increase in immobility in FSL vs. FRL vehicle groups (Fig. 2A; $p < 0.0001$), and a significant decrease in climbing ($p < 0.0001$) and swimming ($p < 0.0001$) behavior (Fig. 2B & C). Significantly decreased immobility ($p = 0.001$) together with increased climbing behavior ($p = 0.006$) was observed in the FSL IMI group vs. the FSL vehicle group (Fig. 2A & B). GM tended to reverse immobility in FSL rats, ($p = 0.37$), and did not reverse reduced climbing behavior. IMI failed to alter swimming behavior vs. FSL vehicle control (Fig. 2C), although a significant increase in swimming behavior was noted in the FSL GM (50 mg/kg) group ($p = 0.005$) vs. FSL vehicle control (Fig. 2C).

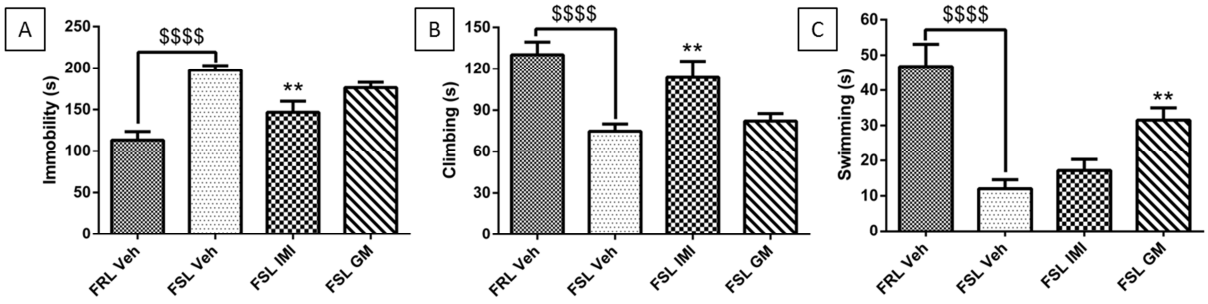


Figure 2. Effect of chronic administration of IMI (20 mg/kg) and GM (50 mg/kg) compared to vehicle treatment in FSL rats, as well as the strain difference between the FRL and FSL vehicle control groups on A: Immobility, B: Climbing and C: Swimming behavior.

($^{\$\$\$\$}p < 0.0001$ vs. FRL Veh, **p < 0.01 vs. FSL Veh; one-way ANOVA with the Bonferroni post-hoc test).

3.3.2.3 Monoamine analysis

Noradrenaline: MHPG levels were below the limit of detection and could not be reported. A one-way ANOVA demonstrated significant treatment*NA interactions in the frontal cortex ($F(3.44) = 43.03$, $p < 0.0001$, Fig. 3A) and hippocampus ($F(3.44) = 18.86$, $p < 0.0001$, Fig. 3D).

NA levels did not differ between FSL and FRL vehicle groups in either brain region (Fig. 3A & D). However, FSL IMI significantly decreased NA levels in the frontal cortex ($p < 0.0001$, Fig. 3A) and hippocampus ($p < 0.0001$, Fig. 3D) vs. the FSL vehicle group. No significant difference was noted between the FSL and FRL vehicle and the GM (50 mg/kg) groups in the frontal cortex ($p = 0.09$, $p = 0.15$, respectively) or hippocampus ($p = 0.97$, $p < 0.99$, respectively).

Serotonin: One-way ANOVA demonstrated significant treatment*5-HT interactions in the frontal cortex ($F(3.44) = 19.70$, $p < 0.0001$, Fig. 3B) but less so in the hippocampus ($F(3.44) = 2.705$, $p = 0.06$, Fig. 3E). No significant differences were found with regard to 5-HT between the FSL and FRL vehicle groups in both brain regions ($p = 0.23$, $p = 0.96$, respectively). The Bonferroni post-hoc tests of the data showed that IMI ($p < 0.0001$) and GM (50 mg/kg, $p < 0.0001$) significantly decreased 5-HT levels in the frontal cortex (Fig. 3B), although only IMI ($p = 0.02$) significantly decreased 5-HT in the hippocampus vs. FSL vehicle control (Fig. 3E), with GM having no effect in this regard ($p = 0.82$).

5-HIAA: A one-way ANOVA demonstrated significant treatment*5-HIAA interactions in the frontal cortex ($F(3.44) = 19.62$, $p < 0.0001$, Fig. 3C) and hippocampus ($F(3.44) = 16.59$, $p < 0.0001$, Fig. 3F). The Bonferroni post-hoc testing showed a significant increase in 5-HIAA in the hippocampus ($p = 0.004$) but not in the frontal cortex in FSL vs. FRL vehicle-treated animals. The FSL GM (50 mg/kg) group showed significantly increased 5-HIAA levels in the

frontal cortex ($p < 0.0001$, Fig. 3C) and hippocampus ($p = 0.03$, Fig. 3F) vs. the FSL vehicle group, while the FSL IMI group presented with significantly reduced hippocampal 5-HIAA levels vs. the FSL vehicle group ($p = 0.014$, Fig. 3F), without affecting frontal cortical levels (Fig. 3C).

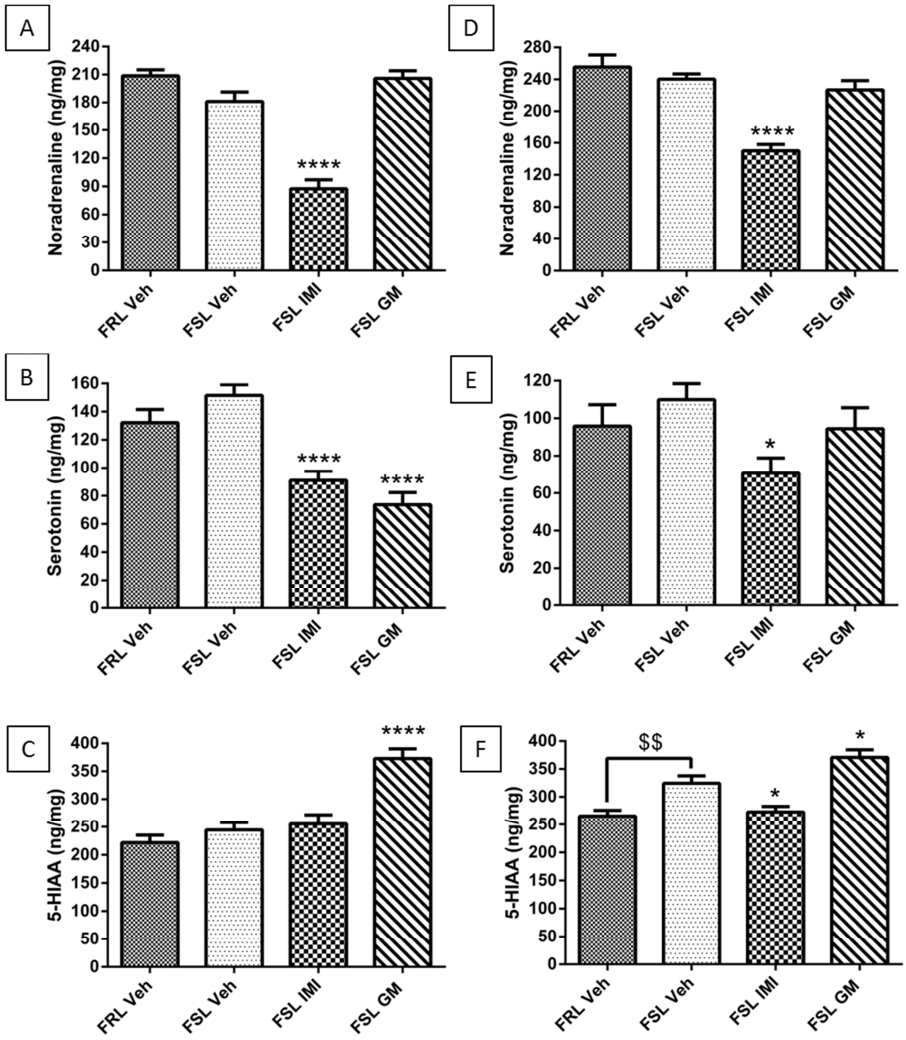


Figure 3. NA, 5-HT and 5-HIAA levels in the frontal cortex (A), (B), (C) and hippocampus (D), (E), (F) of vehicle-treated FRL rats, and FSL rats treated with the various drugs, as indicated.

($^{\$\$}p < 0.01$ vs. FRL Veh, $*p < 0.05$ vs. FSL Veh; $****p < 0.0001$ vs. FSL Veh; one-way ANOVA, the Bonferroni post-hoc test).

3.3.2.4 Hippocampal lipid peroxidation

A one-way ANOVA revealed a significant treatment*lipid peroxidation interaction ($F(3.44) = 3.30$, $p = 0.03$).

The Bonferroni post-hoc testing indicated a significant increase in lipid peroxidation in FSL vs. FRL vehicle-treated groups ($p = 0.02$). A trend to decreased hippocampal lipid

peroxidation was observed in the FSL IMI (p = 0.08) and FSL GM (50 mg/kg) groups (p = 0.08) vs. the FSL vehicle control group (Fig. 4).

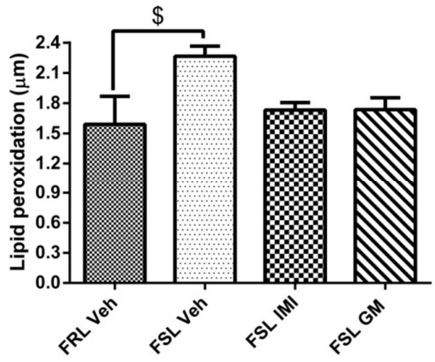


Figure 4. Lipid peroxidation in the hippocampus of FSL vs. FRL vehicle-treated controls, and the effect of chronic administration of imipramine (IMI) (20 mg/kg) and GM (50 mg/kg) compared to the FSL vehicle-treated control group. ($^{\$}p < 0.05$ vs. FRL Veh, one-way ANOVA, the Bonferroni post-hoc test).

3.3.2.5 Immune-inflammatory cytokines

A one-way ANOVA revealed a significant treatment*IL-10 interaction ($F(3.40) = 21.85$, $p < 0.0001$) but no significant treatment*TNF-α interaction ($F(3.42) = 2.40$, $p = 0.08$).

The Bonferroni post-hoc testing found that neither TNF-α (Fig. 5A) nor IL-10 (Fig. 5B) was elevated in FRL and FSL vehicle groups ($p > 0.99$ in both cases). TNF-α remained unaltered in any treatment group (IMI: $p > 0.99$, GM: $p = 0.73$, Fig. 5A) vs. FSL vehicle control. However, IL-10 was significantly elevated in the FSL IMI ($p = 0.0004$) and FSL GM (50 mg/kg) groups (Fig. 5B, $p < 0.0001$).

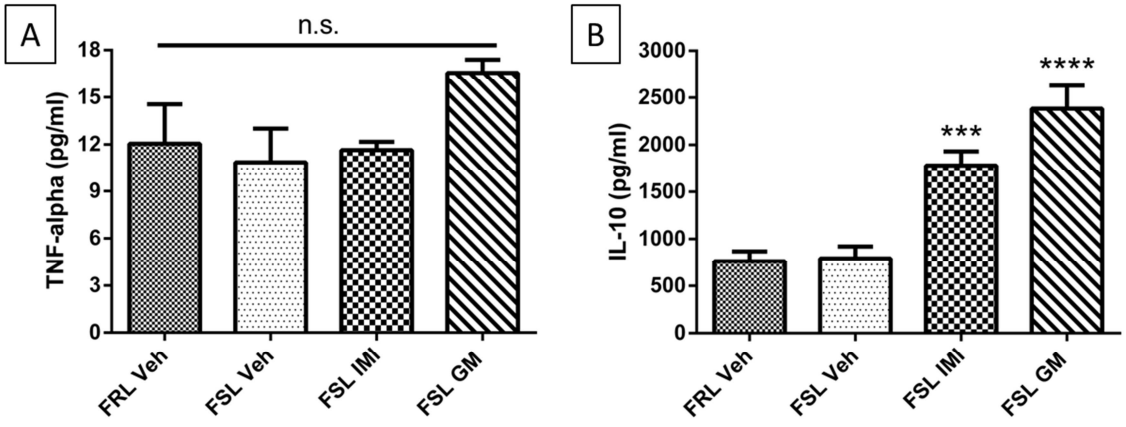


Figure 5. Plasma TNF-α (A) and IL-10 (B) levels in FSL vs. FRL vehicle-treated controls, and the effect of chronic administration of IMI (20 mg/kg) and GM (50 mg/kg) in FSL rats vs. the FSL vehicle group.

(***$p < 0.001$ vs. FSL Veh, ****$p < 0.0001$ vs. FSL Veh, one-way ANOVA, the Bonferroni post-hoc test).

3.4 Discussion

The most important findings of the current study are that GM displayed a statistically significant acute antidepressant-like effect at a dosage of 50 mg/kg. Acute GM presented with an underlying noradrenergic mode of action, as seen by increased climbing behavior. After chronic administration it is transformed to a serotonergic related mechanism when the swimming movement became dominant. FSL rats presented with depressive-like behavior associated with lipid peroxidation in the frontal cortex and hippocampus, although TNF-α, IL-10 and monoamine levels remained unaltered. As expected, acute and chronic IMI was antidepressant in this model and selectively bolstered noradrenergic activity, with an associated increase in anti-inflammatory IL-10 and a tendency to reverse lipid peroxidation. Chronic treatment of FSL rats with IMI resulted in significantly reduced 5-HT and NA in both the frontal cortex and the hippocampus, with GM also reducing 5-HT levels in the frontal cortex while it increased cortical and hippocampal 5-HIAA. Chronic GM did not alter NA levels in either brain region. Finally, chronic GM treatment reduced hippocampal lipid peroxidation (although not significantly, $p = 0.08$) while it significantly elevated plasma IL-10 levels.

Results from both the acute dose-range analysis as well as the chronic study showed no significant effect of any treatment on the locomotor activity of FSL rats in the OFT. These results are important as it indicates that any differences in immobility observed in the FST cannot be attributed to manipulation stress and/or drug-induced changes in locomotor activity.

This is the first dose-related study with GM that investigated its antidepressant-like effects. The acute dose-range analysis with GM identified a dose of 50 mg/kg to have the most prominent antidepressant-like properties. The absence of any adverse locomotor effects suggests that this dose is not acutely toxic, and to be without prominent sedating and/or other motor activating or inhibitory effects that may complicate interpretation of the FST data. A dosage of 100 mg/kg, administered orally, appeared neuroprotective against 3-nitropropionic acid that induced Huntington's disease-like symptoms in rats (Mehan *et al.*, 2014:1). Indeed, FSL rats manifest pronounced reductions in climbing and swimming behaviors, indicative of reduced noradrenergic and serotonergic processes, respectively (Cryan *et al.*, 2005:547). These responses are required to maintain adequate coping in the face of adversity (forced swim stress) and to counter behavioral despair that is typical of the FSL rat. Importantly, IMI (20 mg/kg) and GM (50 mg/kg, but not the higher doses) significantly reversed immobility in FSL rats and significantly increased climbing *but not* swimming behavior (Fig. 1A & B, respectively). These findings are congruent with a noradrenergic mode of action for IMI, which is regarded as a preferential inhibitor of NA re-

uptake (Brand *et al.*, 2015:324). Moreover, these findings also link the antidepressant-like action of GM to a preferential noradrenergic response, at least when administered acutely. Interestingly, neither compound was associated with enhanced swimming behavior, seemingly relegating 5-HT to a lesser role in the acute antidepressant-like effects of IMI and GM (Fig. 1C). A dose of 50 mg/kg GM was therefore deemed effective and utilized in the chronic studies.

In the chronic treatment study, vehicle-treated FSL animals presented with significantly increased immobility together with decreased climbing and swimming behavior compared to their FSL controls (Fig. 2). These findings are in agreement with that observed in the acute study, and reaffirms that FSL rats have reduced noradrenergic and serotoninergic activity that may underlie its prominent depressive-like behavior, evidenced by assuming an immobile posture that resembles behavioral despair in MDD (Lucki, 1997:523). Corresponding to the acute dose-range analysis, chronic treatment with IMI (20 mg/kg) significantly reversed immobility in FSL rats, and selectively increased climbing behavior (Fig. 2A & B respectively). These findings for IMI agree with data found in previous studies (Harvey *et al.*, 2010:1580; Morley-Fletcher *et al.*, 2004:841). Again the absence of any effect on swimming behavior (Fig. 2C) is perhaps unusual, considering the dual noradrenergic and serotonergic mode of action of IMI (Baldessarini, 2006:429), although similar findings have been described previously (Schramm *et al.*, 2001:4875; Harvey *et al.*, 2010:1580). Chronic GM did not significantly reduce immobility (Fig. 2A; $p = 0.3734$), suggesting that the 50 mg/kg dose shown to be effective in acute treatment may not be optimized for chronic treatment. Interestingly, while acute GM did not affect climbing behavior in FSL rats (Fig. 1B), chronic administration significantly increased swimming behavior (Fig. 2C), thus suggesting the presence of an underlying serotonergic mechanism of action and warranting re-evaluation of its behavioral effects.

To evaluate the correlation between the above behavioral data and an underlying neurobiological mechanism, monoamine levels were analyzed in FSL and FRL rats that received vehicle, as well as FSL rats after chronic treatment with GM and IMI. Contradictory to the biogenic amine hypothesis, FSL rats showed an *increase* in monoamines as well as normalization of these levels by antidepressant treatment (Malkesman *et al.*, 2006:17). Despite the behavioral differences regarding serotonergic related swimming behavior between the FRL and FSL vehicle groups, 5-HT and 5-HIAA levels were not significantly elevated in the frontal cortex and hippocampus in FSL rats compared to FRL animals (Fig. 3), with 5-HIAA significantly elevated in the hippocampus (Fig. 3F). Importantly, IMI *and* GM reversed this increase in 5-HT in the frontal cortex (Fig. 3B), with just IMI significantly decreasing hippocampal 5-HT levels (Fig. 3E). This may explain the relative lack of

serotonergic behavior displayed by IMI in the FST. Of particular significance, GM significantly elevated 5-HIAA levels in both these brain regions (Fig. 3C & F). Previous results have indicated that chronic treatment with desipramine (a TCA derivative of IMI) reduced 5-HT as well as 5-HIAA levels in the prefrontal cortex and hippocampus of FSL rats (Zangen *et al.*, 1997:2477), which is in line with the IMI results presented here. It is possible that a reduction in 5-HT release from nerve terminals may result in a compensatory increase in 5-HT synthesis via a feedback mechanism (Adell *et al.*, 1988:1678). Thus, IMI increases the synaptic availability of NA that in turn leads to the desensitization of α_2 receptors that control the release of 5-HT (Mongeau *et al.*, 1997:145) (Fig. 5.2 & 5.3). Recent work has also revealed a decrease in the density of α_2 adrenoceptors in FSL rats (Landau *et al.*, 2015:97). This may explain our results; although the significant increase in cortical and hippocampal 5-HIAA by GM is clearly more complex. However, the GM-induced reduction in 5-HT (Fig. 3B) and the increase in 5-HIAA (Fig. 3C & F) can possibly be attributed to increased catabolism of 5-HT leading to elevated levels of the 5-HIAA metabolite. Increased 5-HT turnover explains reduced swimming in FSL rats (Fig. 2C), which in turn can be correlated with the significant increase in 5-HT related swimming behavior observed following chronic GM administration to FSL animals (Fig. 2C).

Considering cortical and hippocampal NA levels, FSL rats show a non-significant decrease in NA in the frontal cortex and hippocampus (Fig. 3A & D), which aligns with the biogenic amine hypothesis. However, chronic IMI treatment further and significantly reduced NA levels in both the frontal cortex and hippocampus of FSL animals (Fig. 3A & D). This is in accordance with the results obtained by Zangen et al (1999), demonstrating that chronic antidepressant treatment significantly decreases cortical and hippocampal NA levels in FSL rats (Zangen *et al.*, 1999:243), and which has more recently been described as the basis for antidepressant-induced emotional blunting and cognitive deficits (Harvey & Slabbert, 2014:503). On the other hand, chronic treatment with GM had no attenuating effect on frontal cortical or hippocampal NA levels (Fig. 3A & D). Nevertheless, GM still managed a selective enhancement of climbing behavior after acute treatment, thus indicative of an underlying noradrenergic mechanism. This is especially interesting, as exerting an antidepressant response *without* suppressing cortical monoamines, especially NA (and DA), is increasingly being seen as a desirable property of an antidepressant (Harvey & Slabbert, 2014:503).

The role of the 5-HT_{2C} receptor in mood regulation and antidepressant action has gathered considerable attention (Brand *et al.*, 2015:324). Interestingly, the xanthone constituent of GM, γ-mangosteen, has demonstrated 5-HT_{2C} receptor competitive antagonism *in vivo* (Sukma *et al.*, 2011:450). Since post-synaptic 5-HT_{2C} receptor activation abrogates frontal

cortical release of NA (Brand *et al.*, 2015:324) the initial increase in NA presumed to occur after acute administration of GM (based on climbing data presented in Fig. 1B) could be attributed to $5HT_{2C}$ receptor antagonism resulting in disinhibition of frontal cortical NA release (Fig. 6, points 1 & 2) (Carr & Lucki, 2011:265). Various established antidepressants, including agomelatine, mianserin and mirtazepine, are potent 5-HT_{2C} antagonists (Chenu *et al.*, 2013:275). A recent study of agomelatine demonstrated time-related direct and indirect modulation of monoaminergic neuronal activity in the brain stem (Chenu *et al.*, 2013:275). The latter study highlights that an acute two-day regimen of agomelatine enhances locus coeruleus-NA firing rate (Fig. 6, point 2) without affecting raphe nucleus (5-HT) firing activity (Fig. 6, point 4) (Chenu *et al.*, 2013:275). However, 14 days of agomelatine treatment is associated with an increase in serotonergic firing (Fig. 6, point 6), possibly due to α_{2A} receptor desensitization (Fig. 6, point 5). The corresponding increase in 5-HT firing activity then inhibits NA release possibly via a negative feedback mechanism at 5-HT_{2C} receptors, resulting in normalization of NA levels (Fig. 6, points 7,6,8) (Chenu *et al.*, 2013:275). This would explain increased serotonergic behavior (swimming) following chronic GM administration (but not with IMI), as well as increased 5-HIAA levels in the frontal cortex. It also explains the apparent normalization of frontal cortical and hippocampal NA levels after chronic GM treatment.

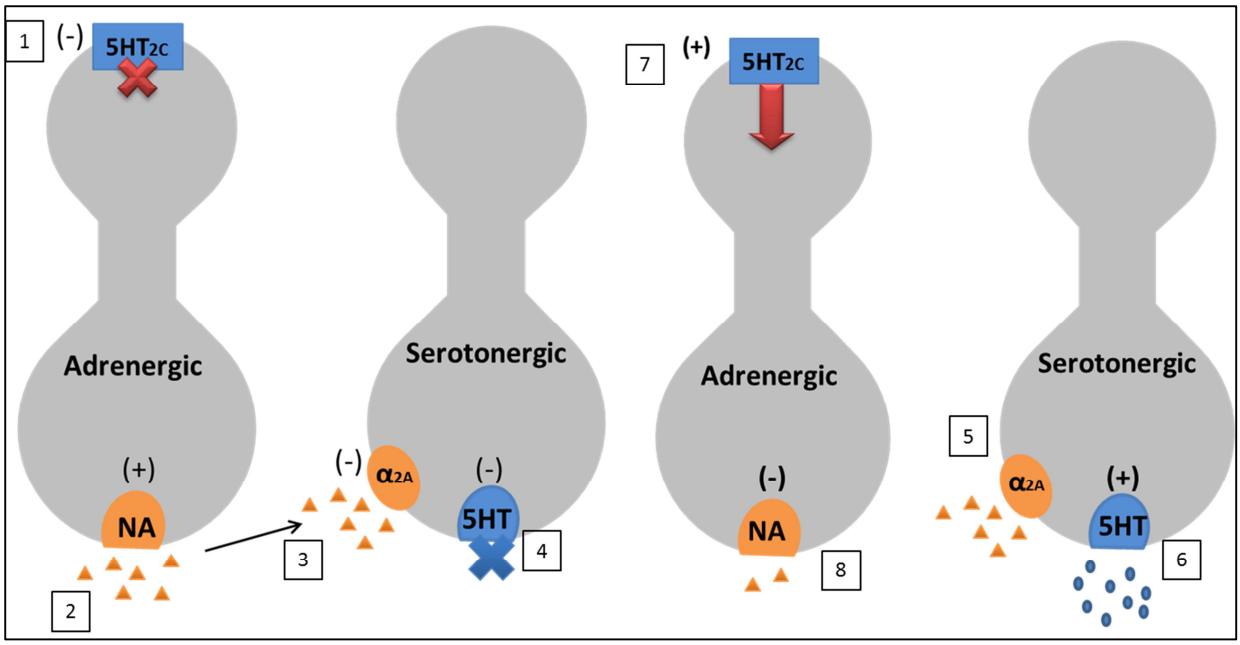


Figure 6. GM (acute mechanism): $5HT_{2C}$ receptor antagonism on the noradrenergic cell body (1) leads to an increase in NA release (2), resulting in the stimulation of inhibitory α_{2A} receptors on serotonergic neurons (3) to reduce 5-HT neurotransmission (4).

GM (chronic mechanism): Negative feedback mechanism, overstimulation and desensitization of α_{2A} receptors on serotonergic neurons (5) leading to an increase in 5-HT release (6) and a stimulation of somatodendritic $5HT_{2C}$ receptors (7) to abrogate and/or normalize NA neurotransmission (8).

GM: *Garcinia Mangostana* Linn., 5HT: serotonin, NA: noradrenaline, (-): inhibitory effect, (+): stimulation

That MDD has been described as a pro-oxidative state (Leonard & Maes, 2012:764), and with GM displaying various redox-related actions (Pedraza-Chaverrí *et al.*, 2009:491), it suggests that the above-described effects of GM on behavior and neurochemistry may occur via direct or indirect normalization of immune-inflammatory processes. The brain contains high levels of polyunsaturated fatty acids, while low concentrations of endogenous antioxidant enzymes (Sarandol *et al.*, 2007:67) make it especially vulnerable to oxidative stress (Maes *et al.*, 2011:676). Any reduction in antioxidant capacity will impair protection against ROS causing damage to fatty acid membrane lipids (Maes *et al.*, 2011:676). Furthermore, altered redox is well-known to engender changes in monoamine synthesis and release (Möller *et al.*, 2013:687), while antioxidants such as NAC are antidepressant (Ferreira *et al.*, 2008:747; Berk *et al.*, 2014:628). Of relevance here, FSL rats present with reduced hippocampal volume and deficits in neuroplasticity not unlike that in MDD (Neumann *et al.*, 2011:1357). Hippocampal lipid peroxidation was significantly elevated in FSL rats while both chronic IMI or GM treatment reversed said lipid peroxidation, although missing significance in both instances (Fig.4; $p = 0.08$ for both). These data not only link hippocampal toxicity to depressive symptoms in the FSL rat, but also show that these pathological changes are amenable to treatment with a conventional antidepressant (IMI) as well as GM. Likewise, previous studies have shown that α-mangosteen as well as IMI reduce hippocampal lipid peroxidation in a dose dependent manner (Pardo Andreu *et al.*, 2010:124; Reus *et al.*, 2010:325).

A precursor for oxidative stress is inflammation (Brand *et al.*, 2015:324). Immune-inflammatory cytokines play a central role in immune signaling and can effectively cross the blood–brain barrier, driving both central and peripheral immune activation (Brand *et al.*, 2015:324). Under our conditions of study, FSL rats did not present with overt changes in TNF-α or IL-10 (Fig .5), despite earlier studies showing significantly increased levels of pro-inflammatory IL-1α in FSL compared to FRL rats (Brand *et al.*, 2015:324). However, while pro-inflammatory TNF-α levels remained unaltered following IMI or GM treatment, both compounds significantly increased IL-10, an anti-inflammatory cytokine (Fig. 5B). The earlier mentioned antidepressant and neurochemical actions induced by both IMI and GM can thus be causally associated with an anti-inflammatory action. In support of this, peripheral TNF-α administration increases central 5-HIAA levels (Clement *et al.*, 1997:981), an observation not unlike that described in our findings (Fig. 3C & E). Interestingly, TNF-α increases serotonin transporter (SERT) activity resulting in increased breakdown of 5-HT to 5-HIAA (van Heesch *et al.*, 2013:191), an action inherently opposite to how antidepressants like IMI work.

3.5 Conclusion

GM displayed dose-dependent antidepressant-like effects after acute treatment. Translational relevance was established by a similar response after chronic treatment, although a dose of 50 mg/kg may need further characterization in order to acquire a more significant response and further dose ranging studies are warranted. Behavioral and regional brain monoamine analysis support early-onset noradrenergic activity following acute GM treatment, although a late emerging bolstering of 5-HT and associated behavior follows long-term administration. GM also displayed some antioxidant and pronounced anti-inflammatory properties by reducing lipid peroxidation and increasing IL-10 activity, respectively, actions that may underlie the aforementioned behavioral and neurochemical changes. Considering that this data was generated in a genetic animal model of MDD and which compared favorably to that produced by a reference antidepressant (IMI), the antioxidant and anti-inflammatory potential of GM suggest that it may be a valuable adjunctive treatment with conventional antidepressants, and warrants further study. These results can be beneficial in the development of a new approach to the treatment of MDD.

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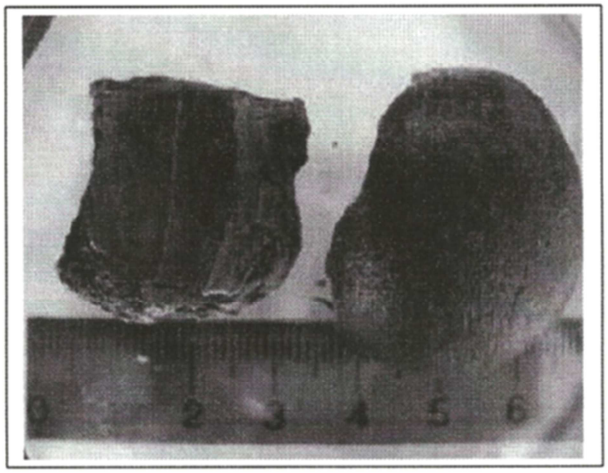
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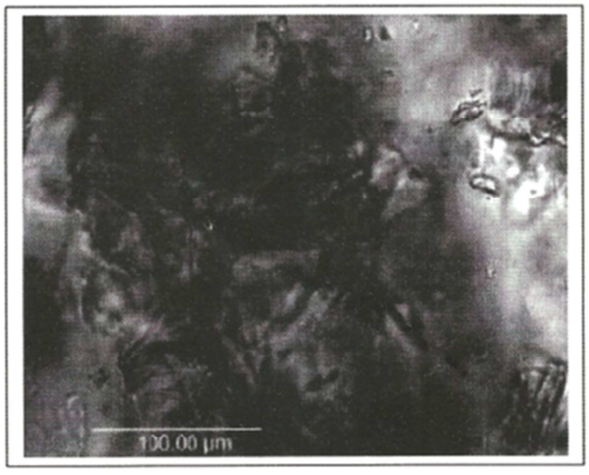
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Chapter 4:
Conclusion and recommendations for future studies

4.1 Introduction

Oxidative stress has a significant contributory role in major depressive disorder (MDD) *(see Chapter 2 section 2.1.5.5)*. Therefore antioxidant compounds have attracted a great deal of attention in the treatment of the illness. A number of clinical (Berk *et al.*, 2008:346; Berk *et al.*, 2014:628) as well as animal studies (Ferreira *et al.*, 2008:747) have provided preliminary evidence in support of this approach. Consequently, as an antioxidant N-acetyl cysteine (NAC) offers significant potential as a means to addressing MDD-related neurobiological changes associated with disordered redox. In fact, studies in our laboratory have confirmed the ability of NAC to reverse oxidative damage *in vivo* (Harvey *et al.*, 2008:508).

Another class of compounds worth studying for their recognized psychotropic and antidepressant-like effects is the xanthones (Negi *et al.*, 2013:1). Of relevance for this particular study, the raw powdered pericarp of the fruit of *Garcinia mangostana* Linn. (GM) was studied with respect to its overall behavioral pharmacology. More specifically it was evaluated with respect to key symptoms of MDD, viz. despair, learned helplessness and cognitive deficits, as well as neurobiological correlates including, redox balance, peripheral markers of immune-inflammation and regional brain monoamine levels in the frontal cortex, striatum and hippocampus. To this end, a genetic animal model of MDD, viz. the Flinders Sensitive Line (FSL) rats, was selected as the subject for this investigation. All of the above effects were compared to NAC (initially considered in this study as a positive antioxidant-related control) as well as to imipramine (IMI), a well-known conventional reference antidepressant (Maubach *et al.*, 2002:609). The FSL rat model presents with robust face, construct and predictive validity for MDD (Overstreet & Wegener, 2013:143) *(see Chapter 2 section 2.3.1).*

4.2 Primary outcomes

4.2.1 The FSL animal model of MDD

Novel object recognition (NORT)

No significant differences were observed between the cognitive effects of FSL and control Flinders Resistant Line (FRL) vehicle-treated animals, as measured in the NORT.

Locomotor activity

A significant decrease in general locomotor activity was observed in FSL rats compared to the FRL counterpart, as determined by the open field test (OFT). Reduced activity is a general manifestation of “sickness behavior” in animals, and is related to malaise observed in MDD.

Forced swim test (FST)

In the acute and chronic treatment study, vehicle-treated FSL animals presented with significantly increased immobility together with decreased climbing and swimming behavior compared to the FRL control rats, thus indicative if depressive-like behavior.

Monoamine analysis

Monoamine analysis demonstrated differences between the FSL and FRL strains, with FSL rats demonstrating a decrease in 3, 4-dihydroxyphenylacetic acid (DOPAC) levels in the frontal cortex, a decrease in noradrenaline (NA) levels and an increase in serotonin (5-HT) levels in the striatum, while hippocampal NA levels were reduced and 5-hydroxyindoleacetic acid (5-HIAA) levels increased. This deficiency of monoaminergic activity as seen in the FSL rat is mostly consistent with the classic monoamine hypothesis of MDD (Berton & Nestler, 2006:137; Schildkraut, 1965:509).

Lipid peroxidation

FSL rats demonstrated significantly increased hippocampal lipid peroxidation compared to FRL rats, suggesting the presence of neuronal damage to membrane lipids due to oxidative stress. The activation of oxidative stress pathways is a key pathophysiological factor in MDD (Maes *et al.*, 2009:27)

Cytokine analysis

No significant difference could be observed between the IL-10 and tumor necrosis factor (TNF)-α levels in FSL and FRL rats. Inflammation is linked to neurotoxicity and neurodegeneration that in turn contributes to the symptoms of MDD. (Maes *et al.*, 2009:27)

4.2.2 Response to chronic NAC treatment

Novel object recognition test

After a fixed dose chronic treatment regime with NAC, no cognitive effects, as measured in the NORT, were observed in either strain, suggesting at least one behavioral level where predictive validity is compromised.

Locomotor activity

No effect was observed on locomotor activity after acute treatment with NAC, thus confirming minimal influence of locomotor effects on the FST. The latter observation indicated the absence of any adverse locomotor effects and confirmed this to be a dose that is not acutely toxic, and to be without prominent sedating and/or other motor activating or inhibitory effects that may complicate interpretation of the FST data.

Forced swim test

After acute treatment of FSL rats, NAC significantly decreased immobility behavior while fully reversing the reduced swimming behavior in FSL vehicle-treated rats, confirming its antidepressant-like actions.

Monoamine analysis

- NAC significantly reduced cortical NA levels in FSL rats as seen in results obtained by Zangen *et al* (1999), demonstrating that chronic antidepressant treatment significantly decreased cortical NA levels in FSL rats (Zangen *et al.*, 1999:243).
- NAC had no significant effect on 5-HT levels in any of the brain regions compared to the vehicle control. Contradictory to the monoamine hypothesis, there is some doubt as to whether an increase in 5-HT is necessary for antidepressant response (Brand *et al.*, 2015:324).
- NAC significantly decreased striatal DA levels in FSL rats with the same trend observed in the frontal cortex. NAC significantly increased the DA metabolite homovanillic acid (HVA) in the hippocampus of FSL animals possibly indicating an increase in DA metabolism.

Lipid peroxidation

NAC did not significantly affect lipid peroxidation in any of the brain regions and in either strain. A trend towards increased lipid peroxidation in the hippocampus was observed in FRL animals, with a reduction noted in FSL rats after NAC treatment. Oxidative stress in itself may mediate altered monoaminergic activity (Möller *et al.*, 2013b:687) therefore by targeting the redox status; NAC may have distinct benefits in treating the illness.

Cytokine analysis

NAC did not significantly affect plasma levels of IL-10 and TNF-α in either strain. There was however a trend towards reduced levels of TNF-α after NAC treatment. Inflammation contributes towards alterations in neurotransmitters as well as neuroendocrine function, synaptic plasticity and behavioral characteristic of MDD (Brand *et al.*, 2015:324).

4.2.3 Response to IMI treatment

Locomotor activity

Results from both the acute analysis as well as the chronic study with IMI showed no significant effect on the locomotor activity of FSL rats in the OFT, thus confirming minimal influence of locomotor effects on the FST.

Forced swim test

Antidepressant-like effects were observed in the FST after acute and chronic treatment with IMI in FSL rats by significantly reducing immobility and increasing climbing behavior. These data confirm its antidepressant-like effects following acute *and* chronic treatment, confirming the robust predictive validity of IMI for MDD.

Novel object recognition

After a fixed dose chronic treatment regime, no cognitive effects, as measured in the NORT, were observed after treatment with IMI, suggesting at least one behavioral level where predictive validity is compromised.

Monoamine analysis

- Monoamine analysis indicated that IMI significantly reduced cortical and hippocampal NA levels in the frontal cortex and hippocampus of FSL rats while significantly increasing NA levels in the striatum. This is in accordance with the results obtained by Zangen *et al* (1999), demonstrating that chronic antidepressant treatment significantly decreases cortical and hippocampal NA levels in FSL rats (Zangen *et al.*, 1999:243).
- IMI also significantly reduced 5-HT levels in the frontal cortex and hippocampus of FSL rats while also reducing hippocampal 5-HIAA levels. The same trend can be observed in the striatum with a trend for IMI to reduce 5-HT levels and increase 5-HIAA levels. It is possible that a reduction in 5-HT release from nerve terminals may

result in a compensatory increase in 5-HT synthesis via a feedback mechanism (Adell *et al.*, 1988:1678).

- Striatal DA levels were significantly decreased in FSL rats while a trend was observed for increased DOPAC levels in both strains. Elevated DOPAC levels may indicate an increase in dopaminergic activity.

Lipid peroxidation

IMI indicated a strong trend towards reducing lipid peroxidation levels that was significantly elevated in FSL rats. By targeting the redox status, monoaminergic activity may be mediated (Möller *et al.*, 2013b:687), contributing to the antidepressant effect of IMI.

Cytokine analysis

After a fixed dose chronic treatment regime in a separate group of animals, IMI significantly increased the anti-inflammatory cytokine, IL-10, but had no effect on the pro-inflammatory cytokine, TNF-α, in the plasma of FSL rats. The anti-inflammatory effect of IMI may contribute to the monoaminergic alterations characteristic of MDD (Brand *et al.*, 2015:324).

4.2.4 Response to GM treatment

Locomotor activity

GM had no significant effect on locomotor activity in FSL rats in the OFT, confirming a lack of effect on locomotor activity and hence the FST data in the FSL rat are unlikely to be adversely influenced by locomotor effects of the compound.

Forced swim test

The FST conducted after the acute treatment regime identified the effective dose for GM to be 50 mg/kg, with GM significantly reversing immobility and increasing climbing behavior in FSL rats. The dose response relationship for GM indicated that at higher dosages (above 150 mg/kg) there were no appreciable antidepressant-like effects in the FST in FSL rats when compared to NAC and IMI as reference antidepressants. Following chronic treatment, an antidepressant-like effect is still evident, although missing significance. However, increased climbing behavior observed in the acute analysis transformed to increased swimming behavior after chronic treatment in FSL rats.

Novel object recognition

After a fixed dose chronic treatment regime, no cognitive effects, as measured in the NORT, were observed after treatment with GM, suggesting at least one behavioral level where predictive validity is compromised.

Monoamine analysis

- GM did not alter NA levels in any of the brain regions in the FSL rats except for a significant increase in NA in the striatum. This is in line with the monoamine deficiency theory that antidepressants increase monoaminergic activity (Berton & Nestler, 2006:137; Schildkraut, 1965:509).
- GM significantly reduced cortical 5-HT levels in FSL animals while increasing 5-HIAA levels in the frontal cortex and hippocampus of FSL rats. It is possible that reduced 5-HT release from nerve terminals may result in a compensatory increase in 5-HT synthesis via a feedback mechanism (Adell *et al.*, 1988:1678).
- GM significantly reduced striatal DA levels with no significant differences observed for GM regarding DOPAC and HVA levels. The significant increase in striatal NA following treatment with GM, may reduce DA release via the stimulation of α_2 inhibitory heteroreceptors on DA terminals in the striatum (Rominger *et al.*, 2010:654)

Lipid peroxidation

GM indicated a strong trend towards reducing lipid peroxidation levels in the FSL rats, narrowly missing significance. By reducing the oxidative stress, GM may mediate monoaminergic activity (Möller *et al.*, 2013b:687).

Cytokine analysis

- GM significantly increased the anti-inflammatory cytokine IL-10 in FSL but had no effect on the pro-inflammatory cytokine, TNF-α. The above-described effects of GM on behavior and neurochemistry may occur via direct or indirect immune-inflammatory processes.

4.3 Secondary outcomes

Antidepressants have no mood uplifting effects in healthy individuals (Kanemaru *et al.*, 2009:363). Thus, any observed efficacy of a novel antidepressant in a "non-depressed" animal model may invalidate the findings. Considering drug treatment response in FRL (i.e. healthy) vs. FSL (i.e. sick or "depressed") animals, the presence of severe pathology such as oxidative stress and inflammation may modify treatment response. The efficacy of an antioxidant like NAC or GM depends on the pathological state of the animal (Emsley *et al.*, 2014:224), and since FSL rats present with noteworthy pathological features associated with oxidative stress (see above), a clear therapeutic advantage should be observed over treatment in a non-pathological "healthy" animal where no obvious pathology exists. There were no differences observed between the FSL and FRL strain with respect to certain pathological markers (i.e. TNF-α, IL-10), which then questions the pro-oxidant state of the FSL rat.

On the other hand, distinct differences in other biomarkers were seen, such as NAC portraying an antioxidant role in FSL rats by reducing lipid peroxidation (although not significantly) while in healthy FRL control animals NAC proved to be a pro-oxidant by slightly increasing lipid peroxidation. This is in-line with Emsley et al. (2014) who posit that the dose and efficacy of an antioxidant is influenced by the redox, or illness, state of the patient. Furthermore NAC significantly increased hippocampal NA levels in FRL rats, while significantly reducing NA in the FSL rats. NAC also significantly increased hippocampal 5-HIAA levels in FRL rats while significantly increasing the DA metabolite HVA in the hippocampus of FSL and FRL animals. Regarding IMI a trend can be observed for reduced 5-HT levels and increase 5-HIAA levels in the striatum of FSL and FRL rats. Striatal DA levels were significantly also decreased in FSL and FRL rats while a trend was observed for increased HVA levels in FRL rats with a decrease in the FSL strain. GM significantly reduced locomotor activity in "healthy" FRL rats but not in FSL rats in the OFT. Moreover, GM significantly reduced cortical 5-HT levels in FSL and FRL animals with a trend for increased 5-HIAA observed in the striatum of FRL rats. GM also significantly increased the anti-inflammatory cytokine IL-10 in FSL and FRL rats.

This suggests that the FSL rat perhaps does not address *all* levels of construct validity (biological basis) in keeping with existing theories of MDD, such as inflammation, redox, monoamine hypotheses etc. The two-way ANOVA with respect to strain effects allowed us to make tentative conclusions from this. Of course the relevance of these differences holds

great interest, and further work is needed to explain the basis and application of these findings.

4.4 Recommendations for future studies

- A possible reason for the negative results obtained in the NORT could be that the rats were too big relative to the size of the NORT boxes, thus complicating the process of distinguishing between actual exploration of the object or accidental unintended contact. NORT conducted in boxes larger than 50 x 50 x 40cm should be explored as various studies have implemented these larger boxes (Ennaceur *et al.*, 1997:509; Reger *et al.*, 2009:672). Furthermore, the measurement with the Noldus program should be further calibrated with standardized settings to avoid variation between students. Time was however limited and we were unable to undertake a comprehensive validation of the method before conducting the study. Further work on this is therefore imperative before definitive conclusions regarding NAC, IMI and GM can be made.
- Other inflammatory cytokines, including interferon-α/λ and other interleukin type cytokines, should be evaluated to access their level of influence by GM. The anti-inflammatory effects of GM can also be more effectively measured in rats by inducing inflammation with lipopolysaccharide or applying a more severe stressor.
- Standard Sprague Dawley rats, as opposed to FRL rats used here as control animals, can be investigated to establish a more prominent difference between the "sick" and "healthy" control animal, especially with regards to the monoamine analysis (Malkesman *et al.*, 2006:17; Zangen *et al.*, 1997:2477; Zangen *et al.*, 1999:243).
- In this study the oral route and hepatic first past metabolism may have restricted the effect of NAC, since we have previously observed pharmacological effects following subcutaneous administration (Möller *et al.*, 2013a:156). Rodent studies demonstrated that NAC is well absorbed from the small intestine but undergoes extensive first-pass metabolism through the liver, while intestinal metabolism of NAC further reduces the bioavailability after oral administration (Liu *et al.*, 2005:193).
- The pro-oxidative state was perhaps not ideally replicated in the FSL rat, possibly due to the absence of an additional stressor noted above, and can be the reason for the ineffectiveness of NAC.
- A broad range of low to moderate dosages for GM should be considered (i.e. 5-100 mg/kg) to determine the optimal antidepressant dosage since there was a clear trend for reduced immobility in the FST at the 50 mg/kg dose. Despite significant efficacy

following acute treatment, chronic treatment efficacy remains the standard for predictive validity.

- An attempt at reformulating GM should be attempted to aid in a simpler administration procedure. Possibilities include the coating of rat pellets or via a water source. The raw powder could possibly also be crushed into smaller particles to aid administration.
- The effect of GM on corticosterone, gamma aminobutyric acid (GABA) and glutamate should be evaluated in an attempt to clarify the monoamine discrepancies observed in this study.
- A future study could compare GM to a serotonergic related antidepressant, in order to further explore its mechanism of action.
- Regarding GM, the kynurenine pathway and the inhibition of indolamine 2,3-dioxygenase (IDO) by IL-10 (Fig.1), can be considered as a possible mechanism whereby GM induced an increase in 5-HIAA and in 5-HT related swimming behavior in the FST after chronic administration.

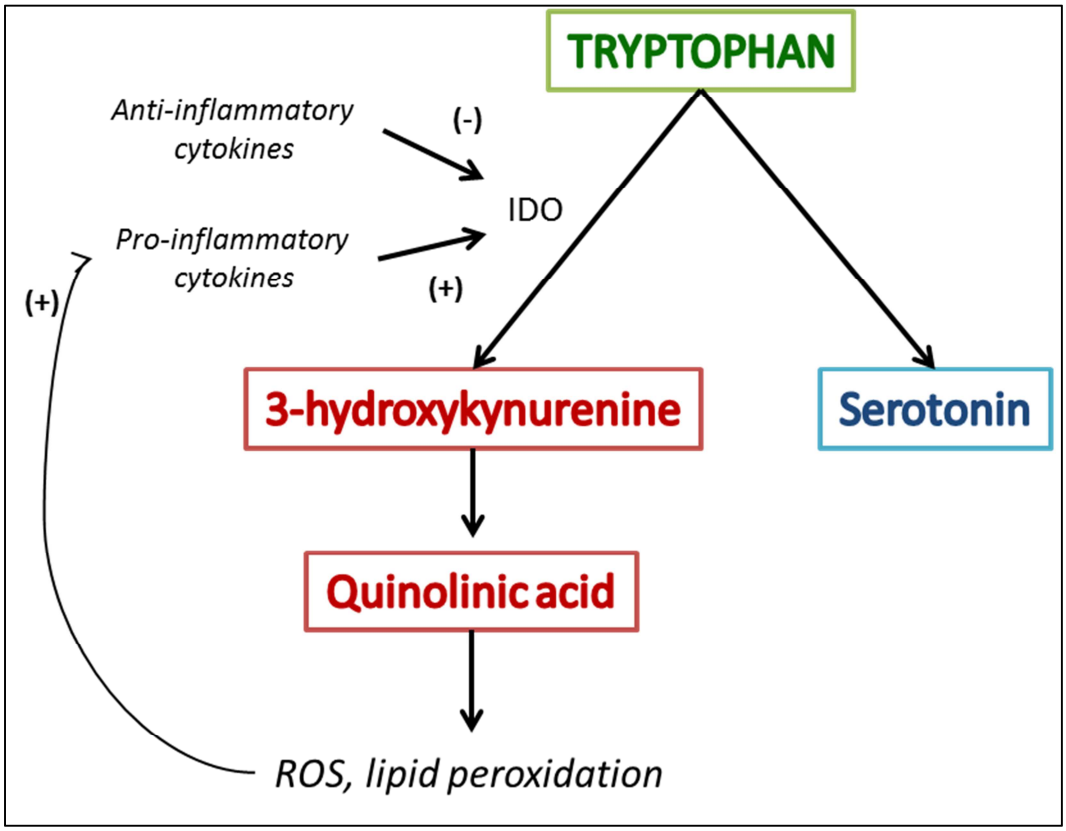


Figure 1. The influence of inflammatory cytokines, oxidative stress and lipid peroxidation on serotonin synthesis by means of the kynurenine pathway. IDO: indolamine 2, 3-dioxygenase, (-): inhibitory effect, (+): stimulation

4.5 Novel findings and conclusion

This study demonstrated various novel behavioral findings. In terms of GM, an acute two-day administration using a translational animal model of MDD found 50 mg/kg to be the most effective antidepressant dosage in the FST, with higher dosages (i.e. 150 and 200 mg/kg) demonstrated no effect. Moreover, the lower dosage of 50 mg/kg further consolidated its antidepressant-like effect by significantly reducing immobility *and* increasing noradrenergic related climbing behavior in the FST. Taking this work to a chronic treatment scenario showed GM to indeed reduced immobility but this missed significance, suggesting that the dose of GM needs to be further optimized with respect to efficacy for chronic treatment. Nevertheless, GM significantly increased serotonergic related swimming behavior in the FST that could be positively correlated to the significant increase observed in 5-HIAA after the chronic administration of GM. This is the first study to investigate the antidepressant effects of GM, and even more relevant being the application in FSL rats, thus providing robust translational relevance for these observations.

Novel biological findings include a strong trend for GM to reduce hippocampal lipid peroxidation levels ($p = 0.08$) that were significantly increased in FSL rats. Chronic treatment with GM also significantly increased the anti-inflammatory cytokine; IL-10, in the plasma of FSL rats, but had no effect on the pro-inflammatory TNF-α. Novel findings regarding the response of monoamine levels to GM after chronic treatment include a decrease in cortical 5-HT transmission with increased 5-HIAA levels in the frontal cortex and hippocampus. Striatal NA levels were also significantly elevated after treatment with GM in FSL rats while DA levels were reduced. The latter observations are novel and have, to the best our knowledge, not been observed in any animal model of MDD.

Considering that GM was analyzed in a genetic animal model of MDD and that it compares favorably to a reference antidepressant (IMI), the antioxidant and anti-inflammatory potential of GM suggest that it may be of value as adjunctive treatment with conventional antidepressants, and warrants further study. These results can be beneficial in the development of a new approach to the treatment of MDD.

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Addendum A: Additional Results

Introduction

This addendum contains additional data and acts as supporting material to the main study. N-acetyl cysteine (NAC) did not demonstrate the anticipated effect and was thus removed as a positive control from the article *(Chapter 3)*, but will be presented in this addendum. An analysis of behavioral and neurochemistry results as a function of strain (Flinders Sensitive Line (FSL) vs. Flinders Resistant Line (FRL) will also be examined, the rationale being to access differences in treatment response as a factor of presenting pathology (i.e. monoamine changes, altered redox, inflammation) and also to establish a genetic basis for the bio-behavioral changes observed. The monoamine analysis of striatal dopamine (DA), serotonin (5-HT), noradrenaline (NA) and their respective metabolites after treatment with imipramine (IMI) and *Garcinia mangostana* Linn. (GM), as well as the results obtained in the novel object recognition test (NORT) and lipid peroxidation analysis in the frontal cortex and hippocampus, did not fit the scope of the article *(Chapter 3)* and are therefore presented in this addendum. Finally, the results of the FRL control rats that were omitted from the article (Chapter 3) in order to streamline the data for possible publication are also presented here.

Addendum A will thus consist of:

A.1: The NAC results for the acute treatment study in the open field test (OFT) and forced swim test (FST), the chronic behavioral results in the NORT, OFT and FST as well as the biological analysis that include the monoamine and immune-inflammatory and oxidative stress related analyses. Furthermore, the behavioral and neurochemical analysis as a function of strain will also be examined.

A.2: The effects of chronic IMI and GM treatment in the NORT, in striatal monoamine analysis and in cortical and striatal lipid peroxidation.

A.3: The effects of chronic IMI and GM on FRL control rats in the OFT, the FST, cortical and hippocampal monoamine analysis, hippocampal lipid peroxidation and plasma cytokine analysis.

Addendum A.1

The main hypothesis (see *Chapter 1.3*) was that treatment of a translational animal model of major depressive disorder (MDD) with NAC (150 mg/kg), as a positive control, would demonstrate:

- Antidepressant activity in FSL rats after acute treatment using the OFT and the FST.
- Pro-cognitive and antidepressant-like activity as tested in the NORT, the OFT and the FST after 14 days of chronic treatment.
- FSL animals would present with cortical, striatal and hippocampal alterations in 5-HT, 5-hydroxyindoleacetic acid (5-HIAA), NA, 3-methoxy-4-hydroxyphenylglycol (MHPG), DA, 3,4-dihydroxyphenyl-acetic acid (DOPAC) and homovanillic acid (HVA) levels vs. FRL animals, which would be reversed following chronic treatment with NAC.
- Reversal of FSL associated elevation in markers of oxidative stress and immune-inflammation.
- Antidepressant-like effects would not be observed in NAC treated FRL rats to provide more convincing evidence of efficacy for the tested compounds to evaluate differences in treatment response as a factor of presenting pathology (i.e. monoamine changes, altered redox, inflammation).

A.1.1 Materials and methods

Male FSL rats and a corresponding behavioral control line, FRL rats, were used for the current study as described previously in *Chapter 3, section 2.1*.

NAC (Sigma Aldrich, Kempton Park, South Africa) was dissolved in double distilled water and administered at a fixed dose of 150 mg/kg by oral gavage with a 20 gauge bird needle.

A.1.1.1 Study design

A.1.1.1.1 Acute study

An acute treatment study with NAC was initially performed in FSL and FRL rats. This allowed a proof of concept to be established, i.e. that NAC has antidepressant-like effects.

In the acute study, NAC treatment was compared to a FRL vehicle group as well as a FSL vehicle group receiving water. Three treatment groups were set up, with each having 6 rats per group. Two groups (one with FRL and the other with FSL rats) received water, acting as drug naïve controls. The other group of FSL rats received 150 mg/kg of NAC. Each group was treated three times – 24 hours, 6 hours and 1 hour before the behavioral tests commenced. These tests included the locomotor test (using the OFT arena) and the FST.

A.1.1.1.2 Chronic study

The chronic study consisted of a behavioral and a biological cohort, performed in both FRL and FSL rats. The treatment group received 150 mg/kg/day NAC (n = 12 FSL, n = 12 FRL). As this data formed part of a larger study that required two vehicle types, this needed to be controlled for. One treatment group received 0.1% xanthan gum (vehicle for *Garcinia mangostana* Linn. (n = 6 FSL, n = 6 FRL) and another water (vehicle for NAC; n = 6 FSL, n = 6 FRL). Analysis between these groups returned no significant differences and were therefore combined and hereafter referred to as the "vehicle" group. Treatment lasted for 14 days. On day 15 the rats that formed part of the behavioral cohort were then subjected to sequential behavioral testing, taking place 12 hours after the last treatment and at the start of the dark cycle (Liebenberg *et al.*, 2010:137). The behavioral tests included the NORT (for the measurement of the cognition), the OFT (for measurement of locomotor activity) and the FST (for measurement of the antidepressant-like effects). The FST was conducted at 08:00 the next morning. Behavioral testing was performed according to a sequential protocol validated previously (Mokoena *et al.*, 2015:1). Observations and analysis of data using rats subjected to the FST indicated that exposure to the OFT does not affect behavior in the FST (Overstreet *et al.*, 2005:739).

Rats used in the neurochemical cohort were randomly divided into the same treatment groups as the behavioral study. On day 15 the animals were sacrificed by decapitation with trunk blood and brain (frontal cortex, striatum and hippocampus) harvested for the various neurochemical tests, as described in *section A.1.1.3*.

A.1.1.2 Behavioral tests

Following the vehicle/drug treatment for the acute analysis and the behavioral cohort of the chronic treatment, the NORT described below, and the OFT and the FST (described in *Chapter 3 section 2.4.1 & 2*), were performed.

A.1.1.2.1 Novel object recognition test

The NORT is used to access visual learning and memory in rats. A dysfunction of these cognitive processes is evident in MDD (American Psychiatric Association, 2013:970). This test relies on the premise that rats prefer exploration of a novel object relative to a familiar one. If the test shows an increase in exploration time of a novel object it would indicate improved cognitive function (Ennaceur & Delacour, 1988:47). The NORT were evaluated as described previously (Grayson *et al.*, 2007:31), and modified by ourselves (Möller *et al.*, 2013:156).

During an acquisition trial (on day 13 of treatment) rats were exposed to a 10 minute habituation session in a (50 x 50 x 40 cm) plexiglas NORT box two days prior to the day of testing (Mokoena *et al.*, 2015:1). Experimental trials followed on day 15 where each rat was exposed to two identical objects (A1 and A2) for a period of 5 minutes (trial 1). The rats were then returned to their home cage for a 90 minute inter-trial interval. The entire box was cleaned with 10% ethanol and both objects removed (Möller *et al.*, 2013:156). After the 90 minutes, the retention trial followed where one object was replaced with an identical familiar copy and the other with a novel unfamiliar object (B) (trial 2). After the retention trial, the rats were returned to the test box to explore the familiar and novel object for 5 minutes. The experiments were filmed and recorded for subsequent behavioral analysis (Möller *et al.*, 2013:156). Object exploration included sniffing, licking or touching the objects with forepaws. The exploration time (s) of each object in each trial were recorded and scored manually by an observer blind to the study, using two stopwatches (Grayson *et al.*, 2007:31). Essentially, more exploration time at the novel object reflects accurate recollection that the familiar object has been presented before, thus prompting greater exploration of the novel object.

A.1.1.3 Biological studies

Quantification of 5-HT, 5-HIAA, NA, MHPG, DA, DOPAC and HVA regional brain monoamine analysis as well as lipid peroxidation and peripheral cytokine levels were performed as described previously in *Chapter 3* section 2.5.2 & 3.

A.1.1.4. Statistical analysis

For the acute study a one-way analyses of variance (ANOVA) was implemented followed by the Bonferroni post-hoc multiple comparison test. In the chronic cohorts, multiple comparisons were analyzed by two-way ANOVA followed by the Bonferroni post-hoc test evaluating the interaction with the treatment as well as with the rat strain. Grubbs' test was used to identify outliers. GraphPad Prism® (version 5.00, San Diego California, U.S.A.) was used for all statistical analyses and graphical representation. Data are presented as mean ± SEM with a value of $p < 0.05$ regarded as statistically significant.

A.1.2. Results

A.1.2.1 Acute analysis

A.1.2.1.1 Open field test

A one-way ANOVA of the data showed no significant treatment*distance moved interaction (F (2.15) = 3.26, P = 0.07, data not shown). Consequently no effect on locomotor performance was noted between any of the treatment groups.

A.1.2.1.2 Forced swim test

A one-way ANOVA of the data revealed a significant treatment*immobility (F (2.25) = 18.99, p < 0.0001); treatment*climbing (F (2.25) = 15.38, p < 0.0001) and treatment*swimming interaction F (2.25) = 6.84, p = 0.004). Bonferroni post-hoc test indicated a significant increase in immobility in the FSL vehicle group (p < 0.0001) compared to the FRL vehicle group, which was significantly reversed by NAC treatment in the FSL NAC group (p = 0.003, Fig. 1A). The FSL vehicle group displayed a significant decrease in climbing behavior compared to the FRL vehicle group (p < 0.0001), although NAC treatment was unable to reverse this decrease in the FSL NAC group (p > 0.99, Fig. 1B, Bonferroni post-hoc test). With regards to swimming behavior, the FSL vehicle group displayed a significant decrease compared to the FRL vehicle group (p = 0.01), while NAC treatment significantly and completely reversed this decrease in the FSL NAC group (p = 0.006) (Fig. 1C, Bonferroni post-hoc test).

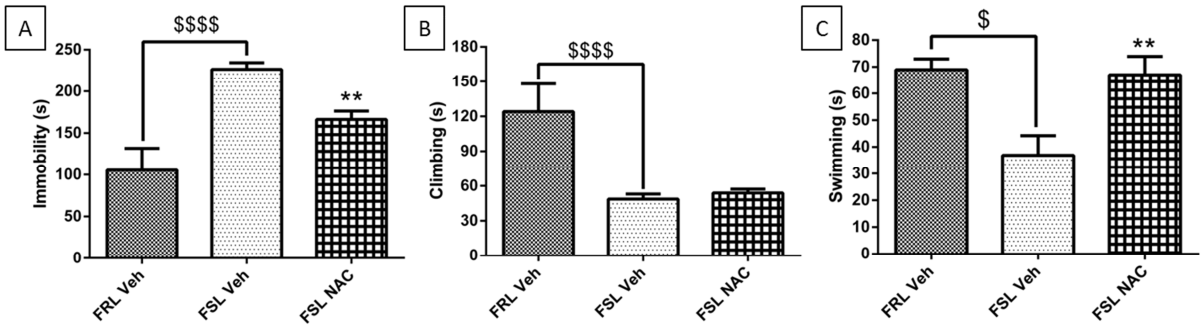


Figure 1. The effect of acute administration of NAC (150 mg/kg) versus vehicle (Veh) (control) in FSL rats as well as the difference between the FRL and FSL vehicle control groups with regards to A: Immobility, B: Climbing and C: Swimming behavior. ($^{\$}p < 0.05$ vs. FRL Veh, $^{\$\$\$\$}p < 0.0001$ vs. FRL Veh, **p < 0.01 vs. FSL Veh; one-way ANOVA with Bonferroni post-hoc test). Refer to the text for the exact P-values.

A.1.2.2 Chronic behavioral study

A.1.2.2.1 Novel object recognition test

A two-way ANOVA of the data showed no significant treatment*strain interaction (F (1.44) = 0.8, p = 0.38). There was also no significant effect of treatment or strain on the time spent exploring the novel object (F (1.44) = 0.07, p = 0.79; F (1, 44) = 3.1, p = 0.09, respectively) Fig. 2).

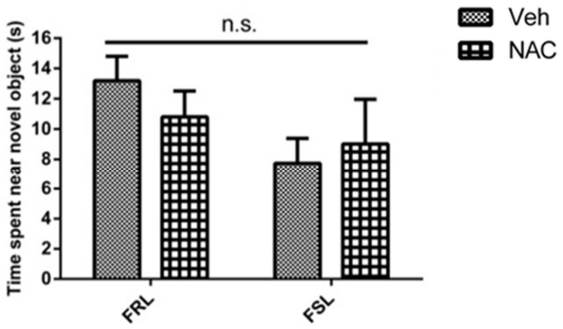


Figure 2. The effect of chronic administration of NAC (150 mg/kg) on the time spent exploring a novel object in FSL and FRL rats compared to vehicle control groups (two-way ANOVA with Bonferroni post-hoc test), n.s. = not significant.

A.1.2.2.2 Open field test

A two-way ANOVA of the data showed no significant treatment*strain interaction (F (1.44) = 0.88, p = 0.35). Treatment demonstrated a significant effect on the locomotor activity (F (1.44) = 15.69, p = 0.0003) while strain had no significant effect (F (1.44) = 1, p = 0.32). Bonferroni post-hoc test indicated no significant differences between the FSL NAC and FRL NAC group (p > 0.99) or between the FSL NAC group and the FSL vehicle group (p = 0.23, Fig. 3). The FRL and FSL vehicle control groups also showed no significant difference in locomotor activity (p > 0.99, Fig. 3). However, the FRL NAC group showed significantly lowered locomotor activity compared to the FRL vehicle control group (p = 0.007, Fig. 3).

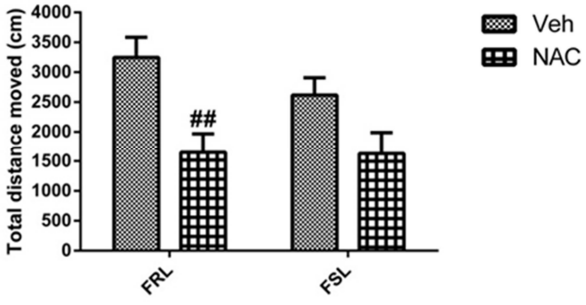


Figure 3. The effect of chronic administration of NAC (150 mg/kg) on the total distance moved in FSL and FRL rats compared to vehicle control groups ($^{\#\#}p < 0.01$ vs. FRL Veh, two-way ANOVA with Bonferroni post-hoc test), Refer to the text for the exact P-values.

A.1.2.2.3 Forced swim test

After chronic administration of NAC, a two-way ANOVA of the data revealed no significant treatment*strain interaction regarding immobility ($F(1.44) = 3.47$, $p = 0.07$); climbing ($F(1.44) = 2.5$, $p = 0.12$); or swimming ($F(1.44) = 0.02$, $p = 0.89$). There was also no effect of the treatment on immobility ($F(1.44) = 1.38$, $p = 0.25$); climbing ($F(1.44) = 0.23$, $p = 0.63$); or swimming ($F(1.44) = 0.05$, $p = 0.82$), but a significant effect of the strain on the behavior was observed for immobility ($F(1.44) = 116.4$, $p < 0.0001$); climbing ($F(1.44) = 61.4$, $p < 0.0001$); and swimming ($F(1.44) = 42.5$, $p < 0.0001$).

Immobility: Bonferroni post-hoc test indicated a significant difference in the FSL vehicle group compared to the FRL vehicle group ($p < 0.0001$; Fig. 4A) and between the FSL NAC and FRL NAC groups ($p < 0.0001$; Fig. 4A). No significant difference were observed between the FSL NAC and the FRL NAC groups and their respective vehicles of the same strain ($p = 0.22$ and $p > 0.99$, respectively).

Swimming: A significant difference in the FSL vehicle group compared to the FRL vehicle group ($p = 0.0002$; Fig. 4B), and between the FSL NAC and FRL NAC groups ($p = 0.0003$; Fig. 4B) was noted after Bonferroni post-hoc testing. No significant difference was observed between the FSL NAC and the FRL NAC groups and their respective vehicles of the same strain ($p > 0.99$, for both groups).

Climbing: Bonferroni post-hoc test indicated a significant difference in the FSL vehicle group compared to the FRL vehicle group ($p = 0.0004$; Fig. 4C) and between the FSL and FRL groups that received NAC ($p < 0.0001$; Fig. 4C). No significant difference was observed between the FSL NAC group vs. the FSL vehicle group ($p = 0.91$) or the FRL NAC groups and the FRL vehicle group ($p > 0.99$).

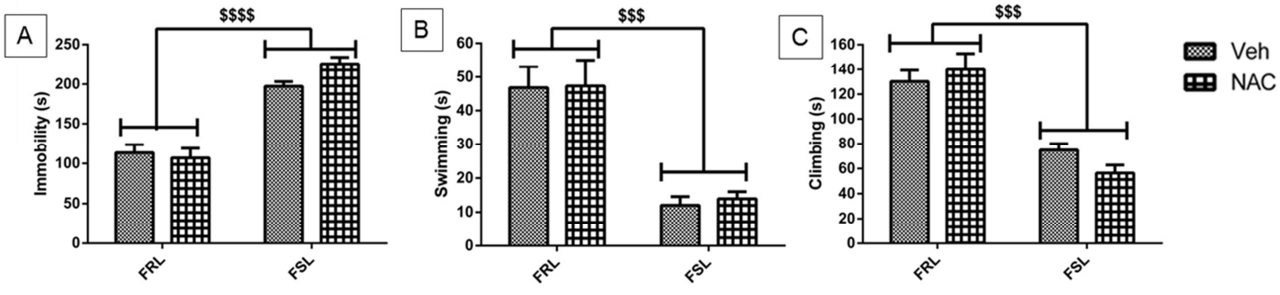


Figure 4. The effect of chronic administration of NAC (150 mg/kg) in FSL and FRL rats on A: Immobility, B: Swimming and C: Climbing behavior. ($^{\$\$\$\$}p < 0.0001$ vs. FRL Veh and FRL NAC respectively, $^{\$\$\$}p < 0.001$ vs. FRL Veh and FRL NAC respectively; two-way ANOVA with Bonferroni post-hoc test). Refer to the text for the exact P-values.

A.1.2.3. Chronic biological analysis

A.1.2.3.1 Monoamine analysis

Noradrenaline: MHPG levels were below the limit of detection, and could not be reported. A two-way ANOVA demonstrated significant treatment*strain interactions in the hippocampus ($F(1.44) = 69.19$, $p < 0.0001$) but not in the frontal cortex ($F(1.44) = 0.006$, $p = 0.94$) or in the striatum ($F(1.44) = 3.24$, $p = 0.08$). Treatment with NAC had a significant effect on NA in the frontal cortex ($F(1.44) = 22.57$, $p < 0.0001$), but not in the striatum ($F(1.44) = 0.68$, $p = 0.41$) or in the hippocampus ($F(1.44) = 2.87$, $p = 0.09$). A significant effect of the rat strain on NA was observed in the frontal cortex ($F(1.44) = 10.3$, $p = 0.003$), in the striatum ($F(1.44) = 7.76$, $p = 0.008$) and in the hippocampus ($F(1.44) = 80.93$, $p < 0.0001$).

Bonferroni post-hoc test showed that the FSL NAC and FRL NAC groups had significantly decreased NA levels in the frontal cortex ($p = 0.008$ and $p = 0.01$ respectively, Fig. 5A) compared to their vehicle control groups. Bonferroni post-hoc testing indicated no significant differences between any of the groups with regards to striatal NA levels except between the FRL and FSL vehicle control groups ($p = 0.01$, Fig 5B). Hippocampal levels of NA was significantly elevated in FRL NAC rats ($p < 0.0001$) while it was significantly lowered in the FSL NAC group ($p = 0.0002$) compared to the control of each strain (Fig. 5C). No significant difference could be observed between the FRL and FSL vehicle animals ($p > 0.09$), although significantly lowered NA levels was evident in the FSL NAC group compared to FRL NAC ($p < 0.0001$, Fig. 5C).

Serotonin: Two-way ANOVA of treatment*strain interactions demonstrated no significance in the frontal cortex ($F(1.44) = 0.0006$, $p = 0.98$), in the striatum ($F(1.44) = 0.12$, $p = 0.73$) or in the hippocampus ($F(3.44) = 1.21$, $p = 0.28$). A significant effect of NAC treatment was observed on 5-HT in the frontal cortex ($F(1.44) = 6.29$, $p = 0.02$), but not in the striatum ($F(1.44) = 2.09$, $p = 0.16$) or hippocampus ($F(1.44) = 2.90$, $p = 0.09$) while there was significant strain effects in the striatum ($F(1.44) = 25.01$, $p < 0.0001$) and hippocampus ($F(1.44) = 5.92$, $p = 0.02$) but not in the frontal cortex $F(1.44) = 0.02$, $p = 0.88$).

Bonferroni post-hoc test indicated no significant difference in 5-HT levels in the frontal cortex and hippocampus of the different treatment groups (Fig. 5D & F). However in the striatum a significant increase in 5-HT levels was observed in the FSL vehicle ($p = 0.01$) as well as the FSL NAC groups ($p = 0.003$) compared to the FRL vehicle and FRL NAC groups respectively (Fig. 5E).

5-HIAA: Treatment*strain interactions by means of a two-way ANOVA demonstrated significance in the hippocampus ($F(1.44) = 5.61$, $p = 0.02$) but not in the frontal cortex (F

(1.44) = 1.34, p = 0.25) or in the striatum (F (1.44) = 1.79, p = 0.19). Significant effects of treatment on 5-HIAA levels was observed in the frontal cortex (F (1.44) = 6.95, p = 0.01) and hippocampus (F (1.44) = 7.75, p = 0.008) but not in the striatum (F (1.44) = 0.02, p = 0.89) while the strain had no effect on 5-HIAA levels in the frontal cortex (F (1.44) = 0.34, p = 0.56), in the striatum (F (1.44) = 3.73, p = 0.06) or the hippocampus (F (1.44) = 0.66, p = 0.42).

Bonferroni post-hoc test of the data showed no significant differences in cortical 5-HIAA levels between any of the treatment groups (Fig. 5G), although a significant increase in 5-HIAA levels was evident in the hippocampus of the FRL NAC group compared to the FRL vehicle group (p = 0.004, Fig. 5I). No post-hoc test was conducted on the striatal 5-HIAA levels due to no significance demonstrated after conducting the two-way ANOVA.

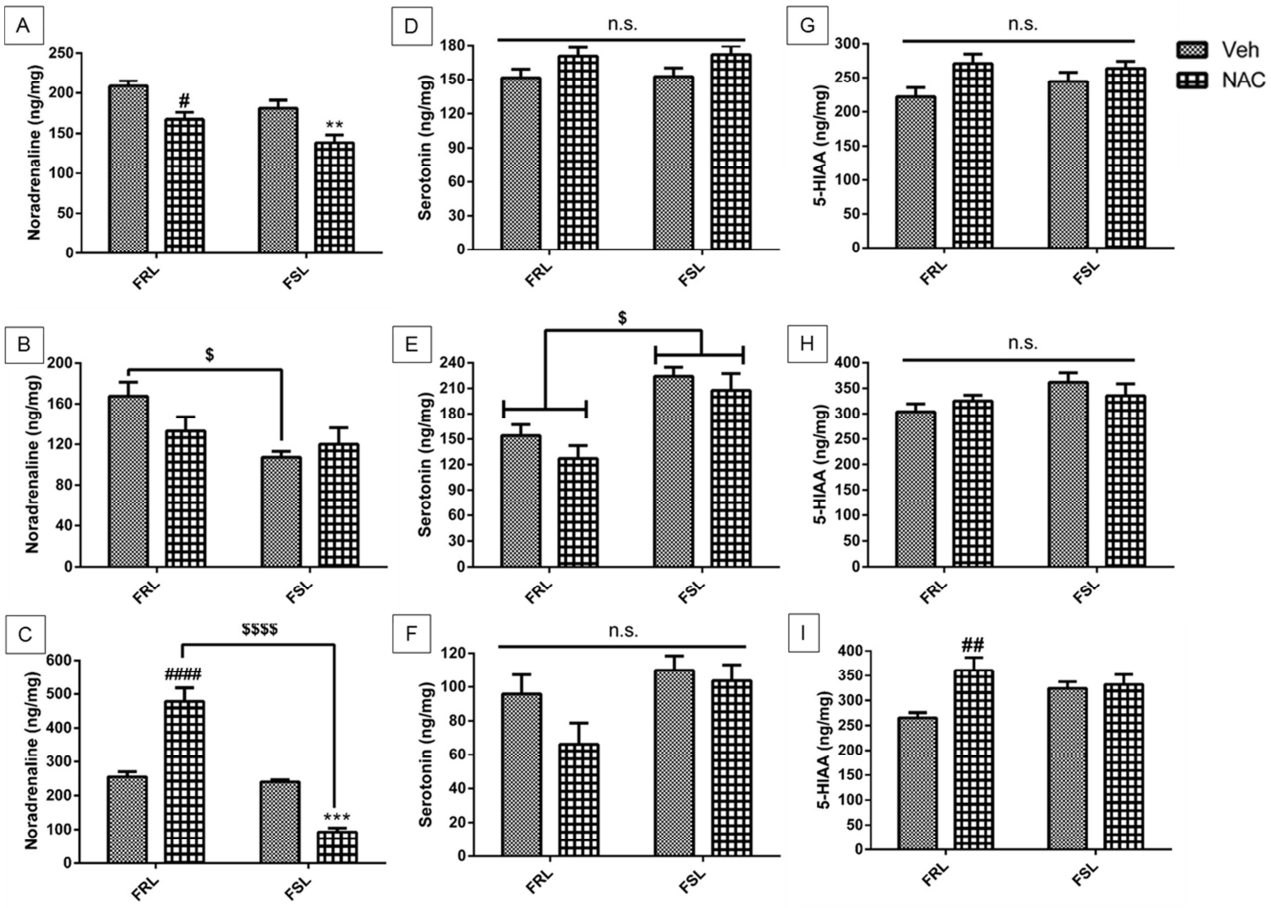


Figure 5. NA, 5-HT and 5-HIAA levels in the frontal cortex (A), (D) and (G), striatum (B), (E) and (H) and in the hippocampus (C), (F) and (I) of FSL and FRL rats following treatment with either NAC (150 mg/kg) or vehicle. ($^{\$}p < 0.05$ vs. FRL Veh, $^{\$\$\$\$}p < 0.0001$ vs., FRL Veh, $^{\#}p < 0.05$ vs. FRL Veh, $^{\#\#}p < 0.01$ vs. FRL Veh, $^{\#\#\#\#}p < 0.0001$ vs. FRL Veh, $^{**}p < 0.01$ vs. FSL Veh, $^{***}p < 0.001$ vs. FSL Veh; two-way ANOVA, Bonferroni post-hoc test). Refer to the text for the exact P-values.

Dopamine: Two-way ANOVA of treatment*strain interaction demonstrated significance in the striatum (F (1.43) = 8.52, p = 0.006) but not in the frontal cortex (F (1.43) = 1.02, p = 0.32) or

in the hippocampus (F (1.44) = 0.002, p = 0.97). A significant effect of treatment on DA levels was observed in the striatum (F (1.43) = 29.55, p < 0.0001) but not in the frontal cortex (F (1.43) = 1.15, p = 0.29) or in the hippocampus (F (1.44) = 0.15, p = 0.7). Similarly, a significant strain effect on DA levels was observed in the frontal cortex (F (1.44) = 5.44, p = 0.02) but not in the striatum (F (1.44) = 0.07, p = 0.79) or the hippocampus F (1.44) = 0.43, p = 0.51).

Bonferroni post-hoc test of the data showed a significant decrease in striatal DA levels in the FSL NAC group (p > 0.0001, Fig. 6B) when compared to the FSL vehicle group, but no significant differences in DA levels could be found among any of the groups in the frontal cortex (Fig. 6A) while no post-hoc test was conducted on the hippocampal DA levels (Fig. 6C) due to no significance observed in the two-way ANOVA.

DOPAC: Treatment*strain interactions by means of a two-way ANOVA demonstrated significance in the frontal cortex (F (1.44) = 6.14, p = 0.02) and in the striatum F (1.44) = 5.83, p = 0.02) but not in the hippocampus (F (1.44) = 0.003, p = 0.96). A significant effect of treatment on the DOPAC levels were observed in the hippocampus (F (1.44) = 7.93, p = 0.007) but not in the frontal cortex (F (1.44) = 1.64, p = 0.21) or the striatum F (1.44) = 0.46, p = 0.49). A significant effect of strain on DOPAC was observed in the striatum (F (1.44) = 11.69, p = 0.001), but not in the frontal cortex (F (1.44) = 3.13, p = 0.08) or hippocampus (F (1.44) = 0.62, p = 0.44).

A Bonferroni post-hoc test of the data showed a significant decrease in the cortical levels of DOPAC in the FSL vehicle group (p = 0.03) when compared to the FRL vehicle group (Fig. 6D) as well as a significant decrease in the striatal DOPAC of the FSL NAC group (p = 0.001) compared to the FRL NAC control group (Fig. 6E). No significant differences in DOPAC were observed between any of the groups in the hippocampus (Fig. 6F).

HVA: Two-way ANOVA of treatment*strain interaction only demonstrated significance in the striatum (F (1.44) = 4.37, p = 0.04) but not in the frontal cortex (F (1.44) = 2.57, p = 0.12) or in the hippocampus (F (1.44) = 0.06, p = 0.81). A significant effect of treatment on HVA levels were observed in the hippocampus (F (1.44) = 20.15, p < 0.0001) but not in the frontal cortex (F (1.44) = 1.61, p = 0.21) or the striatum (F (1.44) = 0.01, p = 0.91). Similarly, a significant effect of strain on HVA levels was evident in the frontal cortex (F (1.44) = 4.18, p = 0.05) and striatum (F (1.44) = 18.17, p = 0.0001) but not in the hippocampus F (1.44) = 2.29, p = 0.14).

Bonferroni post-hoc test of the data showed no significant differences in HVA levels in the frontal cortex between the different treatment groups (Fig. 6G), although a significant decrease in HVA levels was observed in the striatum of FSL NAC (p = 0.0003, Fig. 6H)

compared to FRL NAC animals. A significant increase in hippocampal HVA was noted in FRL NAC and FSL NAC groups (p = 0.01 and p = 0.03, respectively, Fig. 6I) compared to their vehicle control groups.

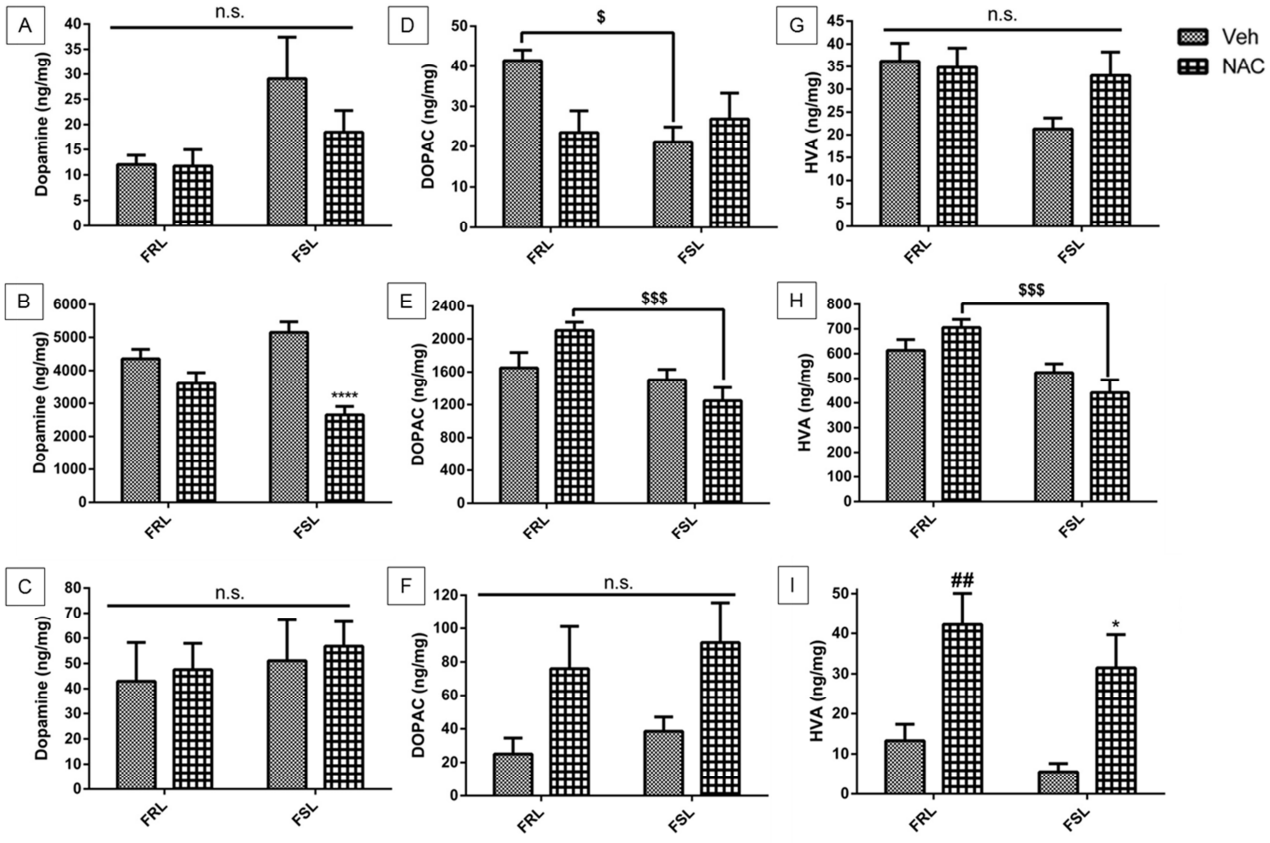


Figure 6. The levels of DA and its metabolites DOPAC and HVA in the frontal cortex (A), (D), (G), striatum (B), (E), (H) and in the hippocampus (C), (F), (I) of FSL and FRL rats following treatment with NAC (150 mg/kg) or vehicle. ($^{\$}p < 0.05$ vs. FRL Veh, $^{\$\$\$}p < 0.001$ vs. FRL Veh, $^{\#\#}p < 0.01$ vs. FRL Veh, *p < 0.05 vs. FSL Veh, ****p < 0.0001 vs. FSL Veh; two-way ANOVA, Bonferroni post-hoc test). Refer to the text for the exact P-values.

A.1.2.3.2. Lipid peroxidation

Treatment*strain interactions by means of a two-way ANOVA demonstrated significance in the frontal cortex (F (1.44) = 6.1, p = 0.02, Fig. 7A) but not in the striatum F (1.44) = 0.35, p = 0.56, Fig. 7B) or in the hippocampus (F (1.44) = 3.23, p = 0.08, Fig. 7C). Treatment did not significantly affect lipid peroxidation in the frontal cortex (F (1.44) = 0.005, p = 0.94), in the striatum F (1.44) = 0.002, p = 0.96) or the hippocampus (F (1.44) = 0.32, p = 0.57), while a significant effect of strain was noted in the hippocampus (F (1.44) = 4.68, p = 0.04) but not in the frontal cortex (F (1.44) = 0.58, p = 0.45) or striatum F (1.44) = 1.33, p = 0.25).

Bonferroni post-hoc test of the data showed no significant differences in lipid peroxidation levels in the frontal cortex (Fig. 7A) between any of the treatment groups while no post-hoc test was conducted on the striatal lipid peroxidation (Fig. 7B) due to no significance in the

two-way ANOVA. However, a significant increase in hippocampal lipid peroxidation was observed in the FSL vehicle group (p = 0.05, Fig. 7C) when compared to the FRL vehicle group. NAC tended to reverse the latter but failed to reach significance (p = 0.61; Fig. 7C).

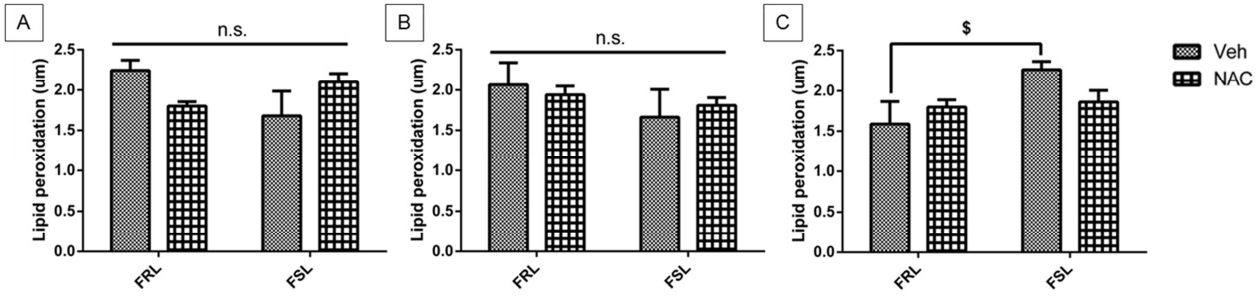


Figure 7. The effect of chronic administration of NAC (150 mg/kg) or vehicle in FSL and FRL rats on lipid peroxidation in the A: frontal cortex, B: striatum and C: hippocampus. ($^{\$}p < 0.05$ vs. FRL Veh, two-way ANOVA, Bonferroni post-hoc test), n.s. = not significant. Refer to the text for the exact P-values.

A.1.2.3.3 Immune-inflammatory cytokines

Two-way ANOVA of treatment*strain interactions demonstrated no significance in the plasma levels of IL-10 (F (1.39) = 0.13, p = 0.73) or TNF-α (F (1.42) = 0.35, p = 0.56). No significant effect was observed in cytokine levels after NAC treatment with respect to plasma IL-10 (F (1.39) = 0.50, p = 0.48) and TNF-α levels (F (1.42) = 1.94, p = 0.17) while the effect of strain also did not alter the data significantly with regard to IL-10 (F (1.39) = 0.40, p = 0.53) or TNF-α levels (F (1.42) = 0.01, p = 0.92).

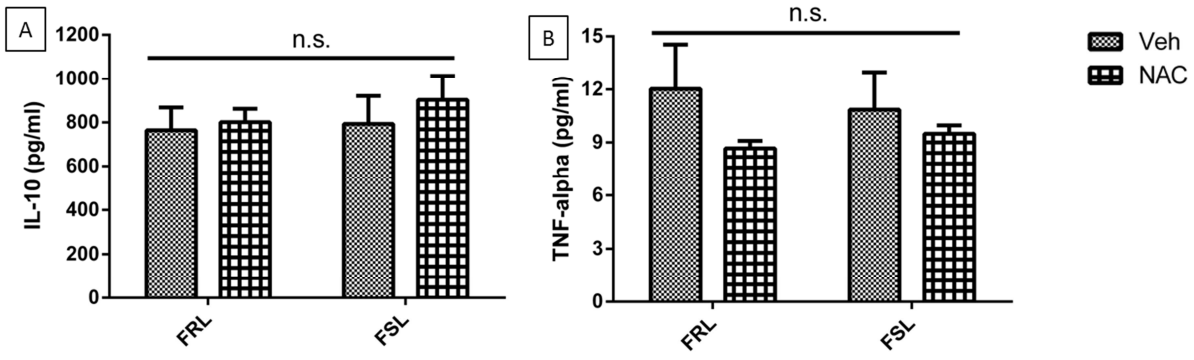


Figure 8. The effect of chronic administration of NAC (150 mg/kg) or vehicle in FSL and FRL rats on: A, IL-10 and B, TNF-α levels in the plasma (two-way ANOVA, Bonferroni post-hoc test), n.s. = not significant. Refer to the text for the exact P-values.

A.1.3 Discussion

The most important findings from this study were that FSL rats present with significant depressive-like symptoms i.e. increase immobility, together with a decrease in swimming and climbing behavior in the FST. Although acute treatment with NAC significantly

decreased immobility behavior while fully reversing the reduced swimming (but not climbing) behavior observed in the FSL rats *(Chapter 3),* chronic treatment as described here had no effect. Furthermore, selecting a suitable dose for NAC based on the literature (Ferreira *et al.*, 2008:747; Smaga *et al.*, 2012:280; Möller *et al.*, 2013:156) provided agreement of its antidepressant-like effects at a dose of 150 mg/kg. However, applying this same dose in a chronic treatment paradigm delivered less emphatic results. Although FSL rats still presented with significant depressive-like symptoms i.e. increased immobility, together with decreased swimming and climbing behavior in the FST, no difference could be observed between the two strains in the NORT or after analysis of IL-10 and TNF-α in the plasma. Lipid peroxidation was influenced by the strain with FSL rats displaying an increase in lipid peroxidation compared to FRL rats. Monoamines differed between the strains with FSL rats demonstrating a significant decrease in cortical DOPAC and significant reductions in striatal NA together with an increase in 5-HT in the same brain region. Chronic treatment of FSL and FRL rats with NAC showed no behavioral effects in the NORT or in the FST and no biological alterations in lipid peroxidation, IL-10 or TNF-α. FRL rats treated with NAC demonstrated a significant reduction in locomotor activity and the same trend was observed in the FSL animals treated with NAC ($p = 0.23$) when compared to their respective vehicle rats. Monoamine analysis noted that NAC significantly reduced cortical NA in FRL and FSL rats as well as DA in the striatum of FSL animals. A reduction in striatal DOPAC and HVA was also observed in FSL rats that received NAC when compared to the FRL rats receiving the same treatment. In the hippocampus NA was significantly increased in FRL rats but decreased in FSL animals compared to their respective vehicle controls. Hippocampal HVA was elevated in both strains while increased 5-HIAA levels was noted in FRL rats compared to their respective vehicle animals. Considering the above findings and the lack of response, especially in the behavioral cohort of the study, a decision was made to remove NAC as a positive control from the chronic study.

Results from the acute and chronic studies showed no significant changes in locomotor activity (OFT) between the FSL rats in the different treatment groups except for NAC reducing locomotor activity in the “healthy” FRL control rats after chronic treatment. For the most part these results indicate that any differences observed in the FST cannot be attributed to changes in locomotor activity, although reduced locomotor activity following NAC administration to FRL animals suggests cautious interpretation as this may generate a false negative result in the FST.

To our knowledge, no studies have looked at the antidepressant-like effects of NAC following chronic treatment in an animal model of MDD. Since all antidepressants require

chronic and not acute treatment for clinical efficacy, it was imperative that we undertake a chronic treatment paradigm in order to establish predictive validity for NAC (the ability of the animal model to predict the theorized outcome). To this end, we first undertook an acute treatment pilot study with NAC to confirm a viable dose for use in the chronic study.

The acute treatment study verified the antidepressant-like effect of NAC using a dose of 150 mg/kg, as suggested by earlier work in the literature (Ferreira *et al.*, 2008:747; Smaga *et al.*, 2012:280; Möller *et al.*, 2013:156). First, the depressive phenotype of FSL rats was established, viz. increased immobility and reduced swimming and climbing behaviors. Thereafter NAC was found to significantly reverse the increase in immobility in FSL rats and to increase swimming behavior, compared to FSL animals receiving vehicle (Fig. 1A & C respectively). Swimming behavior in the FST is related to underlying serotonergic mechanisms (Cryan *et al.*, 2005:547), indicating that the antidepressant-like effects of NAC is mediated through a bolstering of serotonergic pathways, at least when administered acutely. However, climbing behavior, which is linked to an underlying noradrenergic mechanism (Cryan *et al.*, 2005:547), was not significantly altered by acute treatment with NAC (150 mg/kg) in FSL rats (Fig. 1B), confirming a selective action on 5-HT. Based on this acute treatment pilot study, it seemed reasonable to assume that a dose of 150 mg/g/day would suffice for application in the chronic study.

MDD does not present as a single symptom but as a syndrome (see *Chapter 2, section 2.1.2*). Consequently the chronic study looked at more than just learned helplessness or despair in the FST (Lucki, 1997), but at deficits in memory (in the NORT) as an additional behavioral measure (Ennaceur & Delacour, 1988:47). Firstly, evaluation of cognitive function in the NORT did not deliver any significant differences, although a trend towards a decrease in novel object exploration time is evident in the stress sensitive FSL animals compared to the FRL controls, as would be expected. A possible reason for this negative result could be that the rats were too big relative to the size of the NORT boxes, thus complicating the process of distinguishing between actual exploration of the object or accidental unintended contact. Time was however limited and we were unable to undertake a comprehensive validation of the method before testing NAC. Further work on this is therefore imperative before definitive conclusions regarding NAC can be made.

FSL rats demonstrated a significant increase in immobility together with a decrease in climbing and swimming behavior, thereby reaffirming their stress-sensitive and depressive-like behavior, as already noted in the acute study above. Moreover, chronic NAC was unable to reverse increased immobility and reduced climbing and swimming in FSL rats, which was an unexpected finding. Indeed, the increased swimming behavior following NAC treatment

observed in the acute analysis now disappeared. These findings suggest that acute and chronic doses are not necessary equivalent, and further work on the basis for this difference is required.

Studies in rodents have found that oral administration of NAC has very low total serum concentrations when measured an hour after administration (Dickey *et al.*, 2008:235) and that the half-life of orally administered NAC is 6-40 minutes (Liu *et al.*, 2005:193; Dickey *et al.*, 2008:235). NAC is well absorbed from the small intestine but undergoes extensive first-pass metabolism through the liver, while intestinal metabolism of NAC further reduces its bioavailability after oral administration (Liu *et al.*, 2005:193). In this study the oral route and hepatic first past metabolism may have restricted the effect of NAC, since we have previously observed pharmacological effects following subcutaneous administration (Möller *et al.*, 2013:156).

With regard to the monoamine data, an increase in striatal 5-HT together with decreased NA was observed after NAC treatment (Fig. 5E & B respectively). The reduced NA levels may lead to the disinhibition of inhibitory α_2 adrenoceptors on 5-HT nerve terminals resulting in an increase in 5-HT neurotransmission as observed in the FSL rats (Maura *et al.*, 1982:272). Recent work has also revealed a decrease in the density of inhibitory α_2 adrenoceptors in FSL rats (Landau *et al.*, 2015:97). DOPAC metabolites were reduced in the frontal cortex which is probably due to the trend showing increased cortical DA ($p = 0.13$, Fig. 6A). A previous study found no differences in DA or DOPAC levels in the frontal cortex of FSL rats (Zangen *et al.*, 1999:243). The monoamine levels in the FSL rat therefore do portray certain aspects of MDD. There was however a general lack of changes across most of the other monoamines. Various trends could be observed including increased DA and decreased HVA in the frontal cortex and in the striatum. A trend was also noted in the decrease in cortical NA together with an increase in 5-HIAA in the frontal cortex and in the striatum. It is thus difficult to assure the validity of the model with respect to the monoaminergic changes observed between the FSL and FRL strains.

After chronic administration of NAC to FRL and FSL animals, NAC significantly reduced cortical NA in FRL and FSL rats. This was in line with a previous study where antidepressant treatment reduced NA in FSL rats in the same brain region (Zangen *et al.*, 1999:243). Hippocampal NA was also altered by NAC administration, with an increase observed in FRL rats but a reduction in FSL rats. These strain differences are important as it does support predictive validity to some extent, especially the realization that the pharmacological properties of antioxidants may differ according to the redox (and illness) state of the

organism and the presence of pathology (Emsley *et al.*, 2014:224). MDD is widely associated with dysfunction of the frontal cortex and hippocampus which play an important role in cognitive function (Ohashi *et al.*, 2002:131). The hippocampo-prefrontal cortical pathway and its activation by NA plays an important role in regulating long-term potentiation, a process involved in memory (Lim *et al.*, 2010:1219). This mechanism may in part underlie some of the effects resulting from the noradrenergic alterations described above.

The FSL rat has previously shown an *increase* in monoamines as well as normalization of these levels by antidepressant treatment (Malkesman *et al.*, 2006:17). Although mechanistically different to traditional antidepressants, such a mechanism may ultimately explain the reduction in striatal DA as well as the cortical and hippocampal NA levels after NAC treatment (Fig. 6B and 5A & C, respectively).

Hippocampal HVA was elevated in both strains while increased 5-HIAA levels was noted in FRL rats compared to their respective vehicle animals. Elevated hippocampal HVA levels (Fig. 6I) may indicate an increase in dopaminergic activity which allows for the stimulation of D_2 heteroreceptors on serotonergic cell bodies (Fig. 9 B.2) leading to an increase in 5-HT release (Chenu *et al.*, 2013:275), Fig. 9 B.3) and subsequent metabolism resulting in increased 5-HIAA levels.

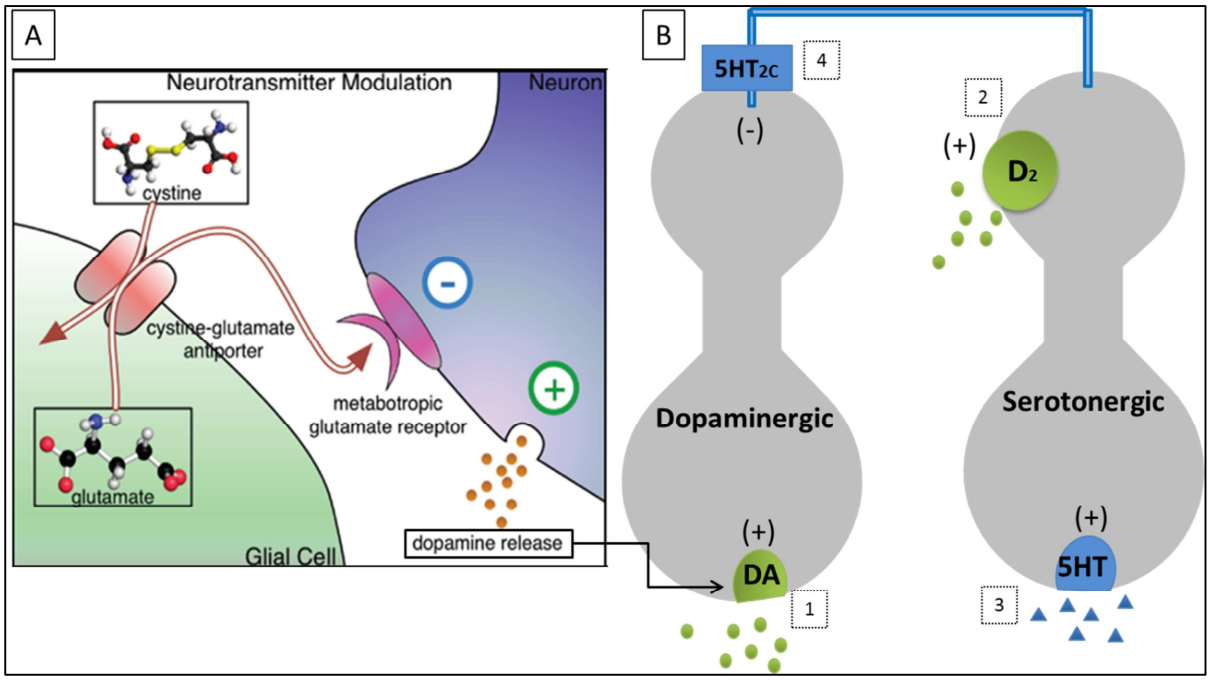


Figure 9. NAC induces DA release and subsequent 5-HT increase via D_2 stimulation.

NAC increases the activity of cystine–glutamate antiporter, resulting in an increase in the activation of glutamate receptors on inhibitory neurons which facilitates DA release (Dean *et al.*, 2011:78) and an associated increase in 5-HT transmission (Chenu *et al.*, 2013:275)**.**

The brain is highly vulnerable to oxidative stress due to the high levels of polyunsaturated fatty acids and low concentrations of endogenous antioxidant enzymes (Eren *et al.*, 2007:1188; Maes *et al.*, 2011:676; Sarandol *et al.*, 2007:67). Due to the reduced antioxidant capacity the defence against oxidative stress is impaired resulting in damage to fatty acid membrane lipids (Maes *et al.*, 2011:676). Moreover, MDD has been described as a pro-oxidative state (Leonard & Maes, 2012:764), which ideally should be replicated in the FSL rat. Indeed, bio-analysis demonstrated that FSL rats receiving vehicle had significantly elevated levels of hippocampal lipid peroxidation when compared to the FRL vehicle group (Fig. 7C). Moreover, hippocampal damage in FSL rats described here, as well as structural brain changes described by other investigators (Neumann *et al.*, 2011:1357), correlates with hippocampal shrinkage so often described in MDD (Falkai *et al.*, 2013:1). FSL rats treated chronically with NAC (150 mg/kg) showed a definite trend in reduced lipid peroxidation (Fig. 7C), albeit not significantly so. In other animal models, especially those related to oxidative stress such as tardive dyskinesia e.g. (Harvey *et al.*, 2008:508) chronic NAC is distinctly advantageous in versing these changes. A few studies examining the oxidative status of FSL rats found elevated levels of the pro-oxidant nitric oxide but only after the animals was subjected to stress e.g. the escapable stress/ inescapable stress paradigm (Wegener *et al.*, 2010:461) or ozone exposure (Mokoena *et al.*, 2010:125). Our study did not involve a stress inducing aspect and therefore the oxidation status of the FSL animals was possibly not the same as in previous studies.

Immune-inflammatory cytokines exert a central and peripheral role in immune system signaling while MDD is widely described as an inflammatory state (Brand *et al.*, 2015:324). Unfortunately, we were unable to demonstrate a similar scenario in FSL animals, with plasma IL-10 and TNF-α levels unaltered vs. FRL control animals. Furthermore, neither strain nor the treatment with NAC had a significant effect on IL-10 or TNF-α levels. However, a previous study has shown that NAC reduces TNF-α in rat models of traumatic brain injury (Dean *et al.*, 2011:78).

Another objective of this study was to consider the drug treatment response in FRL (i.e. healthy) vs. FSL (i.e. sick or “depressed”) animals. The basis for this question is firstly to validate the findings for MDD, and secondly to establish whether NAC’s efficacy may be dependent on the pathological state. In other words, does the presence of severe pathology such as oxidative stress and inflammation modify NAC’s response? Although it would have been ideal to access illness severity as a function of pathology (redox and immune status) vs. behavior for *each* animal using an appropriate correlation analysis, we undertook a preliminary analysis using a two-way ANOVA, as reported in the results, and drew tentative

conclusions from this. Based on this, we noted that NAC portrayed an antioxidant role in FSL rats by reducing lipid peroxidation (although not significantly) while in healthy FRL control animals NAC showed to be a pro-oxidant by slightly increasing lipid peroxidation (Fig. 7C). The antioxidant potential of NAC may therefore be dependent on the oxidative state. Furthermore NAC had a different effect in FRL and FSL rats regarding hippocampal NA (Fig. 5C) and striatal DOPAC (Fig. 6E) and HVA (Fig. 6H) by demonstrating an increase in these neurotransmitters in FRL rats while decreasing them in the FSL animals (although not always significantly). NAC administration also displayed different effects in hippocampal 5-HIAA levels by significantly increasing 5-HIAA in FRL animals with no effect on FSL rats (Fig. 5I).

Antidepressants have no mood uplifting effects in healthy individuals (Kanemaru *et al.*, 2009:363), while antidepressants are not always effective in managing severe MDD (Thase, 2002:95). Hence, a less effective antidepressant that shows efficacy in the FST using a *normal healthy* animal, such as the FRL rat, may be less effective when challenged with a pathological (translational) animal model, such as the FSL rat. This would also access differences in treatment response as a factor of presenting pathology (i.e. monoamine changes, altered redox, inflammation etc.) and genetic predisposition. Furthermore, there has been some debate as to whether an antioxidant performs differently depending on the redox status of the organism (Emsley *et al.*, 2014:224), with dose and response dependent on the pathological state of the subject (i.e. illness progression and illness severity). Based on our preliminary data, it can be said that this study did not demonstrate *significant* differences in NAC response in FRL and FSL rats, although subtle differences were evident. These differences could be due to dose and route of administration. Based on the subtle strain effect for NAC, its response may have been amplified with the addition of a stressor, leading to a worsened pathophysiological state, with increased redox-inflammatory imbalance that would be more amenable to treatment with NAC.

A.1.4. Conclusion

The response to NAC in FSL animals, both at behavioral and neurobiology level, was disappointing in that even after promising results in the acute study less emphatic results were obtained after chronic administration (NORT, FST). NAC also demonstrated no significant effect on lipid peroxidation or cytokine levels. Monoamine analysis demonstrated that NAC significantly reduced cortical NA in FRL and FSL rats as well as striatal DA in FSL animals. Striatal DOPAC and HVA reduction could also be observed in FSL rats that received NAC while NA was significantly increased in FRL rats but decreased in FSL animals in the hippocampus compared to their respective vehicle controls. Hippocampal

HVA was elevated in both strains while increased 5-HIAA levels was noted in FRL rats compared to their respective vehicle animals. Based on the above findings and the general lack of response, especially in the behavioral cohort of the study, it was decided to remove NAC as a positive antioxidant control from the chronic study. The chronic study would therefore remain controlled by imipramine, which not only is an effective antidepressant in humans and in rats (Abraham *et al.*, 1963:286; Klerman & Cole, 1965:101; Chenu *et al.*, 2013:275), but has also demonstrated antioxidant-like effects (Reus *et al.*, 2010:325).

These shortcomings in efficacy may be due to the use of the oral route for NAC and the effect of hepatic first past metabolism, as well as issues such as the sensitivity of the FST for acute vs. chronic treatment paradigms. Another aspect is that the precise dosage for NAC in MDD remains elusive, while there is a paucity of chronic treatment studies in animals, especially in translational animal models. The *in vivo* use of antioxidants and the specific dose needed has attracted a great deal of controversy in numerous studies (Emsley *et al.*, 2014:224; Seybolt, 2014:222). The prediction of the therapeutic effects of an antioxidant in the treatment of MDD (and in animals) is complex due to the fact that antioxidants may act either as a pro- or antioxidant depending on the redox status of the organism (Tylicki *et al.*, 2003:303; Flora, 2009:191), which in turn relates to illness state (or severity). Based on our findings NAC demonstrated an antioxidant effect in FSL rats by reducing lipid peroxidation (although not significantly) while in healthy FRL control animals NAC showed to be a pro-oxidant by slightly increasing lipid peroxidation. The oxidative state of the animals may therefore predict the antioxidant potential of NAC. Stress increases the oxidative state (Wegener *et al.*, 2010:461) which may therefore induce a pro-oxidative state and improve the effectiveness of NAC. Further work in this area is definitely warranted.

Addendum A.2

The main hypothesis for this study (see *Chapter 1.3*) was that the treatment of a translational animal model of MDD (FSL rats) with GM and IMI, as a positive control, will demonstrate:

- Pro-cognitive activity as tested in the NORT after 14 days of chronic treatment.
- FSL animals will present with an alteration in striatal levels of 5-HT, 5-HIAA, NA, DA, DOPAC and HVA levels vs. FRL animals, which will be reversed following chronic treatment with IMI and GM.
- Reversal of FSL associated elevation in markers of oxidative stress.
- Antidepressant-like effects will not be observed in IMI treated FRL rats to provide more convincing evidence of efficacy for the tested compounds to evaluate differences in treatment response as a factor of presenting pathology.

A.2.1 Materials and methods

A.2.1.1 Animals

Male FSL rats and a corresponding behavioral control line, FRL rats, were used for the current study as described previously in *Chapter 3 section 2.1*.

A.2.1.2 Drug treatment

Imipramine hydrochloride (Sigma Aldrich, Kempton Park, South Africa) was dissolved in double distilled water and administered at a fixed dose of 20 mg/kg by oral gavage (Monleon *et al.*, 1995:453; Brand, 2011:1; Gigliucci *et al.*, 2014:1349). The GM powder (Industrial Analytical, Kyalami, South Africa) was incorporated in a 0.1% xanthan gum solution for dispersion.

A.2.1.3 Study design

The study consisted of a behavioral and a neurochemical cohort, performed in both FRL and FSL rats as described previously in *Chapter 3 section 2.3*.

A.2.1.4 Behavioral tests

Following the vehicle/drug treatment for the behavioral cohort of the chronic treatment, the NORT, described above in *section A.1.2.2.1* was performed.

A.2.1.5 Biological studies

The quantification of 5-HT, 5-HIAA, NA, DA, DOPAC and HVA in striatal tissue were performed as described previously in *Chapter 3 section 3.2.3.* DA, DOPAC and HVA levels in the hippocampus and frontal cortex were below the limit of detection, and could not be reported.

A.2.1.6 Statistical analysis

A two-way analyses of variance (ANOVA) was followed by the Bonferroni post-hoc test comparing all groups to the FSL vehicle control group. Grubbs' test was used to identify outliers. GraphPad Prism® (version 5.00, San Diego California, U.S.A.) was used for all statistical analyses and graphical representation. Data are presented as mean ± SEM with a value of $p < 0.05$ regarded as statistically significant.

A.2.2 Results

A.2.2.1 Chronic behavioral study

A.2.2.1.1 Novel object recognition test

A two-way ANOVA of the data showed no significant treatment*strain interaction ($F(3, 44) = 1.821$, $p = 0.1572$). Not the treatment or the strain significantly affected novel object exploration time ($F(2.66) = 0.54$, $p = 0.59$; $F(1.66) = 3.42$, $p = 0.07$, respectively). Consequently, no effect on novel object recognition was evident for FSL or FRL rats following IMI as well as the 50 mg/kg dose of GM, compared to the respective vehicle groups or between the two strains (Fig. 10).

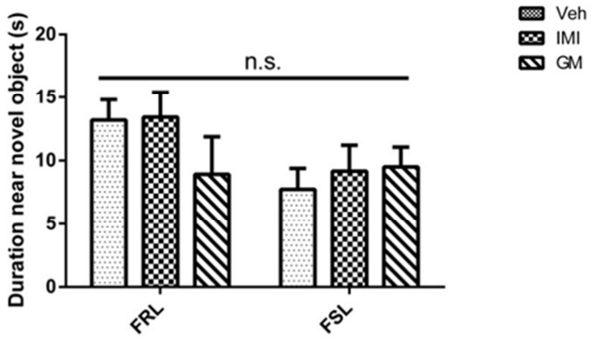


Figure 10. Effect of chronic administration of vehicle, IMI (20 mg/kg) as well as the GM (50 mg/kg) to FRL and FSL rats on the time spent exploring the novel object compared to the FSL vehicle group (two-way ANOVA, Bonferroni post-hoc test), n.s = not significant.

A.2.2.2. Chronic biological analysis

A.2.2.2.1 Striatal monoamine analysis

Serotonin: Two-way ANOVA of treatment*strain interaction demonstrated no significance in the striatum ($F(2.66) = 0.58$, $p = 0.56$) and although no significant effect of treatment on the 5-HT levels was observed ($F(2.66) = 3.07$, $p = 0.05$), a significant effect of strain was evident ($F(1.66) = 21.27$, $p < 0.0001$). Bonferroni testing showed no significant interaction between the groups except for a significant increase in 5-HT in FSL vs. FRL vehicle treated groups ($p = 0.02$, Fig. 11A).

5-HIAA: Two-way ANOVA of treatment*strain interaction demonstrated significance in the striatum ($F(2.65) = 3.92$, $p = 0.02$). A significant effect could also be observed with regard to treatment ($F(2.65) = 5.43$, $p = 0.007$) and strain ($F(1.65) = 5.65$, $p = 0.02$) on 5-HIAA levels. No difference could be seen between FSL and FRL control groups ($p = 0.43$) or between FSL IMI ($p > 0.99$) and FSL GM ($p = 0.06$) groups vs. the FSL control group (Bonferroni post-hoc test). There was however a significant increase in 5-HIAA in the FRL IMI group ($p = 0.01$) vs. the FRL control group (Bonferroni post-hoc test, Fig. 11B).

Noradrenaline: Two-way ANOVA of treatment*strain interaction demonstrated significance in the striatum ($F(2.64) = 4.91$, $p = 0.01$) and although no significant effect was noted on NA levels regarding the treatment ($F(2.64) = 10.35$, $p = 0.0001$), the strain affected it significantly ($F(1.64) = 2.58$, $p = 0.11$). A Bonferroni post-hoc test showed that NA was significantly lower in FSL vehicle when compared to FRL vehicle animals ($p = 0.01$) that in turn was significantly increased by both IMI ($p = 0.0006$) and GM ($p = 0.0001$) treatment, respectively (Fig. 11C).

Dopamine: Two-way ANOVA of treatment*strain interaction demonstrated significance in the striatum ($F(2.66) = 5.959$, $p = 0.004$). A significant treatment effect on DA levels was observed ($F(2.66) = 35.52$, $p < 0.0001$) while the rat strain had no significant effect ($F(1.66) = 0.04$, $p = 0.84$). Bonferroni post-hoc test of the data showed a significant decrease in DA levels in the FSL IMI ($p < 0.0001$, Fig. 11D) and the FSL GM groups ($p > 0.0001$, Fig. 11D) compared to the FSL vehicle group, although no significant difference in DA levels between the FSL and FRL vehicle control rats was observed ($p = 0.49$, Fig. 11D). The FRL IMI group also demonstrated a significant decrease in DA compared to the FRL control group ($p = 0.0001$, Fig. 11D).

DOPAC: Two-way ANOVA showed no significant treatment*strain interaction ($F(2.65) = 0.48$, $p = 0.62$). A significant effect of NAC treatment on DOPAC levels was observed ($F(2.65) = 4.03$, $p = 0.02$) while no significant effect was observed relevant to the strain (F

(1.65) = 3.77, p = 0.06). There was no significance between any of the groups with regards to striatal DOPAC (Fig. 11E).

HVA: A two-way ANOVA showed no significant treatment*strain interaction (F (2.65) = 2.99, p = 0.06) or a significant effect of treatment on HVA levels. The strain however did significantly affect these levels (F (1.65) = 3.61, p = 0 < 0.0001). There was no significance between any of the groups in the striatum except between the FSL groups treated with IMI (p = 0.0002) and GM (p = 0.001) compared to their FRL counterparts receiving the same treatment (Fig. 11F).

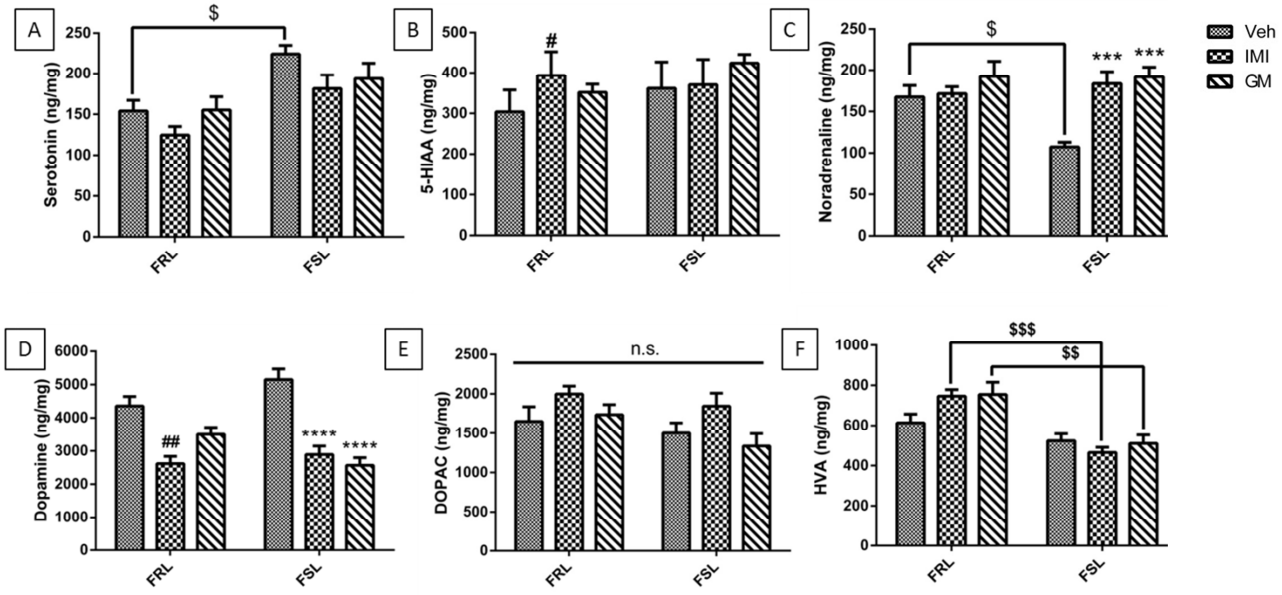


Figure 11. The levels of 5-HT, 5-HIAA, NA, DA, DOPAC and HVA in the striatum of FSL and FRL rats following treatment with vehicle, or IMI (20 mg/kg) and GM (50 mg/kg). ($^{\$}p < 0.05$ vs. FRL Veh, $^{\$\$}p < 0.01$ vs. FRL Veh, $^{\$\$\$}p < 0.001$ vs. FRL Veh, $^{\#}p < 0.05$ vs. FRL Veh, $^{\#\#}p < 0.01$ vs. FRL Veh, ***p < 0.001 vs. FSL Veh, ****p < 0.0001 vs. FSL Veh; two-way ANOVA, Bonferroni post-hoc test), n.s = not significant. Refer to the text for the exact P-values.

A.2.2.2.2 Lipid peroxidation

Treatment*strain interactions by means of a two-way ANOVA demonstrated no significance in the frontal cortex (F (2.66) = 1.68, p = 0.19, Fig. 12A) or in the striatum (F (2.66) = 0.67, p = 0.51, Fig. 12B). Neither treatment nor strain significantly affected lipid peroxidation in the frontal cortex (F (2.66) = 1.97, p = 0.15; F (2.66) = 0.89, p = 0.41, respectively) or in the striatum (F (1.66) = 3.39, p = 0.07; F (1.66) = 1.09, p = 0.29, respectively).

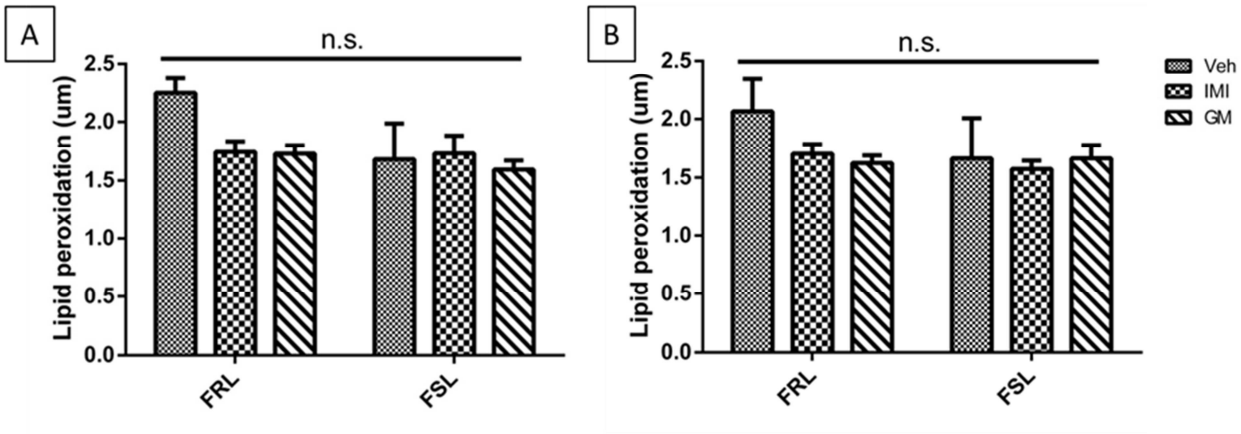


Figure 12. Lipid peroxidation in the frontal cortex (A) and striatum (B) of FSL and FRL rats and the effect of chronic administration of IMI (20 mg/kg) and GM (50 mg/kg). (Two-way ANOVA, Bonferroni post-hoc test), n.s = not significant.

A.2.3 Discussion

Evaluation of cognitive function by means of the NORT did not deliver any significant differences although a trend towards a decrease in novel object exploration time was observed in FSL vs. FRL vehicle treated controls. A slight increase in novel object exploration was observed following treatment with IMI (20 mg/kg) or GM (50 mg/kg), although this was not significant. We therefore could not incorporate the NORT data into *Chapter 3*, or use it as part of our strategy to address the earlier mentioned objectives. Possible reasons for the lack of usable results are the large size of the rats relative to the size of the NORT boxes, thus complicating the process of distinguishing between actual exploration of the object or accidental unintended contact. As noted earlier, further validation of this method is recognized. However, time constraints prevented this from happening within the time lines of this study and will be pursued at a later stage.

Previous work has demonstrated that FSL rats show an increase in monoamine levels as well as normalization of these levels by antidepressant treatment (Malkesman *et al.*, 2006:17). Increased striatal DA levels (although not significant) in FSL rats compared to FRL control animals was significantly reversed by GM and IMI, while significantly increased striatal 5-HT levels in FSL rats compared to the FRL control animals was partially decreased by GM and IMI (although not significantly). Significantly reduced striatal NA in FSL vs. FSL controls was also significantly reversed by both IMI and GM. These findings indicate the targeting of monoamine signaling by both IMI and GM. IMI is a dual 5-HT and NA reuptake inhibitor which explains its reversal of lowered NA in FSL rats. Its action to reverse elevated 5-HT in FSL rats may rest more on re-establishing synaptic feedback mechanisms after chronic treatment, e.g. up-regulating 5-HT_{1A} autoreceptors (Zanoveli *et al.*, 2005:543).

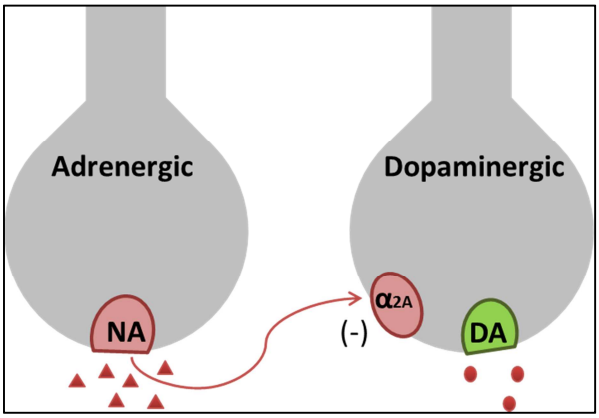


Figure 13. An initial stimulation of synaptic release of NA may reduce DA neurotransmission via inhibitory α_{2A} receptors (Rominger *et al.*, 2010:654).

Concerning the pronounced striatal DA reduction following both IMI and GM, the significant increase in striatal NA that ensues following these two drugs may reduce DA release via the stimulation of α_2 inhibitory heteroreceptors on DA terminals in the striatum (Rominger *et al.*, 2010:654), Fig. 13). DOPAC, HVA and 5-HIAA levels were not influenced by the strain or by the treatment with IMI or GM.

IMI decreased DA in the FSL as well as the "healthy" control animals, which raises questions as to the validity of these changes for efficacy in MDD. A possible explanation can be the increase of DA in the frontal cortex of the FRL rats (data not shown, some values under the limit of detection) resulting in reduced striatal DA levels, following a mechanism based on the DA hypothesis of schizophrenia (Howes & Kapur, 2009:549). In fact, previous research has noted that administration of a DA agonist to the frontal cortical area reduces DA metabolites in the striatum (Howes & Kapur, 2009:549). Concerning the significantly raised striatal levels of 5-HIAA in FRL rats following IMI treatment, sustained blockade of the 5-HT transporter (SERT) by IMI and a resultant elevation in 5-HT over time may engender a reactive up-regulation of 5-HT metabolism leading to greater production of its primary metabolite, 5-HIAA. This would suggest that some synaptic effects of IMI may not necessarily be related to its therapeutic effects, or alternatively that IMI may have noteworthy pharmacological effects in healthy individuals and that questions their role in its therapeutic effects.

There were no significant differences observed in lipid peroxidation in the frontal cortex and in the striatum. Further study is warranted to explore the reasons. The cortical and striatal lipid peroxidation data was therefore omitted from the article in *Chapter 3*.

A.2.4 Conclusion

Cognitive difficulties, as observed in MDD (Maes *et al.*, 2009:27), was not significantly affected by the strain of the rat or treatment with IMI or GM. Further studies need to evaluate object recognition memory using an improved method, which among others may involve the use of a larger NORT box, to use younger rats or alternatively to consider performing the test in mice. With regard to monoamine changes and their association with pathology, IMI (20 mg/kg) and GM (50 mg/kg) increased NA neurotransmission while reducing DA release in the striatum. Further studies are needed to explore the precise mechanism of GM by exploring its effects on the various serotonergic, noradrenergic and dopaminergic receptors and subtypes. Analysis of cognitive function by means of the NORT demonstrated no significant differences although a trend for reduction of novel object exploring behavior was observed in the FSL rats. Striatal monoamine analysis demonstrated significant differences between the two strains which included increased 5-HT and reduced NA in the FSL vehicle rats compared to the FRL vehicle animals. In the FSL animals IMI and GM significantly increased DA and reduced NA compared to the FSL vehicle rats while HVA levels were also reduced by both treatment groups when compared to the FRL counterparts receiving the same treatment. In “healthy” FRL rats IMI treatment demonstrated an increase in 5-HIAA as well as a reduction in striatal DA. Monoamine signaling is therefore targeted by both IMI and GM but further investigation is necessary to analyze the inherent monoamine differences between FSL and FRL rats and the contribution of monoamine differences to the construct validity of this animal model. This data was excluded from the article in *Chapter 3* due to the restricted scope of the article and to raise its impact. Evaluation of cortical and striatal lipid peroxidation levels showed no significant differences, and thus these data were also not included in the article in *Chapter 3*.

Addendum A.3

The main hypothesis for FRL control animals was that the treatment with GM and IMI, as a positive control, will demonstrate no different effect from that observed for the FRL vehicle group.

A.3.1 Materials and methods

A.3.1.1 Animals

Male FRL rats were used for the current study as described previously in *Chapter 3*.

A.3.1.2 Drug treatment

Imipramine hydrochloride (Sigma Aldrich, Kempton Park, South Africa) (IMI) was dissolved in double distilled water and administered at a fixed dose of 20 mg/kg by oral gavage (Brand, 2011:1; Gigliucci *et al.*, 2014:1349; Monleon *et al.*, 1995:453). The GM powder (Industrial Analytical, Kyalami, South Africa) was incorporated in a 0.1% xanthan gum solution for dispersion.

A.3.1.3 Study design

The study consisted of a behavioral and a neurochemical cohort, performed in FRL rats as described previously in *Chapter 3 section 2.3*.

A.3.1.4 Behavioral tests

Following the vehicle/drug treatment for the behavioral cohort of the chronic treatment, the OFT and the FST (*described in Chapter 3.2.4.1 & 2*), were performed.

A.3.1.5 Biological studies

The quantification of 5-HT, 5-HIAA, NA, DA, DOPAC and HVA in cortical and hippocampal tissue were performed as described previously in *Chapter 3 section 2.5.2.*

A.3.1.6 Statistical analysis

A one-way ANOVA was followed by the Bonferroni post-hoc test using multiple comparisons. Grubbs' test was used to identify outliers. GraphPad Prism® (version 5.00, San Diego California, U.S.A.) was used for all statistical analyses and graphical representation. Data are presented as mean ± SEM with a value of $p < 0.05$ regarded as statistically significant.

A.3.2 Results

A.3.2.1 Open field test

A one-way ANOVA of the data showed a significant treatment*locomotor activity interaction (F (2.33) = 10.45, p = 0.0003). Bonferroni post-hoc test indicated a significant decrease in locomotor activity in GM compared to the FRL vehicle (p = 0.0004) and to the IMI group (p = 0.004, Fig. 14).

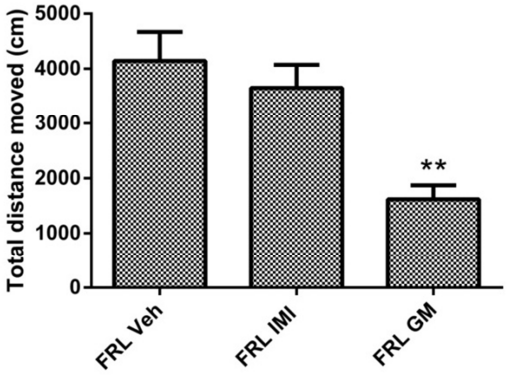


Figure 14. Effect of chronic administration of vehicle, IMI (20 mg/kg) as well as the GM (50 mg/kg) to FRL rats on locomotor activity (**p < 0.01 vs. FRL Veh; one-way ANOVA, Bonferroni post-hoc test). See the text for exact P-values.

A.3.2.2 Forced swim test

After chronic administration of IMI and GM, a one-way ANOVA of the data revealed no significant treatment*behavior interaction regarding immobility (F (2.33) = 0.83, p = 0.44); climbing (F (2.33) = 0.36, p = 0.7); or swimming (F (2.33) = 0.67, p = 0.52). The FST behaviors (data not shown) were therefore not affected by treatment with IMI or GM.

A.3.2.3 Monoamine analysis

Noradrenaline: MHPG levels were below the limit of detection, and could not be reported. A one-way ANOVA demonstrated no significant treatment*NA interactions in the frontal cortex (F (2.33) = 3.17, p = 0.05, data not shown) or in the hippocampus (F (2.33) = 2.74, p = 0.08, data not shown). Cortical and hippocampal NA were therefore not affected by treatment with IMI or GM.

Serotonin: A one-way ANOVA demonstrated significant treatment*5-HT interactions in the frontal cortex (F (2.33) = 9.45, p = 0.0006) and in the hippocampus (F (2.32) = 11.05, p = 0.0002). Bonferroni post-hoc testing indicated a significant decrease in cortical 5-HT after

treatment with IMI (p = 0.0004, Fig. 15A) while in the hippocampus both the IMI (p = 0.01) and GM group (p = 0.0002, Fig. 15B) significantly reduced 5-HT.

5-HIAA: A one-way ANOVA demonstrated significant treatment*5-HIAA interactions in the frontal cortex (F (2.33) = 6.09, p = 0.006) but not in the hippocampus (F (2.33) = 2.19, p = 0.13, Fig. 15D). Bonferroni post-hoc testing indicated a significant increase in 5-HIAA in the frontal cortex of IMI treated FRL rats (p = 0.005, Fig. 15C) compared to the FRL control. No post-hoc test was conducted on hippocampal 5-HIAA levels based on the result of the one-way ANOVA.

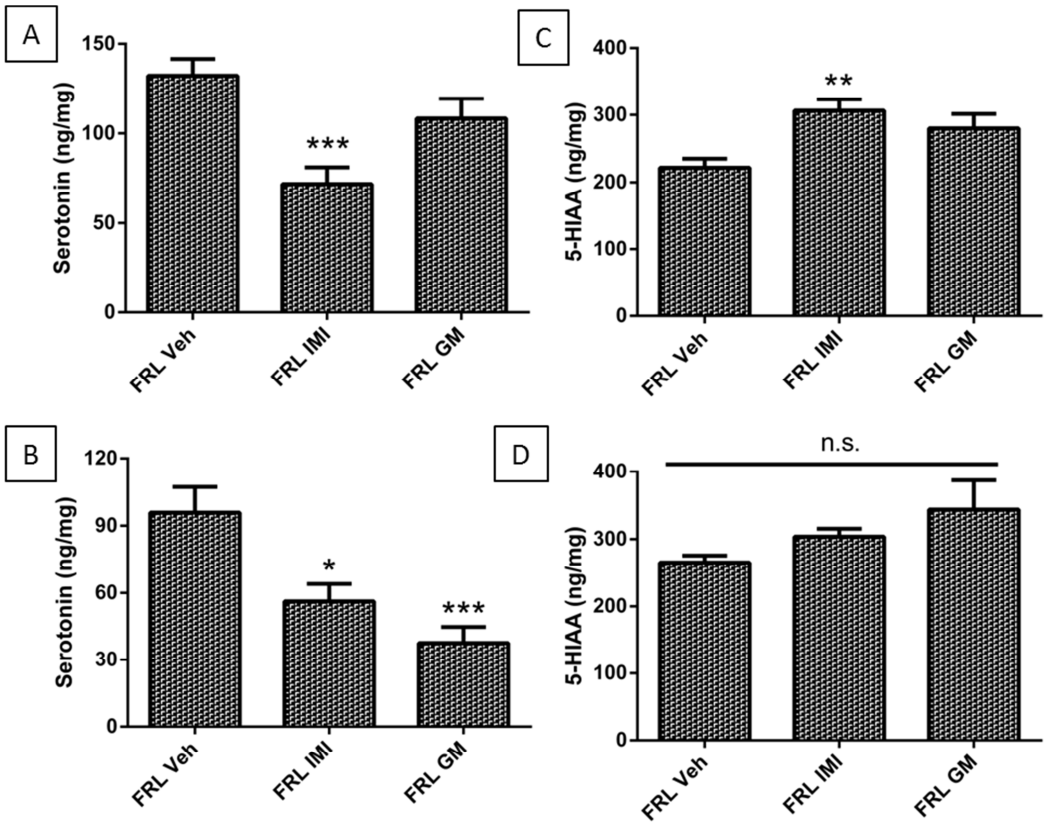


Figure 15. 5-HT and 5-HIAA levels in the frontal cortex (A), (C) and hippocampus (B), (D) of FRL rats.

(*p < 0.05 vs. FRL Veh; **p< 0.01 vs. FRL Veh; ***p< 0.001 vs. FRL Veh; one-way ANOVA, Bonferroni post-hoc test), n.s. = non-significant. See text for the exact P-values.

A.3.2.4 Hippocampal lipid peroxidation

A one-way ANOVA demonstrated no significant treatment*lipid peroxidation interactions in the hippocampus (F (2.33) = 0.09, p = 0.91, data not shown). Hippocampal lipid peroxidation was therefore not affected by treatment with IMI or GM.

A.3.2.5 Immune-inflammatory cytokines

A one-way ANOVA revealed a significant treatment*IL-10 interaction ($F(2.3) = 13.53$, $p < 0.0001$). Bonferroni post-hoc testing found that IL-10 was significantly increased by administration of GM compared to the FRL vehicle group ($p < 0.0001$) and compared to the FRL IMI group ($p = 0.001$, Fig. 16). TNF-α remained unaltered by any treatment group ($F(2.33) = 0.002$, $p = 0.99$, data not showed).

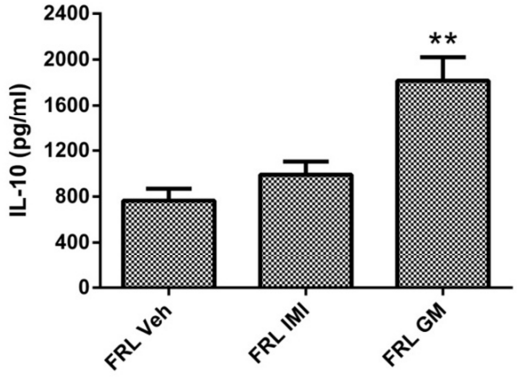


Figure 16. Plasma IL-10 levels in FRL rats after chronic administration of IMI (20 mg/kg) and GM (50 mg/kg). (**p < 0.01 vs. FRL Veh, one-way ANOVA, Bonferroni post-hoc test). See the text for the exact P-values.

A.3.3 Discussion & Conclusion

The most important findings of the current study are that GM significantly reduced locomotor activity in "healthy" FRL rats (Fig. 14) but not in FSL rats as was observed in *Chapter 3 section 3.2.1*. As described above in *section A.1.2.2.2*, the "healthy" FRL group that received NAC also demonstrated reduced locomotor activity after chronic treatment with no effect on FSL animals. There may thus be a possible common element between NAC and GM and this warrants further study. Reduced locomotor activity suggests cautious interpretation as this may generate a false negative result in the FST. In the FST there was no significant differences observed between any of the treatment groups concurrent with the fact that antidepressants have no mood uplifting effects in healthy individuals (Kanemaru *et al.*, 2009:363).

GM and IMI both significantly affected 5-HT levels in FRL rats. IMI significantly reduced cortical 5-HT while increasing 5-HIAA levels in the same brain region (Fig. 15A & C respectively), implying increased turnover of 5-HT to its metabolite. IMI and GM reduced 5-HT levels in the hippocampus although no significant effect was noted for hippocampal 5-

HIAA (Fig. 15B & D respectively). The same trend for 5-HT and 5-HIAA can be observed in *Chapter 3 section 3.2.3.*

There was no effect of IMI or GM treatment on cortical or hippocampal lipid peroxidation or plasma TNF-α analysis in FRL rats. However, plasma IL-10 levels were significantly elevated by GM, in keeping with an anti-inflammatory action of the drug (Fig. 16). This observation suggests that an antioxidant may indeed have antioxidant effects in a non-pathological state, although more definitive studies are required to verify this.

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