Studies on *Garcinia mangostana* Linn as a therapeutic intervention in an immune-inflammatory model of schizophrenia

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“I cannot pretend I am without fear.

But my predominant feeling is one of gratitude.

I have loved and been loved;

I have been given much and I have given something in return;

I have read and travelled and thought and written.

Above all, I have been a sentient being,

A thinking animal, on this beautiful planet,

And that in itself has been an enormous privilege and adventure.”

- Oliver Sacks

ABSTRACT

Schizophrenia is a life-long psychiatric disorder affecting 0.5-1% of the global population. This illness typically presents with severely debilitating clinical features in late adolescence, including positive, negative and cognitive symptoms. It is triggered in a multi-factorial manner by genetic, environmental and neurodevelopmental risk factors, contributing to the complexity of the disease. Prenatal factors are especially prominent in its aetiology, such as obstetric complications, substance abuse and prenatal maternal infection. Although the exact underlying mechanisms of schizophrenia remains unclear, oxidative stress and inflammation pathways have been implicated in its pathophysiology. Since currently-available treatment regimens for schizophrenia are notoriously inadequate for successful management of the clinical syndrome, novel strategies with regards to pharmacotherapy are urgently required. The utility of antioxidant treatment, especially from plant origin, holds substantial interest given the role of oxidative stress in schizophrenia. The pericarp of *Garcinia mangostana* Linn (GML) or mangosteen, an exotic fruit from Southeast Asia, contains numerous bioactive components including the dominant constituent α-mangostin (AM), that are known for their antioxidant and anti-inflammatory activity.

Considering that prenatal inflammation have been associated with an increased susceptibility for schizophrenia, the maternal immune activation (MIA) animal model provides a valuable framework for exploring potential treatment strategies and underlying behavioural and biological deficits in schizophrenia. The rodent MIA model involves exposing a pregnant dam to an infectious agent during gestation to mimic prenatal infection, subsequently inducing a maternal immune response that alters normal neurodevelopment in the offspring and leading to behavioural abnormalities later in life. These altered behaviours bear a striking similarity to many of the positive, negative and cognitive symptoms of schizophrenia. This study aimed to assess the therapeutic effects of GML and AM, as stand-alone or adjunctive treatment to well-known antipsychotic, haloperidol (HAL), on behaviour and plasma and brain immune-inflammatory bio markers related to schizophrenia using a MIA animal model.

Animals were bred and housed at the Vivarium of the North-West University (NWU) and all experiments were approved by the AnimCare animal research ethics committee of the NWU (Ethics approval number NWU-00376-16-A5). In the present study, prenatal immune activation was induced by exposing pregnant Sprague Dawley dams (n=18) to a bacterial endotoxin, lipopolysaccharide (LPS) (100 µg/kg) on gestational days 15 and 16. The male offspring from exposed dams were randomly divided into 6 treatment groups viz. vehicle; HAL (2 mg/kg); GML (50 mg/kg); HAL+GML; AM (20 mg/kg) and HAL+AM, consisting of ±12 rats

per group. Control dams (n=3) and their offspring (n=8) were treated with vehicle. The offspring were treated via oral gavage with the respective drug treatments for 16 days from postnatal day (PND) 52 – 66. On the last two days of treatment, all groups were subjected to the following behavioural tests: (1) social interaction test (SIT) on day 12 of treatment; (2) prepulse inhibition (PPI) on day 13 of treatment (PND 63); (3) open field test (OFT) on day 14 of treatment (PND 64); (4) forced swim test (FST) on day 14 of treatment (PND 64). 36 hours after the last behavioural test, rats were euthanized by decapitation followed by the collection of trunk blood and brain tissue for peripheral and neurochemical analyses. Frontal cortical, hippocampal and striatal lipid peroxidation as well as plasma levels of pro-inflammatory cytokines, interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α), were measured.

The MIA model elicited deficits in all behavioural paradigms studied, including reduced sensorimotor gating in the PPI test, increased locomotor activity in the OFT, depressive-like behaviour in the FST and an increase in social behaviour in the SIT. The MIA-induced deficits in %PPI were only successfully reversed by HAL and HAL+GML treatment. AM was the only treatment to significantly reduce MIA-induced locomotor hyperactivity. MIA-induced depressive-like behaviour was reversed by AM and GML alone and both in combination with HAL, with both combinations being more effective than HAL. Although HAL showed a trend towards antidepressant activity, this did not reach statistical significance. Concerning the SIT, prenatal LPS-challenged offspring showed an uncharacteristic increase in all social behaviours studied, a paradoxical finding when relating this to the well-known social deficits described in schizophrenia. Nevertheless, HAL+GML treatment reversed this finding, suggesting that further study in this regard is necessary. Elevated levels of lipid peroxidation markers were observed in the frontal cortex and striatum (but not hippocampus) of LPS exposed offspring, although only frontal cortical membrane damage was reversed by HAL and AM. Increased plasma concentrations of IL-6 and TNF-α in offspring of immune-compromised dams was reversed by GML, AM, HAL and combinations thereof, although no bolstering effect was observed with the latter.

In conclusion, this study confirms that the MIA model is able to induce behavioural deficits akin to schizophrenia symptomology, except social interaction that needs further investigation, together with peripheral and central redox-inflammatory alterations in the offspring later in life. This suggests that prenatal inflammation may affect the normal process of neurodevelopment and result in increased susceptibility for developing schizophrenia during late adolescence. MIA-induced bio-behavioural alterations showed variable responses to treatment, with HAL, GML and AM, with depressive manifestations showing the best response to GML, AM and a bolstering of response when either are combined with HAL, while GML+AM presents with

some benefit with respect to sensorimotor gating deficits. AM may be a more effective antioxidant than GML *in vivo*, although this does not imply improved therapeutic response. Overall, GML displayed superior effects over AM in combination with HAL and may be of clinical value as an adjunctive treatment to antipsychotic agents for improving the therapeutic outcome of schizophrenia.

Keywords

Schizophrenia, maternal immune activation, cytokines, oxidative stress, antioxidants, Garcinia mangostana Linn, alpha-mangostin.

OPSOMMING

Skisofrenie is ‘n lewenslange psigatriese toestand wat 0.5 -1% van die wêreldbevolking raak. Hierdie toestand presenteer oor die algemeen met ernstige afwykende kliniese kenmerke gedurende laat adolessensie, insluitend positiewe, negatiewe en kognitiewe simptome. Dit word op 'n multi-faktoriële wyse geaktiveer deur genetiese, omgewings- en ontwikkelingsrisikofaktore almal bydraend tot die kompleksiteit van die siekte. Prenatale faktore, soos obstetriese komplikasies, middelmisbruik en prenatale infeksie, is veral prominent in die etiologie. Terwyl die presiese onderliggende patofisiologiese meganismes van skisofrenie onduidelik bly, is daar sterk aanduidings dat oksidatiewe stres en inflammasie ‘n belangrike rol speel in die proses. Huidige beskikbare behandelingsregimens vir skisofrenie blyk egter ontoereikend te wees vir suksesvolle hantering van die kliniese sindroom, en dus word nuwe strategieë met betrekking tot farmakoterapie dringend benodig. Die bruikbaarheid van antioksidatiewe behandeling, veral van plantaardige oorsprong, is van groot belang veral met betrekking tot die rol van oksidatiewe stres in die patofisiologie van skisofrenie. Die perikarp van *Garcinia mangostana* Linn (GML) of mangosteen, 'n eksotiese vrug uit Suidoos-Asië, bevat talle bio-aktiewe komponente, insluitend die dominante bestanddeel α-mangostin (AM), wat bekend is vir hul antioksidatiewe en anti-inflammatoriese aktiwiteit.

In ag genome die feit dat prenatale infeksie geassosieer word met 'n verhoogde vatbaarheid vir skisofrenie, bied die moeder-immuun-aktiveringsdieremodel (MIA) 'n waardevolle raamwerk om moontlike behandelingsstrategieë en onderliggende gedrags- en biologiese veranderings in skisofrenie, te ondersoek. Die MIA-model behels die blootstelling van 'n swanger rot aan 'n aansteeklike agens om prenatale infeksie na te boots. Gevolglik word die moeder se immuunrespons geaktiveer en normale neuro-ontwikkeling in die nageslag verander en kan lei tot gedragsafwykings later in die lewe. Hierdie veranderde gedrag kom opvallend ooreenmet ‘n groot hoeveelheid van die positiewe, negatiewe en kognitiewe simptome van skisofrenie. Die huidige studie het dus ten doel om die terapeutiese effekte van GML en AM of as monoterapie, of addisionele behandeling in kombinasie met ‘n bekende antipsigotiese middel, haloperidol (HAL), te evalueer t.o.v. gedrag asook plasma- en brein-immuun-inflammatoriese bio-merkers geassosieer met skisofrenie, deur gebruik te maak van 'n MIA-dieremodel.

Diere is geteel en gehuisves in die Vivarium van die Noord-Wes Universiteit en alle eksperimente is goedgekeur deur die AnimCare-navorsingsetiekkomitee van die NWU (Etiese goedkeuring nommer NWU-00376-16-A5). In die huidige studie is prenatale immuunaktivering geïnduseer deur swanger Sprague Dawley rotte (n = 18) bloot te stel aan 'n bakteriese

endotoksien, lipopolisakkaried (LPS) (100 µg/kg) op swangerskapdae 15 en 16. Die manlike nageslag van blootgestelde wyfies is willekeurig verdeel in 6 behandelingsgroepe nl. geneesmiddeldraagstof; HAL (2 mg/kg); GML (50 mg/kg); HAL + GML; AM (20 mg/kg) en HAL + AM, bestaande uit ± 12 rotte per groep. ‘n Kontrole groep swanger rotte (n = 3) en hul nageslag (n = 8) is met die geneesmiddeldraagstof behandel. Die nageslag is behandel met die onderskeie behandelings vir 16 dae vanaf postnatale dag (PND) 52-66. Op die laaste twee dae van behandeling is alle groepe onderwerp aan die volgende gedragstoetse: (1) sosiale-interaksie-toets (SIT) op dag 12 van behandeling; (2) prepuls-inhibisie (PPI) op dag 13 van behandeling (PND 63); (3) oop-veldtoets (OFT) op dag 14 van behandeling (PND 64); (4) gedwonge-swem oets (FST) op dag 14 van behandeling (PND 64). Ses en dertig uur na die laaste gedragstoets, is die rotte onthoof, en bloed en breinweefsel vir perifere en neurochemiese bepalings is versamel. Frontale kortikale, hippokampale en striatale lipiedperoksidasie sowel as plasmavlakke van pro-inflammatoriese sitokiene, interleukien-6 (IL-6) en tumornekrosefaktor-α (TNF-α), is gemeet.

Die MIA-model het veranderings in alle gedragsparadigmas veroorsaak, insluitend verminderde sensorimotoriese versperring in die PPI-toets, verhoogde motoriese aktiwiteit in die OFT, depressiewe gedrag in die FST en 'n toename in sosiale gedrag in die SIT. Die MIA-geïnduseerde tekorte in% PPI is slegs suksesvol omgekeer deur HAL en HAL+GML behandeling. AM was die enigste behandeling wat MIA-geïnduseerde lokomotoriese hiperaktiwiteit betekenisvol kon verminder. MIA-geïnduseerde depressiewe gedrag is omgekeer deur AM en GML alleen en beide in kombinasie met HAL, met beide kombinasies meer effektief as HAL alleen. HAL was oneffektief as 'n antidepressant. Met betrekking tot die SIT, het prenatale LPS-blootgestelde nageslag 'n onverwagte toename in alle sosiale gedragsondersoeke getoon, 'n paradoksale bevinding wanneer dit in verband gebring word met die bekende sosiale afwykende gedrag wat vir skisofrenie beskryf word. Nietemin, HAL + GML behandeling het hierdie resultate omgekeer, wat daarop dui dat verdere studie in hierdie verband nodig is. Verhoogde vlakke van lipiedperoksidasie merkers is in die frontale korteks en striatum (maar nie in die hippokampus nie) van LPS-blootgestelde nageslag waargeneem, hoewel slegs frontale kortikale membraanskade omgekeer is deur HAL en AM. Verhoogde plasmakonsentrasies van IL-6 en TNF-α in die nageslag van immuun-gekompromitteerde wyfies is omgekeer deur GML, AM, HAL en kombinasies daarvan, hoewel geen potensiërings-effek met laasgenoemde waargeneem is nie.

Ten slotte bevestig hierdie studie dat die MIA-model in staat is om gedragsafwykings wat kenmerkend is van skisofrenie-simptome, tesame met perifere en sentrale redoks-inflammatoriese veranderinge in die nageslag, te veroorsaak. Dit dui daarop dat prenatale

inflammasie die normale proses van neuro-ontwikkeling kan beïnvloed en kan lei tot verhoogde vatbaarheid vir die ontwikkeling van skisofrenie tydens laat adolessensie. MIA-geïnduseerde bio-gedragsveranderings het ‘n verskeidenheid reaksies op behandeling met HAL, GML en AM getoon.Depressiewe manifestasies het die beste reaksie op GML, AM en 'n versterking van reaksie getoon wanneer dit gekombineer is met HAL, terwyl GML + AM ten opsigte van sensorimotoriese versperringstekorte die beste reaksie gegee het. AM mag dalk 'n meer effektiewe antioksidant as GML *in vivo* wees, hoewel dit nie ‘n beter terapeutiese effek impliseer nie. Oor die algemeen het GML beter effekte as AM in kombinasie met HAL vertoon en kan GML moontlik van kliniese belang wees as 'n addisionele behandeling in kombinasie met antipsigotiese middels om sodoende die terapeutiese uitkoms van skisofrenie te verbeter.

Sleutelwoorde:

Skisofrenie, moeder-immuunaktivering, sitokiene, oksidatiewe stres, antioksidante, *Garcinia mangostana* Linn, alfa-mangostin.

CONGRESS PROCEEDINGS

Excerpts from this study were presented as follows:

A comparative study of *Garcinia mangostana* Linn and α-mangostin vs. haloperidol on selected behaviour in an immune-inflammatory model of schizophrenia

Jana Lotter, Marisa Möller, Olivia M. Dean, Michael Berk, Brian H. Harvey

The results were presented as a podium presentation at the South African Society for Basic and Clinical Pharmacology Congress, 2-5 October 2017, University of the Free State, Bloemfontein, South Africa.

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LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine (serotonin)
A	
ACh	Acetylcholine
AChE	Acetylcholinesterase
AM	α-mangostin
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
C	
Ca^{2+}	Calcium
CAT	Catalase
CNS	Central nervous system
COX	Cyclooxygenase
D	
DA	Dopamine
DNA	Deoxyribonucleic acid
DSM	The Diagnostic and Statistical Manual of Mental disorders
E	
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
EPS	Extrapyramidal symptoms

F

FSL	Flinders Sensitive Line
FST	Forced swim test

G

GABA	Gamma-amino butyric acid
GD	Gestational day
GML	Garcinia mangostana Linn
GPx	Glutathione peroxidases
GSH	Glutathione

H

HAL	Haloperidol

I

IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IL	Interleukin
IV	Intravenously

K

KMO	Kynurenine-3-monooxygenase
KYN	Kynurenine
KYNA	Kynurenic acid

L

LDL	Low density lipoprotein
LI	Latent inhibition

LPS	Lipopolysaccharide
LS	Limbic system

M

MAO	Monoamine oxidase
MDA	Malondialdehyde
mDNA	Mitochondrial DNA
MIA	Maternal immune activation
MRI	Magnetic resonance imaging

N

NA	Noradrenaline
NAC	N-acetyl cysteine
NMDA	N-methyl-d-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthase

O

OFT	Open field test
OXPHOS	Oxidative phosphorylation

P

PBS	Phosphate buffered solution
PCP	Phencyclidine
PEG	Polyethylene glycol
PET	Positron emission tomography
PFC	Prefrontal cortex

PG	Prostaglandin
PND	Postnatal day
Poly I:C	Polyinosinic:polycytidylic acid
PPI	Prepulse inhibition
PUFA’s	Polyunsaturated fatty acids

Q

QA	Quinolinic acid

R

RNS	Reactive nitrogen species
ROS	Reactive oxygen species

S

SD	Sprague Dawley
SEM	Standard error of the mean
SERT	Serotonin transporter
SIT	Social interaction test
SOD	Superoxide dismutase

T

TBARS	Thiobarbituric acid reactive substances
TDO	Tryptophan 2,3-dioxygenase
TLR	Toll-like receptor
TNF	Tumor necrosis factor

V

VTA	Ventral tegmental area

GLOSSARY

Affective flattening: To lack emotional expressiveness. Usually indicated by avoidance of eye contact, unresponsive facial expression and a reduction in general body language.

Alogia: A speech disturbance which may include a decrease in the amount of speech (poverty of speech) or speech that does not convey meaningful information (poverty of content of speech).

Anhedonia: Reduced ability to experience or anticipate pleasure

Apoptosis: Programmed cell death.

Asociality: Diminished interest in, motivation for, and appreciation of social interactions with others

Avolition: Refers to significant failure to engage in goal-directed behaviour.

Hyperprolactinemia: the presence of abnormally high levels of prolactin in the blood

Necrosis: The death of cells or tissue.

Polyinosinic:polycytidylic acid (Poly I:C): A synthetic analogue of double-stranded RNA, associated with viral infection. Binds to TLR-3, leading to cytokine production

Toll-like receptor: a class of proteins that plays an important role in the innate immune system

CHAPTER 1

INTRODUCTION

1.1 Dissertation approach and layout

In this dissertation, the key data will be presented in as a concept article to be submitted for possible publication in a peer review scientific journal (Chapter 3). Any supplementary data will be included in the addenda.

The dissertation format includes:

- 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
- Addenda

1.2 Problem statement

Schizophrenia is a severely debilitating neurodegenerative disorder (Davis *et al.*, 2014), influenced by a number of causative components. This illness affects approximately 1% of the world's population (Anderson & Maes, 2013) and is generally characterized by positive, negative and cognitive symptoms (Meyer, 2013). Positive symptoms include hallucinations, delusions and disordered thoughts whereas negative symptoms consist of flattened affect, impoverished speech, social withdrawal, apathy and anhedonia (Moller, 2007). The negative symptoms closely resemble the core affective symptoms of depression and may be appreciated by considering their mutual manifestations (Moller, 2007), while cognitive deficits include verbal learning, attention and memory impairments (Bowie & Harvey, 2006). Moreover, the treatment outcome for both negative and cognitive symptoms remain suboptimal so that studying novel therapeutic interventions is crucial (Blyler & Gold, 2000; Mishara & Goldberg, 2004; Fusar-Poli *et al.*, 2014).

Several hypotheses have been proposed in an attempt to clarify the aetiology and symptomology of schizophrenia; this confirms the complexity of the disorder and encourages

researchers to focus on understanding the underlying mechanism responsible for schizophrenia development and to develop improved treatments.

Abundant evidence indicates that maternal infection during pregnancy is one of the significant environmental risk factors of neurodevelopmental brain disorders in the off-spring, with schizophrenia being a typical example (Meyer *et al.*, 2009). Subsequently, this evidence links the neurodevelopmental pathology of schizophrenia to activated immuno-inflammatory pathways in utero (Anderson & Maes, 2013). These pathways can include cytokine-associated neuroinflammation, oxidative and nitrosative stress (O&NS) (Bitanihirwe & Woo, 2011), and activation of the neurotoxic tryptophan catabolite (TRYCAT) pathway which in turn may result in glutamatergic dysregulation (Myint & Kim, 2014) and disordered monoaminergic transmission, commonly associated with schizophrenia (Crow *et al.*, 1979; Sumiyoshi *et al.*, 2014).

The treatment of schizophrenia has evolved substantially since discovery of the dopamine D_2 receptor antagonists, haloperidol (HAL) and chlorpromazine in the 1950s (Awouters & Lewi, 2007). Even though the conventional antipsychotic, HAL, demonstrates clinical efficacy in treating the positive symptoms observed in schizophrenia (Beasley *et al.*, 1996) and is still frequently used for this purpose (Dold *et al.*, 2012), it is limited in its efficacy to treat negative and cognitive symptoms.

On account of the significant evidence that implicates redox-immune-inflammatory dysfunction in the aetiology of schizophrenia (Mahadik & Mukherjee, 1996; Fendri *et al.*, 2005; Pérez-Neri *et al.*, 2006; Pedraza-Chaverri *et al.*, 2008), clinical studies have begun to focus on the utility of antioxidants in treating schizophrenia (Cabungcal *et al.*, 2014), while the possible therapeutic benefit of antioxidants of plant origin, specifically xanthones, holds substantial interest.

Extracts of the *Garcinia mangostana* Linn (GML) fruit have displayed a wide range of biological properties *in vitro*, such as antioxidant (Yoshikawa *et al.*, 1994; Jung *et al.*, 2006), cytotoxic (Ho *et al.*, 2002; Wang *et al.*, 2011), anti-inflammatory (Chairungsrilerd *et al.*, 1996; Chen *et al.*, 2008), antibacterial (Phongpaichit *et al.*, 1994; Chomnawang *et al.*, 2009), antifungal (Puripattanavong *et al.*, 2006; Kaomongkolgit *et al.*, 2009) and antitumoral properties (Matsumoto *et al.*, 2004), while recent work has established its possible utility to address central nervous system (CNS) dysfunction, viz. neuroprotective in a Huntington's disease model in rats(Dey & De, 2015) and antidepressant activity in a genetic rodent model of depression (Oberholzer *et al.*, 2017). α-Mangostin (AM), one of the primary active constituents of GML has also been reported to present with noteworthy pharmacological

activity (Sakagami *et al.*, 2005; Nakagawa *et al.*, 2007), especially antioxidant and antiflammatory effects (Jung *et al.*, 2006; Gutierrez-Orozco *et al.*, 2013), while it has shown analgesic effects *in vivo* (Sani *et al.*, 2015). Since altered redox systems and inflammation processes are implicated in schizophrenia development(Moller *et al.*, 2015), and considering the anti-oxidant and antidepressant-like effects of GML pericarp *in vivo* (Oberholzer *et al.*, 2017), this raises the question whether GML pericarp extract may be a potential therapeutic agent to target the dysregulated immune-inflammatory aspects of schizophrenia and so to improve the treatment outcome of the disorder.

Taking into account that schizophrenia is characterized by several behavioural abnormalities, including social withdrawal (Tandon *et al.*, 2009), impaired sensorimotor gating (Cilia *et al.*, 2005) and depressive-like behaviour (negative symptoms) (Chatterjee *et al.*, 2012), modelling these schizophrenia-related behaviours in rodents are crucial before the evaluation of the therapeutic benefits of a novel treatment option is considered.

The maternal immune activation (MIA) model of schizophrenia induces an immune response in the pregnant dam by exposing her to an immunogenic agent that simulates an infection. The resulting pro-inflammatory state in the dam and her foetus alters the normal neurodevelopmental process of the foetus and increases the risk for developing schizophrenia-like bio-behavioural manifestations in the offspring later in life (Meyer *et al.*, 2009). In this study lipopolysaccharide (LPS), a key component of the cell wall of gram negative bacteria, was used as an immune activator (Kirsten *et al.*, 2010; Lin *et al.*, 2012). Ultimately, the prenatal LPS model displays several behavioural and neurochemical alterations in rodents that closely emulate that described in schizophrenia (Ashdown *et al.*, 2006; Zhu *et al.*, 2014; Swanepoel, 2017).

Reduced social interaction has consistently been observed in schizophrenia patients (Tandon *et al.*, 2013), accordingly the social interaction test (SIT) was used to evaluate social interactive behaviour of rodents in this study (Möller *et al.*, 2011). Similarly, schizophrenia is associated with an inability to appropriately filter in-coming sensory input and referred to as a loss in sensorimotor gating, a cognitive deficit that results in the typical fragmentation of reality seen in these patients. To determine sensorimotor gating in rodents, prepulse inhibition (PPI) of the acoustic startle response was used (Möller *et al.*, 2011). This analysis is based on the ability of rodents to reduce a startle when the main acoustic pulse is preceded by a pre-pulse of smaller amplitude. Moreover, given that diminished affect, especially depression, forms part of the negative symptoms of schizophrenia, the depressive-like behaviour of rodents was examined by using the forced swim test (FST). The total time the rodent spends in an immobile

posture has been described as resembling behavioural despair and learned helplessness seen in depression and schizophrenia (Lucki, 1997; Siris, 2000; Castagné *et al.*, 2010).

Considering the role of immune-inflammatory pathways in schizophrenia (Moller *et al.*, 2015), cytokines may play a crucial role in altering foetal brain development. Elevated levels of pro-inflammatory cytokines following maternal immune activation can ultimately cause abnormal neurodevelopment (Ashdown *et al.*, 2006). Interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) are pro-inflammatory cytokines that may induce various adverse effects on the developmental processes in the CNS (Dammann & Leviton, 1998). Plasma levels of IL-6 and TNF-α were therefore analysed in this study as inflammation biomarkers.

Accumulating evidence has also associated schizophrenia with an imbalance in the regulation of endogenous reduction–oxidation (redox) systems, referred to as oxidative stress (Fendri *et al.*, 2006; Bitanihirwe & Woo, 2011; Yao & Reddy, 2011). This imbalance results in increased pro-oxidants, reactive oxygen species (ROS) and reactive nitrogen species (RNS) and diminished antioxidants (Boskovic *et al.*, 2011). High levels of oxidative stress leads to increased lipid peroxidation, that may have detrimental effects in membranes, proteins and genes (Mahadik *et al.*, 2001). Levels of thiobarbituric acid reactive substances (TBARS) provides an indication of lipid peroxidation and have been proven to be higher in patients with schizophrenia (Khan *et al.*, 2002; Arvindakshan *et al.*, 2003; Petronijević *et al.*, 2003). In this study lipid peroxidation was measured as a biomarker of oxidative stress (Harvey *et al.*, 2008; Möller *et al.*, 2011).

1.3 Hypothesis, aims and objectives

Hypothesis:

The study proposed that prenatal inflammatory activation will result in schizophrenia-like behavioural and neurochemical changes in offspring in early adulthood. These changes will include deficits in sensorimotor gating and social interaction and depressive-like behaviour, as well as elevated plasma levels of pro-inflammatory cytokines, viz. IL-6 and TNF-α, and increased regional brain lipid peroxidation, particularly in the striatum, hippocampus and frontal cortex. Both behavioural and neurochemical changes will be reversed or reduced by chronic administration of the reference antipsychotic, HAL, thus affording predictive validity for the model. Moreover, GML and AM as anti-oxidants will be effective in reversing these bio-behavioural changes equivalent to that of HAL while they will have bolstering effects when administered in combination with HAL. Finally, since pharmacological responses to a raw extract can be dependent on the combined presence and ratio of the constituents, the

researcher proposed that the pure compound derived from GML, AM, will display reduced or at best similar bio-behavioural responses compared to GML.

Aims:

The first aim was to establish an immune inflammatory animal model capable of inducing schizophrenia-like behaviour and redox-immune-inflammatory alterations in the offspring. Additionally, the study intended to evaluate whether these changes are reversed following chronic treatment with HAL. Thereafter the effects of GML on the above-mentioned behavioural, peripheral and neurochemical parameters were undertaken and compared to HAL. Together with this treatment objective, the study aimed to assess the effects of AM on bio-behavioural changes in the MIA model and how this compares to GML and HAL. Finally, the augmenting effects (if any) of GML and AM respectively in combination with HAL were examined in reversing the above-mentioned bio-behavioural alterations in the MIA model, compared to HAL alone.

Objectives:

Using an immune inflammatory model based on pre-natal LPS administration in rats, as previously set up in our laboratory (Swanepoel, 2017), our primary objectives were:

- To establish whether the schizophrenia-like behavioural characteristics is evident in the model and accompanied by peripheral and brain immune-inflammatory and redox alterations
- To establish whether chronic HAL treatment can reverse the above-mentioned schizophrenia-like bio-behavioural changes.
- To establish whether GML and AM can reverse the above-mentioned schizophrenia-like behavioural changes in LPS exposed rats, and how this compares to HAL.
- To establish whether GML and AM separately can reverse immune-inflammatory and redox changes in LPS exposed rats, and how this compares to HAL.
- To establish whether GML and AM separately can be used as adjunctive therapy to improve the treatment response to HAL in LPS exposed rats considering the above-mentioned bio-behavioural parameters.
- To compare the treatment response of GML on the above-mentioned bio-behavioural changes to that of a pure xanthone component of GML, i.e.AM.

1.4 Project layout

A total of 18 pregnant Sprague Dawley (SD) dams were exposed to LPS on gestational days (GD) 15 and 16. The male offspring from dams (n=74) were divided into six treatment groups that received oral dosing of the following: saline (1ml/kg po), HAL (2 mg/kg po) (Gao *et al.*, 1997; Schmitt *et al.*, 1999; Schleimer *et al.*, 2005; Terry *et al.*, 2007); GML (50mg/kg po) (Oberholzer, 2017), HAL + GML (at the previously mentioned doses), AM (20mg/kg po) (Li *et al.*, 2011) and HAL+ AM (at the previously mentioned doses) (Fig. 1). Control dams (n=3) were treated with saline and male offspring (n=8) from these dams were also treated with saline. Variation in the control group may be less provided that they did not receive any intervention, such as a treatment or stressor, therefore the number of animals were less in the control group compared to the treatment groups. Additionally, the reduced control group is in line with the "reduce" fragment of ethical principles. After treatment, all groups were subjected to a battery of behavioural tests that follow a specific sequence to minimise stress on the animals, viz. (1) SIT on day 12 of drug administration (PND 62), (2) PPI on day 13 of treatment (PND 63) and (3) FST on day 14 of treatment (PND 64). The animals were euthanized (via decapitation) 36 hours after the last behavioural test for the collection of trunk blood and brain tissue (frontal cortex, striatum and hippocampus). All tissue samples were collected and stored at -80 °C until the day of analysis. A complete flow chart of the study design is illustrated in Figure 1.

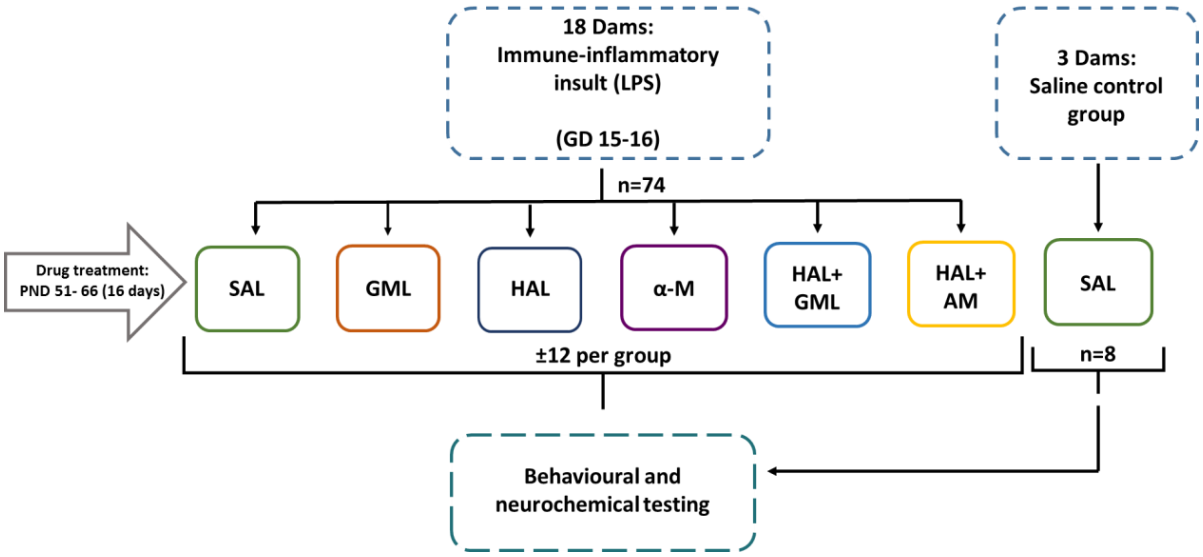

Figure 1: The flow chart of the study design. 18 Dams were exposed to LPS on gestational days 15 -16 to induce immune activation. Offspring from LPS exposed were then divided in six groups for drug treatment, viz. Saline, GML, HAL, HAL+ GML, AM and HAL+ AM. Control dams (n = 3) were treated with saline and male offspring from these dams were also treated with saline. Behavioural tests included SIT, PPI and FST. Peripheral and neurochemical analyses consisted of plasma cytokine analysis and regional brain lipid-peroxidation analysis.

1.5 Expected outcomes

- LPS exposed pregnant dams will produce offspring that present with schizophrenia-related behaviours, in particular deficits in social interaction and sensorimotor gating, as well as depressive-like manifestations in adulthood.

- Offspring of prenatal LPS-exposed dams will be associated with late life presentation of immunological and redox changes in adulthood that will be linked to the observed behavioural manifestations.

- HAL treatment will reverse the above mentioned bio-behavioural changes in the off-spring of prenatal LPS-exposed dams.

- GML and AM will reverse the above mentioned bio-behavioural changes in the offspring of prenatal LPS-exposed dams and will be as effective as HAL in this regard.

- Combining GML and HAL will be more effective than HAL alone in reversing the above mentioned bio-behavioural changes in the off-spring of prenatal LPS-exposed dams.

- Combining AM and HAL will be more effective than HAL alone in reversing the above mentioned bio-behavioural changes in the off-spring of prenatal LPS-exposed dams.

- GML will have similar or superior bio-behavioural effects compared to AM.

1.6 Ethical approval

All experiments and procedures were approved by the AnimCare animal research ethics committee (NHREC reg. number AREC-130913-015) of the North-West University (NWU). The housing of animals and procedures performed were in accordance with the code of ethics in research, training and testing of drugs in South Africa and complied with national legislation (Ethics approval number NWU-00376-16-A5). Animals were bred and housed at the Vivarium (SAVC reg. number FR15/13458; SANAS GLP compliance number G0019) of the Pre-Clinical Drug Development Platform of the NWU. The study was performed with consideration of the ARRIVE guidelines, as described by Kilkenny (Kilkenny *et al.*, 2010).

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CHAPTER 2

LITERATURE REVIEW

2.1 Introduction: Schizophrenia

"Dementia praecox" was the term first used by Emil Kraepelin (1856–1926), a German psychiatrist, to describe the mental malfunction and disturbed behaviour known in the present day as schizophrenia (Kraepelin & Beer, 1992). The term "schizophrenia" was derived from the Greek words *schizein* and *phren* by Dr. Eugen Bleuler implying a "split mind/soul/spirit" (Ashok *et al.*, 2012). Bleuler was a Professor in psychiatry at the University of Zurich and emphasized that "*splitting of psychic functioning is an essential feature of schizophrenia*" (Kuhn & Cahn, 2004). In the book "Schizophrenia: Straight Talk for Family and Friends" Maryellen Walsh compares the neuronal abnormalities in schizophrenia to a telephone switchboard: "*In most people the brain's switching system works well. Incoming perceptions are sent along appropriate signal paths, the switching process goes off without a hitch, and appropriate feelings, thoughts, and actions go back out again to the world… in the brain afflicted with schizophrenia… perceptions come in but get routed along the wrong path, or get jammed, or end up at the wrong destination*" (Walsh, 1985).

Schizophrenia is a severe psychiatric disorder, normally with a life-long course (Tandon *et al.*, 2008b), that effects roughly 1% of the world's population (Anderson & Maes, 2013) and is among the top ten global causes of disability (Mathers *et al.*, 2008). This debilitating disease normally surfaces in late adolescence, early adulthood (Gogtay *et al.*, 2011) and can reduce a patient's lifespan with 15–30 years (Bushe *et al.*, 2010). Even though a fragment of the excessive mortality rate can be attributed to unnatural deaths such as suicide (Palmer *et al.*, 2005); natural causes including cardiovascular, respiratory and cancer related deaths are increasingly contributing to the premature mortality observed in schizophrenia patients (Brown *et al.*, 2010; Bushe *et al.*, 2010; Morden *et al.*, 2012).

The predominant characteristics of schizophrenia include distortions in perception, cognition and behaviour, communication difficulties and social impairment (Harris *et al.*, 2013). These diverse collection of characteristics can be classified in three fundamental symptom groups, namely positive, negative and cognitive symptoms (Meyer, 2013). Positive symptoms include hallucinations, delusions and disordered thoughts whereas negative symptoms consist of flattened affect, impoverished speech, social withdrawal, apathy and anhedonia (Möller, 2007). The negative symptoms closely resemble the core

symptoms of depression and may be appreciated by considering their mutual manifestations (Möller, 2007). Lastly, cognitive symptoms are characterized by memory impairment and attention deficits (Meyer, 2013).

Several causative components can be considered when focusing on the aetiology of schizophrenia, a combination of genetic and environmental elements can contribute to abnormal neurodevelopment and brain dysfunction associated with schizophrenia (Walker *et al.*, 2004). Although the exact nature of this complex disorder remains unknown, numerous hypotheses have been proposed in an attempt to clarify the aetiology of schizophrenia, they include monoamine neurotransmitter abnormalities (Sumiyoshi *et al.*, 2014), alterations in γ-aminobutyric acid (GABA) and glutamate function (Schwartz *et al.*, 2012), oxidative stress (Bitanihirwe & Woo, 2011), mitochondrial dysfunction (Rajasekaran *et al.*, 2015) and inflammation (Anderson & Maes, 2013). All of these hypotheses will be elaborated on later in this chapter.

2.2 Clinical features and symptoms

Schizophrenia-like symptoms have been noted in ancient literature, though it was believed that demonic possession or godly punishment was to blame for the observed bizarre behaviour (Kyziridis, 2005). In the present day, the “divine madness” better known as schizophrenic syndrome is associated with a varied range of symptoms affecting perception, emotion, and judgement; a human’s primary processes (Ross *et al.*, 2006).

The clinical manifestations of schizophrenia typically appear in young adulthood (Thompson *et al.*, 2004), even though the disease can originate during early neurodevelopment (Rapoport *et al.*, 2005b). The full onset of schizophrenia is normally preceded by a prodromal period that consist of mood symptoms, cognitive impairment and psychotic symptoms (Lieberman *et al.*, 2001). Schizophrenia is ultimately diagnosed by the manifestation of distinctive symptoms (viz. positive, negative and cognitive symptoms), as mentioned earlier (Meyer, 2013).

Positive symptoms are frequently referred to as psychotic symptoms and are generally the most prominent set of symptoms (Gonzalez-Burgos *et al.*, 2011), whereas the negative symptoms are less observable and closely resemble the core symptoms of depression (Möller, 2007). Several cognitive deficits shapes the third collection of symptoms and can severely affect the functional status of the patient (Harvey & Keefe, 2001). Diefendorf and Kraepelin (1923) expressed that the link between the disorder’s observable symptoms and the unobservable psychopathology is crucial in the fundamental understanding of

schizophrenia. However to make the connection, the three symptom groups should be examined separately.

2.2.1 Positive symptoms

Positive symptoms are the most defining feature of schizophrenia and primarily include hallucinations and delusions (Figure 1) that can be illustrated as abnormal perceptions and irrational beliefs, respectively (Fletcher & Frith, 2009). Slade and Bentall (1988) depicted a hallucination as a perception that (a) arises without an appropriate stimulus, (b) has the full impact of an equivalent actual perception and (c) is not responsive to direct and voluntary control by the experiencer. Auditory hallucinations are the most common and occur in approximately 60% of schizophrenia patients (Slade & Bentall, 1988) and contribute to ongoing disability and morbidity (Fitzgerald *et al.*, 2006). Moreover, the risk of suicide attempts can surface due to these hallucinated voices (Falloon & Talbot, 1981). A delusion can be described as a false belief firmly adopted by the believer despite evidence that the belief is deceptive, where the individual is completely unresponsive to reason (Garety & Freeman, 1999; Langdon & Coltheart, 2000). Additionally, paranoia, conceptual disorganized behaviour and thought processes (Figure 1) can also occur as part of the acute psychotic state (Nekovarova *et al.*, 2015).

2.2.2 Negative symptoms

The term “negative” indicates loss; in the case of schizophrenia, the loss of pleasure, motivation, social interaction, emotional expression and speech are observed (Millan *et al.*, 2014). Originally theorised by Wagman (1988), primary negative symptoms form an integral part of the disorder whereas the secondary symptoms can be a result of external factors such as antipsychotic treatment, co-morbid depression and social withdrawal leading to insufficient environmental stimulation (Kaiser *et al.*, 2011; Foussias *et al.*, 2014). Recent research are focusing more on the major domains of negative symptoms, including blunted affect, alogia, asociality, anhedonia, and avolition (Kirkpatrick *et al.*, 2006). The above-mentioned symptoms (Figure 1) are closely connected, however different neurobiological mechanisms may be responsible for different domains and could therefore demand different treatment options (Kaiser *et al.*, 2011). Currently available treatments have been shown to improve positive symptoms (Leucht *et al.*, 2012) and although a recent meta-analysis study found that numerous treatments displayed statistically significant effects on negative symptoms, the outcome was not clinically noticeable (Fusar-Poli *et al.*, 2014). Moreover, a review from Buckley and Stahl (2007) found that pharmacological treatment to date for

negative symptoms has been greatly disappointing. Consequently, negative symptoms persist in patients, even with optimal clinical care (Wagman, 1988).

2.2.3 Cognitive symptoms

The third cluster of symptoms viz. neurocognitive impairments, has been recognized as a central feature of schizophrenia (Heinrichs & Zakzanis, 1998; Barch & Ceaser, 2012; Keefe & Harvey, 2012). As presented in Figure 1 this includes problems in processing speed, attention/vigilance, working memory, verbal and visual learning and reasoning, (Kuperberg & Heckers, 2000; Green, 2006). These deficits in cognitive ability can compromise everyday functioning (Green, 1996) and are strongly associated with a reduction in the quality of life of the patient (Alptekin *et al.*, 2005; Keefe & Harvey, 2012) and have therefore become a critical treatment target in schizophrenia (Gold, 2004). Minimal evidence is available on the therapeutic benefit of antipsychotics on cognitive disturbances and may suggest that cognitive symptoms are independent from the other symptomatic domains of schizophrenia. Aforementioned emphasizes the need for the development of cognition specific treatments (Gold, 2004).

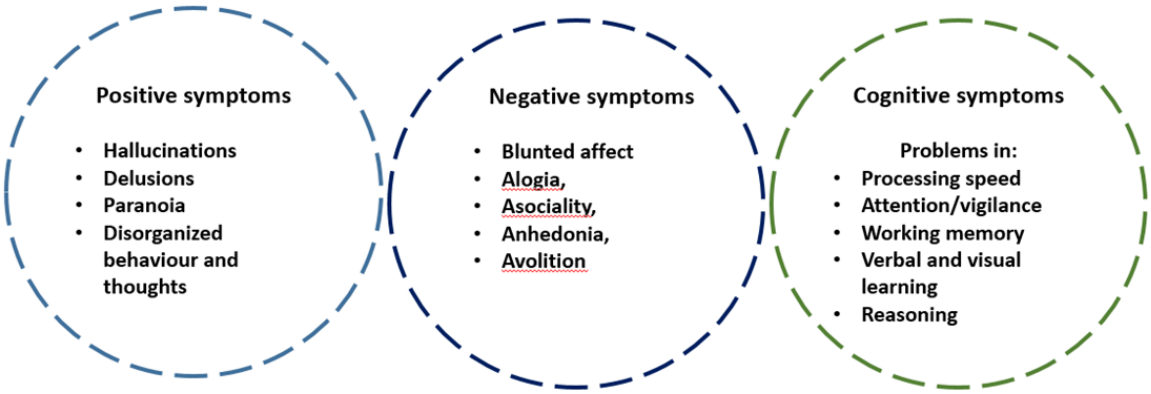

Figure 1: The three symptom clusters of schizophrenia (Adapted from: Nekovarova et al., 2015; Kirkpatrick et al., 2006; Green, 2006)

2.3 Diagnosis of schizophrenia

The definitions and diagnostic criteria for schizophrenia have evolved through the various editions of the Diagnostic and Statistical Manual of Mental Disorders (DSM) over the past

decades. The proposed diagnostic criteria in the latest edition, DSM-5, include two (or more) of the following characteristic symptoms (Criterion A):

1. Delusions
2. Hallucinations
3. Disorganized speech
4. Disorganized or catatonic behaviour
5. Negative symptoms i.e. alogia, asociality, anhedonia, and avolition

Each of these characteristic symptoms should be present for at least one month and one of these symptoms should include 1-3 (Tandon *et al.*, 2013). Furthermore, Criterion B describes interpersonal and occupational dysfunction; the persistence of disturbances is explained in Criterion C and Criterion D and E depicts the exclusion of schizoaffective and mood disorders in addition to general and substance associated conditions (Association, 2013).

2.4 Epidemiology and aetiology of schizophrenia

MacMahon and Pugh (1970) defines epidemiology as the study of determinants and distribution of disease. The distribution of a disorder refers to new cases of the disorder (incidence) together with the total new and existing cases (prevalence), whereas the determinants of disease, regarding schizophrenia, consists of environmental and genetic risk factors (Tandon *et al.*, 2008a). Aforementioned determinants are in continuous interaction and is crucial in the aetiology of schizophrenia (Tsuang *et al.*, 2004). A meta-analysis of published studies done by McGrath et al. (2008) revealed an annual median incidence rate of 15.2/100,000 and displayed significant variations in incidence within the population, with a higher risk for developing schizophrenia associated with urbanicity, migration, and male gender (McGrath *et al.*, 2004; McGrath & Sasser, 2009).

Looking at prevalence studies, Saha *et al.* (2005) found prevalence estimates for schizophrenia in the range of four to seven per 1,000 persons with prevalence of schizophrenia higher in migrants than native-born individuals, but no significant difference between sexes or urban and rural settings . Even though these figures may not mirror the precise extent of variation between various populations, it emphasizes the universal impact of this disorder.

Schizophrenia is highly inheritable with heritability estimated between 60-80 % (Sullivan *et al.*, 2003; Lichtenstein *et al.*, 2009), indicating that genetic factors alone can't account for the complex aetiology of this disorder (Kirkbride & Jones, 2010; Svrakic *et al.*, 2013).

However, having a family member diagnosed with schizophrenia significantly increases the risk of developing the disorder (Tandon *et al.*, 2008a). Consistent confirmation of the genetic architecture of schizophrenia have been provided by numerous twin and adoption studies. Aforesaid studies exhibiting a rate of concordance of roughly 46-53% for twins who share 100% of their genetic material (Kendler, 1983; Gottesman, 1991) and found that adopted offspring of biological mothers with schizophrenia had an increased risk for schizophrenia development compared to adopted offspring of control mothers (Heston, 1966; Lowing *et al.*, 1983; Tienari *et al.*, 2000; Tienari *et al.*, 2003).

Although a high fraction of vulnerability for schizophrenia is gene-dependant, numerous environmental exposures can contribute to the risk of developing schizophrenia. Early environmental insults such as obstetric complications and pre-natal malnutrition and infection are likely to participate in the aetiology of schizophrenia (Susser & Lin, 1992; Brown & Derkits, 2009) attributable to morphological and molecular changes in the foetal brain (Boksa, 2010). Stressful life events can also play a role in the onset of schizophrenia in susceptible individuals (Norman & Malla, 1993). In relation to this it was found that cortisol can alter neurotransmitter activity and long-term elevated levels of cortisol can consequently impact brain structure and function (Walker *et al.*, 2004), resulting in more severe symptoms in schizophrenia (Walder *et al.*, 2000). Furthermore, several substances can induce psychosis and substance abuse can precipitate relapse of an existing psychotic disorder (Breakey *et al.*, 1974). Identifying these risk factors for schizophrenia can therefore assist in understanding the complex interaction between the environmental elements and genetic predispositions.

2.5 The pathophysiology of schizophrenia

2.5.1 Neuroanatomy

Since there are such a wide variety of symptoms in schizophrenia, it is difficult to point out a specific regional brain deficit in the disorder. However, the prefrontal cortex (PFC) has long been implicated in schizophrenia (Kraepelin, 1919; Piercy, 1964) and especially dorsal PFC neuronal pathology have proved to be fundamentally involved in the disorder (Petrides *et al.*, 1993; Selemon & Goldman-Rakic, 1999; Lewis *et al.*, 2004). Several dorsal PFC abnormalities have been reported in post-mortem studies such as gray matter deficits, including reductions in the quantity and activity of dorsal PFC interneurons (Benes *et al.*, 1991) and reduced inhibitory inputs onto the axonal processes of dorsal PFC pyramidal

neurons from the prefrontal cells (Woo *et al.*, 1998). Moreover, Breier *et al.* (1992) examined the morphologic features of the prefrontal cortex using magnetic resonance imaging (MRI) and found significantly reduced prefrontal volumes and prefrontal white matter in schizophrenic patients compared to healthy controls. Thompson (2002) also illustrated dynamic grey matter reduction in schizophrenic patients over a 5 year period by using MRI scans to create 3D brain maps of the process (Figure 2). In addition to morphologic defects, accumulating evidence suggests reduced activity in the PFC (hypofrontality) that results in hypo-function of essential pathways, including dopamine (DA) and glutamate pathways (Stahl, 2007). Researchers attempted to clarify the origin of the hypofrontality by analysing other brain regions that are innervated by the PFC, such as the hippocampus (Pantelis *et al.*, 1992).

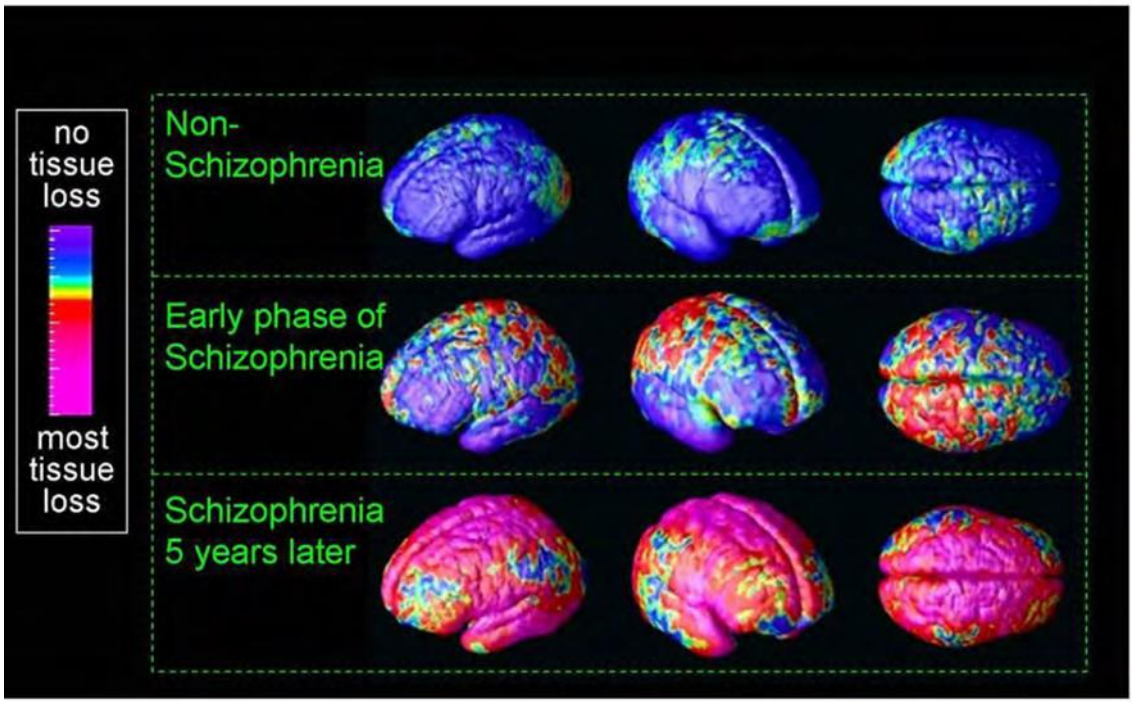

Figure 2: Obtained from MRI scans over a period of time, these images illustrate severe grey matter loss in schizophrenia and its progression over time (Thompson, 2002)

Histological, molecular, structural and neuropsychological studies as well as functional neuroimaging confirmed the involvement of the hippocampus in the pathophysiology of schizophrenia (Gothelf *et al.*, 2000; Harrison, 2004). A meta-analysis performed by Adriano *et al.* (2012) found significant hippocampal volume reduction in schizophrenic patients compared to healthy individuals, which is in line with abovementioned studies. Furthermore

the study revealed that the volume reduction observed in patients with chronic schizophrenia was identical to that seen in first-episode schizophrenic patients, indicating the possible neurodevelopmental origin of this disease (Adriano *et al.*, 2012). The hippocampus is a crucial area of the brain implicated in memory formation and information processing (Leonard, 2004) and may be associated with the cognitive impairments of schizophrenia rather than the psychotic symptoms (Harrison, 2004). However, Phillips *et al.* (2003) proposed that impairments in the amygdala, hippocampus, and parahippocampal gyrus could, depending on the particular processes dysfunction, cause flattened affect, anhedonia, or delusions.

Another potential contribution to deficits in schizophrenia involves dysfunction of frontal–striatal circuitry (Frith & Done, 1988; Pantelis & Brewer, 1995; Pantelis & Brewer, 1996). The striatum receives inputs from the cortex, thalamus, hippocampus, and amygdala and plays a vital role in procedural learning of motor habits (Simpson *et al.*, 2010). The negative symptoms of schizophrenia associated with reduced motivation including anhedonia and flattened effect can also be linked to impairments in the ventral striatum (Breiter *et al.*, 2001; Phillips *et al.*, 2003). Moreover, this region of the brain is considered to play a part in learning associations and can potentially be responsible for positive symptoms, more specifically delusions (Kapur, 2003). In addition to increased striatal DA activity (Brisch et al., 2014), striatal dysfunction can result in deficits in attention and cognitive control that occur in schizophrenia (Simpson et al., 2010). Ultimately, the opposing dopaminergic changes in the frontal cortex and striatum emphasises the complexity of the disorder, especially with regard to pharmacological management.

2.5.2 Neurochemistry

2.5.2.1 Monoamine hypotheses in schizophrenia

2.5.2.1.1 Dopamine

The most renowned hypothesis of schizophrenia is the dopamine (DA) hypothesis, which proposes excessive striatal DA activity (responsible for the positive symptoms) (Carlsson & Lindqvist, 1963) and deficient frontal-cortical DA activity (responsible for the negative symptoms) (Davis & Kahn, 1991), as mentioned earlier. Initial indication for the involvement of DA in psychosis was the observation that stimulants such as amphetamine, which trigger DA release, can induce psychotic symptoms (Angrist & Gershon, 1970; Harris & Batki, 2000). Furthermore, the therapeutic efficacy of dopamine D_2 receptor antagonists, such as haloperidol, to address the positive symptoms of schizophrenia strongly confirms the

implication of DA dysregulation in the underlying pathology (Seeman *et al.*, 1975; Seeman *et al.*, 1976). However, a critical limitation in the DA hypothesis is that D_2 receptor antagonists are unsuccessful in treating the negative and cognitive symptoms present in schizophrenia (Miyamoto *et al.*, 2005).

DA binds to a group of G-protein-coupled receptors following pre-synaptic release, namely D_1, D_2, D_3, D_4 and D_5-receptors (Tritsch & Sabatini, 2012) located pre- and post-synaptically. The inability to treat abovementioned symptom groups could be attributed to alterations of frontal cortical D_1 receptors and additional receptor abnormalities (Weinberger, 1987; Davis & Kahn, 1991; Castner & Goldman-Rakic, 1999). Moreover, post-mortem studies have revealed increased density of D_2 receptors in the striatum of schizophrenic patients (Guillin *et al.*, 2007). However, it has been proposed that the DA hypothesis is more likely based on increased dopamine transmission following presynaptic dysregulation rather than receptor number and alterations (Kapur, 2003).

DA neurotransmission follows four axonal projection pathways after DA synthesis, including nigro-striatal; mesolimbic; mesocortical; and tuberoinfundibular pathways (Figure 3) (Vallone *et al.*, 2000). These pathways are implicated in crucial brain functions, such as learning, memory and motivation, often impaired in schizophrenia (Kahn *et al.*, 1994; Cohen *et al.*, 2002; D'Esposito, 2007). The heightened DA in the mesolimbic is commonly responsible for the positive symptoms (1) while negative, cognitive and affective symptoms follows DA hypoactivity in the mesocortical pathway (2) (Horvitz, 2000; Wise, 2000). Numerous neuroimaging studies also exhibited increased synthesis and release of dopamine (Reith *et al.*, 1994; Hietala *et al.*, 1995; Dao-Castellana *et al.*, 1997; Abi-Dargham *et al.*, 1998; Lindström *et al.*, 1999) together with elevated levels of synaptic dopamine during psychotic episodes (Abi-Dargham *et al.*, 2000; Gjedde & Wong, 2001). Dopamine in the tuberoinfundibular pathway, on the other hand is responsible for prolactin secretion of from the anterior pituitary gland via dopamine neurons located in the mediobasal hypothalamus (the ‘tuberal region’) that project to the median eminence (the ‘infundibular region’) where dopamine is released. (Meisenzahl *et al.*, 2007). This could result in antipsychotic medications to cause hyperprolactinaemia primarily by blocking D_2 receptors on the anterior pituitary and can have secondary endocrine and metabolic consequences such as reduced bone mineral density (O'Keane, 2008). Ultimately, the variety of brain regions and pathways involved in schizophrenia suggests that dopamine cannot be exclusively responsible for the manifestations seen in schizophrenia.

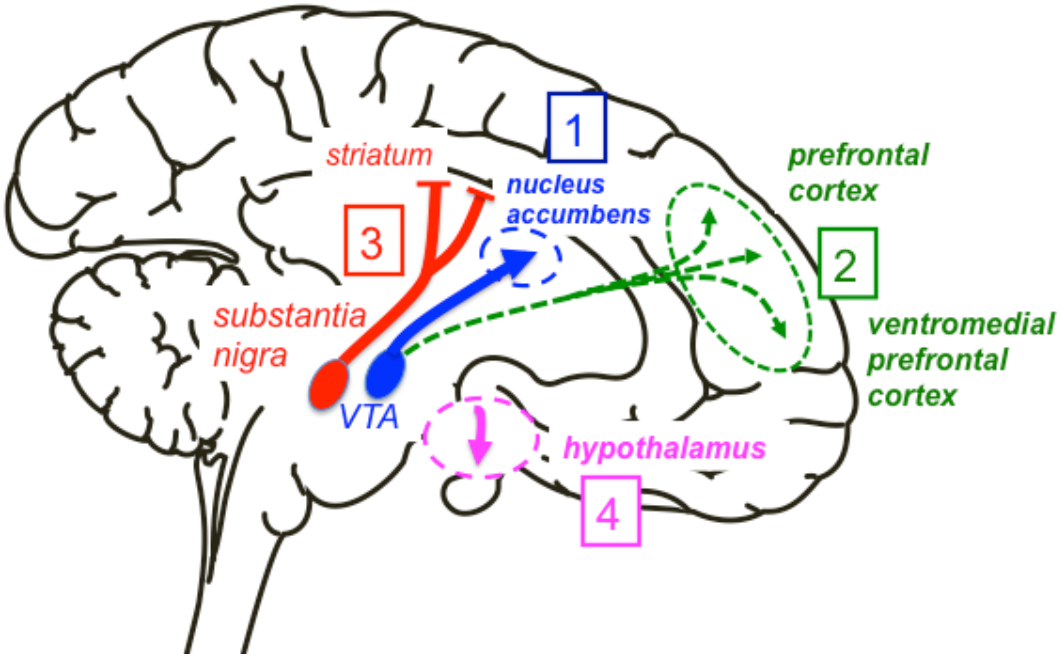

Figure 3: The four dopaminergic pathways: (1) mesolimbic, (2) mesocortical, (3) nigrostriatal and (4) tuberoinfundibular

2.5.2.1.2 Serotonin

Additional neurotransmitter systems were considered following the discovery of clozapine, a multi-potent antagonist on serotonin $(5HT)_{2A/C,}$ D_2 and adrenergic α_{2c} receptors (Meltzer *et al.*, 1996). Clinical studies soon demonstrated that this antipsychotic proved to be more effective against negative symptoms when compared to traditional antipsychotics, and has since been instrumental in raising the level of importance of 5-HT in schizophrenia and its treatment. The 5-HT hypothesis originated in the 1900's when Woolley and Shaw (1954) suggested that 5-HT transmission may be increased in schizophrenia patients after finding that 5-HT agonists such as lysergic acid diethylamine (LSD) induced hallucinations in patients (Woolley & Shaw, 1954), although varying somewhat from psychosis seen in schizophrenia. In addition to hyper-5-HTergic activity, increased $5\text{-}HT_{1A}$ receptor density has been found in the PFC of schizophrenia patients (Sumiyoshi *et al.*, 1996). Given that the activation of $5\text{-}HT_{1A}$ receptors can have detrimental effects on working memory (Buhot, 1997), it suggests the essential role that 5-HT may play in the regulation of cognitive behaviour. Furthermore, 5-HT associated modulation of glutamatergic signalling via $5\text{-}HT_{1A}$ receptors localized on dendritic spine neurons (Yuen *et al.*, 2005) has also been suggested to underlie a central role in psychosis. Subsequently, 5-HT indirectly modulates the effect of glutamate on DA release in the PFC and therefore links the 5-HT and DA hypotheses with the GABA-glutamate hypothesis of schizophrenia (discussed later in section 2.5.2.2).

2.5.2.1.3 Noradrenaline

With regards to noradrenaline (NA), the NA system was suggested to play a critical role in schizophrenia as early as 1971 (Stein & Wise, 1971; Hartmann, 1976; Hornykiewicz, 1982). NA neurons are abundantly distributed throughout the brain and have been implicated in various brain processes, specifically the so-called “higher brain functions” such as arousal, attention, anxiety, fear, stress, memory association, learning, psychomotor behaviour, and several neuroendocrine and autonomic functions (Kety, 1970; Laverty, 1974; Amaral & Sinnamon, 1977; Moore & Bloom, 1979; Mason, 1981). Considering these functions, imaging studies have implied NA hyperactivity in the temporal lobe/limbic system related to hallucinatory (positive) symptoms and hypoactivity of NA in the prefrontal cortex associated with negative symptoms (Andreasen *et al.*, 1992; Silbersweig *et al.*, 1995). In support of this, Lechin and van der Dijs (2005) successfully utilized clonidine, a drug that limits noradrenergic and adrenergic over-activity in the central nervous system (CNS), to treat psychotic episodes, suggesting the involvement of NA hyperactivity in psychotic symptoms. Indeed, recent preclinical studies have provided robust evidence that antagonism of the α_{2C} adrenoceptor has pronounced pro-cognitive and antipsychotic-like effects, as assessed in a validated animal model of schizophrenia, and that this noradrenergic receptor be given serious consideration as a novel therapeutic approach to treating schizophrenia (Uys *et al.*, 2016).

2.5.2.2 GABA-Glutamate-NMDA Alterations in Schizophrenia

Altered 5-HTergic, NAergic, and DAergic pathways that are accountable for certain symptoms of schizophrenia, are a direct result of disordered GABA−glutamate interactions (Coyle *et al.*, 2003). Glutamatergic neurons are the major excitatory pathways connecting various brain regions that have been associated with schizophrenia, including the cortex, limbic system, and thalamus (Goff & Coyle, 2001), whereas GABA inhibits postsynaptic neurons. Cortical GABAergic neurotransmission is a crucial part of cognitive processes (Inan *et al.*, 2013), therefore irregular GABA transmission might clarify the cognitive abnormalities described in schizophrenia. Impaired glutamatergic neurotransmission was initially considered as a potential mechanism of schizophrenia after finding that dissociative anaesthetics, which function as N-methyl-D-aspartic acid (NMDA) receptor antagonists, can induce psychosis (Javitt, 1986). The behavioural effects of these drugs closely resemble the key symptoms of schizophrenia (Kantrowitz & Javitt, 2010). These findings suggest NMDA hypofunction in schizophrenia and is adequate to account for certain features of the illness.

Primary glutamate neurons in the PFC activate NMDA receptors on inhibitory GABA interneurons to regulate DA activity. These GABA interneurons then form synapses with secondary cortical glutamate neurons that is responsible for the downstream release of neurotransmitters (DA, NA, 5HT) in the ventral tegmentum area (VTA), limbic system (LS) and striatum (Moller *et al.*, 2015). Hypo-glutamatergia as a result of NMDA receptor dysfunction in the PFC of schizophrenia patients will lead to inadequate inhibition of the glutamate−GABA−glutamate neuronal circuit, causing excessive DA release in the VTA, LS, and striatum, finally responsible for the positive symptoms (Schwartz *et al.*, 2012). However, a hyperactive secondary glutamate neuron can be the result of insufficient GABA tone in addition to defective NMDA receptors on cortical GABA interneurons. This overactive secondary neuron can then overstimulate another GABA interneuron which is connected to DA neurons that regulates the release of striatal DA and subsequently inhibits the mesocortical pathway activity of DA by releasing excessive inhibitory GABA (Stahl, 2007). The inhibition of the mesocortical DA pathway results in underactive frontal cortical DA projection and is responsible for hypodopaminergic and negative symptoms (Schwartz *et al.*, 2012).

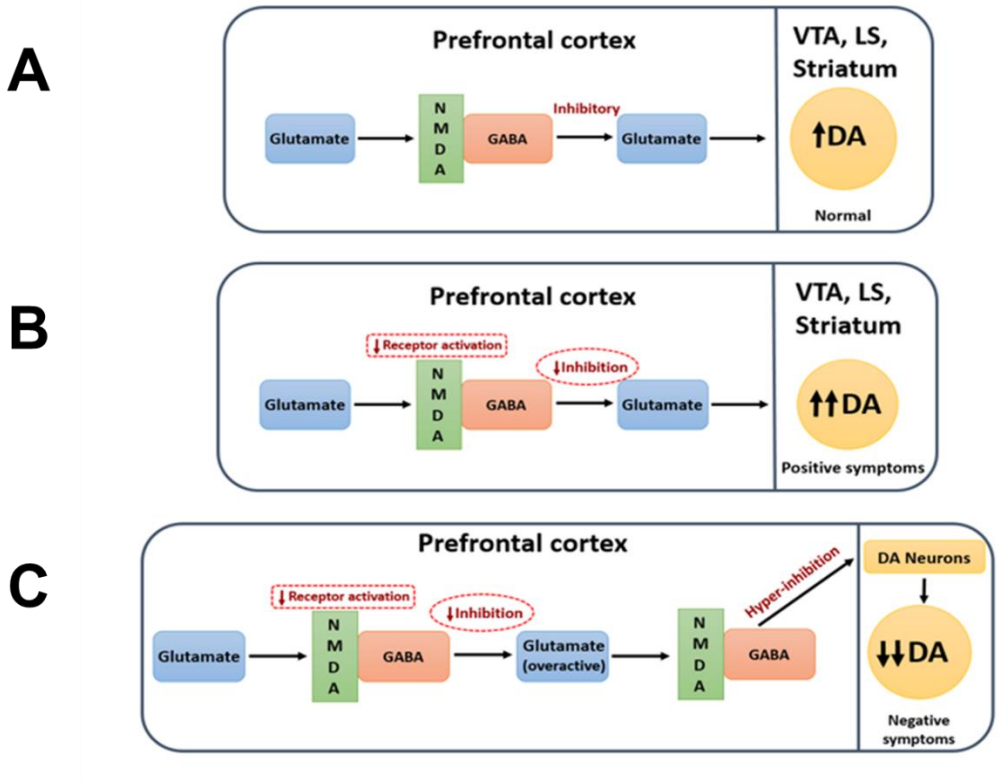

Figure 4: Normal glutamate-GABA-glutamate-DA neurocircuitry (A) compared to altered glutamate-GABA-glutamate-DA neurocircuitry responsible for the positive (B) and negative symptoms (C) in schizophrenia (Adapted from Moller *et al*. (2015)).

2.5.2.3 Mitochondrial dysfunction

Mitochondria are cytoplasmic organelles that play a crucial role in regulating cellular metabolic functions such as supplying energy, calcium homeostasis, redox signalling, and apoptotic cell death and is therefore vital to neurodevelopment (Rajasekaran *et al.*, 2015). These organelles have a separate genome—the mitochondrial DNA (mDNA) — and supply the oxygen for the production of adenosine triphosphate (ATP) via oxidative phosphorylation (OXPHOS) that fuels energy-dependent intracellular processes (Green *et al.*, 2011), although mainly regulated by DNA factors. The intricate processes of neurodevelopment are dependent on mitochondria to provide energy for proliferation and differentiation of neural stem cells. Neurons are particularly energy dependent and thus hypersensitive to changes in mitochondrial function (Su *et al.*, 2010). Irregularity of mitochondrial function can result in abnormal energy production leading to underdeveloped neurons and therefor abnormalities in neuronal connectivity, neurotransmission, and myelination (Rajasekaran *et al.*, 2015). These abnormalities are likely to contribute to cognitive deficits and neurodegeneration that are commonly associated with schizophrenia (Schon & Manfredi, 2003; Picard & McEwen, 2014).

Mitochondria also form microdomains with calcium (Ca^{2+}) influx sites to buffer cytosolic Ca^{2+} (Rizzuto & Pozzan, 2006). This is a critical mechanism since Ca^{2+} activates several biological processes with high energy demands. Excess mitochondrial Ca^{2+} however, can have harmful effects on mitochondrial capability and can result in apoptosis, otherwise known as programmed cell death (Orrenius *et al.*, 2003). Intra-mitochondrial proteins, such as cytochrome c and pro-caspases, which regulate apoptosis can be released following events that trigger excessive Ca^{2+} influx, decreased mitochondrial membrane potential, or DNA damage from oxidative stress (Clay *et al.*, 2011). The release of these proteins activate a complex cascade and will ultimately achieve apoptosis, a mechanism that has been suggested may clarify the neuroprogressive nature of schizophrenia (Glantz *et al.*, 2006). *In vivo* clinical neuroimaging studies have noted progressive grey matter loss early in the disorder, thus confirming the damaging effects of apoptosis on neuronal and glial cells that may underlie the neurodegenerative nature of the illness and evident in schizophrenia patients (Jarskog *et al.*, 2005).

Mitochondria, more specifically the mitochondrial respiratory chain in the inner mitochondrial membrane, are one of a number of sources (Andreyev *et al.*, 2005) of reactive oxygen species (ROS) produced as a by-product of normal cellular activity (Chance *et al.*, 1979). These products are normally detoxified by several endogenous antioxidant systems (Lenaz, 2012). However, mitochondrial malfunction can lead to an increased production of ROS that

will have detrimental effects through alterations in both cellular macromolecules and the redox state of factors involved in signal transmission, thus leading to abnormalities in neurotransmitter signalling pathways (Lenaz, 2012). Indeed, oxidative stress in its own right is associated with altered monoamine release (Bitanihirwe & Woo, 2011) that may drive the symptoms of the disorder.

2.5.3 Oxidative stress

Significant evidence abounds indicating the presence of oxidative damage in schizophrenia (Mahadik & Mukherjee, 1996; Fendri *et al.*, 2005; Pérez-Neri *et al.*, 2006). Oxidative stress is an imbalance in the regulation of the endogenous reduction–oxidation (redox) system, resulting in an excess pro-oxidants and diminished antioxidants (Boskovic *et al.*, 2011). These pro-oxidants, including reactive oxygen species (ROS and reactive nitrogen species (RNS)), can cause untold damage to cell lipids, proteins, enzymes, carbohydrates and deoxyribonucleic acid (DNA) (Halliwell & Gutteridge, 2015) through lipid peroxidation (Horton *et al.*, 1987). Luckily, numerous enzymatic and non-enzymatic pathways function as endogenous antioxidant defence mechanisms to compensate for the production of ROS and RNS (Nordberg & Arner, 2001), and include the antioxidant enzymes superoxide dismutase (SOD), glutathione-peroxidase (GPx), and catalase (CAT), and the non-enzymatic antioxidants glutathione (GSH), vitamin E, vitamin C and β-carotene. However, the deficiency of these antioxidant defences and the high rate of oxidative metabolic activity in the brain in addition to the excessive amount of metals (e.g. iron, zinc, copper and manganese) that can catalyze the formation of ROS/RNS, increases the brain's vulnerability to oxidative damage (Rougemont *et al.*, 2002; McQuillen & Ferriero, 2004). The above-mentioned antioxidants are capable of inhibiting the initiation of ROS chain reactions (Gilgun-Sherki *et al.*, 2001; Halliwell, 2001) and thereby provides endogenous protection against ROS and RNS (Lushchak, 2012). Post-mortem studies have detected significantly reduced GSH levels in the PFC of schizophrenia patients (Do *et al.*, 2000; Gawryluk *et al.*, 2011). Moreover, studies in first-episode schizophrenia patients also discovered a negative correlation between plasma SOD activity and the positive symptoms of schizophrenia (Wu *et al.*, 2012) along with low levels of total antioxidant status, catalase, glutathione peroxidation, as well as a positive correlation between GSH levels and cognitive function (Martínez-Cengotitabengoa *et al.*, 2012).

Thus, an important burgeoning avenue of research is to provide a possible connection between redox dysregulation and the underlying mechanism of schizophrenia. Interestingly pre-clinical and clinical studies have found that N-acetyl cysteine (NAC), a glutathione replenishing antioxidant that also acts on glutamate NMDA receptors, has important

adjunctive effects when combined with an antipsychotic (Berk *et al.*, 2008; Möller *et al.*, 2013). These findings provide support for innovative augmentation strategies with antioxidants in the treatment of schizophrenia.

2.5.4 Inflammation

The complex process of inflammation is crucial for survival, unfortunately the harmful effects of the immune response can occasionally overshadow its protective purpose if not held in check (Zweifach *et al.*, 2014). Evidence that prenatal infection and foetal inflammation may contribute to the development of schizophrenia in the off-spring (Brown & Derkits, 2010) has linked the neurodevelopmental pathology of schizophrenia to activated immuno-inflammatory pathways (Anderson & Maes, 2013). These pathways can include activated microglia (Monji *et al.*, 2009), oxidative and nitrosative stress (O&NS) (including lowered antioxidants such as GSH) (Bitanihirwe & Woo, 2011), and activation of the neurotoxic tryptophan catabolite pathway through cytokines that in turn would result in glutamatergic dysregulation (see Figure 5) (Myint & Kim, 2014) and subsequently to disordered monoaminergic transmission, as noted earlier.

Microglia are antigen presenting cells in the CNS that primarily produce and release pro-inflammatory cytokines after activation (Smith *et al.*, 2012). These cells respond rapidly to minimal pathological changes in the brain and may therefore participate directly in neuronal degeneration (Monji *et al.*, 2009). Certain immunological microglial activators such as lipopolysaccharide (LPS) are present in the CNS after exposure to viral or bacterial pathogens (Monji *et al.*, 2009) resulting in activated microglia that release copious amounts of free radicals and pro-inflammatory cytokines in response to cytokines. Cytokines are glycoproteins, proteins, or peptides that, in addition to their inflammatory functions, play a critical role in signalling pathways in the CNS (Hopkins & Rothwell, 1995). Pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-1 (α + β), IL-12 and IL-6, are known to cause neuronal degeneration, white matter abnormalities and decreased neurogenesis (Figure 5) (Monje *et al.*, 2003; Iosif *et al.*, 2006; Monji *et al.*, 2009). In support of this, Al-Asmari and Khan (2014) found elevated levels of IL-1β, IL-6 and TNF-α in patients with schizophrenia. However, IL-1β, IL-6, and TGF-β appears to be state-associated markers, with significantly heightened levels during acute psychosis, but normalized levels following antipsychotic treatment (Miller *et al.*, 2011). In contrast, IL-4, and IL-10 are considered anti-inflammatory cytokines that minimize the immune-inflammatory response (Potvin *et al.*, 2008). Blood IL-10 levels were found to be significantly diminished in relapsed schizophrenic inpatients (Miller *et al.*, 2011). Additionally, Meyer *et al.* (2008b) found that enhanced expression of IL-10 reduces the behavioural and

neurochemical effects of prenatal immune activation in animals. On the other hand, in the absence of an immune response stimulus, elevated levels of IL-10 in the foetal environment can contribute to behavioural deficits in the offspring, thus emphasizing the critical balance between pro- and anti-inflammatory cytokines during pregnancy (Meyer *et al.*, 2008b).

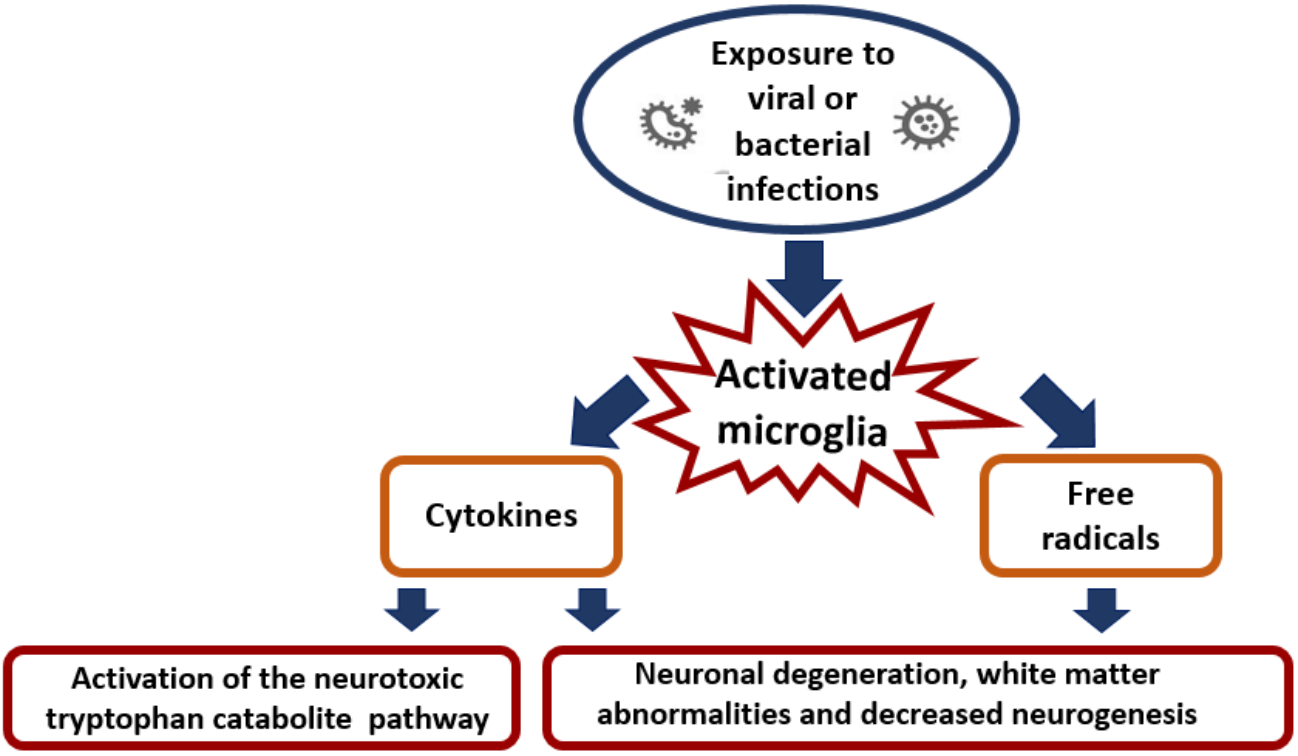

Figure 5: The potential role of microglia and cytokines in schizophrenia: Activated microglia release pro-inflammatory cytokines and free radicals that may contribute to higher susceptibility for schizophrenia

2.5.4.1 Linking pre-natal inflammation to schizophrenia

Pre-natal exposure to viral or bacterial infections are known to cause disturbances in the foetal environment, subsequently manipulating the normal course of brain development (Rees & Harding, 2004; Rees & Inder, 2005). This interference during critical periods of neurodevelopment can lead to certain structural and functional brain abnormalities later in life and have been associated with an increased risk of schizophrenia in the offspring (Weinberger, 1987; Rapoport *et al.*, 2005a; Meyer, 2013). However, the link between maternal infection and a higher susceptibility for schizophrenia does not seem to be restricted to a single infectious agent (Meyer *et al.*, 2008a). This has led to Gilmore and

Jarskog (1997) propose that critical mediators, such as pro-inflammatory cytokines, associated with the maternal immune response may play a pivotal role in altering normal neurodevelopment. Preclinical (Burns *et al.*, 1993; Pousset, 1994) and clinical studies (Mousa *et al.*, 1999) presented the expression of pro-inflammatory cytokines and cytokine receptors during foetal brain development, implying critical roles for these molecules in the neurodevelopmental process. Gilmore *et al.* (2004) also found that the cytokines IL-1b, IL-6, and TNF-α significantly reduced the development of dendrites in embryonic cortical neurons. In addition to this, systematic elevations in plasma IL-6 could sensitize amphetamine-induced locomotion and alternate dopamine turnover resulting in the neurotransmitter dysregulation associated with schizophrenia (Zalcman *et al.*, 1994; Zalcman *et al.*, 1999). Moreover, it has been confirmed that IL-6 can cross the placenta in rodents and humans (Zaretsky *et al.*, 2004; Dahlgren *et al.*, 2006), although IL-1b and TNF-α displayed minimal placental transmission (Zaretsky *et al.*, 2004). However, elevated levels of TNF-α and IL-8 levels have been observed during the second and third trimesters of pregnancy and have been linked to psychotic symptoms in the offspring (Buka *et al.*, 2001; Brown *et al.*, 2004).

2.5.5 The tryptophan catabolite pathway

Tryptophan is an amino acid obtained through external sources and is essential for several metabolic pathways (Chen & Guillemin, 2009), including 5-HT synthesis (Allegri *et al.*, 2003). The tryptophan catabolite pathway, also known as the kynurenine pathway, embodies a key route for the metabolism of tryptophan. As seen in Figure 6, tryptophan is catabolized by enzymes, tryptophan-2,3-dioxygenase (TDO) and indoleamine-2,3-dioxygenase (IDO), into kynurenine (KYN) (Stone *et al.*, 2003; Möller *et al.*, 2012; Moller *et al.*, 2015). Following the degradation of tryptophan to KYN, metabolites of this pathway includes among others the neurotoxic quinolinic acid (QA) and the neuroprotective kynurenic acid (KYNA) (Barry *et al.*, 2008). QA is an endogenous NMDA receptor agonist that can mediate NMDA neuronal damage and dysfunction. KYNA, however, is a NMDA receptor antagonist and can modulate the neurotoxic effects of QA in addition to disrupting excitatory amino-acid neurotransmission (Heyes *et al.*, 1992). Last mentioned neuroactive metabolites can then initiate alterations in glutamatergic, GABAergic, DAergic and NAergic neurotransmissions, which in turn can lead to changes in neuronal-glial network and consequently neuropsychiatric manifestations (Myint & Kim, 2014). Furthermore, both IDO and TDO divert tryptophan catabolism away from 5-HT synthesis, resulting in decreased 5-HT, possibly involved in the depressive-like/ negative symptoms commonly observed in schizophrenia (Stone *et al.*, 2003).

Activated microglia, ROS, as well as pro-inflammatory cytokines such as interferon-γ (IFNγ) can be significant mediators of tryptophan catabolism by amplifying the activity of IDO, resulting in the above mentioned manifestations (Yasui *et al.*, 1986; Carlin *et al.*, 1987; Heyes *et al.*, 1993; Anderson & Maes, 2013). These findings support the relationship between an inflammatory state, oxidative stress, tryptophan metabolism, 5-HTergic neurotransmission in the neurobiology of schizophrenia.

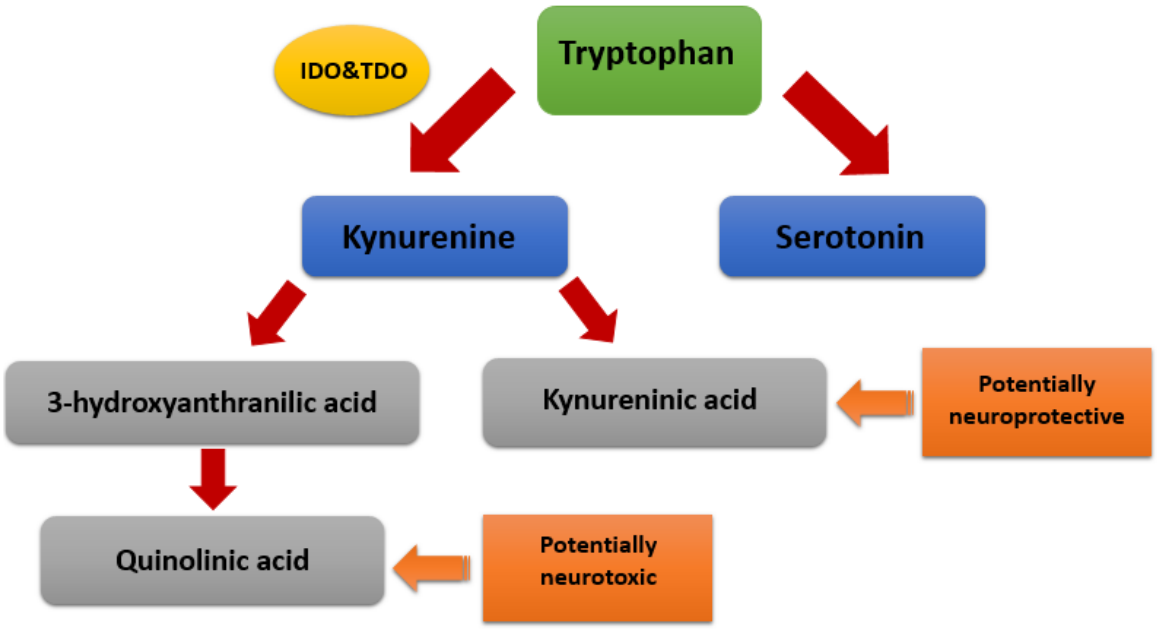

Figure 6: Tryptophan metabolism via the kynurenine pathway (IDO: Indoleamine 2,3-dioxygenase; TDO: Tryptophan 2,3-dioxygenase).

2.6 Animal models of schizophrenia

Replicating the symptomology and neurobiology associated with schizophrenia is crucial for the investigation of pathological mechanisms underlying the disease and for the development of novel treatment strategies. The complexity of schizophrenia, however, creates a challenge for modelling schizophrenia in animals. Firstly, the genetic nature of psychiatric disorders in combination with the environmental risk factors that contribute to the susceptibility of schizophrenia, complicate the process of developing a model that takes into account the gene-environment interplay (Ayhan *et al.*, 2016). Furthermore, although several models have been developed to mimic schizophrenia-related behaviour, various symptoms observed humans (e.g., hallucinations, delusions) cannot be evidently established in animals (Nestler & Hyman, 2010). Ultimately, an important limitation of animal models is that even though there are

sufficient correlates in animals, (e.g., reduced social behaviour, motivation, executive function, and working memory), the correspondence may only be an estimate (Nestler & Hyman, 2010).

In order to assess the reliability of an animal model as a useful tool for preclinical research, it should be validated in accordance with certain criteria as seen in Figure 7, namely (1) construct, (2) face and (3) predicative validity (Jones *et al.*, 2011).

Construct or etiologic validity refers to the method of constructing a model and its relevance to the human disorder (Nestler & Hyman, 2010). To achieve this, the researcher would have to be able to replicate the particular pathological processes that result in the human illness in the animal and thus to reproduce core features of the disease (Chadman *et al.*, 2009). Animal models with construct validity may be generated by manipulating genetic factors, prominent proteins, neural circuits or neurochemical pathways known to be responsible for the illness (Nestler & Hyman, 2010). However, this is rarely achieved due to the elusive and extremely complex aetiology of schizophrenia (Purcell *et al.*, 2009). Additionally, exposing the animal to well-validated environmental stressors that are considered to play a part in pathogenesis of schizophrenia could also be used to create a model with construct validity (Nestler & Hyman, 2010). Such models usually employ an early-life neurodevelopmental stressor, such as maternal/prenatal infection and/or inflammation models(Moller *et al.*, 2015) and the social isolation rearing model (Moller *et al.*, 2015), and where bio-behavioural manifestations related to schizophrenia are evaluated in the off-spring later in life. However, since the underlying neurobiology of schizophrenia remains elusive, construct validity remains the weakest or most speculative of the three criteria.

Face validity signifies the similarity between the behavioural features exhibited in the animal model with the symptoms observed in humans with schizophrenia (Rand, 2004). A few behaviours associated with increased dopamine such as hyperlocomotion and stereotypic behaviour in animals have been related to the psychotic or positive symptoms of schizophrenia (Lipska & Weinberger, 2000). Although the negative symptoms are exceptionally complicated to mimic and assess in animals, the close resemblance of depression to these symptoms provide a possibility of using behavioural tests of depression e.g. the forced swim test and sucrose preference test to evaluate despair and anhedonia observed in schizophrenia (Ellenbroek & Cools, 2000). Reduced social interaction in animals have also been consistently associated with the social withdrawal seen in schizophrenia patients (McGlashan & Fenton, 1992; Meyer & Feldon, 2010). Furthermore, cognitive impairments in schizophrenia can be observed in animals through models of learning and memory such as the novel object recognition test (Ennaceur & Delacour, 1988). Finally, deficits in information processing, sensorimotor gating and attention and which are well-described in schizophrenia (Strauss *et*

al., 1993; Perry & Braff, 1994), can be measured with prepulse inhibition (PPI) and latent inhibition (LI) tests.

The final criterion for animal models is predictive validity, also referred to as pharmacological validity which requires that a model responds predictively to known and effective pharmacological treatment of the human disorder, thus in a manner predicting the outcome of such treatment in humans (Nestler & Hyman, 2010). In the ideal situation, the model would respond effectively to well-known antipsychotics and will furthermore reveal the effects of novel treatments, while at the same time it may be non-responsive to agents that are also ineffective in humans, e.g. anxiolytics. Ultimately, animal models of complex psychiatric disorders such as schizophrenia are valuable instruments for preclinical research to study the relationship between the biological basis and behavioural features of the disease, its treatment and their interaction with environmental factors.

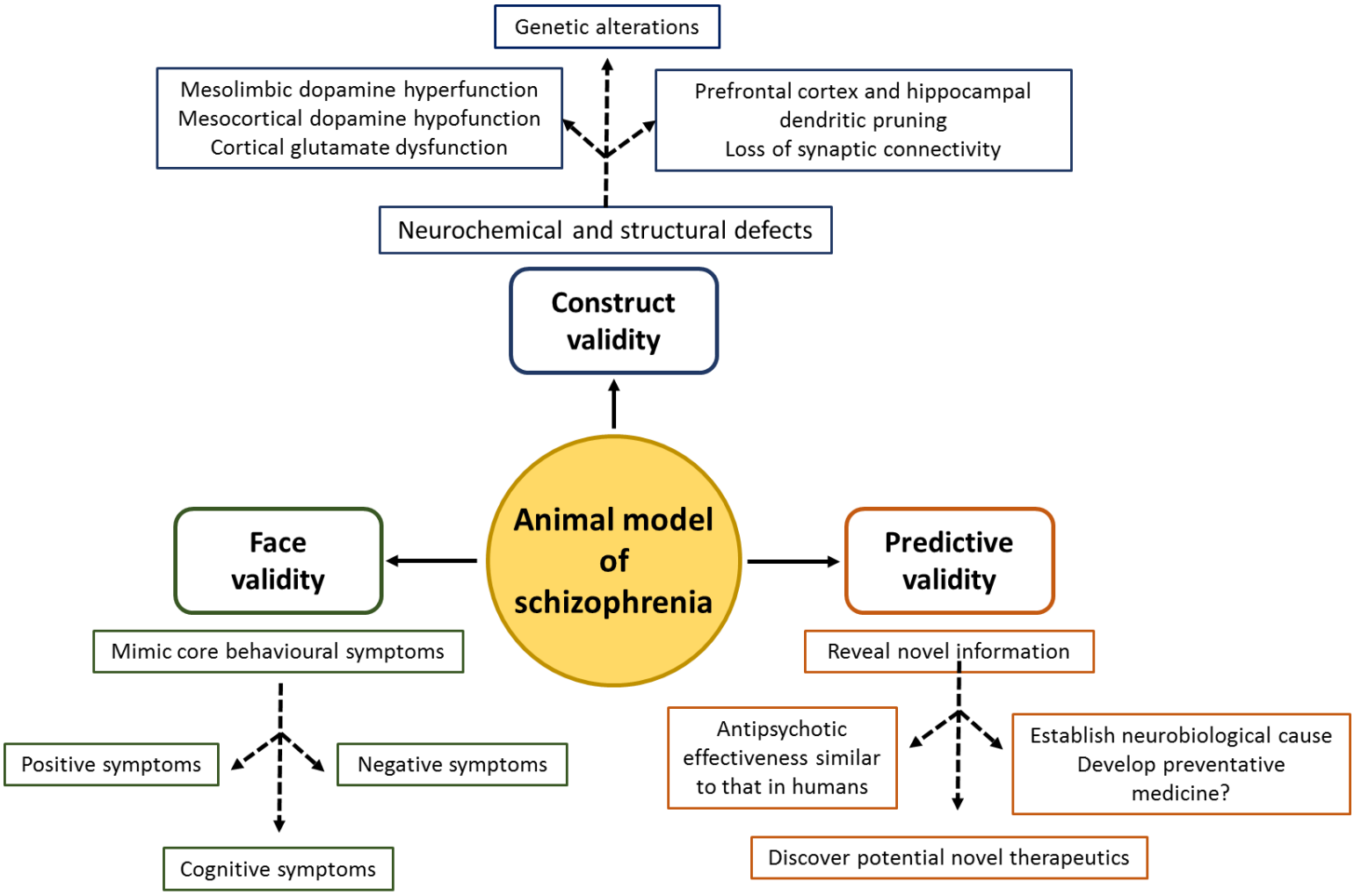

Figure 7: A summary of the criteria for the validation of reliable animal models (Adapted from Jones *et al*., 2011).

2.6.1 Pre-natal inflammation models in animals

Abundant evidence indicates that maternal infection during pregnancy is one of the significant environmental risk factors of neurodevelopmental brain disorders occurring in the off-spring, such as schizophrenia (Meyer *et al.*, 2009). Environmental insults during early brain development can negatively alter the normal process of neurodevelopment (Rees & Inder, 2005). The development of rodent models have supplied robust evidence for a pivotal relationship between prenatal exposure to infectious and/or immune activating agents and the development of various brain dysfunctions later in life (Meyer *et al.*, 2009). The maternal immune activation (MIA) model produced several pharmacological and behavioural changes associated with schizophrenia in the adult offspring (Zuckerman & Weiner, 2005; Ozawa *et al.*, 2006). An immune-inflammatory response can be induced in animals by numerous immunological activators, which include polyinosinic:polycytidylic acid (poly I:C) (Ozawa *et al.*, 2006; Wolff & Bilkey, 2010; Van den Eynde *et al.*, 2014), the direct administration of cytokines (Tohmi *et al.*, 2004) and lipopolysaccharide (LPS) (Kirsten *et al.*, 2010; Lin *et al.*, 2012). LPS is the key component of the outer membrane of Gram-negative bacteria and is recognized by toll-like receptor-4 (TLR-4). It is used to mimic bacterial infection and leads to fever and inflammation (Jenkins, 2013) by activating the production and release of pro-inflammatory cytokines, including IL-1b, IL-6, and TNF-α (Meyer *et al.*, 2005; Boksa, 2010; Baharnoori *et al.*, 2012). Cytokines also have important effects on CNS development, including effects on neuronal survival, differentiation and apoptosis, expression of transmitters and neurotrophins and excitotoxicity in the developing brain (Boksa, 2010).

The LPS inflammatory animal model displays a number of important manifestations akin to schizophrenia, including increased immobility in the forced swim test (FST) (Lin *et al.*, 2012), percentage prepulse inhibition (%PPI) deficits (Borrell *et al.*, 2002), working memory impairments, and increased locomotor activity (Jenkins, 2013) in the offspring. Working memory performance is cardinal for the thought process and impairments thereof may possibly be responsible for the cognitive dysfunction consistently observed in patients with schizophrenia (Castner *et al.*, 2004). The immobility seen in the FST displays the despair in the animal in the face of acute exposure to an adverse environment and is similar to that observed in depression, while deficits in %PPI reflect dysfunction of sensorimotor gating, which are both well-described in schizophrenia (Kulhara *et al.*, 1989; Möller *et al.*, 2011). Previous studies in our laboratory have indicated that prenatal LPS can significantly

compromise %PPI in the off-spring, as well as induce other behavioural anomalies such as social anxiety (Swanepoel *et al.*, 2017). The use of a well-established pre-natal inflammation model can assist with the clarification of certain aspects of the underlying mechanism and dysfunction in the brain in schizophrenia development.

2.7 Treatment of schizophrenia

2.7.1 Typical antipsychotics

The discovery of the antipsychotic effects of the dopamine D_2 receptor antagonists, haloperidol and chlorpromazine in the 1950s and its success in treating the positive symptoms of schizophrenia, fuelled further research into potential treatment options related to the targeting of dopamine receptors. The typical or first generation antipsychotics all have a high affinity for D_2 receptors and recent imaging studies have demonstrated a high-level of limbic cortical and striatal D_2 receptor occupancy following therapeutic doses of typical antipsychotics (Bigliani *et al.*, 1999; Xiberas *et al.*, 2001). Although first generation treatments are effective in treating the psychotic symptoms of schizophrenia and preventing the recurrence thereof (Miyamoto *et al.*, 2002), they are less effective in managing the cognitive and negative symptoms associated with the illness (Spohn & Strauss, 1989; Meltzer *et al.*, 1996). In addition to this, prominent adverse effects including extrapyramidal symptoms (EPS) and tardive dyskinesia accompany these drugs, reducing their appeal from the patient's point of view and prompting concerns over poor adherence and its negative effect on long-term outcome. Nevertheless, despite the above drawbacks these agents are both affordable and effective. Because of this, there is perceived benefit in adjunctive treatment where another agent that targets specific pathological processes implicated in schizophrenia is combined with the typical agent in order to bring about a superior treatment response but that is economically viable, such as combining typical agents with antioxidants like NAC (Rapado-Castro *et al.*, 2017), as well as neuroprotectants like lithium, aspirin, minocycline, statins, leptin and melatonin(Dodd *et al.*, 2013). Turning to the natural pharmacopoeia may have significant benefits in this regard (Singh *et al.*, 2010; Fedotova *et al.*, 2017; Nabavi *et al.*, 2017).

2.7.2 Atypical antipsychotics

Newer or 2nd generation antipsychotics are generally referred to as atypical agents due to their improved efficacy to treat the negative symptoms of the disorder while displaying less motor and endocrine-related side-effects (Leucht *et al.*, 2013). A meta-analysis completed by Davis *et al.* (2003) concluded that atypical treatments such as clozapine, risperidone and

olanzapine are superior to first generation antipsychotics with regards to treatment response. The causal mechanism of their improved response remains unexplained, but can be connected to how they target specific neurotransmitter pathways involved in the biology of schizophrenia. Such pathways include 5-HT, acetylcholine (Ach), glutamate and noradrenaline, in addition to the critical dopaminergic modulation (Kinon & Lieberman, 1996). The crucial role of dopaminergic receptor blockade for antipsychotic effects is undeniable. However, serotonergic modulation similarly shows great potential in the treatment of schizophrenia, especially by bolstering the action of D_2 receptor antagonists, and includes 5-HT_{2A} receptor antagonists, 5-HT_{1A} receptor antagonists or agonists, 5-HT_{2c} receptor partial agonists and 5-HT_3 receptor antagonists (Horacek *et al.*, 2006). The combination of 5-HT_{2A} and D_2 receptor antagonism, with higher affinity for 5-HT_{2A} than D_2 receptors, have been found to increase dopamine output to the PFC and striatum, thus providing improvement in various cognitive and negative symptoms of the illness while at the same time providing a rescuing effect on D_2-antagonism induced EPS (Horacek *et al.*, 2006). Although atypical antipsychotics are recommended as first-line treatment (Gaebel *et al.*, 2005) and appear to be more tolerable and effective than conventional treatments (Geddes *et al.*, 2000), they still have the potential of inducing adverse effects such as hyperlipidemia, weight gain, or sexual and cardiac dysfunction (ÜÇOk & Gaebel, 2008), while they are also expensive. Furthermore, 40–70% of patients treated with clozapine demonstrates unsatisfactory response to this drug, while clozapine remains the most effective of the newer antipsychotics (Remington *et al.*, 2005). Evidently, it is essential to focus on new treatment strategies targeting pathological mechanisms underlying schizophrenia. One such approach is to target redox-inflammatory pathways.

2.7.3 Other considerations in treatment

Substantial evidence implicates inflammatory pathways, altered antioxidant activity and reduced glutathione levels in schizophrenia (Yao *et al.*, 1998; Yao *et al.*, 2006; Raffa *et al.*, 2009; Kirkpatrick & Miller, 2013). Consequently, anti-inflammatory agents have been suggested as an adjunctive therapeutic strategy to reduce the severity of symptoms in schizophrenia (Sommer *et al.*, 2012). Recent rodent studies have demonstrated that NAC is able to reverse schizophrenia-related electrophysiological, morphological, and behavioural abnormalities in mice with GSH deficits (Otte *et al.*, 2011; Cabungcal *et al.*, 2013). Furthermore, NAC normalizes social behaviour and reduces striatal lipid peroxidation in an immune-inflammatory animal model of schizophrenia (Swanepoel *et al.*, 2017) and to also reduce deficits in startle response (%PPI) in a neurodevelopmental model of schizophrenia (Möller *et al.*, 2013). Recently studies have begun to focus on the clinical

utility of antioxidants in treating schizophrenia (Emsley *et al.*, 2014), including of plant origin (Magalhães *et al.*, 2016).

2.7.3.1 Plants in psychopharmacology

Plants have been used as herbal remedies for centuries and although treatment options have shifted to more synthetic options over the years, the ancient use of plants has lead researchers to investigate the therapeutic properties of plants over the past decade (Carlini, 2003; Sarris, 2007). Several studies have suggested a role for herbal medicine in the treatment of psychological disorders such as depression or anxiety (Beaubrun & Gray, 2000; Ernst, 2006; Saki *et al.*, 2014). Medicinal plants such as *Crocus sativus (C. sativus)* (saffron) and *Rhodiola rosea (R. rosea)* (roseroot), displayed promising antidepressant activity and also anxiolytic activity due to mood elevating effects (Saki *et al.*, 2014). With regards to schizophrenia, studies revealed that traditional Chinese herbal medicine combined with antipsychotics might have a beneficial impact on the treatment outcome in patients (Rathbone *et al.*, 2007). Overall, minimal robust research is available on herbal treatment for psychotic disorders such as schizophrenia and examining other plant properties such as antioxidant activity may reveal valuable treatment options.

2.7.3.2 Xanthones

Xanthones are antioxidants that have been observed for their broad variety of effects. They are a group of organic compounds that are isolated from higher plants, fungi, ferns, and lichens and show great potential for research due to their medicinal properties (Negi et al., 2013). Mangiferin, a xanthone that functions as a monoamine oxidase inhibitor (Negi et al., 2013), has been reported to have CNS stimulatory effects (Lin et al., 1984) and to have high antioxidant (Jung et al., 2006; Okonogi et al., 2007) and anti-inflammatory activity (Mandal *et al.*, 1992; Park *et al.*, 2006; Chen *et al.*, 2008). Considering the involvement of the CNS, ROS and activated immune response in the pathology of schizophrenia, these compounds have promising possibilities for treating psychosis, at least at a mechanistic level.

2.7.3.3 *Garcinia mangostana* Linn (GML)

GML or mangosteen, is an evergreen fruit tree originating from Indonesia (Garrity et al., 2013). The fruit, and in particular the pericarp or rind of the fruit contains over 85 components including polyphenols such as xanthones, prodelophinidins anthocyanins, procyanins, tannins and epicatechins, of which the best characterized are α-, β-, and γ-mangostin, garcinone E, 8-deoxygartanin, and gartanin (Pedraza-Chaverri et al., 2008). Extracts of the fruit have displayed a wide range of biological properties *in vitro*, such as

antioxidant (Yoshikawa et al., 1994; Jung et al., 2006), cytotoxic (Ho et al., 2002; Wang et al., 2011), anti-inflammatory (Chairungsrilerd et al., 1996; Chen et al., 2008), antibacterial (Phongpaichit et al., 1994; Chomnawang et al., 2009), antifungal (Puripattanavong et al., 2006; Kaomongkolgit et al., 2009) and antitumoral properties (Matsumoto et al., 2004; Moongkarndi et al., 2004). Recent chronic treatment studies undertaken on raw GML in our laboratory have displayed dose-dependent antidepressant-like effects at 50mg/kg/d (Oberholzer *et al.*, 2017).

2.7.3.4 Alpha-mangostin (AM)

AM, an extracted xanthone derivative from GML, has been reported to present with noteworthy pharmacological activity (Gopalakrishnan et al., 1980; Vlietinck et al., 1998; Sakagami et al., 2005; Nakagawa et al., 2007). Li and colleagues (2011) showed that AM following oral administration is poorly bioavailable, although analgesic effects have been documented at acute oral doses of 30-100mg/kg (Sani et al., 2015). Although its possible psychotropic actions remain untested, the antioxidative activity of AM reveals potential for neuroprotection against oxidative stress and hence to be of value in redox-based disorders. However, it is difficult to locate the beneficial pharmacological activity of GML due to the numerous components present in the pericarp. Isolating separate components of GML may shed light on where the pharmacological activity lies or reveal that the response is more related to a balanced synergism between all constituents than due to one or more of the isolated ingredients. Comparing raw GML to one of its well-characterised active constituents (AM) will provide detail on the benefits of the whole plant extract vs. the isolated components.

2.8 Synopsis

The debilitating symptoms and chronic course of schizophrenia leads to several social, clinical and economic implications (Chisholm *et al.*, 2008). Furthermore, considerable comorbidities and high mortality rates are associated with the illness (Schoepf *et al.*, 2014). Although there are a broad variety of antipsychotic treatments available, the outcome remains suboptimal with regards to their efficacy, adverse effects and tolerability. In addition, schizophrenia patients require lifelong treatment in order to manage the disorder (Citrome *et al.*, 2015). The complexity of the neuropathogical pathways underlying schizophrenia in combination with environmental influences additionally complicates the development of novel treatment options. The involvement of oxidative and inflammation pathways in schizophrenia and the costly nature of current antipsychotic regimens have led

to the consideration of natural treatments with anti-inflammatory and anti-oxidant properties (Magalhães *et al.*, 2016).

In this particular study, a suspension of the dried pericarp of GML will be studied, together with one of its primary active constituents, AM, both known to have anti-inflammatory and anti-oxidant effects (Jung *et al.*, 2006; Chen *et al.*, 2008). Given the role of oxidative stress in schizophrenia, and that depressive behaviour is a key symptom in the disorder, with GML having already demonstrated antidepressant-like effects (Oberholzer *et al.*, 2017), GML and AM may harbour useful therapeutic benefits in schizophrenia. Since clinical studies are restricted to adjunctive treatment for ethical reasons, it would be imperative to investigate the putative benefits of GML and AM as an add-on therapy to known antipsychotic treatment. Since atypical agents like clozapine are already functioning at a higher level of efficacy with respect to all symptoms of schizophrenia, assessing these plant extracts in combination with a 1st generation agent such as haloperidol seems a logical first choice for initial testing.

Research have revealed a strong neurodevelopmental foundation in schizophrenia and has emphasised the crucial interaction between genetic susceptibility and environmental insults such as exposure to pre-natal inflammation (Lewis & Levitt, 2002; Miller *et al.*, 2013). Therefore, in this study we will utilize the MIA animal model to induce schizophrenia-like behaviour and neurochemical alterations in the offspring in order to examine the effects of GML and AM alone thereon vs. haloperidol and in combination with haloperidol. To this end, alterations in schizophrenia-related behaviours, such as SI, PPI and FST, will be analysed, together with associated effects on pro- and anti-inflammatory cytokines and oxidative stress.

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CHAPTER 3

MANUSCRIPT

This chapter presents a concept article for submission to *The International Journal of Neuropsychopharmacology* (IJNP), an appropriate peer-reviewed scientific journal. The current chapter was prepared according to the guidelines to the author for this journal (Addendum C).

The instructions for the preparation of the manuscript are outlined on the journal website: https://academic.oup.com/ijnp, under “Author Guidelines”.

The manuscript title, contributing authors and affiliations will appear on the following page. The abstract, highlights and keywords will be presented on a single page, followed by the main body of the manuscript according to the following structure: Introduction, Materials and Methods, Results and Discussion, Conclusions, Acknowledgements, References, Legends to Figures. To benefit the reader all figures have been inserted in the text and not at the end of the manuscript as required by the journal. The heading numbers and page numbers for this chapter will align with the dissertation.

J.S. Lotter assisted with the design of the study with the help of B.H. Harvey and M. Möller, conducted the behavioural and neurochemical analyses, undertook the statistical analyses and prepared the first draft of the manuscript. B.H. Harvey and M. Möller supervised the study and assisted in the interpretation of the study data, and will finalize the manuscript for publication. Michael Berk assisted with the finalization of the manuscript.

All co-authors have granted permission for the article to be submitted for the purpose of the MSc (see Addendum D).

Studies on haloperidol and adjunctive α-mangostin or raw *Garcinia Mangostana* Linn pericarp on bio-behavioural markers in an immune-inflammatory model of schizophrenia

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Abstract

Background: Schizophrenia is a severe brain disorder that is associated with neurodevelopmental insults, such as prenatal inflammation, that introduce redox-immune-inflammatory alterations and risk for psychotic symptoms later in life. The aim of this study was to examine the therapeutic effects of *Garcinia mangostana* Linn (GML) and α-mangostin (AM) alone and as adjunctive treatment with haloperidol (HAL) on schizophrenia related bio-behavioural alterations in a maternal immune-activation (MIA) model.

Methods: Sprague Dawley dams were exposed to lipopolysaccharide (LPS) on gestational days 15 and 16. The male offspring were treated from PND 52 - 66 with the following: vehicle; haloperidol (HAL; 2 mg/kg); GML (50 mg/kg); HAL+GML, AM (20 mg/kg) and HAL+AM. Control dams and control offspring were treated with vehicle. On PND's 64- 65, prepulse inhibition (PPI) of startle, locomotor activity and depressive-like behaviour was assessed, followed by assay of frontal-cortical lipid peroxidation and plasma pro-inflammatory cytokine levels, viz. interleukin-1 (IL-1) and tumour necrosis factor-α (TNF-α).

Results: MIA-induced deficits in sensorimotor gating was reversed by HAL and HAL+GML. MIA-induced depressive-like behaviour was reversed by AM and GML alone and both in combination with HAL, with the combinations more effective than HAL. MIA-induced cortico-striatal lipid peroxidation was reversed by HAL and AM, with elevated IL-6 and TNF-α levels restored by GML, AM, HAL and combinations thereof, although no bolstering effect was observed with the latter.

Conclusion: Prenatal LPS-induced schizophrenia-like bio-behavioural alterations in the offspring are variably responsive to HAL, GML and AM, with depressive manifestations showing the best response to GML, AM or when combined with HAL. AM may be a more effective antioxidant than GML *in vivo*, although this does not imply improved therapeutic response.

Keywords:

Schizophrenia, prenatal inflammation, oxidative stress, *Garcinia mangostana* Linn, alpha-mangostin.

3.1 Introduction

Schizophrenia is a severe psychiatric disorder with a chronic course, affecting approximately 1% of the global population (Harris et al., 2013). This debilitating disease is characterised by clinical features manifesting in early adulthood (Rapoport et al., 2005), including positive (hallucinations and delusions), negative (social withdrawal, apathy and anhedonia) and cognitive symptoms (working memory deficits, attention disorders and altered information processing) (Simpson et al., 2010). Despite decades of research on the neurobiological and genetic aspects of schizophrenia, the underlying etiological mechanisms remain elusive (Meyer, 2013). Regardless of significant developments in pharmacotherapy, the treatment outcome remains suboptimal (Lindenmayer et al., 2009), especially with regards to negative and cognitive symptoms (Erhart et al., 2006; Buckley & Stahl, 2007).

Several hypotheses have been suggested in an attempt to elucidate the complex pathology of schizophrenia. The neurodevelopmental hypothesis has provided a valuable framework for the relationship between pathologic processes during early brain development and aberrations associated with schizophrenia later in life (Weinberger, 1986; Owen et al., 2011). The developmental origin of schizophrenia might be a result of the interaction between genetic predisposition and early life environmental vulnerability factors such as prenatal exposures related to malnutrition, substance abuse, obstetric complications, season of birth and infection and catalysed by later stresses such as substance abuse, social defeat and trauma (McGrath & Welham, 1999; Cannon et al., 2002; Kirkbride et al., 2012; Davis et al., 2016).

A body of epidemiological evidence has suggested a link between viral or bacterial maternal infection during pregnancy and the increased risk for developing schizophrenia in the offspring (Brown & Derkits, 2009; Brown, 2012). However, the increased susceptibility for schizophrenia may be a critical consequence of the immune activation common to the infectious process rather than the infectious agent itself (Penner & Brown, 2007; Patterson, 2009). Several preclinical studies have demonstrated biological and behavioural abnormalities in rodents following prenatal maternal exposure to lipopolysaccharide (LPS) (Kirsten et al., 2010; Lin et al., 2012), polyinosinic:polycytidylic acid (poly I:C)(Ozawa et al., 2006; Wolff & Bilkey, 2010; Van den Eynde et al., 2014), human influenza virus (Shi et al., 2003) and cytokines (Tohmi et al., 2004; Smith et al., 2007). LPS, an endotoxin derived from the cell wall of Gram-negative bacteria, mimics an infection by activating the synthesis and release of pro-inflammatory cytokines, including interleukin-1β (IL-1b), IL-6, and tumour necrosis factor-α (TNF-α) (Meyer et al., 2005; Boksa, 2010; Baharnoori et al., 2012) and is known to lead to various behavioural,

neurochemical and inflammatory changes akin to that noted in schizophrenia (Borrell et al., 2002; Basta-Kaim et al., 2011a; Zhu et al., 2014).

In addition to inflammation processes, oxidative stress mechanisms have been recognised to play a key role in psychiatric pathophysiology (Ng et al., 2008; Bitanihirwe & Woo, 2011). The increased production of reactive oxygen species (ROS) and reduced antioxidants as a result of oxidative imbalance have been observed in people with schizophrenia and may contribute to the neuroprogression of the disorder (Mahadik & Mukherjee, 1996; Mahadik et al., 2001; Reddy et al., 2003; Boskovic et al., 2011). In this regard, antioxidant treatment with N-acetyl cysteine (NAC) has proved to have therapeutic benefits in clinical studies (Berk et al., 2008; Carmeli et al., 2012) as well as in preclinical animal models (Möller et al., 2013a; Cabungcal et al., 2014; Swanepoel, 2017).

The anti-inflammatory and antioxidant activity of herbal bioactive compounds have been widely observed, particularly in a group of polyphenols referred to as xanthones (Gutierrez-Orozco & Failla, 2013). *Garcinia mangostana* Linn (GML) is an exotic fruit native to Southeast Asia, known to contain constituents including xanthones, flavonoids, triterpenoids, and benzophenones (Chin & Kinghorn, 2008). Extracts of the fruit have exhibited antioxidant (Yoshikawa et al., 1994; Jung et al., 2006), anti-inflammatory (Chairungsrilerd et al., 1996a; Chen et al., 2008), antibacterial (Chomnawang et al., 2009) and antidepressant effects (Oberholzer et al., 2017). In particular, α-mangostin (AM), a primary component of GML, also presents with substantial pharmacological properties (Sakagami et al., 2005; Nakagawa et al., 2007), while having moderate inhibitory effects on the serotonin $5HT_{2A}$ receptor and cyclic adenosine monophosphate (cAMP) phosphodiesterase (Chin & Kinghorn, 2008).

The aim of this study was to establish whether a maternal immune activation (MIA) model is capable of inducing schizophrenia-like behaviour and redox-inflammatory alterations in the offspring of afflicted dams and to evaluate whether these changes can be reversed by a reference antipsychotic, haloperidol (HAL). Additionally, the study assessed the effects of GML and AM to similarly reverse these alterations and whether they are able to augment the response to HAL. To this end, behavioural assays aimed at assessing typical schizophrenia-related cognitive and negative symptoms, viz. sensorimotor gating and depressive-like symptoms, and their association with changes in plasma and brain inflammatory and redox markers, were performed.

3.2 Methods and materials

3.2.1 Chromatographic fingerprinting of raw GML

In order to determine the authenticity and constituents of GML, separation of prenylated xanthones found in GML was achieved utilizing reversed-phase high-performance liquid chromatography (HPLC) with diode-array detection (DAD), as described in an earlier study from our laboratory Oberholzer and colleagues (2017), but reproduced here for examination purposes.

3.2.2 Animals

Pregnant female Sprague Dawley (SD) dams were used during the prenatal phase of the study. Male pups born from pregnant dams were then used for the remainder of the study after weaning (PND 21).

Animals were bred, supplied, and housed at the Vivarium (SAVC reg. no. FR15/13458; SANAS GLP compliance no. G0019) of the Pre-Clinical Drug Development Platform (PCDDP) of the North-West University (NWU) in identical cages containing corncob, under conditions of constant temperature (22 ± 1°C) and humidity (50 ± 10%) with a 12:12-h light/dark cycle (lights on 06:00 to 18:00). Food and water were provided ad libitum in the home cage, with corncob changed at least once a week.

All experiments were approved by the AnimCare animal research ethics committee (NHREC reg. no. AREC-130913-015) of the NWU. All animals were maintained and all procedures performed in accordance to the code of ethics in research, training and testing of drugs in South Africa and complied with national legislation (Ethical approval numbers: NWU-00376-16-A5 and NWU-00147-14-A5). The study design and procedures were according to the ARRIVE Guidelines (Kilkenny et al., 2010).

3.2.3 Study design

The exposure and treatment lay-out is presented in Fig. 1. The one group of dams (n=18) received LPS from gestational days (GDs) 15–16 as part of the MIA model with control group dams (n=3) received saline from GDs 15-16. These GDs were chosen on the grounds of a previous study showing decreased foetal demise at this stage, as well as the correlation of this period with second trimester human pregnancy, suspected to be a critical period for the development of schizophrenia (Fortier *et al.*, 2007). Male offspring (± 4 per dam) from the above groups, an estimate according to breeding and birth data provided by the NWU

Vivarium, were used in the remainder of the study. Cross fostering was not performed, as per findings of Fortier and colleagues (2007).

The male offspring from LPS-exposed dams (n=70) were randomly divided into six treatment groups as described by Iturria, 2011 and received oral dosing of the following: saline (1 ml/kg), haloperidol (2 mg/kg po) (Gao *et al.*, 1997; Schmitt *et al.*, 1999; Schleimer *et al.*, 2005; Terry *et al.*, 2007); GML (50mg/kg po) (Oberholzer *et al.*, 2017), haloperidol + GML (HAL+GML) (at the previously mentioned doses), α-mangostin (AM) (20 mg/kg po) (Li *et al.*, 2011) and haloperidol+ α-mangostin (HAL+AM) (at the previously mentioned doses).Control dams produced a total number of 8 male offspring (n=8) that received oral dosing of saline. Variation in the control group may be less provided that they did not receive any intervention, such as a treatment or stressor, therefore the number of animals were less in the control group compared to the treatment groups. Additionally, the reduced control group is in line with the "reduce" fragment of ethical principles. The respective drug treatments continued for 16 days from PND 51 – PND 66, according to Oberholzer and colleagues (Oberholzer *et al.*, 2017). During the last two days of treatment, all groups were subjected to a battery of behavioural tests that follow a specific sequence to minimise stress on the animals, viz. (1) prepulse inhibition (PPI) of startle on day 13 of treatment (PND 63), (2) the open field test (OFT) on day 14 of treatment (PND 64) and the forced swim test (FST) on day 14 of treatment (PND 64). 36 hours later the animals were euthanized via decapitation and trunk blood and brain tissue collected and stored at -80 °C until the day of neurochemical analysis.

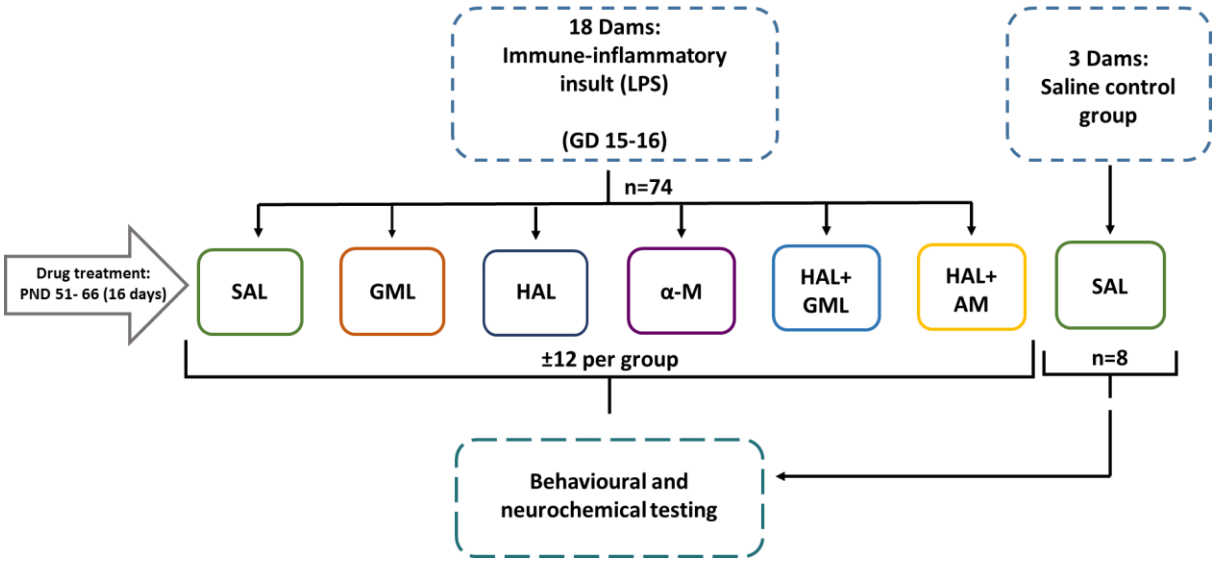

Figure 1: Schematic diagram of the study layout

3.2.4 Drugs and treatment

LPS (100 µg/kg) from *Escherichia coli* (*E.coli)* (Sigma-Aldrich, Johannesburg, South Africa) was dissolved in saline and administered subcutaneously (SC) to pregnant dams on GD 15-16 (Fortier *et al.*, 2007; Baharnoori *et al.*, 2012). HAL (2 mg/kg) (Sigma-Aldrich, Johannesburg, South Africa) was dissolved in a minimum volume of glacial acetic acid, then further diluted with distilled water and the pH adjusted using 10 N NaOH to 6-6.25 and administered by oral gavage (Gao *et al.*, 1997). The dose of HAL was selected for oral dosing specifically, and in line with an earlier study from our laboratory (Harvey *et al.*, 2008). The ground dried pericarp of GML fruit (Industrial Analytical, Kyalami, South Africa) was mixed in a 0.1% xanthan gum solution to aid suspension and administered by oral gavage, at a dose of 50 mg/kg, as described in an earlier study (Oberholzer, 2017). AM (Sigma-Aldrich, Johannesburg, South Africa) was dissolved in polyethylene glycol (PEG) 400 vehicle (PEG 400:water ratio= 6:4, v/v), as previously described (Han *et al.*, 2015) at a dose of 20 mg/kg po (Li *et al.* (2011).

3.2.5 Behavioural analyses

3.2.5.1 Prepulse inhibition (PPI)

Prepulse inhibition (PPI) is used to determine deficits in sensorimotor gating which is well-described in schizophrenia (Braff *et al.*, 2001). The test involves the ability of rodents to reduce a startle response to an auditory pulse when a pre-pulse of smaller amplitude precedes the main pulse. Typically, when a pre-pulse is presented to an animal, the startle response to the next higher amplitude pulse is reduced. This behavioural response is typical of patients with schizophrenia, and is representative of cognitive fragmentation (Swerdlow *et al.*, 2006). PPI was assessed in illuminated and ventilated sound-attenuated startle chambers (SR-LAB, San Diego Instruments, San Diego, USA) (Möller *et al.*, 2011). In brief, a speaker mounted above the test cylinder inside the startle chamber provides acoustic noise bursts, both the sound bursts and the detection of startle responses are delivered by the SR-LAB software running on a designated computer. Startle amplitudes are defined as the average of 100x1 ms stabilimeter readings collected at stimulus onset. The stabilimeter was calibrated before each session.

The procedure was adapted from previously described methods by Möller *et al.* (2011). Briefly, the startle session began with a 5-min acclimatization period, during which a 68-dB background noise level was maintained throughout the session; the basal startle response was then measured with 10 trials of a single 40 ms 120 dB white-noise as a startle stimulus; after this, 80 trials of randomly delivered pulses, including 20 trials of 120 dB PULSE-ALONE

trials, 50 PREPULSE trials and 10 trials with no pulse was delivered. A final 10 trials of single 40 ms 120 dB PULSE-ALONE startle stimuli was then supplied. After the testing session, the percentage PPI (% PPI) for the four pre-pulse intensities was calculated as: % PPI = [100-(startle response for PREPULSE + PULSE trial) / (startle response for PULSE ALONE trial) x 100].

3.2.5.2 Open Field test (OFT)

The FST (see below) was preceded by an OFT to examine the locomotor activity of the rodents to ensure that changes in swim motivation were based on an antidepressant response and not due to an indirect effect of the drug on locomotor activity, as per Liebenberg *et al.* (2010). Moreover, the assessment of motor activity in the OFT could be a valuable indicator for underlying neurotransmitter alterations in rodents (van den Buuse, 2009). This concept is based on the framework that subcortical dopaminergic hyperactivity in rodents will result in increased locomotor activity and is used to model dopamine-linked behaviours in animal models of schizophrenia (van den Buuse, 2009). This test included placing the rats individually in an open field arena (1 m x 1 m) and their behaviour recorded on video for 5 minutes to measure the distance moved. The total distance moved (cm) was scored using EthoVision XT® software (Noldus Information Technology, Wageningen, Netherlands).

3.2.5.3 Forced Swim test (FST)

The FST is a validated method for screening for depressive-like behaviours in rodents and to screen for antidepressant properties following drug treatment (Porsolt *et al.*, 2000). Given that the negative symptoms of schizophrenia are closely related to depressive behaviour (Pokorski & Warzecha, 2011), while schizophrenia is often co-morbid with major depressive disorder (Gozdzik-Zelazny *et al.*, 2011), this test was used to evaluate the effect of prenatal LPS exposure as well as the response to the various drug treatments with respect to such depressive-like behaviours. Moreover, GML has been noted to have antidepressant-like properties in a genetic animal model of depression that is equivalent to imipramine (Oberholzer *et al.*, 2017), so it was incumbent to assess whether such efficacy incorporated other illness models presenting with a mood component.

When rats are placed in an inescapable water cylinder they firstly perform escape-directed movements. Once all escaping attempts fail, they assume an immobile posture that has been described as resembling behavioural despair seen in depression (Lucki, 1997). On the other hand, after treatment with an antidepressant compound, the animals persist in escape-driven

behaviour (Porsolt *et al.*, 2000). The FST was performed as previously described by Liebenberg *et al.* (2010).

Briefly, two hours after the OFT (Liebenberg *et al.*, 2010) and after an hour of habituation in the testing room, the rats were placed into Perspex® cylinders (diameter 18cm, height 40cm). Each cylinder contained 30 cm of water with the temperature maintained at 25°C (Porsolt *et al.*, 2000). Behaviour was recorded over a total period of 7 minutes, with the first and last minute not considered in the analysis (see Oberholzer *et al.*, 2017). In a 15 minutes pre-swim, initial vigorous escape-oriented movements (swimming, struggling, climbing) eventually progress to an immobile posture, as noted earlier. When these rats are re-tested 24 h later in the final 7 min swim (Porsolt *et al.*, 2000), they assume this immobile posture more quickly (Cryan *et al.*, 2002). Immobility time will be scored primarily, which is considered as floating behaviour with only the necessary movements being made to keep the rat's head above the water. Escape behaviours are defined as swimming behaviour, defined as horizontal movements throughout the swim cylinder, and struggling or climbing behaviour, defined as upward-directed movements of the forepaws along the side of the swim chamber (Liebenberg *et al.*, 2010). The latter behaviours are regarded as an indication of escape-directed or coping behaviours mediated either by serotonergic (swimming) or noradrenergic (struggle/climbing) mechanisms (Cryan *et al*., 2002). These behavioural components were expressed in terms of the amount of time (s) spent performing this behaviour.

3.2.6 Neurochemical and redox-immune-inflammatory analyses

3.2.6.1 Brain tissue and plasma preparation

36 hours after the final behavioural analysis, rats were euthanized by decapitation, after which trunk blood was collected into pre-chilled, 4 ml vacutainer tubes (SGVac) containing K_2EDTA solution as anticoagulant. Additionally, after decapitation the frontal cortex and striatum were dissected out on an ice-cooled glass slab as described previously (Toua *et al.*, 2010; Möller *et al.*, 2013b). Briefly, after removing the olfactory bulb from the cortex and then cutting around the anterior tip of the corpus callosum, the frontal cortical tissue was be dissected out. With dissection of the striatum, the dorsal side of the brain was placed facing upwards, the two cerebral hemispheres were then split and the striatum dissected with the corpus callosum as external limits and the external walls of the lateral ventricles as internal limits. Liquid nitrogen was used to fix the above brain regions and stored at -80°C until the day of analysis. The tissue was pre-split into aliquots for use in the different assays to avoid freeze-thaw-freeze changes and possible deactivation of components. On the day of assay, the striatum and frontal cortex were removed from the freezer, weighed and allowed to thaw on ice. A 10 %

tissue homogenate was then prepared in a phosphate buffered saline (PBS) using a Teflon homogenizer (Möller *et al.*, 2013b).

3.2.6.2 Lipid peroxidation analysis

The Parameter™ TBARS assay from R&D Systems (Minneapolis, USA; catalogue number KGE013) was used to analyse lipid peroxidation in brain tissue (Swanepoel, 2017). This assay is based on the reactivity of the end product of lipid peroxidation, malondialdehyde (MDA), with TBA. In an environment comprising high temperature and low pH, MDA reacts with TBA in a nucleophilic addition reaction to produce a red, fluorescent 1:2 MDA:TBA product, which is then extracted with butanol and the absorbance read at 532 nm using a Bio-Tek FL600 Microplate Fluorescence Reader (Bio-Tek, Instruments, Inc., 381 Highland Park, Winooski, VT, USA).

3.2.6.3 Cytokine measurement

Plasma levels of a pro-inflammatory cytokines were determined by enzyme-linked immunosorbent assay (ELISA) (according to manufacturer's protocol). Plasma TNF-α was measured by means of the Rat TNF-α ELISA MAX™ Deluxe Set (catalogue number 438204) from Bio Legend (San Diego, USA), with IL-6 measured using the Rat IL-6 ELISA Kit (catalogue number E-EL-R0015) from Elabscience®. In both cases a Bio-Tek FL600 Microplate Fluorescence Reader (Bio-Tek, Instruments, Inc., 381 Highland Park, Winooski, VT, USA) was used to determine optical density, measured at 450 nm.

3.2.7 Statistical analyses

Unpaired Student's t-test was used to compare the MIA model with a control group. One-way factorial analysis of variance (ANOVA) and Bonferroni post-hoc tests were used for the statistical analyses of FST scores, brain lipid peroxidation levels, and cytokine analyses. For analysis of %PPI data, two-way ANOVA with repeated measures was used with Bonferroni post-hoc tests. Normal distribution of the variables was assessed with a Q-Q plot and histogram for all variables in each treatment group. All data were normally distributed and expressed as the mean ± standard error of the mean (SEM), with a p-value of <0.05 considered statistically significant. Lastly, in cases where no statistical significance was evident following the ANOVA, statistical analysis was subsequently followed by Cohen's d calculations to establish the effect-size and practical significance. Cohen's d indicates the standardized difference between two means, describing medium ($0.5 \geq d < 0.8$), large ($0.8 \geq d < 1.3$) and very large ($d \geq 1.3$) effect sizes. Only very large and large effect sizes will be

indicated in the graphs and text. All data were analysed and graphics prepared using GraphPad Prism 7, San Diego California, USA.

3.3 Results

3.3.1 GML fingerprinting

The GML chromatogram performed in a previous study from our group (Oberholzer *et al*., 2017) is reproduced in Fig. 2. This produced two prominent peaks that were identified as α-mangostin at ~25 min and γ-mangostin at ~20 min. The GM extract used in this study was found to contain 117 mg/g α-mangostin and 11 mg/g γ-mangostin (i.e. 11.7% and 1.1%). α- and γ-Mangostin are therefore the major GML components observed in the fingerprint.

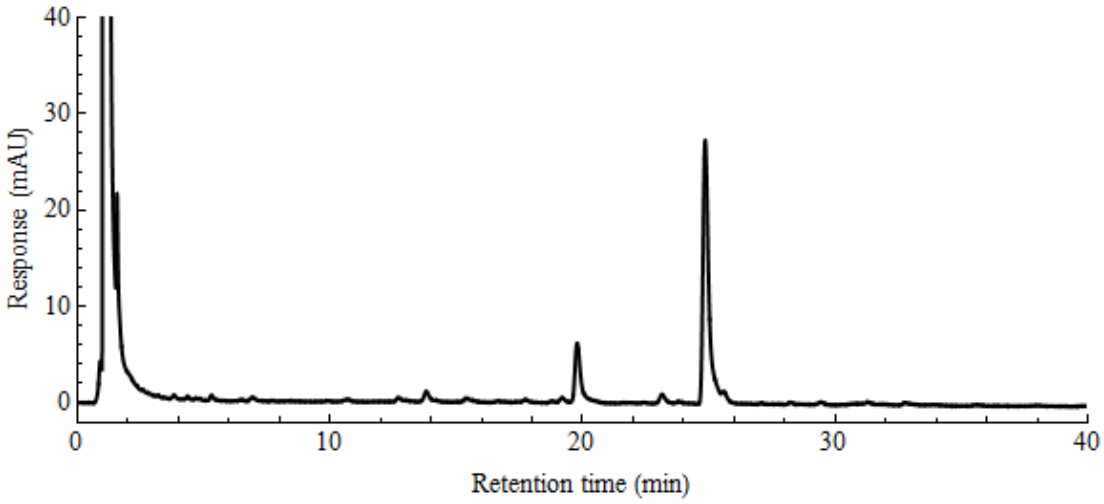

Figure 2: Chromatographic fingerprint of the raw GML extract used in this study. The peak identities are γ-mangostin at ~20 min and α-mangostin at ~25 min, with the solvent peak on the far left. Figure courtesy of (Oberholzer *et al*., 2017).

3.3.2 Prepulse inhibition of acoustic startle

When considering the LPS model alone, with regards to basal startle analysis in the LPS exposed rats compared to the vehicle control group (data not shown), two-way ANOVA with repeated measures for each startle block displayed no significant prenatal exposure x startle block interaction ($F(3, 72) = 0.3115$, $p = 0.8170$) along with no significant main effect of prenatal exposure on startle amplitude ($F(1, 72) = 0.1587$, $p = 0.6916$), but a significant main effect of startle blocks on startle amplitude was observed ($F(3, 72) = 17.54$. $p < 0.0001$). Bonferroni post hoc testing indicated a significant decrease ($p < 0.05$) in startle amplitudes

from block 1 to block 4 in the control (saline + vehicle), and the LPS-exposed group, indicating habituation during the PPI test procedure in these groups (data not shown).

When considering drug treatment in the LPS model, two-way ANOVA with repeated measures for each startle block in all the LPS exposed groups receiving the respective treatment or vehicle indicated a significant treatment x startle block interaction ($F (15, 165) = 2.21$, $p = 0.007$) as well as a significant main effect of startle block ($F (3, 33) = 36.15$, $p < 0.001$), but no significant main effect of treatment ($F (5, 55) = 1.18$, $p = 0.32$) on startle amplitude (data not shown). Bonferroni post hoc testing on the startle amplitude in all the LPS exposed groups receiving the respective treatments or vehicle indicated that all the groups had a significant decrease ($p < 0.05$) in startle amplitude from block 1 to 4 (data not shown). Bonferroni post hoc testing also revealed no significant differences between any of the exposed and treatment groups at the respective startle blocks.

Regarding %PPI, when considering the LPS model alone (Fig. 3) comparing the LPS exposed group to the vehicle group, one-way ANOVA with repeated measures for the different prepulse intensities showed no significant prepulse intensity x exposure interaction ($F (3, 33) = 0.06537$ $p = 0.9778$). However, a significant main effect of prenatal exposure was observed ($F (1, 11) = 33.76$, $p = 0.0001$) on %PPI. The LPS exposed control group (LPS + vehicle) presented with significant deficits in %PPI at 72 dB ($p = 0.0031$), 76 dB ($p = 0.0120$), 80 dB ($p = 0.0112$) and 84 dB ($p = 0.0134$) when compared to the control group (saline+ vehicle) (Fig. 3).

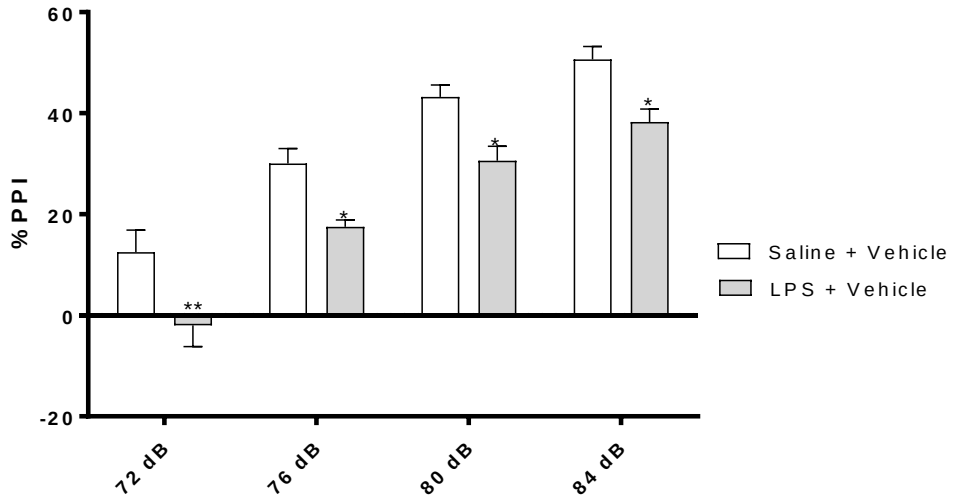

Figure 3: Sensorimotor gating with regards to %PPI at the indicated prepulse intensities in the LPS exposed group compared to the control group (One-way ANOVA with repeated measures, Bonferroni post hoc test). *$p < 0.05$, **$p < 0.01$ vs Saline + Vehicle.

When considering drug treatment in the LPS model (Fig 4), one-way ANOVA revealed a significant main effect of treatment at 72-dB ($F(5, 66) = 5.163$, $p = 0.0005$), 76-dB ($F(5, 66) = 4.831$, $p=0.0008$), 80-dB ($F(5, 66) = 4.616$, $p = 0.0011$) and 84-dB ($F(5, 66) = 4.04$, $p = 0.0029$) on %PPI in all the LPS exposed groups receiving the respective treatments or vehicle. Bonferroni post hoc testing demonstrated that HAL significantly reversed the %PPI deficits in the LPS-exposed rats at 72-dB ($p = 0.0082$) (Fig 4a) and 76-dB ($p = 0.0343$) (Fig 4b). However, a very large effect-size was observed in the LPS exposed HAL-treated rats compared to their LPS vehicle-treated controls at 80-dB ($d = 0.17$) and 84-dB ($d = 0.16$) (Fig 4c and d respectively). The combination treatment of GML+HAL successfully reversed %PPI deficits at all four of the prepulse intensities: 72-dB ($p = 0.0144$), 76-dB ($p = 0.0022$), 80-dB ($p = 0.0043$) and 84-dB ($p = 0.0150$) vs the LPS exposed control group (Fig 4a-d respectively). However, no significant differences were observed in the LPS exposed rats treated with GML alone, AM alone or the combination of HAL+AM vs the LPS + vehicle exposed group (Fig 4a-d).

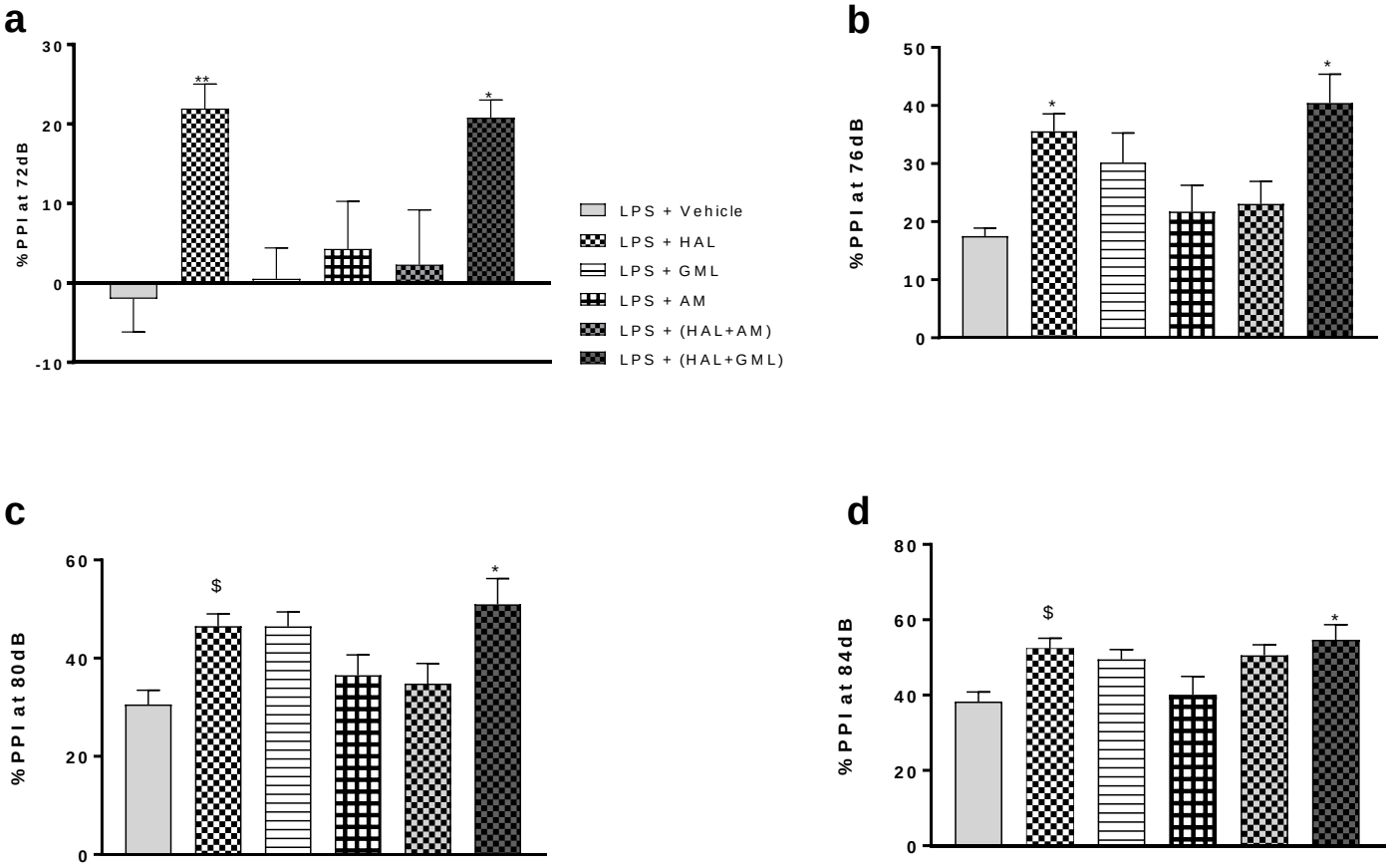

Figure 4: Sensorimotor gating with regards to %PPI at (a) 72dB, (b) 76dB, (c) 80dB and (d) 84dB in the LPS exposed groups receiving saline and the various drug treatments as indicated (One-way ANOVA with repeated measures for the different dB intensities, Bonferroni post hoc test). *$p < 0.05$, **$p < 0.01$ vs LPS + Vehicle. $ $d \geq 1.3$ vs. LPS + Vehicle (Cohen's d value).

3.3.3 Open field test

When considering the LPS model alone, unpaired student's t-test revealed a significant increase in the locomotor activity of the LPS exposed group (LPS + vehicle) compared to the saline control group (saline + vehicle) ($p = 0.0385$) (Fig 5a).

When considering drug treatment in the LPS model, a one-way ANOVA of the OFT data in the LPS exposed groups receiving the respective treatments or vehicle revealed a significant main effect of treatment on total distance moved ($F (5, 68) = 4.632$, $p = 0.0011$). Bonferroni post-hoc tests revealed no significant differences in the total distance moved in LPS exposed treatment groups receiving HAL, GML, HAL+GML and HAL+AM when compared to the LPS exposed control group (LPS + vehicle) (Fig. 5b). However, a significant decrease in locomotor activity was observed in the LPS exposed group treated with AM ($p = 0.0036$) compared to the control group (LPS + vehicle) (Fig 5b)

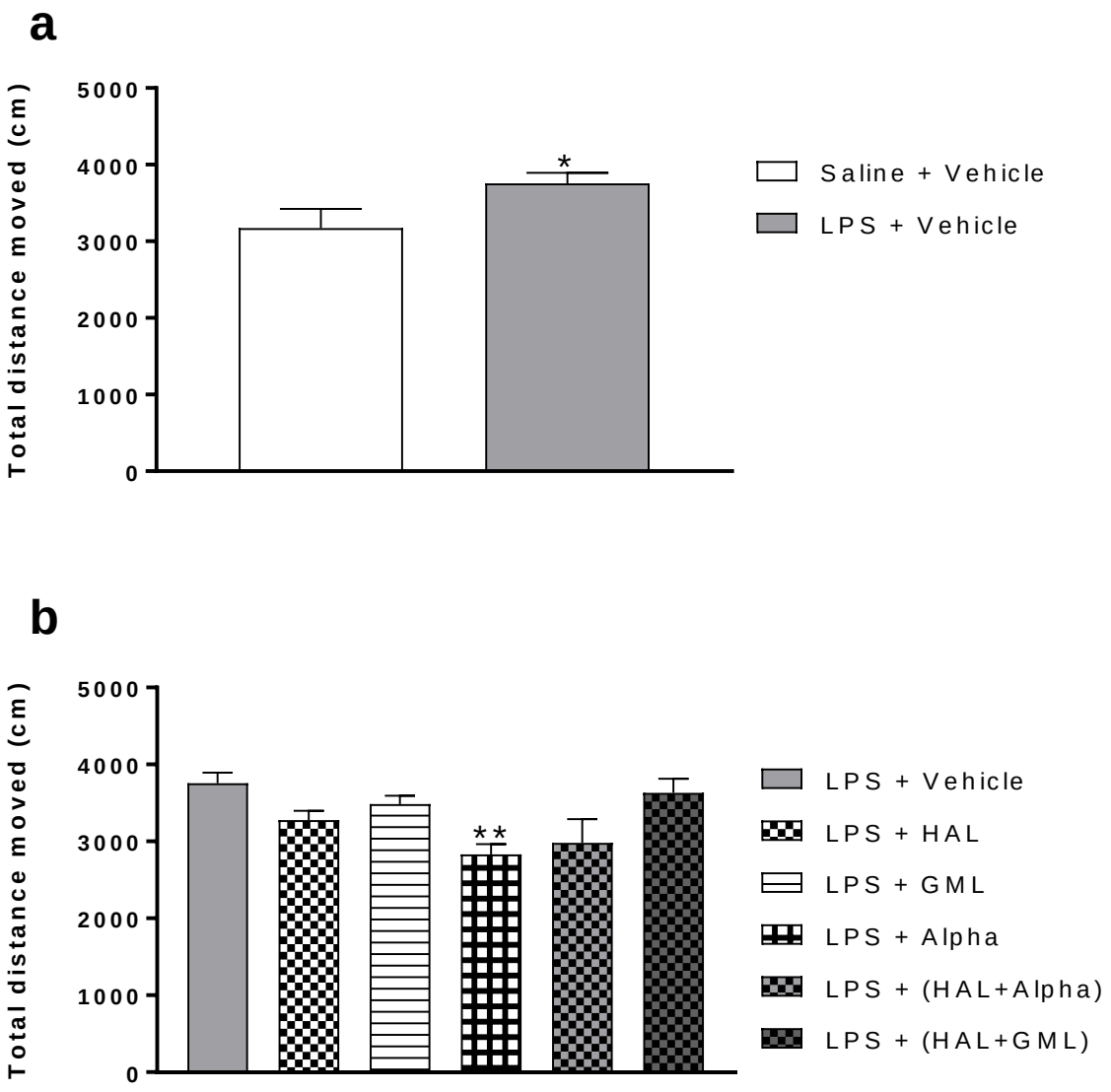

Figure 5: Locomotor activity (total distance moved in cm), analysed in the OFT in rats (a) prenatally exposed to LPS or saline respectively and treated with vehicle (Unpaired Student's t-test). *$p < 0.05$, vs Saline + Vehicle and (b) prenatally exposed to LPS and treated with the various drugs as indicated (One-way ANOVA, Bonferroni post hoc test). **$p < 0.01$ vs Saline + Vehicle.

3.3.4 Forced swim test

When considering the LPS model alone (Fig. 6), unpaired student's t-test revealed a significant increase in immobility in the LPS exposed rats (LPS + vehicle) when compared to the control group (saline + vehicle) ($p < 0.0001$) (Fig. 5a). A significant decrease in both the swimming ($p = 0.0002$) and struggling ($p < 0.0001$) behaviours were also observed in the LPS exposed

group (LPS + vehicle) compared to the control group (saline + vehicle) (Fig. 6b and c respectively).

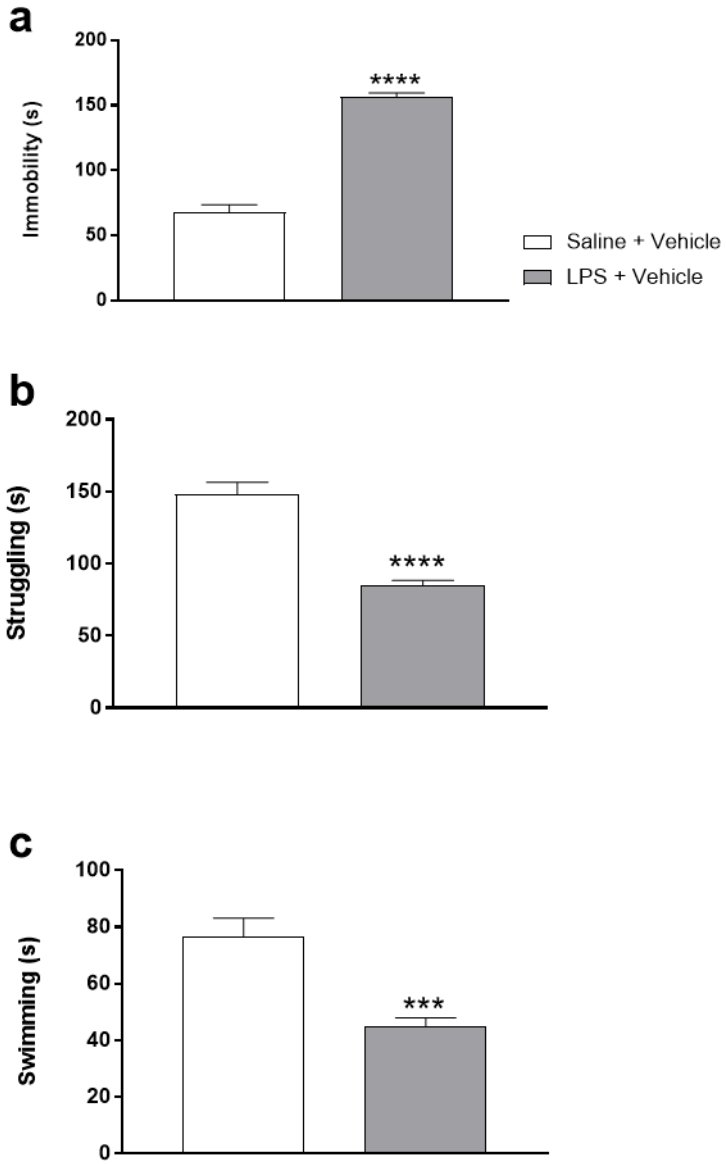

Figure 6: The Forced swim test (FST) with regards (a) Immobility, (b) Struggling and (c) Swimming behaviour in the LPS exposed group and the control group (Unpaired Student's t-test). ***p < 0.001, ****p < 0.0001 vs Saline + Vehicle.

When considering drug treatment in the LPS model (Fig. 7), a one-way ANOVA of the LPS treatment groups revealed a significant main effect of treatment on: immobility ($F(5, 64) = 14.88$, $p < 0.0001$); climbing ($F(5, 64) = 16.75$, $p < 0.0001$) and swimming ($F(5, 64) = 15.05$ $p < 0.0001$). Bonferroni post-hoc testing indicated a significant decrease in immobility in all the LPS exposed treatment groups receiving GML ($p < 0.0001$), AM ($p = 0.0052$), HAL+GML ($p <$

0.0001) and HAL+AM ($p < 0.0001$) compared to the LPS exposed control group (LPS + vehicle) (Fig. 7a). Despite a trend towards lowering immobility, the effect of HAL alone did not reach significance (Fig. 7a). However, treatment with GML alone ($p = 0.0344$); HAL+GML ($p = 0.0003$) and HAL+ AM ($p = 0.0312$) showed a significantly greater decrease in immobility when compared to the HAL treated LPS exposed group (Fig. 7a).

With regards to struggling behaviour in the LPS exposed rats, HAL ($p = 0.0045$), HAL+GML ($p < 0.0001$) and HAL+AM ($p < 0.0001$) displayed a significant increase in struggling compared to the LPS exposed control group (LPS + vehicle) (Fig. 7b). The combination treatment of HAL+GML in the LPS rats displayed significantly greater increased struggling behaviour ($p = 0.0449$) when compared to the HAL treatment alone in the LPS rats (Fig. 7b).

Swimming behaviour was significantly increased in the LPS groups receiving GML ($p < 0,0001$) and AM ($p < 0,0001$) treatment compared to the LPS exposed control group (Fig. 7c). A significant increase in swimming behaviour was observed between the LPS exposed group receiving HAL alone vs. both the LPS exposed groups receiving HAL+GML ($p = 0.0040$) or HAL+ AM ($p = 0.0031$) respectively (Fig. 7c).

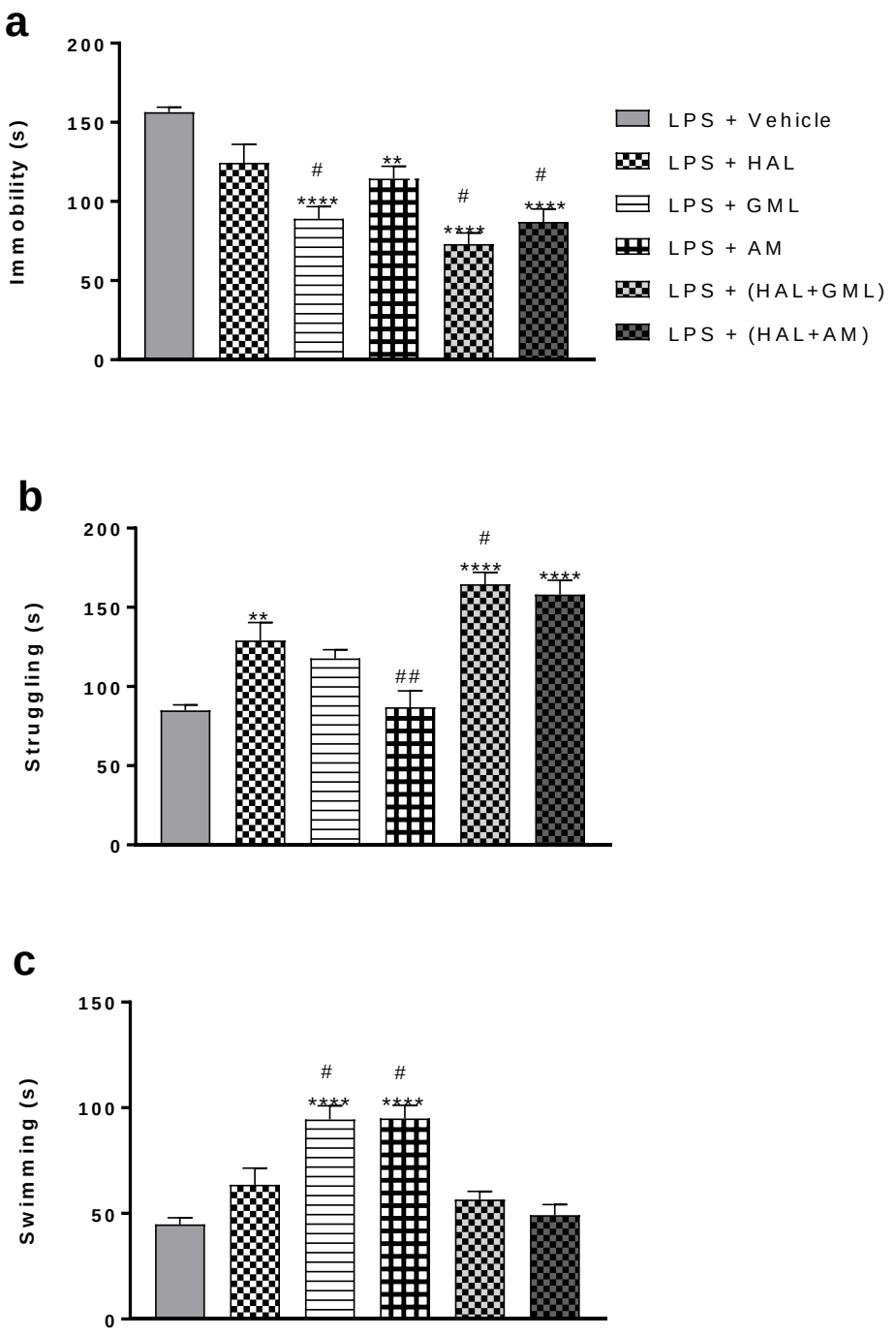

Figure 7: The Forced swim test (FST) with regards (a) Immobility, (b) Struggling and (c) Swimming behaviour in rats exposed to LPS prenatally and receiving saline and the various drug treatments as indicated (One-way ANOVA, Bonferroni post hoc test). **p < 0.01, ****p < 0.0001 vs LPS + Vehicle; #p < 0.05, ##p < 0.01 vs. LPS + HAL.

3.3.5 Regional brain lipid peroxidation

When considering the LPS model alone (Fig. 8), frontal cortical MDA levels were significantly increased in the LPS exposed rats (LPS+ vehicle) ($p = 0.0304$), compared to the saline control group (Saline + vehicle) (Fig. 8a). In the striatum, significantly elevated levels of MDA were also observed in the LPS exposed rats (LPS+ vehicle) ($p < 0.0001$) in comparison with the saline control group (Saline + vehicle) (Fig. 8b).

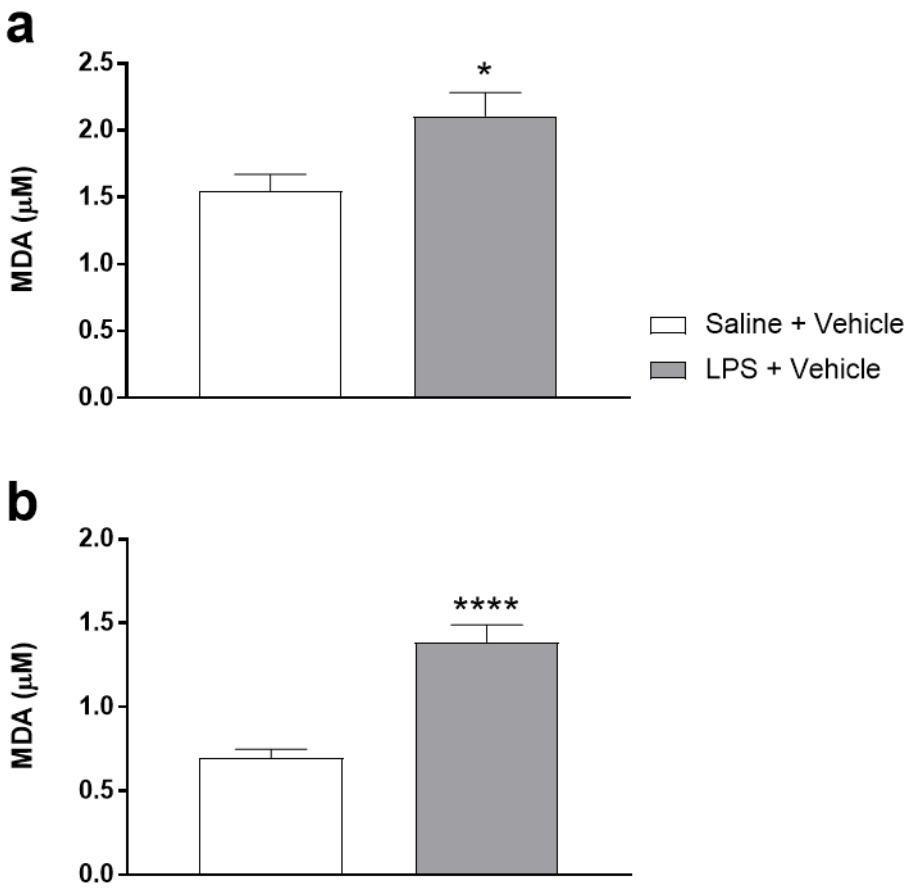

Figure 8: Lipid peroxidation as malondialdehyde (MDA) in (a) frontal cortex and (b) striatum in rats prenatally exposed to LPS or saline respectively and treated with vehicle (Unpaired Student's t-test). *$p < 0.05$, ****$p < 0.0001$ vs Saline + Vehicle.

When considering drug treatment in the LPS model (Fig. 9), one-way ANOVA showed a significant main effect of treatment on lipid peroxidation in the frontal cortex ($F (5, 65) = 5.873$, $p = 0.0002$), but no significant effect in the striatum ($F (5, 64) = 2.173$, $p = 0.0680$). Bonferroni post-hoc analysis revealed that treatment with HAL ($p = 0.0008$), and AM ($p = 0.02$) significantly reduced frontal cortical MDA levels in LPS exposed rats, compared to the LPS exposed control group (LPS+ vehicle), but was unaffected by any of the other LPS exposed

treatment groups (LPS+GML, LPS+HAL+GML and LPS+HAL+AM) vs the LPS exposed control group (Fig. 9a). GML and GML+HAL showed a trend towards reducing MDA levels, with a large effect-size observed in the LPS exposed rats treated with GML (d = 0.1) compared to their vehicle-treated controls (Fig 9a). Finally, none of the respective treatments showed a significant reduction in striatal MDA levels in the LPS exposed rats compared to the LPS exposed control group (Fig. 9b).

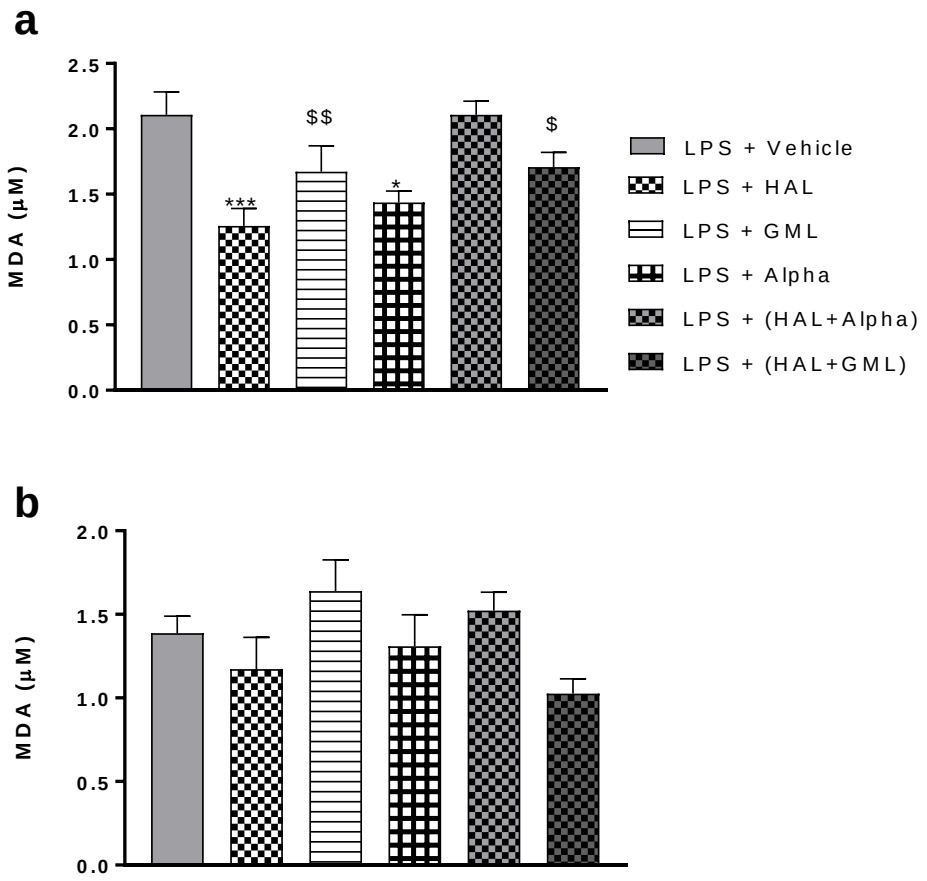

Figure 9: Lipid peroxidation as malondialdehyde (MDA) in (a) frontal cortex and (b) striatum in rats exposed to LPS prenatally and treated as indicated (One-way ANOVA, Bonferroni post hoc test). *p < 0.05, ***p < 0.001 vs Saline + Vehicle. $ d = 0.5 ≥ d < 0.8, $$ d = 0.8 ≥ d < 1.3 vs. LPS + Vehicle (Cohen's d value).

3.3.6 Cytokines

IL-6: When considering the LPS model alone (Fig. 10a), unpaired Student's t-test revealed that plasma IL-6 levels were significantly elevated in the LPS exposed group (LPS+ vehicle) when compared to the saline control group (saline + vehicle) (p = 0.0005) (Fig 10a). When considering drug treatment in the LPS model (Fig. 10b), one-way ANOVA revealed a

significant main effect of treatment on IL-6 ($F(5, 63) = 10.86$, $p < 0.0001$) in the LPS exposed and treated groups. Bonferroni post-hoc analyses showed that treatment with HAL ($p = 0.0001$); GML ($p < 0.0001$); AM ($p < 0.0001$) and HAL+GML ($p = 0.0015$) significantly reversed elevated levels of IL-6 in the LPS exposed groups. However HAL+ AM had no significant effect on IL-6 plasma levels in the LPS exposed animals compared to the vehicle control (Fig 10b).

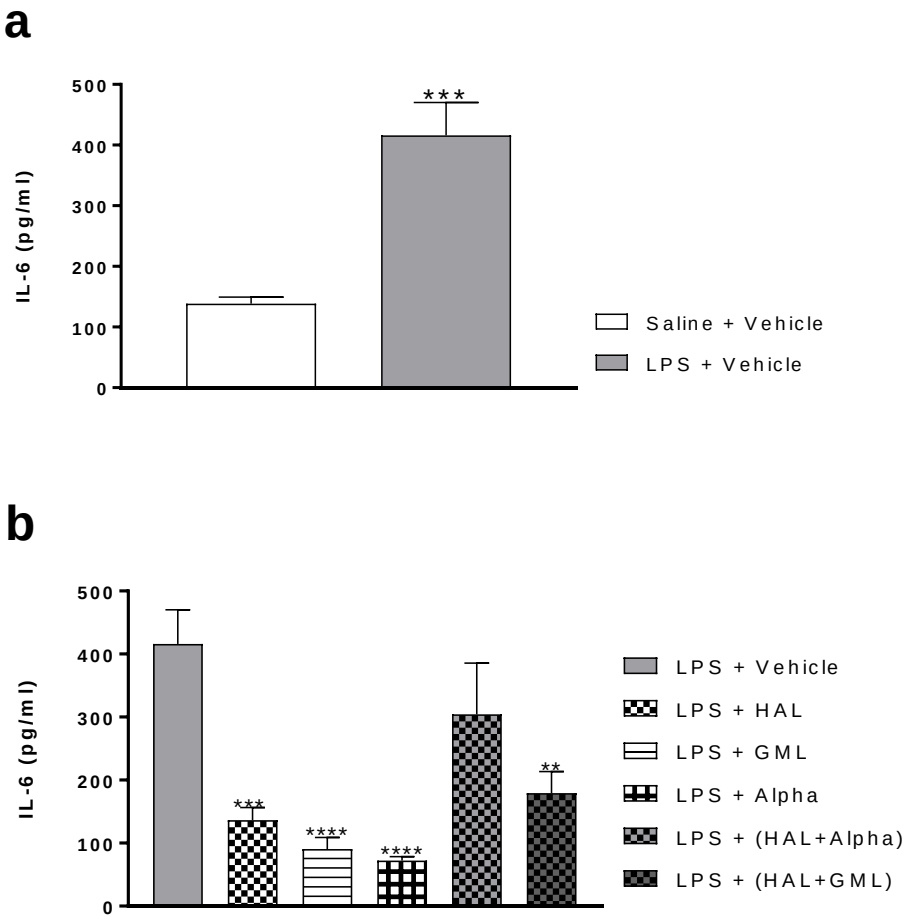

Figure 10: Plasma IL-6 levels in rats (a) prenatally exposed to LPS or saline respectively and treated with vehicle (Unpaired Student's t-test) ***$p < 0.001$ vs Saline + Vehicle and (b) prenatally exposed to LPS and treated as indicated (One-way ANOVA, Bonferroni post hoc test). **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ vs Saline + Vehicle

TNF-α: When considering the LPS model alone (Fig. 11a), unpaired Student's t-test displayed significantly elevated plasma TNF-α levels in the LPS exposed group (LPS+ vehicle) when compared to their saline control group (saline + vehicle) ($p = 0.0411$) (Fig 11a). When considering drug treatment in the LPS model (Fig 11b), one-way ANOVA revealed a significant main effect of treatment on TNF-α levels ($F(5, 64) = 6.555$, $p<0.0001$) in the LPS exposed groups receiving the respective treatments (Fig 11b). Bonferroni post-hoc testing displayed

that GML treatment successfully reversed the elevated TNF-α levels in the LPS exposed rats when compared to the LPS exposed control group (LPS+ vehicle) ($p < 0.0001$) (Fig 11b). In addition, GML was significantly more effective in decreasing plasma TNF-α levels in LPS exposed animals than the HAL treated LPS exposed group (LPS+HAL) ($p = 0.0078$) (Fig 11b). Treatment with HAL+GML also significantly reduced TNF-α plasma levels in LPS exposed animals in comparison to the LPS exposed control group ($p = 0.0037$) (Fig 11b). The remaining three treatment groups viz. HAL; AM and HAL+ AM showed no significant reduction in plasma levels of TNF-α when compared to the LPS exposed control group (LPS+ vehicle) (Fig 11b).

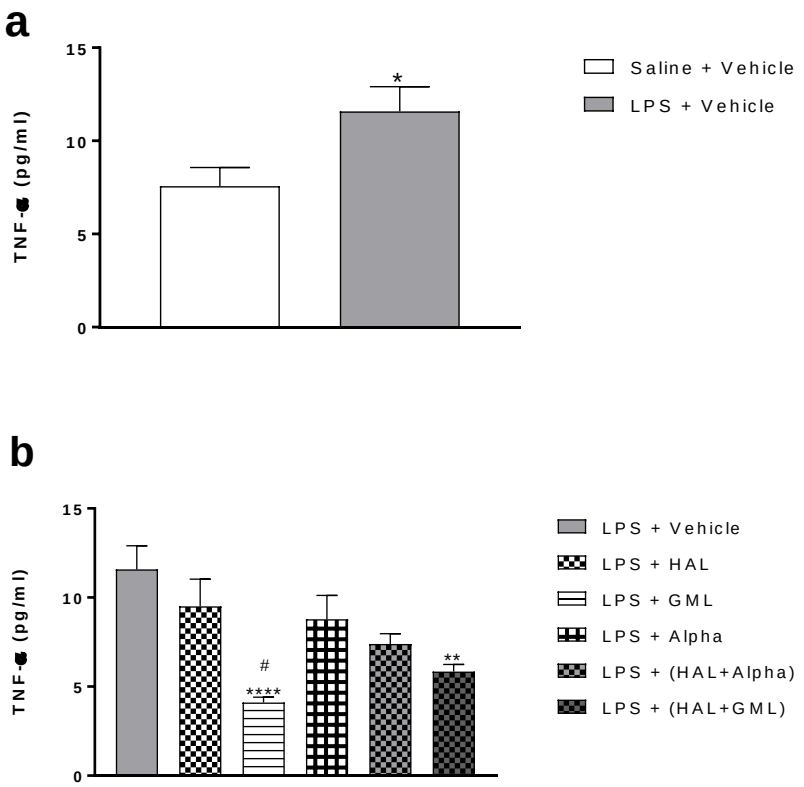

Figure 11: Plasma TNF-α levels in rats (a) prenatally exposed to LPS or saline respectively and treated with vehicle (Unpaired Student's t-test). *$p < 0.05$ vs Saline + Vehicle and (b) prenatally exposed to LPS and treated as indicated (One-way ANOVA, Bonferroni post hoc test). **$p < 0.01$, ****$p < 0.0001$ vs Saline + Vehicle; #$p < 0.05$ vs LPS + HAL.

3.4 Discussion

The key findings of this study is that prenatal exposure to an infectious agent (LPS) can induce schizophrenia-like bio-behavioural changes later in life in the offspring, specifically reduced sensorimotor gating and depressive-like behaviour together with evidence for peripheral and

brain oxidative stress (elevated lipid peroxidation) and inflammation (elevated plasma pro-inflammatory cytokines). HAL significantly reversed MIA-induced deficits in %PPI, although failed to fully reverse depressive manifestations. However, HAL was able to reverse cortical (but not striatal) lipid peroxidation as well as elevated plasma IL-6 in LPS offspring. On the other hand, GML and AM alone reversed depressive-like behaviours in these animals but not deficits in %PPI. However, GML+HAL effectively reversed %PPI and depressive-like symptoms, with GML+HAL and AM+HAL was significantly more effective than HAL alone in as far as antidepressant-like effects are concerned. Considering concomitant effects on redox-inflammatory biomarkers, AM alone successfully reversed cortical (but not striatal) lipid peroxidation and elevated plasma IL-6 levels in LPS offspring, whereas GML was successful in reversing both IL-6 and TNF-α elevations in the plasma of LPS offspring. Correspondingly, the combination of HAL+GML was effective in reversing elevated levels of pro-inflammatory cytokines (IL-6 and TNF-α).

3.4.1 PPI

Deficits in sensorimotor gating, as assessed by measuring %PPI to startle, reflects important clinical features observed in schizophrenia, such as cognitive impairment and deficient information processing that underlies the fragmentation of reality central to the illness (Braff *et al.*, 2001). In line with previous findings (Borrell *et al.*, 2002; Ozawa *et al.*, 2006; Fortier *et al.*, 2007; Basta-Kaim *et al.*, 2011b; Swanepoel, 2017), this study shows (Fig. 3) that prenatal exposure to LPS significantly compromised %PPI in the offspring in late adolescence, congruent with the characteristic pathology of schizophrenia. Importantly, treatment with the typical antipsychotic, HAL (2 mg/kg po x 16 days), was effective in reversing said LPS-induced %PPI deficits at two of the prepulse intensities tested, with a large effect size observed at 80dB and 84dB (Fig. 4). These findings are consistent with a previous report that chronic oral HAL treatment restores auditory PPI deficits following prenatal LPS exposure (Romero *et al.*, 2007), with acute treatment also displaying improved PPI in several other schizophrenia models (Binder *et al.*, 2001; Kusljic *et al.*, 2006; Hadamitzky *et al.*, 2007). However, HAL alone failed to improve deficits in %PPI after sub-chronic dosing in a social isolation reared (SIR) model of schizophrenia (Uys *et al.*, 2016). These opposing reports could be related to the HAL dose and specific model of schizophrenia under study. Nevertheless, that overactive dopaminergic processes have been suggested to underlie the reduction of %PPI (Ralph *et al.*, 2001) may explain the observed ability of HAL as a dopamine D_2 receptor antagonist to reverse sensorimotor gating deficits in the LPS model of schizophrenia (Romero *et al.*, 2010). Treatment with GML and AM, at the doses indicated, failed to reverse %PPI deficits following prenatal LPS exposure. However, of note is that GML in combination with HAL was effective

in reversing LPS-induced reductions in %PPI, although no augmentation was evident when combined with HAL. None of the other treatments were effective in reversing sensorimotor gating deficits, suggesting that neither GML nor its isolated constituent, AM, present with distinct effects on schizophrenia–like deficits, as demonstrated by HAL.

3.4.2 OFT

Locomotor hyperactivity have been proposed to represent some of the positive symptoms of schizophrenia, in particular psychotic agitation (Powell *et al.*, 2009). The locomotor activity of animals prenatally exposed to LPS was significantly increased when compared to the saline exposure control group (Fig. 5). This behaviour may be due to the hyperdopaminergic transmission commonly seen in schizophrenia (Minassian *et al.*, 2010), however this is only speculation since DA was not analysed in this study. Moreover, AM treatment was the only treatment group to reduce locomotion in the LPS exposed offspring. Since AM did not alter %PPI, which is a more robust expression of schizophrenia-like behaviour, and considering reports that AM potentially possess sedative properties in the CNS of rodents (Shankaranarayan *et al.*, 1979), suggests that the putative “antipsychotic-like” effects of AM presented in this assay are most likely a false positive. Indeed, AM may act as a selective and competitive histamine antagonist (Chairungsrilerd *et al.*, 1996b), thus presenting with possible sedative effects. With the other treatment groups showing no effects on locomotor activity in LPS-exposed animals, findings described in the FST can be regarded as being uncomplicated by undesirable motor effects.

3.4.3 FST

Since the negative symptoms of schizophrenia closely resemble the basic symptoms of depression (Kring *et al.*, 2013), an animal behavioural test for depression, the FST, was used to assess depressive-like manifestations in the MIA model. In fact, the FST is also a test for behavioural despair and is anticipated to signify an absence of motivational behaviour commonly seen in schizophrenic patients (Chatterjee *et al.*, 2012). Moreover, GML has been noted to have antidepressant-like properties in a genetic animal model of depression that is equivalent to imipramine (Oberholzer *et al*., 2017), so it was incumbent to assess whether such efficacy incorporated other illness models presenting with a mood component. In this study, LPS exposed offspring presented with significant depressive-like behaviours in the FST later in life (Fig. 6a), while also demonstrating significantly reduced active coping behaviours, viz. swimming and climbing (Fig. 6b,c). These findings are in agreement with studies by Enayati *et al.* (2012) who showed that maternal LPS exposure in late gestation resulted in increased depressive behaviour in the FST in the male offspring, while O'connor *et al.* (2009)

also reported that peripheral LPS administration generates a distinct depressive-like behavioural syndrome.

Although the FST has been developed to be specific for antidepressant action, and hence valid for screening for antidepressant drugs (Porsolt *et al.*, 2000), non-antidepressants such as antipsychotics may also present with antidepressant activity (Fava *et al.*, 2012; Han *et al.*, 2013; Roberts *et al.*, 2016). This is not deemed contrary to the ethos of the model, since the primary illness that antipsychotics are used for, viz. schizophrenia, also presents with a depressed mood component. Indeed, negative affect, comprising affective flattening, alogia, anhedonia, asociality and avolition (lack of motivation) is included in the DSM-5 classification of the negative symptom cluster of the illness (Tandon *et al.*, 2013).

In contrast to previous clinical (Heinz *et al.*, 1998) and preclinical studies(Weiner *et al.*, 2003) that found HAL to induce depressive behaviour (Heinz *et al.*, 1998; Weiner *et al.*, 2003), the study found HAL treatment to decrease MIA-associated immobility in the FST, albeit non-significantly (Fig. 7a), as well as significantly increasing climbing behaviour (Fig. 7b), suggesting antidepressant-like properties that are underscored by a noradrenergic basis (Cryan *et al.*, 2002). However, the latter could not be verified as regional brain monoamine analysis was not performed in this study. In agreement with a recent study performed in Flinders Sensitive Line (FSL) rats (Oberholzer *et al.*, 2017), GML was successful in reversing depressive behaviour in prenatally LPS challenged off-spring, significantly decreasing immobility and increasing swimming behaviours in this model (Fig 7a, b). Interestingly, it was also noted previously that GML bolsters struggling behaviour in the FST in FSL depressed rats, which was correlated to regional brain NA changes (Oberholzer *et al*., 2017). Of particular note, GML had superior antidepressant-like effects when compared to HAL (Fig. 7a, c). AM also exhibited significant antidepressant-like properties regarding immobility and swimming behaviour in the LPS model, although not as marked as GML alone (Fig 7a, c). Moreover, GML and AM displayed significant augmenting effects when given in combination with HAL, presenting with markedly improved reversal of immobility and struggling in comparison to HAL alone (Fig 7a, b).

Overall, these data clearly support the antidepressant-like effects of GML and AM, and supportive of earlier findings describing the antidepressant-like effects of GML using another animal model of depression (Oberholzer *et al*., 2017). Cognitive impairments and negative symptoms are both major contributors to functional disability in schizophrenia (Green, 1996), often requiring adjunctive treatment to improve the outcome. Not tested in the earlier work, here the study found GML to have marked abilities as an adjunctive treatment to bolster the response to a known psychotropic, in this case HAL, at least as an antidepressant. This work

shows that GML and AM have similar potential as a stand-alone and/or adjunctive treatment with HAL when targeting a possible mood component of schizophrenia. However, GML shows greater potential as an adjunctive treatment with respect to deficits in sensorimotor gating, suggesting the therapeutic benefits of raw GML may be a result of the combined activity of components in the pericarp rather than the properties of an isolated ingredient such as AM.

In view of the evident role for oxidative imbalance in schizophrenia (Bitanihirwe & Woo, 2011) together with the poor treatment outcome and costly nature of currently available antipsychotics (Novick *et al.*, 2010; Keefe & Harvey, 2012; Haddad *et al.*, 2014), clinical utility of antioxidants such as GML as a supplementary treatment may improve the management of the disorder (Magalhães *et al.*, 2016). For example, treatment with the antioxidant, NAC, has been found to improve cognitive function (Phensy *et al.*, 2017) and to bolster the effects of clozapine in preclinical schizophrenia studies (Möller *et al.*, 2013a). Considering this and our findings, treatment with GML in combination with a relatively inexpensive antipsychotic such as HAL may offer tangible clinical benefits for schizophrenia, in particular the depressive symptoms, concurring with the findings of Oberholzer *et al.* (2017). The behavioural characteristics induced in the MIA offspring following maternal exposure to LPS are congruent with numerous other studies (Borrell *et al.*, 2002; Fortier *et al.*, 2007; Romero *et al.*, 2007; Coyle *et al.*, 2009), including altered sensorimotor gating, enhanced locomotor activity and depressive-like behaviour. These data therefore confirm the face validity of the prenatal LPS (MIA) model of schizophrenia, and allows further exploration of the drug studies at a bio-molecular level.

3.4.4 Oxidative markers

Accumulating evidence have implicated oxidative damage in the pathophysiology and deteriorating course of schizophrenia (Bitanihirwe & Woo, 2011; Davis *et al.*, 2014) Moreover, schizophrenia patients present with increased plasma lipid peroxidation (Akyol *et al.*, 2002; Khan *et al.*, 2002; Arvindakshan *et al.*, 2003), while elevated lipid peroxidation products might be associated with certain clinical features of schizophrenia (Gama *et al.*, 2008). In agreement with previous findings (Zhu *et al.*, 2007; Swanepoel, 2017), exposure to prenatal LPS resulted in significantly increased cortico-striatal lipid peroxidation (elevated MDA) (Fig. 8a,b). An earlier study demonstrated similar behavioural abnormalities encompassing cognitive and motivational deficits together with dysfunction of the prefrontal cortex in a mouse model of oxidative stress (Johnson *et al.*, 2013). Importantly, here the study shows that HAL treatment significantly reduced MDA levels in the frontal cortex, but showed no significant effect in the striatum of LPS exposed offspring.

Similar to our results, previous reports found that HAL reduced MDA levels in the cortex of a healthy rat brain (Reinke *et al.*, 2004). Interestingly, dopamine contributes to oxidative stress by lowering levels of the antioxidant, glutathione (GSH), while this reduction in GSH can be blocked by D_1/D_2 receptor antagonists (Grima *et al.*, 2003; Bitanihirwe & Woo, 2011). Considering the brain's vulnerability to oxidative damage (Rougemont *et al.*, 2002; McQuillen & Ferriero, 2004), the dopamine blocking effects of HAL may explain the reduction in lipid peroxidation observed. On the other hand, some previous clinical reports have found *elevated* levels of MDA in the serum of HAL treated patients (Padurariu *et al.*, 2010), while this has also been described in animal studies following chronic HAL-treatment (Parikh *et al.*, 2003; Pillai *et al.*, 2007; Harvey *et al.*, 2008). However, such an increase in striatal oxidative stress has been ascribed to a neurotoxic effect of HAL (Harvey *et al.*, 2008) which may not be relevant under our conditions of study. Alternatively, these conflicting results may be attributable to different species used for the studies (rats or humans), difference in tissues (brain regions and plasma), the duration of the disorder or distinctive therapeutic features such as dose and duration of treatment (Padurariu *et al.*, 2010). What is noteworthy is that despite any significant effects on striatal MDA (Fig. 9b), HAL *still* enabled a reversal of both LPS-induced depressive and sensorimotor gating deficits (Fig. 4, 6), suggesting that the benefits of HAL presented here are likely to be related to improving integrity of the frontal cortex (Fig. 9a). In fact, the frontal cortex is involved in a variety of cognitive processes including working memory, behavioural flexibility, attention (Goto *et al.*, 2010) and has been implicated in the development of depression (Davidson *et al.*, 2002; Drevets *et al.*, 2008).

Although *in vitro* studies have revealed that extracts of GML possess potential antioxidant activity (Puripattanavong *et al.*, 2006; Weecharangsan *et al.*, 2006; Chomnawang *et al.*, 2007; Zhao *et al.*, 2010), GML had no effect on MDA levels in the frontal cortex or striatum, albeit a small effect size improvement in the frontal cortex (Fig. 9a). In a recent animal study, Oberholzer and colleagues found chronic GML treatment to reverse elevated hippocampal lipid peroxidation in FSL rats (Oberholzer *et al.*, 2017). However, the reasons for this may be due to the difference in genetic and neurodevelopmental nature of the models used as well as the different brain regions analysed. Interestingly, AM significantly reduced LPS-induced frontal cortical lipid peroxidation, which perhaps implies that the inherent antioxidant properties of AM are more prominent than that of raw GML, at least when assessed *in vivo*. Previous studies have found that AM protects against redox alterations by modulating GSH levels (Márquez-Valadez *et al.*, 2012), as well as to act as a free radical scavenger (Williams *et al.*, 1995) and to inhibit low density lipoprotein (LDL) oxidation (Mahabusarakam *et al.*, 2000). Given that AM is the dominant bioactive xanthone present in the pericarp of GML (Fig. 2;

(Oberholzer *et al.*, 2017), these findings may propose that the abovementioned antioxidant activity observed following GML may more be attributable to the presence of AM.

In the case of AM, its beneficial effects in the FST may be linked to the above-described antioxidant effects. Indeed, other antioxidants such as NAC demonstrate antidepressant-like effects in the FST (Ferreira *et al.*, 2008). However, considering the beneficial effects of GML in the FST and PPI test, especially as stand-alone and/or adjunctive treatment with HAL, it is clear that its beneficial effects, unlike for AM, may not be completely centred around its possible anti-oxidant capabilities, but the synergistic effects of the various other xanthones present in GML. This idea and the neuropharmacological properties of these individual components remain to be investigated. These findings may also be linked to a recent report that an aqueous extract of GML displayed protective properties against acetylcholinesterase (AChE) dysfunction in mice (Phyu & Tangpong, 2014). AChE hydrolyses the neurotransmitter acetylcholine (ACh) (Phyu & Tangpong, 2014) and considering the fact that increased ACh activity may play a role in depressive symptoms and cognitive abilities in humans and animal models (Higley & Picciotto, 2014), this may provide the positive effects of GML observed in this study. What is noteworthy is that neither GML nor AM offered any benefits in combination with HAL with regard to redox markers, confirming again that the beneficial effects of GML on LPS-induced behaviour may be associated with other mechanisms rather than antioxidant activity alone.

3.4.5 Inflammatory markers

Alterations in immune-inflammatory processes have been extensively reported in schizophrenia, as evinced by the detection of elevated levels of pro-inflammatory cytokines (Miller *et al.*, 2011). These cytokines play a crucial role in the development of the CNS (Zhao & Schwartz, 1998; Deverman & Patterson, 2009) and are implicated in the pathogenesis of neurodevelopmental disorders such as schizophrenia (Potvin *et al.*, 2008; Na *et al.*, 2014). Elevated levels of IL-6 and TNF- α have previously been reported in schizophrenia and have been implicated in the symptomatology of the illness (Erbağci *et al.*, 2001). In accordance with previous studies (Urakubo *et al.*, 2001; Swanepoel, 2017), prenatal exposure to LPS resulted in elevated levels of pro-inflammatory cytokines, IL-6 and TNF-α (Fig. 10a, 11a), while these changes parallel the increase in lipid peroxidation in the cortex and striatum. Thus, inflammation is likely to be causally associated with brain damage in the LPS model. That HAL treatment successfully reduced elevated IL-6 levels is consistent with clinical findings that HAL normalizes elevated IL-6 plasma levels in patients with schizophrenia (Maes *et al.*, 1995; Maes *et al.*, 1997). Similarly, both GML and AM treatment reduced elevated plasma IL-6 levels, with GML but not AM also reducing TNF-α levels in the LPS model. Such an anti-inflammatory

effect of GML may be associated with its inhibition of prostaglandin E_2 (PGE_2) synthesis (Nakatani *et al.*, 2002b; Reanmongkol & Wattanapiromsakul, 2008). During immune-inflammatory processes, COX is responsible for prostaglandin (PG) formation and elevated levels of PGE_2 can affect the functions of neurons, microglia/macrophage and lymphocytes (Weissmann, 1993). Consequently, the interplay between PGE_2 and other factors in the CNS, including pro- and anti-inflammatory cytokines, is expected to modulate the outcome of immune-inflammatory responses (Nakatani *et al.*, 2002b).

Studies on AM have also shown it to decrease inflammatory cytokines following LPS induction (Liu *et al.*, 2012), to inhibit IL-2 release (Kasemwattanaroj *et al.*, 2013) and to suppress IL-6 expression (Yiemwattana & Kaomongkolgit, 2015). Treatment with a combination of HAL and GML but not HAL + AM, successfully reversed elevated IL-6 and TNF-α levels in the LPS model, although not more so than HAL alone. These findings could be attributed to the immunosuppressive effects of HAL (Song *et al.*, 2000) and the anti-inflammatory properties of GML (Nakatani *et al.*, 2002a; Chomnawang *et al.*, 2007). As noted in the lipid peroxidation data, neither GML nor AM offered any benefits in combination with HAL vs. HAL monotherapy, which reaffirms the earlier notion that any benefits offered with adjunctive treatment, especially with GML, are not immediately related to effects on inflammatory-redox mechanisms.

3.4.6 Concluding remarks

Prenatal exposure to LPS induced behavioural and immune-redox alterations akin to schizophrenia in the offspring later in life, specifically late adolescence/early adulthood, replicating the typical age of clinical onset of schizophrenia in patients (Jones, 2013). These alterations include deficits in sensorimotor gating, depressive-like behaviour and elevated oxidative and inflammatory biomarkers. Immune activation and pro-inflammatory cytokines related to inflammatory processes are notable etiological factors in schizophrenia (Na *et al.*, 2014). Furthermore, redox pathways are implicated in the aetiology of schizophrenia through lipid peroxidation and oxidative damage to proteins and DNA (Boskovic *et al.*, 2011) and may contribute to the neuroprogressive nature of the disorder (Wood *et al.*, 2009). These attributes confirm the face and construct validity of the model for schizophrenia, while the reversal of these bio-behavioural changes with HAL confirms its predictive validity.

Chronic treatment with GML and AM failed to impact on sensorimotor gating deficits in the model, although dose-response studies could well address this more effectively. However, both GML and AM not only displayed significant antidepressant-like properties in the model

but also bolstered the antidepressant-like response to HAL. These behavioural data suggest that the place of GML and AM in treatment may be to specifically address the depressive symptoms of schizophrenia. However, since only GML+HAL was effective in addressing %PPI deficits in the model, these data do not suggest that AM may have value in treating schizophrenia. Indeed, GML has been noted as providing parity with imipramine as an antidepressant (Oberholzer *et al*., 2017), while it is currently under investigation in the clinic for the treatment of bipolar disorder (Ashton *et al.*, 2016). Whether AM is able to confer psychotherapeutic benefits remains to be confirmed in more elaborate dose-response and other studies.

Reversal of the elevation in TNF-α and IL-6 levels in a MIA model by GML and AM confirms an anti-inflammatory basis to how GML and AM exert their antidepressant effects (Chomnawang *et al.*, 2007; Oberholzer *et al.*, 2017). Although GML was ineffective in reducing lipid peroxidation in the frontal cortex, AM displayed a significant reduction in frontal cortical lipid peroxidation, confirming the protective effect of AM against oxidative stress (Márquez-Valadez *et al.*, 2012; Fang *et al.*, 2016). Finally, these data confirm the idea that a combination of GML and an antipsychotic may offer important therapeutic benefits, with GML especially offering benefits as an adjunctive treatment in the treatment of schizophrenia.

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CHAPTER 4

SUMMARY, CONCLUSION AND RECOMMENDATIONS

4.1 Discussion of results

Schizophrenia is a devastating mental disorder that generally presents with various symptom clusters in late adolescence (Do *et al.*, 2009). Although currently-available antipsychotics are successful in treating the positive symptoms (delusions and hallucinations), their effects against cognitive dysfunctions (impaired executive function, working memory and attention) and negative symptoms (social withdrawal, anhedonia and avolition) remain unsatisfactory, which strongly contributes to the poor management of the clinical syndrome (Millan *et al.*, 2014). What makes the treatment of schizophrenia even more challenging is the complex pathological mechanisms and the multifactorial basis of the illness, influenced by the interaction of genetic, environmental and developmental risk factors (Rapoport *et al.*, 2012; Sullivan *et al.*, 2012; Millan, 2013).

Early life adversities such as prenatal infection during gestation have been associated with increased susceptibility for schizophrenia (Brown & Derkits, 2010). Consequently, maternal immune activation (MIA) animal models for schizophrenia have become a useful tool in preclinical studies for investigating the neurobiological nature and treatment options in this disorder (Jones *et al.*, 2011). As a consequence of maternal infection, the activation of the immune-inflammatory response during critical periods of neurodevelopment may lead to abnormalities associated with schizophrenia in the offspring. These alterations can be driven by oxidative and nitrosative stress (O&NS) pathways and pro-inflammatory cytokine release in the placenta and foetus (Anderson *et al.*, 2013). This makes sense considering the crucial role for cytokines in the central nervous system (CNS) (Feigenson *et al.*, 2014) and the brain's increased susceptibility to oxidative damage (Bitanihirwe & Woo, 2011). Moreover, people with schizophrenia have presented with elevated plasma concentrations of pro-inflammatory cytokines (Potvin *et al.*, 2008; Miller *et al.*, 2011) and compromised antioxidant processes (Dadheech *et al.*, 2008; Wood *et al.*, 2009).

Bearing in mind not only the inadequate treatment available for schizophrenia, but also the financial implication thereof, urgent novel therapeutic strategies are needed for this disorder. Given the accumulating interest of antioxidant treatment in schizophrenia (Emsley *et al.*, 2014; Magalhães *et al.*, 2016; Rossell *et al.*, 2016; Rapado-Castro *et al.*, 2017), this study evaluated the therapeutic effects of *Garcinia mangostana* Linn (GML) and one of its primary components,

alpha-mangostin (AM), both known to have antioxidant and anti-inflammatory properties (Obolskiy *et al.*, 2009), compared to HAL on biological and behavioural alterations akin to schizophrenia using a MIA model. Moreover, given the interest in adjunctive treatment, this study will also consider the ability of AM and GML to augment the response to HAL.

The bacterial endotoxin, lipopolysaccharide (LPS), was used to induce MIA in this study. Here the study confirmed that the LPS model does in fact induce schizophrenia-like behaviours, as assessed in at least three behavioural tests of relevance to schizophrenia, viz. the prepulse inhibition (PPI) test (to assess sensorimotor gating), open field test (OFT) (to assess locomotor activity) and the forced swim test (FST) (to assess depression-related symptoms) (Chapter 3). Additionally, by measuring peripheral and neurochemical biomarkers viz. interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α) and lipid peroxidation, respectively, the study established that redox-inflammatory alterations implicated in schizophrenia are indeed evident in offspring of dams exposed to prenatal LPS (Chapter 3). That this occurred during the early adolescent period (postnatal days 64 -65) coincides with the critical period of symptom presentation in humans with schizophrenia (Jones *et al.*, 2011). Here, elevated pro-inflammatory cytokines, IL-6 and TNF-α, in the plasma and increased lipid peroxidation was observed in the frontal cortex and striatum (Chapter 3), but not in the hippocampus (Addendum A), of afflicted offspring. Furthermore, the well-known antipsychotic effects of haloperidol (HAL) were evident in the model by reversing MIA-associated sensorimotor gating deficits, depressive-like behaviour, IL-6 levels and frontal cortical lipid peroxidation (Chapter 3). Treatment with both GML and AM had beneficial effects on depressive-like behaviour (Chapter 3), but failed to reverse PPI deficits (Chapter 3) or to alter social behaviour changes in the offspring (Addendum A). While AM reduced MIA-induced lipid peroxidation in the frontal cortex similar to HAL, GML had no effect (Chapter 3), suggesting that the therapeutic effects of GML on altered behaviour may not be solely attributed to antioxidant activity. However, GML treatment effectively reversed MIA-induced elevations in both pro-inflammatory cytokines, whereas AM only exhibited lowering effects on IL-6 (Chapter 3). Finally, the combination treatment of HAL and GML presented with the most promising effects by successfully reversing PPI deficits, depressive-like behaviour (Chapter 3), selected social behaviours (Addendum A) and cytokine alterations (Chapter 3), while both AM and GML displayed noteworthy augmentation effects on depressive-like behaviours in combination with HAL. The data are emphatic in supporting GML and AM as having benefits in treating especially the depressive features of schizophrenia, with GML providing some additional benefits with respect to certain cognitive symptoms such as sensorimotor gating. However, more instructive dose-response analyses is needed to determine whether the observed lack of prominent effects of GML and AM on %PPI are not simply due to an inappropriate dose. In fact, the dose of GML (50 mg/kg/d) was

selected from a previous study that investigated the antidepressant-like possibilities of GML in an animal model of depression (Oberholzer *et al.*, 2017). Nevertheless, the Oberholzer study also showed efficacy of this dose on depression related cognitive deficits, which provides some support that a dose of 50 mg/kg/day *is* appropriate for testing in a MIA model that presents with cognitive, psychotic- and depressive-like features. Similarly, the dose of AM was mainly drawn from the pharmacokinetic studies of Li *et al.* (2011) and Sani *et al.* (2015) (30-100 mg/kg po) who studied the antinociceptive mechanisms of AM. However, with our study being the first attempt at testing AM under chronic treatment conditions, the researcher opted for a dose of 20 mg/kg/d for a chronic treatment protocol. Thus AM also requires more comprehensive analysis using a dose-response analysis before definitive conclusions can be drawn as to its putative antidepressant and anti-psychotic-like effects, or absence thereof.

4.2. Conflicts of interest

J.S. Lotter, B.H. Harvey and M. Möller have no immediate conflicts of interest to declare, other than that disclosed in “Funding” above. Alpha-mangostin was provided by Deakin University. Olivia Dean and Michael Berk are employees of Deakin University.

4.3. Primary objectives with their relevant outcomes

(1) *Objective:* To establish whether the schizophrenia-like behavioural characteristics evident in the model are accompanied by peripheral and brain immune-inflammatory and redox alterations

Outcome: It was observed that prenatal exposure to LPS resulted in deficits in sensorimotor gating, depressive-like behaviour, elevations in frontal cortical and striatal lipid peroxidation, as well as a significant increase in plasma inflammatory cytokine levels. However, the LPS model induced elevated social behaviour rather than the expected diminished social interaction.

(2) *Objective:* To establish whether chronic HAL treatment can reverse the above-mentioned schizophrenia-like bio-behavioural changes.

Outcome: In this study HAL treatment reversed most of the bio-behavioural alterations, including reduced %PPI at particular startle intensities, depressive-like behaviour, frontal cortical lipid peroxidation and elevated IL-6 plasma levels.

(3) *Objective:* To establish whether GML and AM can reverse the above-mentioned schizophrenia-like behavioural changes in LPS exposed rats, and how this compares to HAL.

Outcome: GML and AM was successful in reversing depressive-like behaviour. However, both GML and AM failed to normalize sensorimotor gating deficits. Ultimately, the effect of HAL on schizophrenia-like behaviour was superior to GML and AM.

(4) *Objective:* To establish whether GML and AM separately can reverse immune-inflammatory and redox changes in LPS exposed rats, and how this compares to HAL.

Outcome: The researcher observed that GML successfully reversed elevated cytokine concentrations (IL-6 and TNF-α) in the plasma, while AM only reversed elevated IL-6 plasma levels. Alternatively, frontal cortical redox changes were reduced by AM and not by GML. GML proved to be superior to HAL and AM regarding the improvement the immune-inflammatory alterations in LPS exposed rats. However, GML failed to reverse central redox alterations, whereas HAL and AM effectively reversed lipid peroxidation in the frontal cortex of LPS exposed rats.

(5) *Objective:* To establish whether GML and AM separately can be used as adjunctive therapy to improve the treatment response to HAL in LPS exposed rats considering the above-mentioned bio-behavioural parameters.

Outcome: The simultaneous treatment with GML and HAL displayed beneficial effects in reversing sensorimotor gating deficits, depressive-like behaviour and increased inflammatory cytokines, but was unsuccessful in reversing heightened lipid peroxidation in the brain. Nevertheless, the synergistic effects of the raw GML and the antipsychotic HAL revealed therapeutic potential for the treatment of schizophrenia. On the other hand, the combination treatment of AM and HAL displayed minimal significant effects on immune-redox and behavioural changes in the LPS exposed offspring, with the exception of successful reversal of depressive-like behaviour. Ultimately, the adjunctive effects of GML is superior to AM for the improvement of treatment response to HAL.

In conclusion, the study found that the prenatal LPS model of schizophrenia elicited behavioural deficits that closely resemble distinctive symptoms of schizophrenia, particularly within the cognitive and negative symptom domains. However, the LPS model presented with social behaviour results contrasting our expectations. In order to explain these unexpected findings, further validation of the SIT is required in the MIA model under our study conditions. For the moment, these data were therefore excluded from the concept article (Chapter 3).

Although no single animal model is able to accurately replicate every aspect of the disorder, this study demonstrates that the MIA model can be used as a reliable neurodevelopmental model for schizophrenia. Redox-inflammatory alterations was also observed in this model, confirming the involvement of immune and oxidative pathways in schizophrenia (Bitanihirwe & Woo, 2011). Taken together, the study can confirm that prenatal exposure to infection may alter the normal process of neurodevelopment resulting in the stimulation of pathological pathways implicated in the behaviour akin to schizophrenia, as presented in early adolescent in the rat that coincides with the age of first symptom onset in humans. Furthermore, given the prominent anti-depressive properties of GML and AM observed in this study, these compounds may be of value in treating negative symptoms of schizophrenia, especially which associated with a prominent mood component, in combination with an antipsychotic. Finally, the beneficial effects of adjunctive GML treatment with HAL with respect to both behavioural and biological markers may be of clinical significance to improve the treatment outcome of schizophrenia. A summary of our findings and the mechanism behind the MIA model is presented in Fig.1.

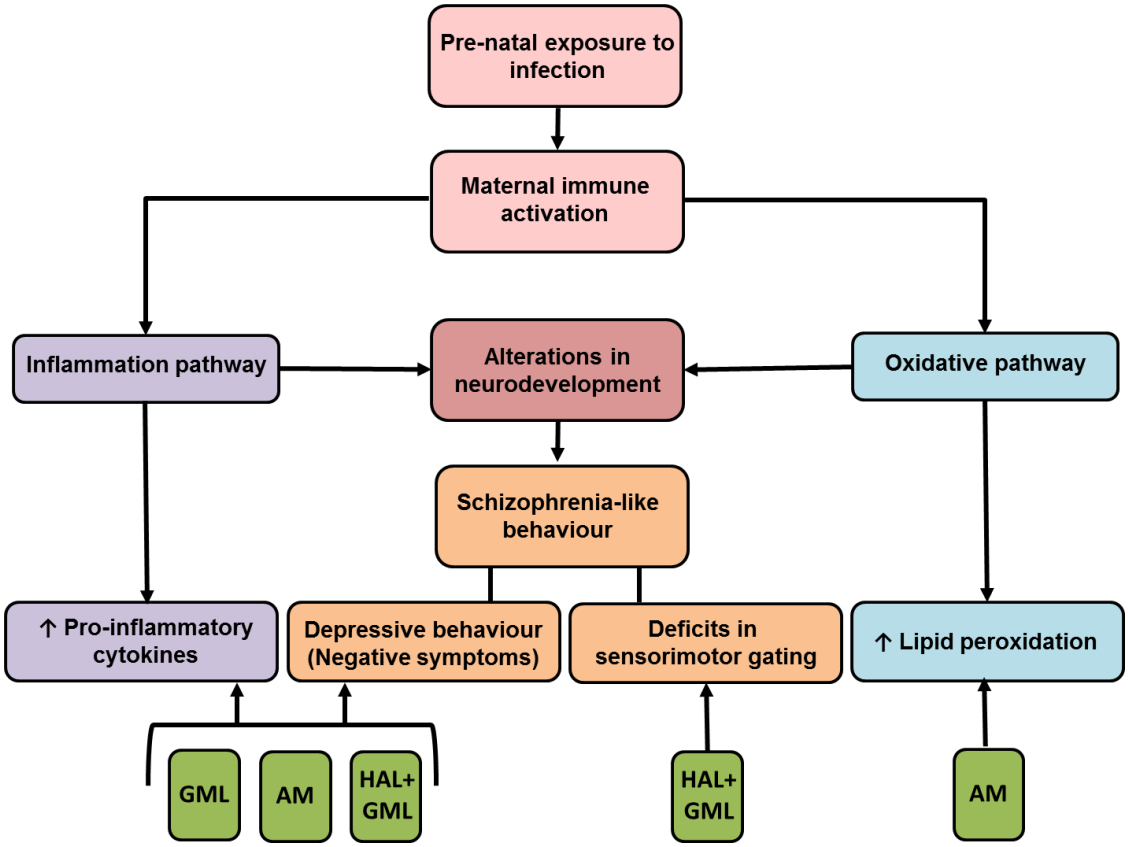

Figure 1: A schematic illustration of the behavioural and biological effects of MIA viz. depressive-like behaviour, deficits in sensorimotor gating as well as elevated pro-inflammatory cytokines and lipid peroxidation. Depressive-like behaviour and increased cytokine levels were reversed by GML, AM and HAL+GML treatment, whereas only AM reduced lipid peroxidation and HAL+GML effectively treated sensorimotor gating deficits.

4.4. Future recommendations

While the current study successfully addressed all of the study objectives presented in Chapter 1, there are numerous limitations which should be addressed in future studies. Recommendations for prospective studies are discussed below:

By including other isolated constituents from the GML pericarp such as β-mangostin and γ -mangostin as treatment in the LPS model, future studies may better elucidate where the pharmacological activity of GML lies.

Given the contributing role of redox pathways in schizophrenia, it is recommended that other biomarkers related to these processes should be investigated in the LPS model in future studies. Antioxidant biomarkers such as NADPH oxidase NOX2, superoxide dismutase (SOD) and glutathione peroxidase (GPx), could be measured to shed more light on the altered

oxidant status in schizophrenia and the antioxidant effect of raw GML and its constituents on these alterations.

Since the behavioural results obtained in this study may be correlated with underlying neurotransmitter alterations and given the critical role of dopamine, serotonin and noradrenaline in the pathology of schizophrenia, the analyses of monoamines could provide valuable information on the effects of GML on regional brain neurotransmitters, especially to support the escape strategies employed by the rats in the FST (i.e. swimming = serotonergic; struggling = noradrenergic).

To confirm the beneficial effects of GML and its constituents on schizophrenia-like behaviour and biology, it is recommended that future studies use other validated animal models of schizophrenia e.g. the social isolation rearing model to evaluate the therapeutic effects of GML.

Since limited data is available on the absorption, bioavailability, disposition, and metabolism of GML and its bioactive constituents, it is recommended that *in vivo* pharmacokinetic studies should be performed in order to obtain knowledge on optimal doses, dosage forms and administration routes for these compounds.

More instructive dose-response analyses are needed to determine whether the observed lack of prominent effects of GML and AM on %PPI and the SIT, or other biological parameters, are not simply a matter of inappropriate dose. Similarly, the dose of AM also requires more comprehensive analysis using a dose-response analysis before definitive conclusions can be drawn as to its putative antidepressant and anti-psychotic-like effects, or absence thereof.

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ADDENDUM A

ADDITIONAL RESULTS

In this addendum additional data that was obtained during the study, but excluded from Chapter 3 (concept article), are presented.

In order to prepare a concept article for publication, certain behavioural and neurochemical data that were less impactful or deemed more validatory in nature, were excluded from the article.

One of the primary objectives of this study was to establish whether schizophrenia-like behavioural characteristics were evident in a MIA model of schizophrenia and to determine the effects of treatment thereon. Deficits in social behaviour, such as social withdrawal i.e. diminished motivation for, or interest in social interactions with others, are well described in schizophrenia and form an integral part of the negative symptoms of the disorder (Millan *et al.*, 2014). The social interaction test (SIT) was therefore originally planned for the study to compliment the measurement of sensorimotor gating and depression-like manifestations in the MIA model, and which earlier studies have used to good effect in the social isolation rearing model (Möller *et al.*, 2011; Möller *et al.*, 2013; Strauss *et al.*, 2014). However, the SIT failed to demonstrate schizophrenia-like social withdrawal in the MIA model. On the contrary, MIA unexpectedly induced a significant increase in social behaviour. In addition, the various treatments displayed minimal effects on social behaviour. Due to time constraints that prevented a comprehensive validation of the SIT in the MIA model, this data was therefore considered inadequate for possible publication purposes and ultimately excluded from the concept article (Chapter 3). However, for the benefit of the dissertation these findings will be presented and briefly discussed in this addendum. For this purpose, the following social behaviours were recorded and scored: the times approaching each other, total distance moved and time spent together.

Another main study objective was to assess redox alterations following maternal immune activation in the offspring and to establish whether treatment can reverse these redox changes. To achieve this, lipid peroxidation was measured with the Parameter™ TBARS assay (described in Addendum B) in brain regions that included the frontal cortex, striatum and hippocampus. While findings pertaining to the former two brain regions are presented in Chapter 3, the lipid peroxidation results obtained from hippocampal tissue are presented here. Considering that the study aimed to examine novel treatment strategies for improvement of

negative and cognitive symptoms of schizophrenia, these data were excluded from chapter 3 because the hippocampal region is mainly related to positive symptoms and has little role to play in the aforementioned poorly treated symptoms (Goghari *et al.*, 2010).

Therefore, the additional data excluded from chapter 3 and presented in this addendum consist of:

- Additional behavioural results as determined in the SIT
- Malondialdehyde (MDA), a product of lipid peroxidation, as assessed in the hippocampus of control and all drug treatment groups.

A.1 Social interaction test

A.1.1 Method

This test involves placing two rodents together in an open field to evaluate spontaneous social activity (Möller, 2009; Möller *et al.*, 2011). Testing took place in an open field arena (70 cm×70 cm×40 cm) and behaviour monitored in a dark room using a digital video camera. The test was initiated by placing two unfamiliar rodents that had received similar treatments, in the centre of the testing arena allowing them 30 s for habituation. Thereafter total social behaviours were scored for 10 min using EthoVision XT software (Noldus Information Technology, Wageningen, Netherlands). The behaviours included: the times approaching each other, total distance moved and time spent together.

Unpaired Student's t-test was used to compare the MIA model with a control group. One-way factorial analysis of variance (ANOVA) and Bonferroni post-hoc tests were used for the statistical analyses of the respective treatment groups vs the LPS control group. Data are expressed as the mean ± standard error of the mean (SEM), with a p-value of <0.05 considered statistically significant.

A.1.2 Results

Considering the MIA model (Fig A-1), unpaired student's t-test revealed a significant increase in social behaviour with regards to (a) the times approaching each other ($p=0.0003$), (b) total distance moved ($p<0.0001$) and (c) time spent together ($p= 0.0001$).

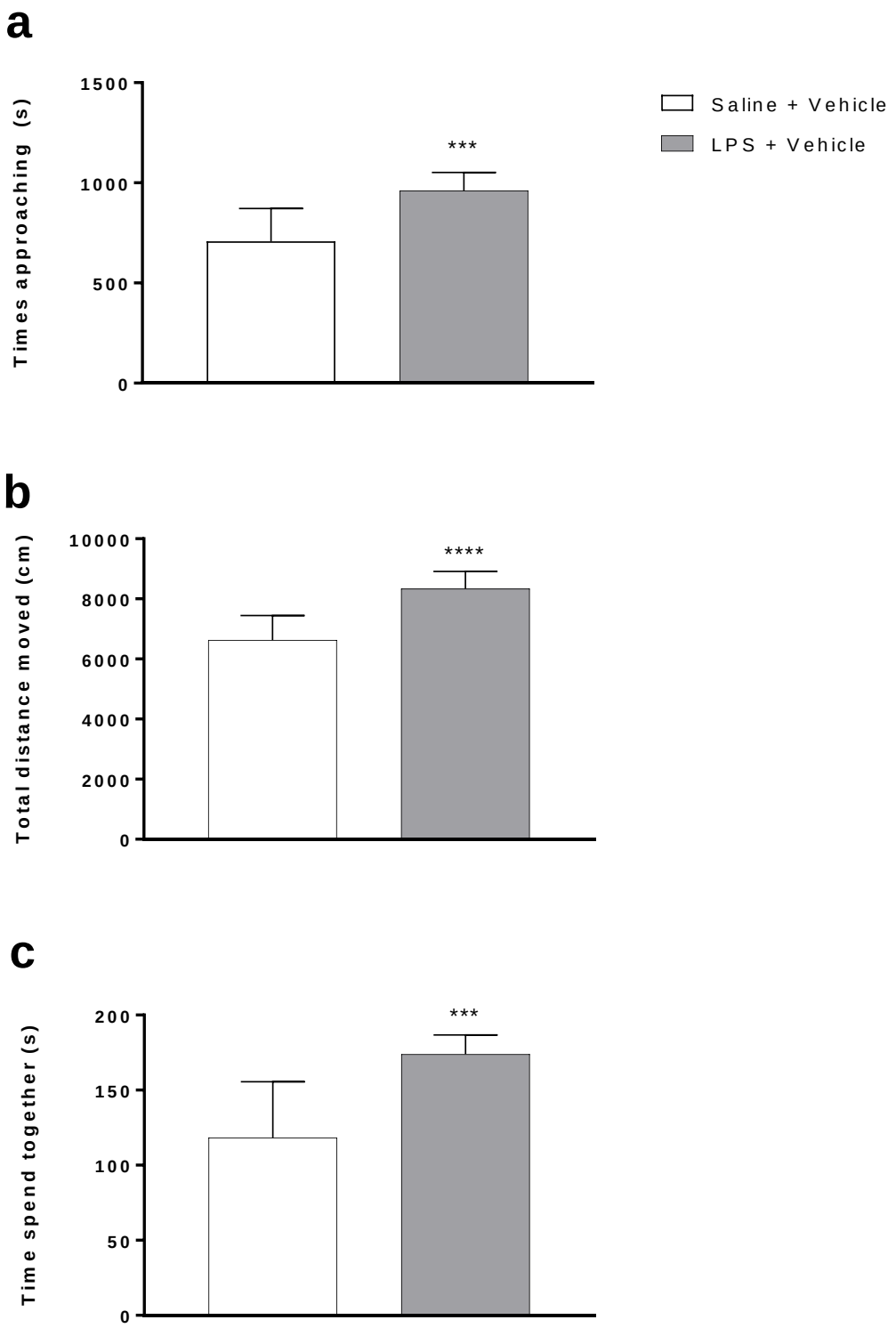

Figure A-1: Social interaction behaviour as (a) the times approaching each other, (b) total distance moved and (c) time spent together in the in the LPS exposed group and the control group.**p<0.001, ***p<0.0001 vs Saline + Vehicle (Unpaired student's test).

Considering the various drug treatments in the MIA model (Fig. A-2), one-way ANOVA of the data revealed a significant main effect of treatment on: (a) times approaching each other ($F(6, 69) = 5.173$, $p= 0.0002$); (b) total distance moved ($F(6, 69) = 14.43$, $p < 0.0001$) and (c) time spent together ($F(6, 69) = 17.92$, $p < 0.0001$). Bonferroni post hoc analysis revealed that both the combination treatment groups HAL+GML ($p = 0.0001$) and HAL+ AM ($p < 0.0001$) significantly reduced the total distance moved when compared to the LPS exposed control group (LPS+ vehicle), however only the GML+HAL treatment group reversed the social behavioural changes as time spent together. Other than that, no treatment groups showed any significant differences in social behaviour in the LPS exposed offspring vs their vehicle-treated controls with respect to (a) times approaching each other; (b) total distance moved and (c) time spent together were observed.

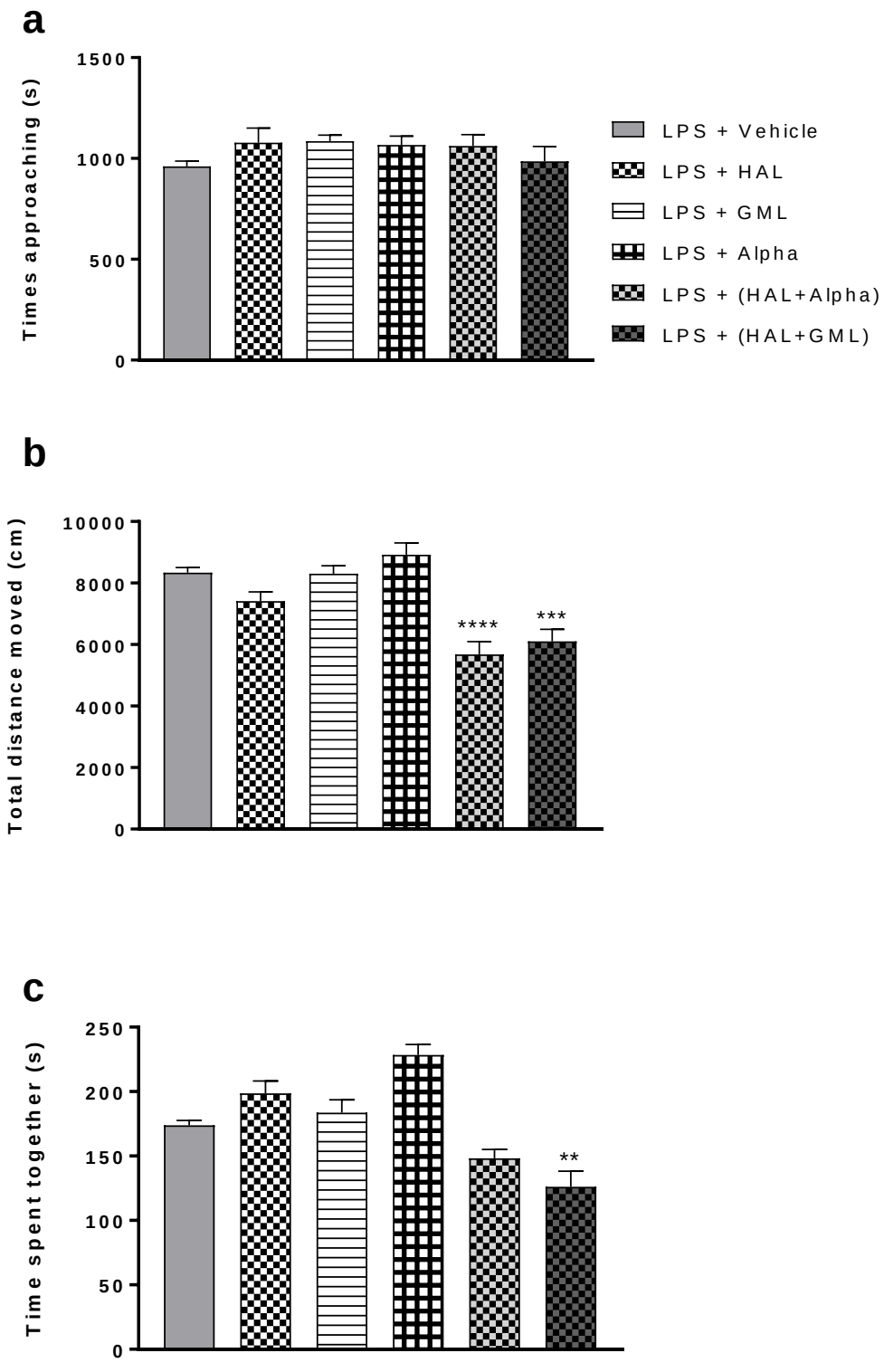

Figure A-2: Social interaction behaviour as (a) the times approaching each other, (b) total distance moved and (c) time spent together in the respective exposure and treatment groups. *p<0.05, **p<0.001, ***p<0.0001 vs LPS + Vehicle; (One-way ANOVA, Bonferroni post hoc test).

A.1.3 Discussion

In this study an increase in social behaviour was observed in the offspring prenatally exposed to LPS in comparison to the saline control offspring. Although these findings are consistent with previous studies (Harvey & Boksa, 2014; Swanepoel, 2017), the results are surprising given that social impairment is a primary negative symptom in schizophrenia (Wilson & Koenig, 2014). In contrast to our results, a reduction in social behaviour in the offspring was reported in other prenatal LPS studies (Burton *et al.*, 2011; Oskvig *et al.*, 2012; Basta-Kaim *et al.*, 2015; Custodio *et al.*, 2016). Given that the standard SIT method applicable to negative symptoms (Moller et al., 2011; 2013) was used, these contradicting results may be attributed to the variation in timing and dose of administration of LPS and the specific strain of rats used in the studies (Foley *et al.*, 2014; Harvey & Boksa, 2014). It must be said that the changes are small, albeit significant, and one wonders about the practical significance of these findings. It can be suggested that the elevated social interaction seen here may be driven by locomotor hyperactivity observed in several rodent models of schizophrenia (Leriche *et al.*, 2003; Duncan *et al.*, 2006; Bradford *et al.*, 2010). Consistent with other animal studies (Mohn *et al.*, 1999; Qiao *et al.*, 2001), The study found that HAL had no effect on the altered social behaviour in the LPS exposed offspring, which is somewhat in line with its lack of clinical efficacy in treating negative symptom schizophrenia (Volavka *et al.*, 2002). Similarly, treatment with GML and AM did not have an effect on elevated social behaviour in the prenatally exposed offspring. However, considering the anti-depressive like effects of GML and AM discussed in chapter 3, these treatment effects may contribute to the seemingly elevated social behaviour described here in the MIA model, given that social impairment is evident in depression (Lewinsohn *et al.*, 2003). Locomotor activity was significantly reduced in the combination treatment of HAL+GML and HAL+AM, which could have potentially orchestrated this response. In fact, HAL is well-known to induce movement-related effects as a result of its potent D_2 receptor antagonism (De Santis *et al.*, 2014), while the possible sedative properties of AM (Shankaranarayan *et al.*, 1979) may underlie its response in this test. Finally, HAL+GML treatment successfully reversed the increased social behaviour with regards to time spent together and could be due to the combination of anti-depressive activity of GML and the antipsychotic and locomotor effects of HAL.

Concluding, until further validation of the SIT is undertaken in the MIA model under our conditions of study to better explain the rather unexpected findings of an increase in SI in prenatally LPS exposed rats, these findings can only be reported and interpreted with caution. This work was thus not included in Chapter 3.

A.2 Hippocampal lipid peroxidation

A.2.1 Method

The Parameter™ TBARS assay from R&D Systems (Minneapolis, USA; catalogue number KGE013) was used to analyse lipid peroxidation in brain tissue (Swanepoel, 2017). This assay is based on the reactivity of the end product of lipid peroxidation, malondialdehyde (MDA), with TBA. In an environment comprising high temperature and low pH, MDA reacts with TBA in a nucleophilic addition reaction to produce a red, fluorescent 1:2 MDA:TBA product, which is then extracted with butanol and the absorbance read at 532 nm using a Bio-Tek FL600 Microplate Fluorescence Reader (Bio-Tek, Instruments, Inc., 381 Highland Park, Winooski, VT, USA).

Unpaired Student's t-test was used to compare the MIA model with a control group. One-way factorial analysis of variance (ANOVA) and Bonferroni post-hoc tests were used for the statistical analyses of the respective treatment groups vs the LPS control group. Data are expressed as the mean ± standard error of the mean (SEM), with a p-value of <0.05 considered statistically significant.

A.2.2 Results

Considering the MIA model, unpaired Student's t-test revealed that there were no significant difference in levels of MDA in the LPS exposed rats (LPS+ vehicle) in comparison to the saline control group (Saline + vehicle) (Fig. A-2). Considering the various drug treatments in the MIA model, one-way ANOVA indicated a significant main effect of treatment on lipid peroxidation ($F(5, 64) = 3.751$, $p=0.0048$). Bonferroni post-hoc analysis showed that only HAL treatment significantly increased MDA levels in LPS exposed rats vs their respective controls (LPS+ vehicle) ($p=0.0422$). No significant differences were observed in any of the treatment groups with respect to hippocampal MDA levels (Fig. A-3).

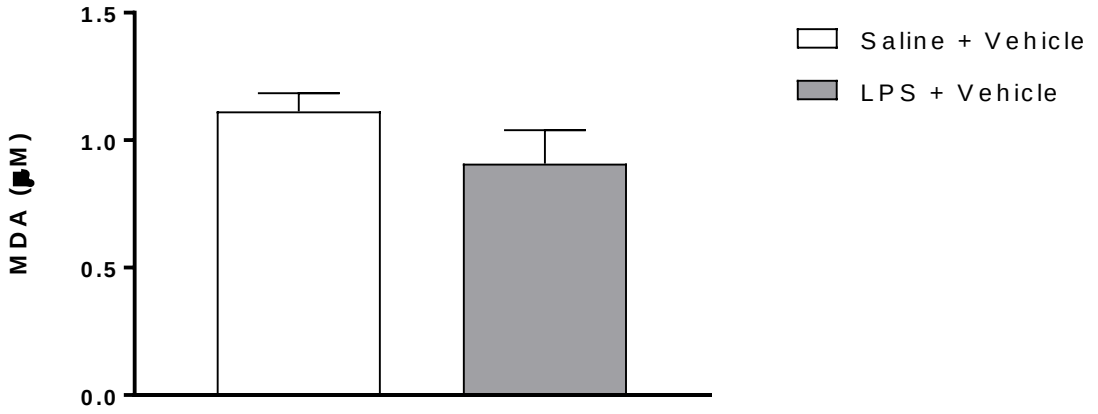

Figure A-3: Lipid peroxidation, assessed as levels of malondialdehyde (MDA), in hippocampus in rats prenatally exposed to LPS or saline respectively and treated with vehicle (Unpaired Student's t-test) vs Saline + Vehicle. $d ≥ 1.3

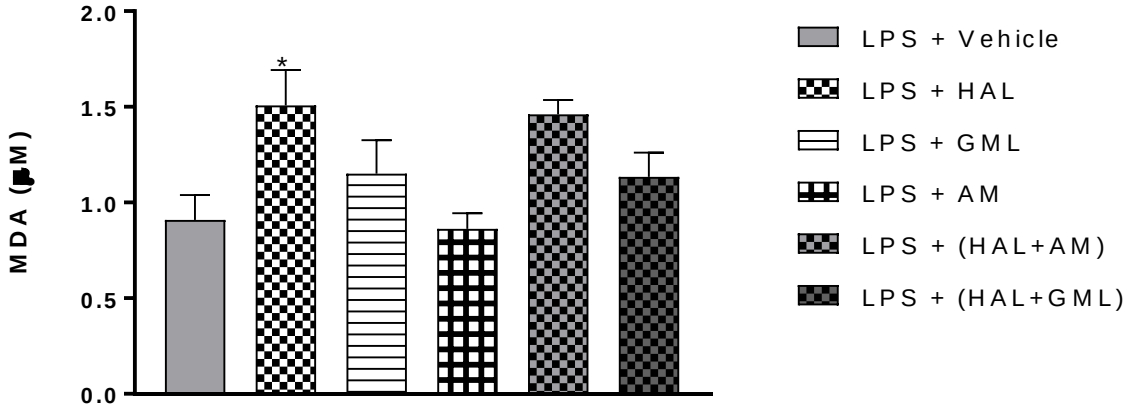

Figure A-4: Lipid peroxidation as malondialdehyde (MDA) hippocampus in rats exposed to LPS prenatally and treated as indicated (One-way ANOVA, Bonferronni post hoc test). *p<0.05 vs Saline + Vehicle.

A.2.3 Discussion

Contrary to previous findings (Zhu *et al.*, 2007; Swanepoel, 2017), LPS exposure failed to induce hippocampal lipid peroxidation in the offspring, no significant difference was observed in MDA levels in the hippocampus of the LPS exposed controls vs the saline exposure controls (Fig. A-2). The lack of effect on lipid peroxidation could possibly be ascribed to the compensating effects of hippocampal antioxidant enzymes following LPS exposure in the offspring. However, that MIA induced depression-like behaviours (Chapter 3), which is a disorder that is strongly dependent on hippocampal pathology, makes this interpretation unlikely. Indeed, Oberholzer (2017) found GML-induced antidepressant effects to be

associated with a reversal of elevated lipid peroxidation in the FSL rat model of depression. Furthermore, HAL have been observed to produce reductions in antioxidant enzyme, superoxide dismutase (SOD) and elevations in lipid peroxidation in the whole brain of rats (Pillai *et al.*, 2007) and may explain the increase in hippocampal MDA concentration following HAL treatment in the LPS exposed offspring. Although Oberholzer *et al.* (2017) demonstrated that raw GML pericarp was effective in reducing hippocampal lipid peroxidation, the study observed no effect of GML on the MDA levels in the hippocampus. These findings are also contradictory to other studies that have reported beneficial effects of N-acetylcysteine (NAC) and other antioxidants on lipid peroxidation (Mahadik *et al.*, 2001; Swanepoel, 2017). As mentioned in chapter 3, the therapeutic effect of GML may not be based exclusively on antioxidant effects alone and suggests that GML possess other properties responsible for the improvement of certain behavioural deficits that still needs to be investigated. For example, the role of monoamines is especially evident (Oberholzer et al., 2017). Moreover, the antioxidant effects of AM was not evident in the reversal of lipid peroxidation in the hippocampus, contradicting previous findings (Andreu *et al.*, 2010) that may suggest inadequate dose of AM used in this study, especially considering relatively low bioavailability following oral administration (Li *et al.*, 2011). In fact, future studies should consider undertaking a dose range analysis with AM.

Concluding, given the relatively insignificant role of the hippocampus in the aspects of schizophrenia studied here, the specific data with regards to this brain region were excluded from Chapter 3. Moreover, further study is warranted on AM to confirm an optimal dose.

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ADDENDUM B

METHODS USED FOR NEUROCHEMICAL AND PERIPHERAL (PLASMA) ANALYSES

This addendum contains the detailed methodology for the following assays:

- Regional brain lipid peroxidation
- Plasma tumor necrosis factor alpha (TNF-α)
- Plasma interleukin-6 (IL-6)

B. 1 Regional brain lipid peroxidation analysis

B.1.1 Introduction

The measurement of lipid peroxidation is usually used as an indication of the involvement of free-radical reactions in cell damage (Gutteridge, 1995) that have been implicated in the pathology of neurodegenerative diseases (Aruoma, 1998). In this study, lipid peroxidation in brain tissue was measured using the Parameter™ TBARS assay from R&D Systems (Minneapolis, USA; catalogue number KGE013).

Lipid structure can be altered by oxidizing agents, generating lipid peroxides that lead to the formation of malondialdehyde (MDA), which can be measured as Thiobarbituric Acid Reactive Substances (TBARS), a convenient method commonly used to determine the relative lipid peroxide content of brain sample sets (Ohkawa *et al.*, 1978; Benzie, 1996). Multi-unsaturated lipids (three or more double bonds) are most likely to form peroxides and are the most reactive in the TBARS assay (Benzie, 1996; Rael *et al.*, 2004; Lykkesfeldt, 2007). Acid treatment of proteins and breakdown of peroxides by heat and acid is required for the release of MDA to facilitate colour development in the TBARS reaction, since free MDA is normally rather low (Gutteridge, 1995; Benzie, 1996; Lykkesfeldt, 2007) (Meagher & FitzGerald, 2000). Removal of protein via precipitation eliminates possible interference of amino acids that may react with thiobarbituric acid (Benzie, 1996; Meagher & FitzGerald, 2000).

The Parameter TBARS assay is a chemical analysis designed to measure TBARS in cell culture supernates, cell lysates, serum, plasma, and urine.

Principle of assay:

In the presence of heat and acid, MDA reacts with TBA to produce a coloured product that absorbs light at 530-540 nm (indicated in Figure B-1). The colour intensity at 532 nm correlates to the level of lipid peroxidation in the sample. Unknown samples are compared to the standard curve.

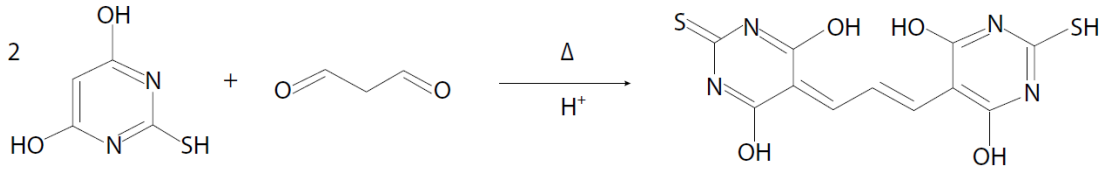

Figure B-1: In the presence of acid and heat, two molecules of 2-thiobarbituric acid react with MDA to produce a coloured end product that can easily be quantified.

B.1.2 Materials

Provided materials:

- Plates, uncoated: Two 96 well microplates (12 strips of 8 wells).
- TBA Reagent: 15 mL of thiobarbituric acid in an aqueous solution.
- TBARS Standard: 1 mL of 500 µM 1,1,3,3-tetramethoxypropane in deionized water.
- TBARS Acid Reagent: 2 vials (15 mL/vial) of 0.6 N trichloroacetic acid in deionized water.
- Plate Sealers: 4 adhesive strips

Required materials:

- Microplate reader (capable of measuring absorbance at 530-532 nm)
- 45-50 °C incubator
- Microcentrifuge capable of ≥ 12,000 x g
- Deionized or distilled water
- Pipettes and pipette tips
- Microcentrifuge tubes for acid treatment
- Test tubes for dilution of standards

B.1.3 Sample preparation:

Preparation of phosphate buffered solution (PBS):

PBS was made up and used for the preparation of brain samples to be used in the TBARS assay. 2.5 litres of a 100x strength solution was prepared by adding 200g NaCl, 5g KCl, and 22.5g Na_2HPO_4 to 2.5 L of double distilled water. 1 part PBS (100x) was diluted with 9 parts double distilled water immediately before use to obtain a 0.01 mM PBS dilution. Previously obtained brain samples were removed from the freezer, left to thaw on ice and weighed. A 10% w/v solution was then made with the brain samples in cold PBS, at a pH of 7.4. The tissue samples were then ultrasonically homogenized (no need for centrifugation prior to acid treatment) and used for the TBARS assay (Župan *et al.*, 2008).

Acid treatment:

Acid treatment is necessary for all samples as this precipitates interfering proteins and other substances for removal by centrifugation, while also catalyzing the TBARS reaction.

1. 300 µL of each sample and 300 µL TBARS Acid Reagent are added to a microcentrifuge tube and mixed.
2. The mixture is then incubated at room temperature for 15 minutes.
3. Sample mixtures are then centrifuged for 4 minutes at ≥ 12,000 x g.
4. The supernate of each sample mixture is carefully removed and retained.
5. Samples are assayed immediately.
6. The concentration read off the standard curve is multiplied by the dilution factor, 2.

B.1.4 Reagent preparation:

All reagents should be at room temperature before use.

TBARS Standard:

1. The standard is converted to MDA by adding 100 µL of TBARS Standard to200 µL of TBARS Acid Reagent.
2. The standard is allowed to sit for a minimum of 30 minutes with gentle agitation. This produces a stock solution of 167 µM.
3. Using a pipette 900 µL of deionized water is placed into the 16.7 µM tube.
4. Using a pipette 500 µL of deionized water is placed into the remaining tubes.
5. The stock solution is used to produce a dilution series (see Fig. B-2 below).
6. Each tube is mixed thoroughly and pipette tips changed between each transfer.

7. The 16.7 µM standard serves as the high standard and deionized water as the 0 µM standard.

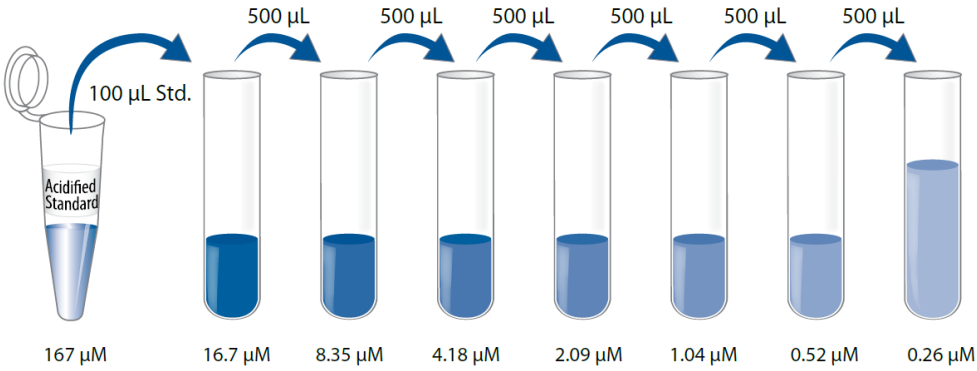

Figure B-2: Dilution series

B.1.5 Assay procedure:

All reagents and samples must be at room temperature before use. Samples, controls, and standards are assayed in duplicate.

1. All reagents, standards, and samples are prepared as mentioned in the previous sections.
2. Excess microplate strips are removed from the plate frame, they are returned to the foil pouch containing the desiccant pack, and resealed.
3. 150 µL of standards and samples are added to each well.
4. 75 µL of TBA Reagent is added to each well.
5. The optical density of each well is pre-read using a microplate reader set to 532 nm.
6. The adhesive strip is used to cover the microplate, which was incubated for 2-3 hours at 45-50 °C.
7. The optical density of each well is determined using a microplate reader set to 532 nm.
8. The pre-reading is then subtracted from the final reading to correct for the sample's contribution to the final absorption at 532 nm.

B.1.6 Calculation of results:

- The optical densities obtained for each standard, sample and control, prior to the incubation with the TBA reagent, are subtracted from the optical densities for the same wells after incubation.

- The corrected duplicate readings for each standard, control, and sample are then averaged.
- Using computer software capable of generating a linear curve fit, a standard curve is created by reducing the data (Figure B-3).
- Given that samples have been diluted, the concentration read from the standard curve need to be multiplied by the dilution factor.

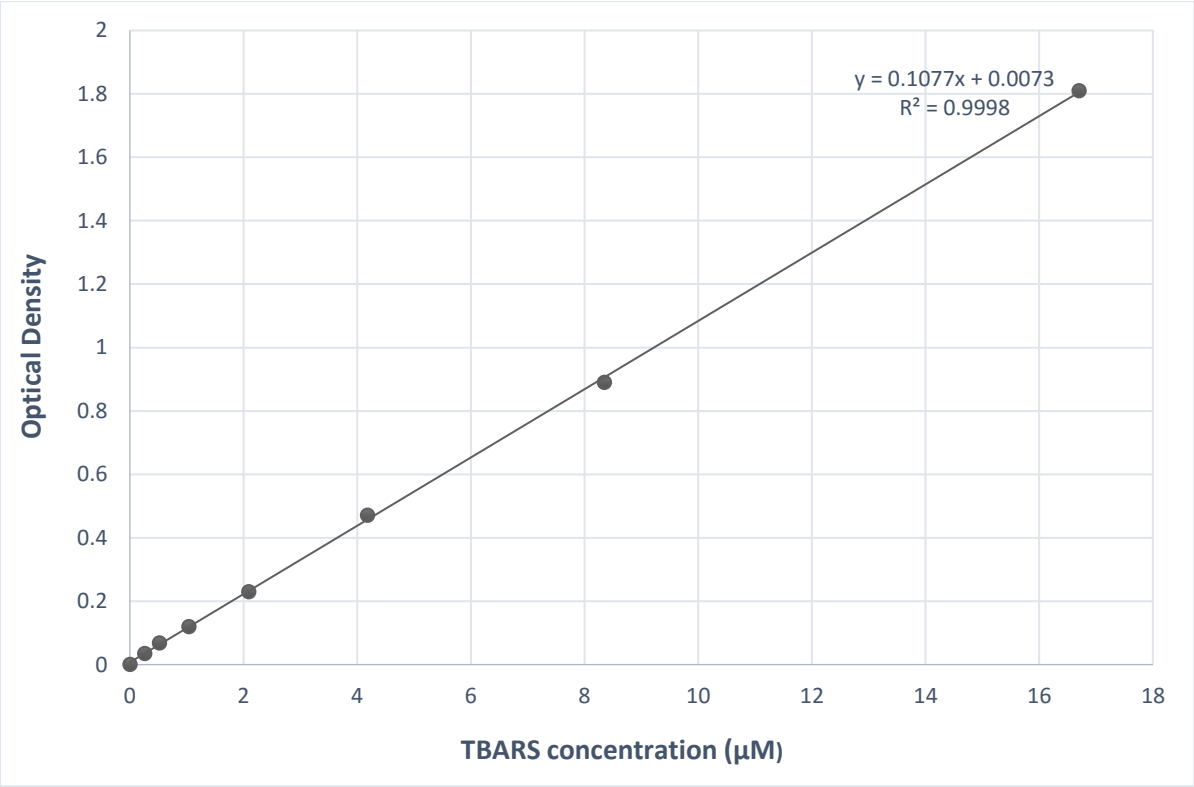

Figure B-3: Standard linear curve for lipid peroxidation.

B.2 Plasma tumor necrosis factor alpha (TNF-α) analysis

B.2.1 Introduction

Plasma tumour necrosis factor-α (TNF-α) was analysed by using the Rat TNF-α ELISA MAX™ Deluxe Set (catalogue number 438204) from Bio Legend (San Diego, USA). TNF-α is a pro-inflammatory cytokine that has effects on both homeostatic and pathophysiological processes in the central nervous system (Olmos & Lladó, 2014).

Method principle:

BioLegend's ELISA MAX™ Deluxe Set is a sandwich Enzyme Linked Immunosorbent Assay (ELISA). A rat TNF-α specific Armenian hamster monoclonal antibody is initially coated on a

96-well plate. Standards and samples are then added to the wells, and TNF-α binds to the immobilized capture antibody. Next, a biotinylated goat polyclonal anti-rat TNF-α detection antibody is added, producing an antibody-antigen-antibody "sandwich". Avidin-horseradish peroxidase is subsequently added, followed by tetramethylbenzidine TMB substrate solution, producing a blue colour in proportion to the concentration of TNF-α present in the sample. Lastly, the stop solution changes the reaction colour from blue to yellow, and the micro-well absorbance is read at 450 nm with a microplate reader.

B.2.2 Materials

Provided materials:

- Rat TNF-α ELISA Capture Antibody (200X)
- Rat TNF-α ELISA Detection Antibody (200X)
- Rat TNF-α Standard
- Avidin-HRP (1,000X)
- Matrix Diluent A (For serum and plasma samples only)
- Substrate Solution A
- Substrate Solution B
- Coating Buffer A (5X)
- Assay Diluent A (5X)
- NUNC Maxisorp™ 96 MicroWell Plates, Uncoated.

Required materials:

- PBS (Phosphate-Buffered Saline): 8.0 g NaCl, 1.16 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.2 g KCl, add deionized water to 1 L; pH to 7.4, 0.2 µm filtered.
- Wash Buffer: PBS + 0.05% Tween-20.
- Stop Solution: Acid solution, e.g. $2NH_2SO_4$
- Plate Sealers: BioLegend Cat. No. 423601 is recommended.
- Deionized water
- A microplate reader (capable of measuring absorbance at 450 nm)
- Adjustable pipettes to measure volumes ranging from 2 µL to 1 mL
- Wash bottle or automated microplate washer
- Log-Log graph paper or software for data analysis
- Tubes to prepare standard dilutions
- Timer
- Absorbent paper

B.2.3 Reagent and plasma preparation:

1. Dilute 5X Coating Buffer A to 1X with deionized water. For one plate, dilute 2.4 mL 5X
   Coating Buffer A in 9.6 mL deionized water.
2. Dilute pre-titrated Capture Antibody 1:200 in 1X Coating Buffer A. For one plate, dilute 60 µL Capture Antibody in 11.94 mL 1X Coating Buffer A.
3. Dilute the pre-titrated Biotinylated Detection Antibody 1:200 in 1X Assay Diluent A. For one plate, dilute 60 µL Detection Antibody in 11.94 mL 1X Assay Diluent A.
4. Dilute Avidin-HRP 1:1,000 in 1X Assay Diluent A. For one plate, dilute 12 µL Detection Antibody in 11.99 mL 1X Assay Diluent A.
5. TMB Substrate Solution is a mixture of equal volumes of Substrate Solution A and Substrate Solution B. Mix the two components immediately prior to use. For one plate, mix 5.5 mL Substrate Solution A with 5.5 mL of Substrate Solution B in a clean container (solution should be clear and colourless).

B.2.4 Assay procedure

1. One day prior to running the ELISA, the Capture Antibody in 1X Coating Buffer is diluted as described in Reagent Preparation. Then, 100 µL of this Capture Antibody solution is added to all wells of the 96-well plate provided in this kit. The plate is sealed and incubated overnight (16-18 hrs) between 2°C and 8°C.
2. First, bring all reagents to room temperature prior to use. All standards and samples are run in duplicate. A standard curve is required for each assay.
3. The plate is washed 4 times with at least 300 µL wash buffer per well and residual buffer blotted by firmly tapping plate upside down on absorbent paper. All subsequent washes are performed similarly.
4. To block non-specific binding, 200 µL 1X Assay Diluent A is added to each well.
5. The plate is then sealed and incubated at room temperature for 1 hour with shaking on a plate shaker (e.g. 500 rpm with a 0.3 cm circular orbit). All subsequent incubations with shaking are performed similarly.
6. While the plate is being blocked, the standards and samples are prepared (sample dilutions are not necessary).
7. The plate is then washed 4 times with wash buffer.
8. For measuring serum and plasma samples: 50 µL Matrix Dilutent A is added to the standard wells and 50 µL Assay Diluent A to the sample wells. Then, 50 µL standard is added to the standard wells or 50 µL sample to the sample wells.
9. The plate is sealed and incubated at room temperature for 2 hours with shaking.

10. The plate is washed 4 times with wash buffer.
11. 100 µL of diluted Detection Antibody solution is added to each well, after which the plate is sealed and incubated at room temperature for 1 hour with shaking.
12. Again the plate is washed 4 times with wash buffer.
13. 100 µL of diluted Avidin-HRP solution is added to each well, followed by sealing the plate and incubating it at room temperature for 30 minutes with shaking.
14. The plate is washed 5 times with wash buffer. For this final wash, wells are soaked in wash buffer for 30 seconds to 1 minute for each wash.
15. 100 µL of freshly mixed TMB Substrate Solution is added and incubated in the dark for 25 minutes, without sealing the plate. Positive wells should turn blue in colour.
16. The reaction is stopped by adding 100 µL of Stop Solution to each well. Positive wells should turn from blue to yellow.
17. Absorbance is read at 450 nm within 15 minutes.

B.2.5 Calculation of results:

Using computer software capable of generating a linear curve fit, a standard curve is created. The data are calculated with computer-based curve-fitting software using a 5- or 4- parameter logistics curve-fitting algorithm.

B. 3 Plasma interleukin-6 analysis

B.3.1 Introduction:

IL-6 was measured in the plasma using the Rat IL-6 ELISA Kit (catalogue number E-EL-R0015) from Elabscience® (Texas, USA).

Method principle:

This kit uses the Sandwich-ELISA method. The microplate provided has been pre-coated with an antibody specific to Rat IL-6. Standards or samples are added to the applicable microplate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for Rat IL-6 and Avidin-Horseradish Peroxidase (HRP) conjugate are added to each microplate well consecutively and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain Rat IL-6, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in colour. The enzyme-substrate reaction is terminated by adding Stop Solution and the colour turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm and the value is proportional to

the concentration of Rat IL-6. The concentration of Rat IL-6 in samples can then be calculated by comparing the OD of the samples to the standard curve.

B.3.2 Materials

Provided materials:

- Micro ELISA Plate (Dismountable)
- Reference Standard
- Concentrated Biotinylated Detection Ab (100×)
- Concentrated HRP Conjugate (100×)
- Reference Standard & Sample Diluent
- Biotinylated Detection Ab Diluent
- HRP Conjugate Diluent
- Concentrated Wash Buffer (25×)
- Substrate Reagent
- Stop Solution
- Plate Sealer

Required materials:

- Microplate reader (capable of measuring absorbance at 450 nm)
- High-precision transferpettor, EP tubes and disposable pipette tips
- 37℃ Incubator
- Deionized or distilled water
- Absorbent paper
- Loading slot for Wash Buffer

B.3.3 Reagent and plasma preparation:

Bring all reagents to room temperature before use. Preheat the Microplate reader for 15 min before OD measurement.

1. **Wash Buffer:** Dilute 30 mL of Concentrated Wash Buffer with deionized or distilled water to prepare 750 mL Wash Buffer.
2. **Standard working solution:** Centrifuge the standard at 10,000×g for 1 min. Add 1.0 mL of Reference Standard &Sample Diluent, let it stand for 10 min and turn it upside down for several times. After it dissolves fully, mix it thoroughly with a pipette. This reconstitution produces a stock solution of 4000 pg/mL. Then make serial dilutions as

needed. The recommended dilution gradient is as follows: 4000, 2000, 1000, 500, 250, 125, 62.50, 0 pg/mL.

3. **Dilution method:** Take 7 EP tubes, add 500 µL of Reference Standard & Sample Diluent to each tube. Pipette 500 µL of the 4000 pg/mL stock solution to the first tube and mix up to produce a 2000 pg/mL stock solution. Pipette 500 µL of the solution from former tube to the latter one in order according to this step (see figure B-4).
4. **Biotinylated Detection Ab working solution:** Calculate the required amount before the experiment (100 µL/well). Centrifuge the stock tube before use, dilute the 100× Concentrated Biotinylated Detection Ab to 1×working solution with Biotinylated Detection Ab Diluent.
5. **Concentrated HRP Conjugate working solution:** Calculate the required amount before the experiment (100 µL/well). Dilute the 100× Concentrated HRP Conjugate to 1× working solution with Concentrated HRP Conjugate Diluent.

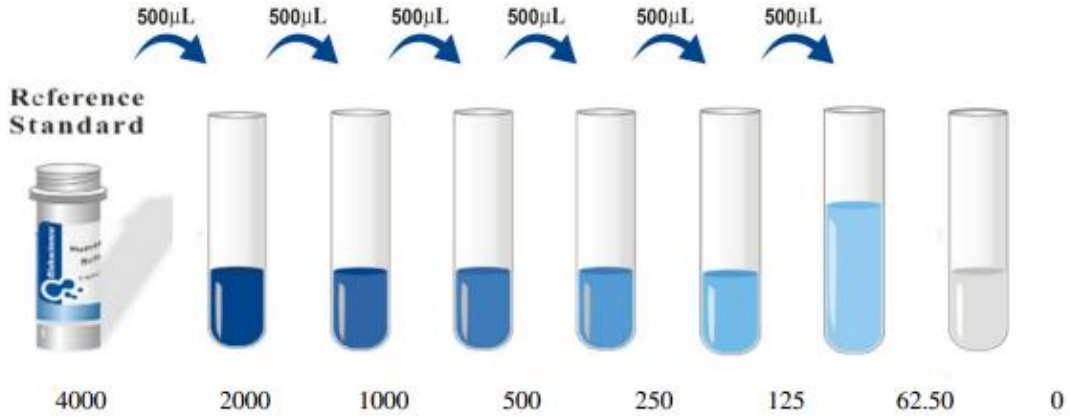

Figure B-4: Dilution series

B.3.4 Assay procedure:

1. The Standard working solution of different concentrations is added to the first two columns: Each concentration of the solution is added into two wells side by side (100 µL for each well). Add samples to other wells (100 µL for each well). Cover the plate with sealer provided in the kit. Incubate for 90 min at 37℃.
2. The liquid of each well is removed, but not washed. Immediately 100 µL of Biotinylated Detection Ab working solution is added to each well and covered with the Plate sealer. It is gently mixed up and incubated for 1 hour at 37°C.
3. The solution is aspirated from each well, 350 µL of wash buffer is added to each well and soaked for 1-2 min. The solution is aspirated from each well and patted dry against clean absorbent paper. Repeat this wash step 3 times.

4. 100 µL of HRP Conjugate working solution is added to each well, covered with the Plate sealer and incubated for 30 min at 37°C.
5. The solution is aspirated from each well and the wash process repeated for five times as conducted in step 3.
6. 90 µL of the Substrate Reagent is added to each well, covered with a new plate sealer and incubated for about 15 min at 37°C. The plate protected from light.
7. 50 µL of Stop Solution is added to each well.
8. The optical density value of each well is determine at once with a micro-plate reader set to 450 nm.

B.3.5 Calculation of results

Average the duplicate readings for each standard and samples, then subtract the average zero standard optical density.

Plot a four-parameter logistic curve on log-log graph paper, with standard concentration on the x-axis and OD values on the y-axis.

If samples have been diluted, the concentration calculated from the standard curve must be multiplied by the dilution factor. If the OD of the sample surpasses the upper limit of the standard curve, this should re-tested after appropriate dilution. The actual concentration is the calculated concentration multiplied by the dilution factor.

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ADDENDUM C

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The instructions for the preparation of the article manuscript are outlined on the journal website: https://academic.oup.com/ijnp, under “Author Guidelines”.

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Examples: More detailed methods, extended data sets/data analysis, tables, or additional figures (including color).

Process: All material to be considered as supplementary material must be submitted at the same time as the main manuscript for peer review. Please indicate clearly the material intended as supplementary material during online submission. Also ensure that the supplementary material is referred to in the main manuscript at an appropriate point in the text. It cannot be altered or replaced after the paper has been accepted for publication. Supplementary material should be submitted online, in its final form.

Please note that supplementary material will not be edited, so ensure that it is clearly and succinctly presented, and that the style of terms conforms with the rest of the paper. Also ensure that the presentation will work on any internet browser.

Acceptable formats: A maximum of 10 files is acceptable to make up the supplementary material unit for the article. The maximum size per file should not exceed 1.5 MBytes, and files must be as small as possible, so that they can be downloaded quickly.

Recommendations:

Pick a common cross-platform (PC, Mac, Linux/UNIX, Amiga etc.) format for your supplementary material to allow the greatest access.

Provide text files in PDF (.pdf), MS Word (.doc), HTML files (.html) or RTF (.rtf) format. Files supplied in Word or RTF may be used to created PDF file.

Provide spreadsheet files in MS Excel (.xls) or CSV format.

Provide image files in .tif, .gif or .jpg format. Images should be a maximum size of 640 x 480 pixels (9 x 6.8 inches at 72 pixels per inch).

Provide sound clips in .mp3 format.

Provide movie clips in .mpg format.

If you require further help or information regarding submission or preparation of supplementary material, please contact the Oxford Journals production department.

ADDENDUM D

LETTERS OF CONSENT TO SUBMIT MANUSCRIPT

NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT
POTCHEFSTROOM CAMPUS

29 November 2017

Dear examiner

MSc THESIS – J LOTTER
PERMISSION TO INCLUDE MANUSCRIPTS FOR EXAMINATION PURPOSES

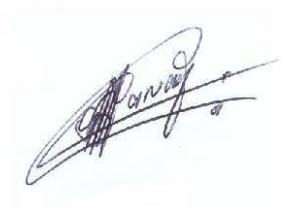
As study leader and senior corresponding author on the article presented in Chapter 3, first authored by Miss Jana Lotter, I hereby approve that the concept manuscript listed below be included as part of the requirements for fulfilment of the MSc. degree, and that this manuscript may be submitted for examination purposes by the candidate.

The article is as follows:

Chapter 3

"Studies on haloperidol and adjunctive α-mangostin or raw Garcinia Mangostana Linn pericarp on bio-behavioural markers in an immune-inflammatory model of schizophrenia"

Sincerely,



Brian H Harvey, PhD

Study leader

NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT
POTCHEFSTROOM CAMPUS

29 November 2017

Dear examiner

MSc DISSERTATION – J LOTTER
PERMISSION TO INCLUDE MANUSCRIPTS FOR EXAMINATION PURPOSES

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The article is as follows:

Chapter 3

"Studies on haloperidol and adjunctive α-mangostin or raw Garcinia Mangostana Linn pericarp on bio-behavioural markers in an immune-inflammatory model of schizophrenia"

Sincerely,

Marisa Möller, PhD

IMPACT
CENTRE FOR INNOVATION IN MENTAL AND PHYSICAL HEALTH AND CLINICAL TREATMENT

DEAKIN UNIVERSITY Barwon Health

29 November 2017

The Post-graduate Examinations Office
North-West University

Dear Sir/Madam

MSc THESIS – J LOTTER
PERMISSION TO INCLUDE MANUSCRIPT FOR EXAMINATION PURPOSES

I hereby approve that the concept manuscript listed below with myself as co-author, be included as part of the requirements for fulfilment of the MSc. degree, and that this manuscript may be submitted for examination purposes by the candidate.

The article is as follows:

CHAPTER 3

"Studies on haloperidol and adjunctive α-mangostin or raw Garcinia Mangostana Linn pericarp on bio-behavioural markers in an immune-inflammatory model of schizophrenia"

Sincerely

Yours sincerely

Michael Berk

MBBCh, MMed (Psych), FF(Psych)SA, FRANZCP, PhD

NHMRC Senior Principal Research Fellow
Alfred-Deakin Professor in Psychiatry at Barwon Health
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DEAKIN UNIVERSITY