

Studies on N-acetyl cysteine as a therapeutic intervention in methamphetamine-associated bio-behavioural changes in an immune-inflammatory model

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The Lord your God has blessed you in all the work of your hands.

Deuteronomy 2:7

Abstract

Purpose:

Schizophrenia is a severe illness, affecting 0.5-1% of the world population. The clinical manifestation of schizophrenia is characterized by three distinct categories of symptoms, namely positive, negative and cognitive symptoms. Schizophrenia has been linked to monoamine alterations, oxidative stress and inflammation. The aetiological and biological processes of schizophrenia are largely unknown, while current treatment regimens remain sub-optimal in clinical practice. The two-hit hypothesis of schizophrenia states that multiple prominent biological events (or “hits”) distributed over various life periods can result in the development of schizophrenia. Moreover, studies report on an increased risk to develop schizophrenia in individuals exposed to infectious agents during the prenatal period. On the other hand, early life methamphetamine (MA) abuse is associated with an increased risk of developing enduring psychosis later in life. Taking this into account, we developed a (1) prenatal inflammation model and a (2) dual-hit model (prenatal inflammation + MA abuse) of schizophrenia to explore the behavioural and neurochemical changes induced by these models. We then incorporated the antioxidant, N-acetyl cysteine (NAC) as a novel treatment, hypothesizing that it will reverse these alterations in the models.

Methods:

Pregnant Sprague-Dawley rats received lipopolysaccharide (LPS) at a dose of 100 µg/kg on gestational day (GD) 15-16 subcutaneously (SC) or saline as control group. After weaning, male pups born from pregnant dams were divided into MA or saline receiving groups. MA was administered at a dose of 0.2 mg/kg escalating daily up to a final dose of 6 mg/kg SC from PND 35 to 50. The prenatal inflammation and the dual-hit groups received treatment with NAC at 150 mg/kg/day or saline SC from PND 51-64. All treatment groups were subjected to a battery of behavioural tests viz. (1) social interaction (SI) on PND 62, (2) novel object recognition (nORT) on PND 63, and (3) %prepulse inhibition (%PPI) on PND 64. One day later the animals were decapitated and trunk blood and brain tissue were collected and stored at -80 °C until the day of biochemical analyses. Analyses included monoamines, pro- and anti-inflammatory cytokines, reactive oxygen species (ROS) and lipid peroxidation.

Results:

Deficits in %PPI were seen in (1) the LPS alone group at prepulses of 74 dB and 84 dB, (2) the MA alone group at 72 dB and 74 dB and (3) the dual-hit model at 72 db, 74 db and 85 db. NAC tended to reverse PPI deficits in the LPS alone and dual-hit models (especially at 84 dB), but only revealed significant results at 72 dB in the dual-hit model. Social interaction was significantly increased in the LPS-alone and the dual-hit models. NAC successfully reversed these alterations. In the nORT, only the double-hit model caused significant deficits in memory function. NAC tended to reverse these changes, but missed statistical significance.

Increased markers of lipid peroxidation were observed in the LPS alone group in the frontal cortex, striatum and hippocampus while NAC reversed these changes in the striatum and hippocampus. Lipid peroxidation was increased in the dual-hit model in the frontal cortex and striatum with NAC reversing these changes in the striatum. The LPS alone and the dual-hit groups revealed significant elevations in plasma ROS levels. NAC successfully reversed this elevation in the prenatal LPS group. Administration of MA alone did not alter markers of oxidative stress.

Frontal cortical dopamine (DA) levels were significantly increased in the LPS alone group and were reversed by NAC treatment. Both the LPS alone and the dual-hit groups had significantly decreased levels of serotonin in the frontal cortex while NAC normalized these deficits. Frontal cortical and striatal noradrenaline (NA) levels were significantly reduced in the LPS alone and dual-hit model, as well as after MA alone administration. NAC treatment reversed these changes in both animal models and in both brain regions.

LPS alone and the dual-hit model significantly reduced levels of the anti-inflammatory cytokine, interleukin-10 (IL-10). LPS alone also significantly increased the pro-inflammatory cytokine, tumor necrosis factor alpha (TNF- α). NAC treatment reversed the decreased levels of IL-10 in the LPS alone and dual-hit models as well as the elevation in TNF- α levels in the LPS alone model.

Conclusion:

Here we provide evidence that both a prenatal inflammation and a dual-hit model cause behavioural changes akin to schizophrenia symptomology. Maternal inflammation induced immune-redox changes, while the dual-hit model only caused noticeable changes in redox status. Irregularities of important monoamine levels confirm that both these schizophrenia models may influence neuro-function. We suggest that prenatal inflammation may alter brain

development and increase the risk of developing schizophrenia. Coupled with a second hit, cognitive changes become more apparent, suggesting that dual-hit aetiology may be involved in the complete manifestation of schizophrenia. NAC showed beneficial effects on behavioural, neurochemical and redox-inflammatory markers, proposing that anti-oxidant treatment may be of value in schizophrenia treatment, and may have value in treating psychosis associated with prior MA abuse.

Keywords: schizophrenia, prenatal infection, two-hit hypothesis, methamphetamine, oxidative stress, N-acetyl cysteine

Opsomming

Doel:

Skisofrenie is 'n ernstige siekte wat 0.5-1% van die wêreld populasie affekteer. Die kliniese manifestasie van skisofrenie word gekarakteriseer deur drie onderskeibare katagorieë simptome, nl. positiewe, negatiewe en kognitiewe simptome. Skisofrenie word geassosieer met monoamien-afwykings, oksidatiewe stres en inflammasie. Die etiologiese en biologiese prosesse van skisofrenie is grootliks onbekend, terwyl huidige behandelingsregimes sub-optimaal bly in kliniese praktyk. Die twee-tref hipotese van skisofrenie stel voor dat verskeie noemenswaardige biologiese gebeurtenisse (of "trefslae"), versprei oor verskeie lewensydperke, tot die ontwikkeling van skisofrenie kan lei. Verder dui studies daarop dat individue wat gedurende die prenatale periode aan aansteeklike organismes blootgestel was, 'n hoër risiko het om skisofrenie te ontwikkel. Aan die ander hand word metamfetamien (MA)-misbruik geassosieer met 'n verhoogde risiko om op die langtermyn psigoses te ontwikkel. Met hierdie agtergrondskennis het ons 'n (1) prenatale inflammasie model en 'n (2) dubbel-tref model (prenatale inflammasie + MA misbruik) ontwikkel om die gedrags- en neurochemiese veranderinge wat deur hierdie modelle veroorsaak word, te ondersoek. Hierna het ons die anti-oksidadant, N-asetielsisteien (NAS) as nuwe behandeling ingesluit, met die verwagting dat dit hierdie veranderinge in die modelle sal omkeer.

Metodes:

Swanger Sprague-Dawley rotte het lipopolisaggaried (LPS) in 'n dosis van 100 µg/kg, of soutwater-oplossing as kontrole, subkutaneus (SK) op swangerskapsdag (GD) 15-16 ontvang. Na spening is die manlike nageslag verdeel in groepe wat of MA of soutwater ontvang het. MA was toegedien teen 'n stygende dosis van 0.2 mg/kg tweemaal daaglik tot 'n finale dosis van 6 mg/kg SK van postnatale dag (PND) 35 tot 50. Beide die prenatale inflammasie groep, sowel as die twee-tref groep het hierna behandeling met NAS ontvang in 'n dosis van 150 mg/kg/dag SK, of soutwater-oplossing, vanaf PND 51-64. Alle behandelingsgroepe was onderwerp aan 'n reeks gedragstoetse, nl. (1) sosiale interaksie (SI) op PND 62, (2) voorwerpherkenning (nORT) op PND 63 en prepuls-inhibisie (PPI) op PND 64. Op PND 65 is die diere onthoof en hulle bloed en breinweefsel geoes en gestoor by -80 °C tot op die dag van neurochemiese analise. Analises het monoamiene, pro- en anti-inflammatoriese sitokiene, reaktiewe oksidatiewe spesies (ROS) en lipiedperoksidasie ingesluit.

Resultate:

Beduidende onderdrukking in %PPI is gesien in (1) die LPS-alleen groep by prepulse van 74 dB en 84 dB, (2) die MA-alleen groep by 72 dB en 74 dB en (3) die twee-tref model by 72 dB, 74 dB en 84 dB. NAC het geneig om %PPI onderdrukking om te keer in die prenatale inflammasie en die twee-tref modelle (veral by 84 dB), maar die effek was slegs statisties betekenisvol in die twee-tref model by 72 dB. Sosiale interaksie het verhoog in die LPS-alleen en die twee-tref model. NAS het hierdie veranderinge suksesvol omgekeer. In die nORT het slegs die twee-tref model onderdrukking van geheue-funksie veroorsaak. NAS het geneig om hierdie veranderinge om te keer, maar het statistiese betekenisvolheid gemis.

Verhoogde merkers van lipiedperoksidase is waargeneem in die LPS-alleen groep in die frontale korteks, striatum en hippokampus en NAS het hierdie veranderinge in die striatum en hippokampus omgekeer. Lipiedperoksidase was verhoog in die twee-tref model in die frontale korteks en striatum en NAS het dit in die striatum omgekeer. Die LPS-alleen en twee-tref groepe het beduidend-verhoogde vlakke van plasma ROS-vlakke geïnduseer. NAS het hierdie verhoging suksesvol omgekeer in die prenatale LPS groep. MA toediening op sy eie het geen veranderinge aan merkers van oksidatiewe stres meegebring nie.

Frontale kortikale dopamien (DA)-vlakke was beduidend verhoog in die LPS-alleen groep, en is met behulp van NAS-behandeling omgekeer. Beide die LPS-alleen en die twee-tref groepe het beduidende onderdrukking van serotonien-vlakke veroorsaak in die frontale korteks. NAS het ook hierdie veranderinge omgekeer. Frontaal-kortikale en striatale noradrenalin (NA) vlakke was onderduk in die LPS-alleen en die twee-tref model, sowel as na MA-alleen toediening. NAS het hierdie veranderinge in beide breinareas in albei skisofrenie diemodelle omgekeer.

Die LPS-alleen en die twee-tref modelle het die vlakke van die anti-inflammatoriese sitokien, interleukin-10 (IL-10), beduidend verlaag. Die LPS-alleen groep het ook die vlakke van die pro-inflammatoriese sitokien, tumor nekrose faktor alfa (TNF- α) beduidende verhoog. NAS behandeling het die verlaagde vlakke van IL-10 in die LPS-alleen en die twee-tref model genormaliseer, en ook die verhoogde TNF- α vlakke in die LPS-alleen model.

Gevolgtrekking:

Hier bewyse ons dus dat beide die prenatale inflammasie en 'n twee-tref model gedragsveranderinge soortgelyk aan skisofrenie simptome kan veroorsaak. Prenatale inflammasie het ook immuun-redoks veranderinge meegebring, terwyl die twee-tref model slegs noemenswaardige verskille in redoks-status veroorsaak het. Onreëlmatighede in die

vlakke van belangrike monoamiene bevestig dat hierdie skisofrenie modelle neuro-funksie kan verander. Ons stel voor dat prenatale inflammasie brein ontwikkeling kan verander en 'n verhoogde skisofrenie-risiko tot gevolg kan hê. Gekoppel met 'n tweede trefslag, raak kognitiewe veranderinge meer prominent, wat moontlik daarop dui dat 'n twee-tref etiologie betrokke mag wees in skisofrenie se totale manifestasie. NAS het voordelige effekte op gedrags-, neurochemiese en redoks-inflammatoriese merkers getoon, wat 'n aanduiding is van die moontlike waarde van anti-oksidatiewe behandeling van skisofrenie, asook die moontlike waarde daarvan in die behandeling van MA-misbruik geassosieerde psigoses.

Sleutelwoorde: skisofrenie, prenatale infeksie, twee-tref hipotese, metamfetamien, oksidatiewe stres, N-asetielsisteïen

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Regret is mostly caused by not having done anything. - Charles Bukowski

Congress proceedings

Excerpts from this study were presented as:

T. Swanepoel, M. Möller, O. Dean, M. Berk, G. Wegener, D.J. Stein, B.H. Harvey

Evaluation of N-acetyl cysteine (NAC) on methamphetamine-associated sensorimotor gating changes in an immune-inflammatory model of schizophrenia

Podium presentation at the South African Society for Basic and Clinical Pharmacology and Toxicology SA congress from 31 August to 2 September 2015 at the Wits Club, Johannesburg, South Africa.

Published work

The following review article has been published:

Möller, M.; Swanepoel, T.; Harvey, B.H.

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List of abbreviations

3HK: 3-hydroxy-kynurenine

3-OHAA: 3-hydroxyanthranilic acid

5-HIAA: 5-hydroxyindoleacetic acid

5-HT: 5-hydroxytryptamine (serotonin)

5-HTT: Serotonin transporter

$\alpha 7$ nAChR: Nicotinic $\alpha 7$ acetylcholine receptor

ACh: Acetylcholine

ADHD: Attention deficit hyperactivity disorder

AKT1: V-akt murine thymoma viral oncogene homolog 1

AMPA: α -amino-3-hydroxy-5-methyl-4-isoazolepropionic acid

ATP: Adenosine triphosphate

BBB: Blood brain barrier

CB1: Cannabinoid receptor 1

CNS: Central nervous system

CSF: Cerebrospinal fluid

CSP: Cavum septum pellucidum

CT: Computed tomography

DA: Dopamine

DAO: D-amino-acid oxidase

DAOA: D-amino-acid oxidase activator

DAT: Plasmalemmal dopamine transporter

DEPPD: N,N-diethyl-para-phenylendiamine

DISC1: Disrupted in schizophrenia 1

DOPAC: 3,4-Dihydroxyphenylacetic acid

dROM: Derivatives of reactive oxygen metabolites

DSM: The Diagnostic and Statistical Manual of Mental disorders

DTNBP1: Dystrobrevin-binding protein 1 (DTNBP1) (Dysbindin)

EDTA: Ethylenediaminetetraacetic acid

ELISA: Enzyme linked immunosorbent assay

ETC: Electron transport chain

GABA: Gamma-amino butyric acid

GAD: Glutamic acid decarboxylase

GD: Gestational day

GIT: Gastro-intestinal tract

GPx: Glutathione peroxidases

GSH: Glutathione

HPLC: High performance liquid chromatography

HPLEC: High performance liquid chromatography with electrochemical detection

HVA: Homovanillic acid

IDO: Indoleamine 2,3-dioxygenase

IFN: Interferon

iGluRs: Ionotropic glutamate receptors

IL: Interleukin

IV: Intravenously

Kainate: 2-carboxy-3-carboxymethyl-4-isopropenylpyrrolidine

KMO: Kynurenine-3-monooxygenase

KYN: Kynurenine

KYNA: Kynurenic acid

LPS: Lipopolysaccharide

LSD: Lysergic acid diethylamide

MA: Methamphetamine

mAChR: Metabotropic muscarine receptors

MAO: Monoamine oxidase

MDA: Malondialdehyde

MDMA: 3,4 methylenedioxymethamphetamine

mDNA: Mitochondrial DNA

mGluRs: Metabotropic glutamate receptors

MHPG: 3-Methoxy-4-hydroxyphenylglycol

MIA: Maternal immune activation

MK-801: Dizocilpine

MRI: Magnetic resonance imaging

Na₂EDTA: ethylenediaminetetraacetic acid disodium salt

NA: Noradrenaline

NAC: N-acetyl cysteine

nAChR: Ionotropic nicotine receptors

NAD Nicotinamide adenine dinucleotide

NET: Noradrenaline transporter

NFκB: Nuclear factor kappa B

NO: Nitric oxide

nORT: Novel object recognition test

NOS: Nitric oxide synthase

NMDA: N-methyl-d-aspartate

NRG1: Neuregulin 1

OC's: Obstetric complications

PANSS: Positive and Negative Symptoms Scale

PBS: Phosphate buffered solution

PCP: Phencyclidine

PET: Positron emission tomography

PFC: Prefrontal cortex

Poly I:C: Polyinosinic:polycytidylic acid

PPI: Prepulse inhibition

PUFA's: Polyunsaturated fatty acids

PV: Parvalbumin

QUIN: Quinolinic acid

rCBF: Regional cerebral blood flow

RELN: Reelin

RGS4: Regulator of G-protein signalling 4

Rh: Rhesus factor

RNS: Reactive nitrogen species

ROS: Reactive oxygen species

SERT: Serotonin transporter

SI: Social interaction

SOD: Superoxide dismutase

TBARS: Thiobarbituric acid reactive substances

TDO: Tryptophan 2,3-dioxygenase

TGF: Transforming growth factor

TLR: Toll-like receptor

TNF: Tumor necrosis factor

VMAT-2: Vesicular monoamine transporter-2

VTA: Ventral tegmental area

WCST: Wisconsin Card Sorting Test

Glossary

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

Apoptosis: Programmed cell death.

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).

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

Cation: A positively charged ion

Cavum septum pellucidum (CSP): A neuro-embryological marker that appears when the septal leaflets in the midline of the brain do not close completely during the first 6 months of life

Centrilobular degeneration: Deterioration at / close to the center of a lobule, e.g. of the liver

Coronal involvement: Involving the crown of the teeth

Endophenotypes: Measurable behavioural, biological or cognitive markers that are more frequently observed in individuals with a disease than in the common population. Frequently used as a diagnostic tool or for aetiological research purposes.

Eye enucleation: The removal of the eye.

Formication: An abnormal skin sensation similar to that of insects crawling in or over the skin

Hemispheric: adjective originating from “hemisphere”. Refers to half of a symmetrical, nearly spherical object.

Interstitial nephritis: A kidney disorder characterized by swelling in between the kidney tubules.

Monozygotic: Identical twins, i.e. twins resulting from one fertilized ovum.

Myoglobinuria: The presence of myoglobin in the urine.

Necrosis: The death of cells or tissue.

Necrotizing angitis: An inflammatory reaction of blood vessels which results in fibrinoid necrosis of tissue, especially of the blood vessel wall.

Nuclear factor kappa B (NFκB): A collection of proteins that play a vital role in inflammation and the stress response via control of gene network expression.

Parvalbumin (PV): Calcium (Ca)-binding albumin proteins of low molecular weight

Plasmalemma: Situated on a plasma membrane.

Pleiotropic: Refers to genes that has multiple phenotypic expressions (or effects)

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

Probandwise: A measure of twin concordance rates. Measures the proportion of twins with an illness who have an affected twin.

Rhabdomyolysis: A disorder in which injury to muscle results in myocyte membrane rupture, causing intracellular contents to leak into the plasma.

Salience: A process whereby events and thoughts come to attract ones attention, motivate action, and inspire goal-directed behaviour because of their association with reward/punishment

Schizoid/schizotypal personality: Personality disorders belonging to the group of cluster A personalities and which shares certain symptoms with schizophrenia

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

Vacuolatype H⁺-pumping ATPase: an ATP-driven enzyme which converts the energy of ATP hydrolysis to electrochemical potential differences of protons across assorted biological membranes via the primary active transport of H⁺

Xerostomia: Abnormal dryness of the mouth.

Chapter 1: Introduction

This dissertation has been composed and submitted in article format for thesis / dissertation submission, as approved by the North-West University. This format includes: (1) an introductory chapter, (2) a chapter with an appropriate literature review, (3) a chapter comprising one full length concept article for submission to a peer-review scientific journal, (4) a recently published review paper (Möller *et al.*, 2015:987) that has been based on this and other work coming from this laboratory, and finally (5) a chapter with concluding remarks with regards to the study, as well as future avenues and recommendations. The article contained in this dissertation has been meticulously prepared to contain the most fresh and relevant data from the study. With the aim to submit and publish this article in a neuroscientific journal, the article will be prepared / modified to suit the author's instructions and in-house style of the particular journal. All other work completed through the course of this study, if any, will be included in the addenda.

1.1 Problem statement:

Up to 1% of the world population is affected by schizophrenia, which is notorious for its progressive nature and complicated aetiology (Laruelle, 2014:97). This neurodegenerative disease is characterized by three main domains of symptoms, namely positive, negative and cognitive symptoms. Positive symptoms include hallucinations, delusions and paranoia, while negative symptoms comprise apathy, depression and social withdrawal (Meyer, 2013:20). Memory impairment, deficits in sensorimotor gating and attention disorders, among others, comprise the cognitive symptoms of the illness (Meyer, 2013:20). In addition, certain patients also present with other symptomalogical manifestations, such as aggression and violent behaviour (Fleischman *et al.*, 2014:3051; Soyka, 2011:913).

As of late, the implication of neurodevelopmental insults in schizophrenia development have extensively been focussed on in the research field (Rapoport *et al.*, 2012:1228). Environmental factors during the prenatal period, e.g. infection, nutritional deficiencies or obstetrical complications, as well as postnatal influences, such as drug abuse or childhood trauma, have all been implicated as possible role-players in schizophrenia development (Brown, 2011:23; Lewis & Levitt, 2002:409). Several infectious agents have been shown to interfere with foetal neurodevelopment, leading to behavioural alterations and brain abnormalities (Remington *et al.*, 2006:1313). This led to the hypothesis that maternal / foetal

immune activation during gestation may contribute to schizophrenia manifestation (Meyer, 2013:20). Both preclinical (reviewed in Möller *et al.*, 2015:987) and epidemiological studies (reviewed in Brown & Derkits 2010:261) have reported on schizophrenia-related changes in offspring prenatally exposed to infection / immune factors. Moreover, the two-hit hypothesis of schizophrenia suggests that a genetic predisposition combined with a specific developmental insult can prime a person for a later-life insult, eventually giving way to schizophrenia presentation (Bayer *et al.*, 1999:543). Seeing that genetic studies remain inconsistent, researchers have suggested that environmental factors during the critical prenatal period may also play the role of first hit (Meyer & Feldon, 2010:285).

Methamphetamine (MA) is a highly addictive central nervous system (CNS) stimulant and is regarded as the second most abused drug globally (Barr *et al.*, 2006:301; Cruickshank & Dyer, 2009:1085). MA-abuse is associated with dopamine (DA) neurotoxicity (Grace *et al.*, 2010:346; Imam & Ali, 2001:952) and redox changes (Darke *et al.*, 2008:253), while associated psychopathologies include psychosis, increased aggression, cognitive deficits and so forth (Darke *et al.*, 2008:253; Granado *et al.*, 2013). These changes may normalize with successful drug rehabilitation, but chronic cases of psychosis have been reported (Chen *et al.*, 2003:1407). Subsequently, studies have proposed that abusers, who experience prolonged psychosis, also exhibited schizophrenia-like symptoms during childhood even before drug abuse (Chen *et al.*, 2005:87). This implies that certain individuals may be susceptible to a second-hit (such as MA-abuse) which could lead to schizophrenia manifestation.

Taking these findings into consideration, we set out to develop two animal models of schizophrenia: a prenatal inflammation model (via prenatal lipopolysaccharide (LPS) administration) and a dual-hit model (via prenatal LPS administration, coupled with postnatal, chronic MA administration). Seeing that schizophrenia and MA-abuse present with oxidative stress, we theorized that an anti-oxidant will have beneficial effects in these animal models. To test this hypothesis, we incorporated N-acetyl cysteine (NAC) in our study, as novel treatment for schizophrenia management.

1.2. Hypothesis, aims and objectives:

Hypothesis:

Changes in redox balance have been noted in prenatal inflammation models of schizophrenia using LPS (Lanté *et al.*, 2007:1231; Zhu *et al.*, 2007:671). Consequently, our first hypothesis was that prenatal LPS administration to rodents will elicit oxidative changes in the brain (lipid peroxidation via measurement of malondialdehyde or MDA) and peripherally (total reactive oxygen species (ROS)) in the offspring. MA has also been shown to cause oxidative stress in animal models (Frey *et al.*, 2006:275; Shivalingappa *et al.*, 2012; Yamamoto & Zhu, 1998:107) and as such we speculated that MA alone will induce similar oxidative changes and that in combination with prenatal LPS, it will worsen these alterations. NAC treatment was only applied to the animal model related specifically to schizophrenia aetiology, i.e. the prenatal LPS model and the dual-hit model (LPS+MA), which were eventually the only models that revealed definite changes in oxidative status thus arguing in favour of using an anti-oxidant to reverse the ensuing bio-behavioural changes. We hypothesized that NAC will be able to significantly reduce or completely reverse oxidative changes in the relevant animal models, and thus have the potential to reverse associated behavioural changes as well.

Studies using various approaches of prenatal inflammation have witnessed schizophrenia-related behaviours in rodents, supporting the face validity of these models (Arsenault *et al.*, 2014; Shi *et al.*, 2003:297; Zuckerman & Weiner, 2005:311). Behavioural changes specifically induced by prenatal LPS administration include deficits in prepulse inhibition (PPI), memory alterations and changes in social interaction (SI) (reviewed in Möller *et al.*, 2015:987). Regarding changes in SI, the literature provides mixed results: several studies found that LPS was able to reduce social behaviour in rodents (Kirsten *et al.*, 2010:240; Oskvig *et al.*, 2012:623), in line with other models of schizophrenia (Möller *et al.*, 2011:471; Rung *et al.*, 2005:827; Shi *et al.*, 2003:297); however, others have found increased levels of social interaction in rodents that received LPS prenatally (Harvey & Boksa, 2014:27). Subsequently, we theorized that the prenatal LPS model, as well as the postnatal MA model will reveal alterations in SI (whether increased or decreased), as well as impairments in %PPI and visual memory (indicated in the novel object recognition test (nORT)). In addition, we expected that the two-hit model, viz. prenatal LPS + postnatal MA, will worsen these behavioural deficits in rodents. We hypothesized that anti-oxidant treatment with NAC will effectively reduce or totally reverse these changes in the applicable animal models.

Regarding neurochemical changes, animal models of prenatal inflammation (reviewed in Möller *et al.*, 2015:987), as well as models of MA abuse (Friedman *et al.*, 1998:35; Kitanaka *et al.*, 2003:63; Suzuki *et al.*, 1997:359) have revealed alterations in the brain concentrations of important monoamines. Consequently, we posited that the LPS-alone model and the MA-alone model will cause significant changes in the brain levels of DA, noradrenaline (NA) and serotonin, when compared to controls. We again expected the dual-hit model to exacerbate these changes. We hypothesized that NAC treatment will reduce or fully reverse monoamine changes in the relevant models.

Finally, prenatal studies using LPS have noted deviations in the inflammatory profile of these animals when compared to controls (reviewed in Möller *et al.*, 2015:987). Studies of MA abuse have also found changes in the levels of pro-and anti-inflammatory markers (Loftis *et al.*, 2011:59; Sekine *et al.*, 2008:5756). Accordingly, we anticipated that both the LPS-alone and the MA-alone models will elicit changes in the peripheral levels of the pro-inflammatory cytokine, tumor necrosis factor- α (TNF- α) and the anti-inflammatory cytokine, interleukin-10 (IL-10). We speculated that the dual-hit model will worsen these alterations. Again, we hypothesized that treatment with NAC will reduce or reverse these changes in the applicable animal models.

Aims:

Our first aim was to establish whether a prenatal LPS model of schizophrenia and a postnatal MA model of psychosis are associated with redox irregularities. Together with this, we aimed to assess whether a dual-hit model of prenatal LPS combined with postnatal MA abuse will exacerbate these oxidative changes. For the treatment leg of the study we only included the models that significantly altered the redox balance in rodents, and which have the specific aim of modelling schizophrenia aetiology. Our second aim was to establish whether the prenatal LPS alone and postnatal MA alone models could induce schizophrenia-related behaviours and regional brain monoamine changes, as well as cause alterations in peripheral pro- and anti-inflammatory cytokine levels. We then aimed to assess whether the dual-hit model could aggravate these changes, when compared to the single-hit models. Finally, we intended to establish whether treatment with an anti-oxidant, NAC, can reduce or reverse both behavioural and biochemical changes in these models.

Objectives:

- Establish whether prenatal LPS administration on gestational day (GD) 15-16 can induce oxidative stress in offspring in later life, akin to redox changes observed in schizophrenia.

- Establish whether postnatal administration of MA (relating to MA abuse) can induce oxidative stress, akin to redox changes observed in schizophrenia.
- Determine whether a dual-hit model can exacerbate oxidative changes in rodents.
- By only including the models that are able to induce redox imbalances, establish whether NAC can reduce or reverse oxidative stress in these models.
- Establish whether a prenatal LPS alone model and a postnatal MA alone model can cause abnormalities in SI, %PPI and visual memory, akin to schizophrenia symptomology, and whether the dual-hit model will worsen these changes.
- Establish whether a prenatal LPS alone model and a postnatal MA alone model can alter monoamine levels in the brain tissue of animals, and whether the dual-hit model will worsen these changes.
- Establish whether a prenatal LPS alone model and a postnatal MA alone model can cause changes in the pro-and anti-inflammatory cytokine profile of rodents, and whether the dual-hit model will worsen these changes.
- Determine whether NAC can reduce or reverse behavioural, redox-inflammatory and neurochemical alterations in the prenatal inflammation and the dual-hit animal models of schizophrenia.

1.3 Project layout:

The study initially consisted of 32 dams, divided into two groups, groups A and B respectively, as follows (see Figure 1):

- A. Saline (n=8)
- B. Immune-inflammatory model (LPS) (n=24).

The dams in groups A and B received either 0.2 ml saline (group A) or LPS (group B) from GD 15-16. These gestational days were chosen on 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 window for the development of schizophrenia (Fortier *et al.*, 2007:270). Male offspring from the above groups were then used for the remainder of the study. Cross fostering was not performed, as per findings of Fortier and colleagues (2007:270).

Using the male offspring from dams in Groups A and B, we attempted to determine the behavioural and biochemical response to chronic early adolescent exposure to MA in

healthy animals (dams receiving prenatal saline) or animals with a history of pre-natal maternal inflammation (dams receiving LPS) (dual-hit model) (Figure 1). Thus, the offspring from both Group A and B was subdivided into either a MA or saline receiving group for 16 days until post natal day (PND) 50 (Strauss *et al.*, 2014:18). MA was administered at a dose of 0.2 mg/kg escalating daily up to a final dose of 6 mg/kg subcutaneous (SC) from PND 35 to 50 (Strauss *et al.*, 2014:18). After MA administration, offspring from group A was housed in home cages until the coinciding day of behavioural tests for group B (see Fig 1). Group B, on the other hand, was further divided into two treatment groups, viz. saline or NAC (150 mg/kg, SC) (Möller *et al.*, 2013:156). Drug treatment continued for 14 days from PND 51 – PND 64 (Möller *et al.*, 2011:471).

After treatment, all groups was subjected to a battery of behavioural tests that follow a specific sequence to minimise stress on the animals, viz. (1) SI on day 12 of drug administration (PND 62), (2) nORT on day 13 of drug administration (PND 63), and (3) %PPI on the last day of drug administration (PND 64) (Möller *et al.*, 2013:156). One day later the animals was decapitated and trunk blood and brain tissue was collected and stored at -80 °C until the day of neurochemical analysis. A complete flow chart of the study design and timeline is illustrated in Figure 1.

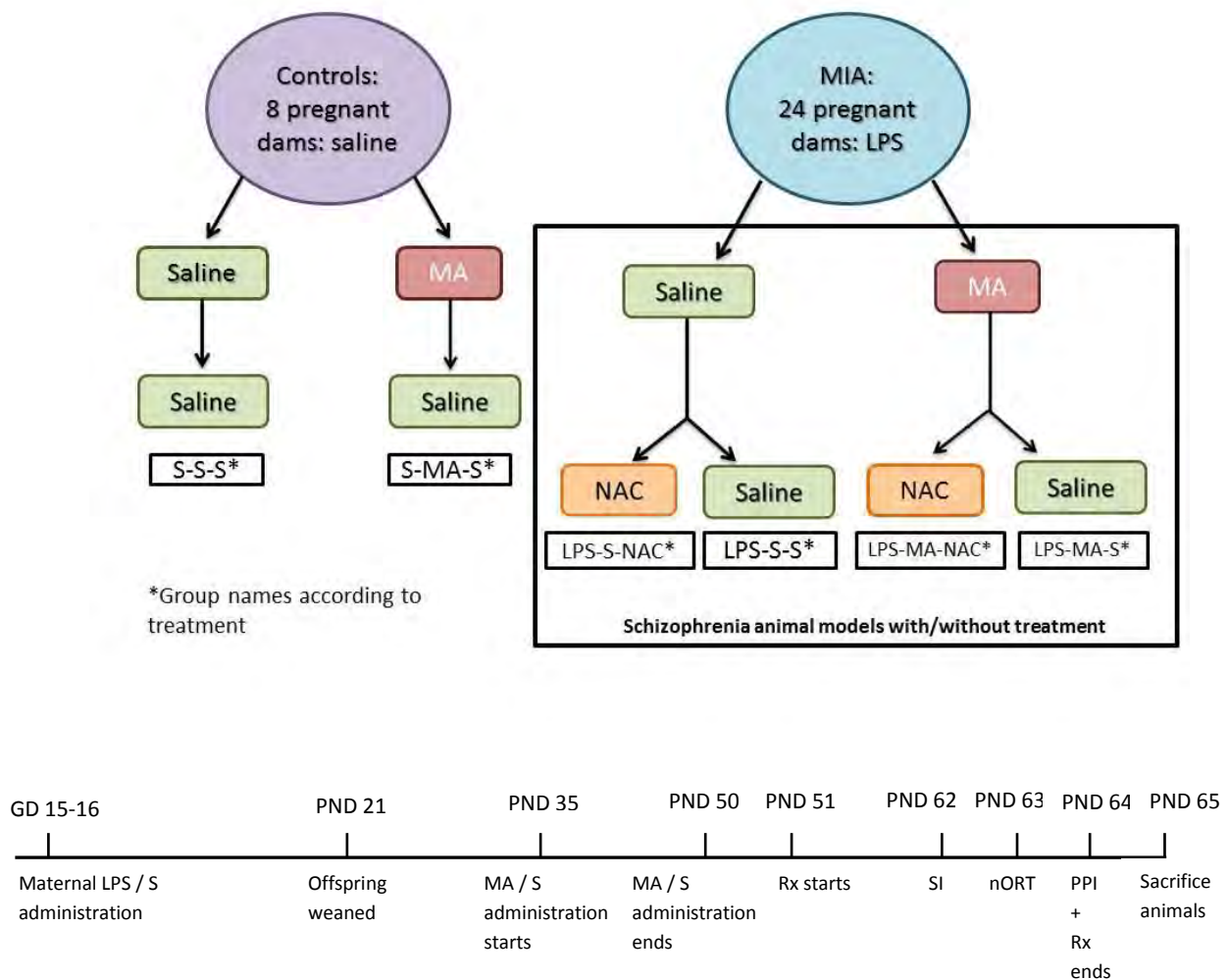


Figure 1: Graphic presentation of the study design and timeline of all treatments and analyses (MIA = maternal immune activation; LPS = lipopolysaccharide; MA = methamphetamine; NAC = N-acetyl cysteine; S: saline; GD: gestational day; PND: postnatal day; Rx: treatment; SI: social interaction; nORT: novel object recognition test; PPI: prepulse inhibition).

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Chapter 2: Literature review

2.1. Introduction: Schizophrenia

The German psychiatrist, Emil Kraepelin (1856–1926) was the first to define schizophrenia, referring to it as “Dementia praecox” (Decker, 2004:248; Piotrowski & Tischauser, 2013). He described the disease as an endless worsening condition with continuous mind degeneration until death (Piotrowski & Tischauser, 2013). The term “schizophrenia” was later attributed to the syndrome by the Swiss psychiatrist, Eugen Bleuler (1857–1939), who in 1908 named it “Die Prognose der Dementia Praecox (Schizophrenie gruppe)” (Kaplan, 2008:305). Bleuler, a dedicated psychiatrist, rejected the view of Kraepelin, believing that persistent deterioration does not always take place, promising more hope for sufferers of schizophrenia (Piotrowski & Tischauser, 2013). In the present day, Piotrowski (2013) defines schizophrenia as “a disorder characterized by disordered thinking and odd perceptions that cause dysfunction in major activities, sometimes including withdrawal from the world, delusions, and hallucinations”.

Schizophrenia is a psychiatric disorder ranked among the world’s top ten causes of long-term disability, characterised by perceptual, cognitive and behavioural disturbances, culminating in impaired social functioning (Harris *et al.*, 2013:752). The annual prevalence of schizophrenia averages 15 per 100 000, and the risk of developing the illness over one's lifetime averages 0.7% (Tandon *et al.*, 2008:1). Schizophrenia does not just affect mental health; patients with a diagnosis of schizophrenia die 12–15 years before the average population, with this mortality difference increasing in recent decades (van Os & Kapur, 2009:635). Thus, schizophrenia causes more loss of lives than do most cancers and physical illnesses (van Os & Kapur, 2009:635). Although some deaths are suicides, approximately 60% of mortalities in schizophrenia can be attributed to effects of physical illness (Brown, 1997:502), such as metabolic syndrome (Harris *et al.*, 2013:752; van Os & Kapur, 2009:635).

Schizophrenia causes disruptions in thought processes, perceptions, and emotions (Maynard *et al.*, 2001:457), while the deviating symptoms of schizophrenia are grouped into three separate clusters, namely positive, negative and cognitive symptoms (Meyer, 2013:20). Positive symptoms include hallucinations, paranoia and delusions, negative symptoms comprise apathy, depression and social withdrawal, while memory impairment and attention disorders comprise the cognitive symptoms of the illness (Meyer, 2013:20).

The effects of this disorder are profound; however there does not appear to be a single neurobiological cause (Maynard *et al.*, 2001:457). It is hypothesized that the pathogenesis of schizophrenia is dependent upon an interaction between genetic and environmental factors (Meyer & MacCabe, 2012:586), although up till now the exact mechanisms of these factors remain a mystery.

The treatment of schizophrenia rests upon the administration of first and second generation antipsychotics (Leucht *et al.*, 2013:951). Despite the wide array of treatments currently available, effective management of all of the symptoms of schizophrenia continues to be an illusory goal, especially with regards to the alleviation of negative symptoms (Buckley & Stahl, 2007:93; Erhart *et al.*, 2006:234). Because of the prevalence, unclear ethology and somewhat ineffectiveness of treatment options for schizophrenia, this field of study is rendered crucial for scientists in numerous different research areas, and especially in pharmacology and drug discovery.

2.1.1. Aetiology and epidemiology

According to the World Health Organisation (WHO), epidemiology can be defined as “the study of the distribution and determinants of health-related states or events (including disease), and the application of this study to the control of diseases and other health problems” (WHO, 2014). A review of studies relating to the spatial distribution (overall incidence and prevalence) of schizophrenia, found a mean incidence rate of 15.2 per 100 000 persons (McGrath *et al.*, 2008:67). Additionally, it was observed that the incidence of schizophrenia tends to be higher in males than females (McGrath *et al.*, 2008:67).

In a review of prevalence studies, Saha and colleagues found a median point prevalence of 4.6 per 1 000, and a lifetime prevalence estimate of 4.0 per 1 000 (Saha *et al.*, 2005:413). This data highlights the widespread impact of this life-altering disease. Interesting is that several studies have drawn an association between childbirth during early spring and winter, showing that this period elevates the risk for developing schizophrenia by up to 10% (Mortensen *et al.*, 1999:603; Schwartz, 2011:785; Torrey *et al.*, 1997:1).

Today schizophrenia is considered to be a polygenic condition with a mixture of contributing environmental risk factors, although the aetiology and pathophysiology have not been fully revealed (Harris *et al.*, 2013:752). Indeed, the core of epidemiology is shaped by the determinants of a disease. When this is applied to schizophrenia, it includes both genetic

and environmental risk factors, with neither of these features contributing to schizophrenia independently (Tsuang *et al.*, 2004:73).

2.1.1. a) Genetic factors:

A role for genetics in the aetiology of schizophrenia has been suggested after a great number of family, twin and adoption studies have noted a role for inheritable factors in disease development (Brown, 2011:23). Thus, an increased risk for schizophrenia has been observed for the identical twin of a schizophrenia patient, while a child born from two schizophrenics has the highest risk (Tsuang, 2000:210). An overall heritability estimate of 82% implies that the majority of the variance in liability is genetic (Cardno *et al.*, 1999:162). In the course of genetic studies, a number of endophenotypes have been suggested for schizophrenia, with at least 15 genetic loci identified that may be connected to schizophrenia (Riley & McGuffin, 2000:23). However, none of these studies have been able to provide replicated data with which specific liable genes may be identified (Owen *et al.*, 2005:518). Presently, the genes that are most frequently implicated in schizophrenia development include (Fig. 1): disrupted in schizophrenia 1 (DISC1) which is responsible for gene expression, intracellular transport and neuronal migration, formation and cell signalling (Hodgkinson *et al.*, 2004:862); V-akt murine thymoma viral oncogene homolog 1 (AKT1) involved in various neural functions, importantly N-methyl-d-aspartate (NMDA) receptor signalling and the expression of long-term potentiation (Chen & Lai, 2011:178); gene G72, D-amino-acid oxidase (DAO) and D-amino-acid oxidase activator (DAOA), all three linked and responsible for the oxidation of D-serine, a co-agonist at NMDA receptors (Madeira *et al.*, 2008:76) and regulator of G-protein signalling 4 (RGS4) which is vital for modulating signaling through G-protein pathways (De Vries *et al.*, 2000:235; Meyer & Feldon, 2010:285; Owen *et al.*, 2005:518). Strong evidence point specifically to genes encoding dysbindin (DTNBP1), a conserved protein widely expressed in the human brain but for which detailed knowledge is still limited (DeRosse *et al.*, 2006:532), as well as neuregulin 1 (NRG1), a pleiotropic growth factor involved in synaptogenesis, gliogenesis, myelination, neuronal migration, neuron-glia communication, and neurotransmission (Li *et al.*, 2006:1995; Meyer & Feldon, 2010:285; Owen *et al.*, 2005:518). However, the evidence is not yet entirely convincing and the field of genetics in schizophrenia remain cryptic. Furthermore, the recorded observations of an increased risk profile in related individuals are not adequate, as relatives share not only genes, but also environments (Tsuang, 2000:210). It is therefore suggested that environmental risk factors may be required in genetically vulnerable individuals in order to lead to the complete expression of schizophrenia (Brown, 2011:23).

2.1.1. b) Environmental factors:

Human anatomy, physiology, and metabolism are directed by several interactions that are predetermined by the individual's genes and his/her environment (Tsuang, 2000:210). Environmental factors are known as powerful disruptors of brain development and as such are recognized as possible causes of neuropsychiatric disorders, such as schizophrenia (Brown, 2011:23). These factors may include drug abuse, brain disorders, psychosocial effects, infections, nutritional deficits, neurotoxins and pre- or peri-natal complications (Fig. 1) (Brown, 2011:23; Tsuang, 2000:210). Notably, most of these environmental factors play a crucial role during pre- and/or perinatal stages of life during critical stages of CNS development (Meyer & Feldon, 2010:285). Indeed, maternal insults during pregnancy have been implicated in the development of a variety of physiological and behavioural changes in the offspring, e.g. low birth weights, cardiovascular and neuroendocrine abnormalities, as well as social, depressive and anxiety-related behaviours (Seckl, 2004:U49; Weinstock, 2001:427). As such, neurodevelopmental factors may be regarded as one of the most influential factors in the development of schizophrenia risk and will therefore be discussed in greater detail under heading 1.4.4: "Neurodevelopmental insults".

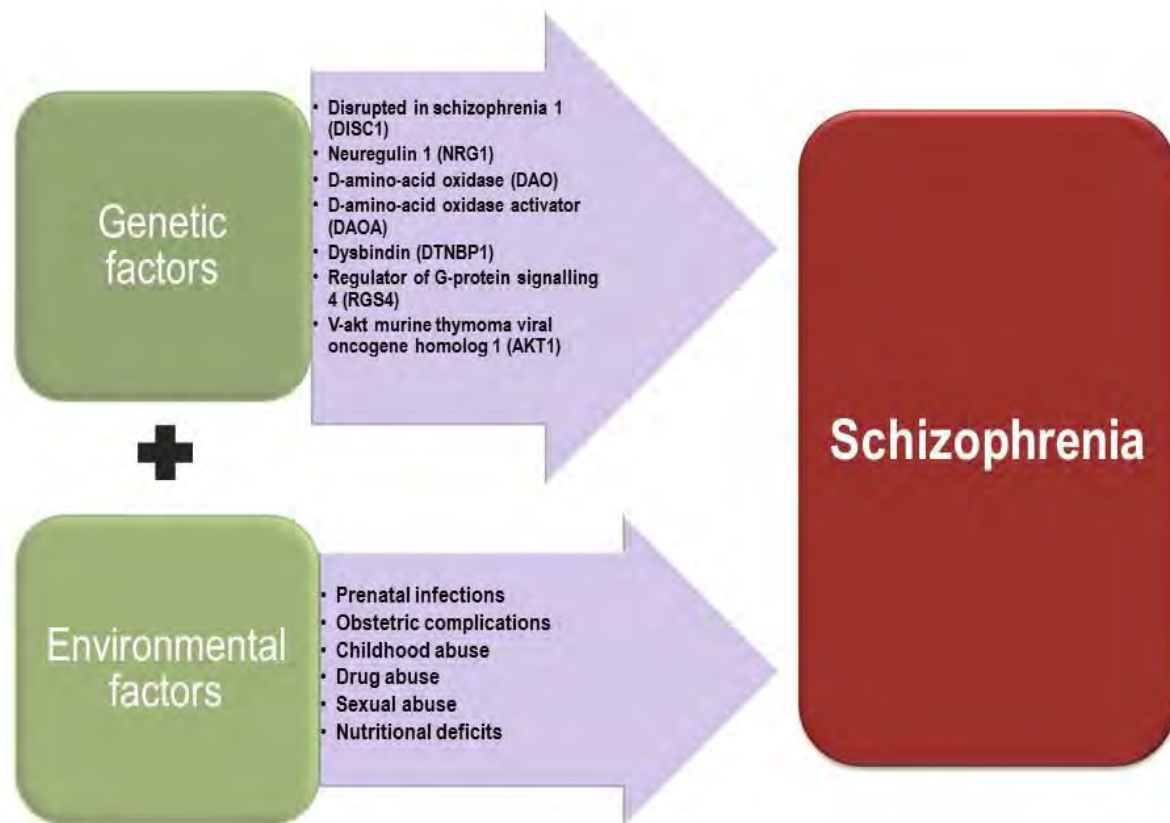


Figure 1: The interplay between several genetic and environmental factors in the development of schizophrenia

2.1.2. Clinical description and symptoms

“The patients... see mice, ants, the hound of hell, scythes and axes. They hear cocks crowing, shooting, birds chirping, spirits knocking... The patient feels himself destined to great things, works beside royalty, can put anyone into prison, speaks many languages, is to be a professor, is getting an inheritance from the Australian Kaiser, possesses fifty estates, millions...” (Decker, 2004:248). This describes extracts from the book, *Dementia Praecox*, wherein the early psychiatrist Kraepelin describes the madness schizophrenia patients must endure. Although this description may seem overly dramatic, it highlights the immense impact this disease has on sufferers.

Schizophrenia is known to originate during early stages of development (Weinberger, 1995:552), with deficits in social interaction, verbal memory, motor skills, and attention during childhood implicated as early signs of disease development (Erlenmeyer-Kimling *et al.*, 2000:188; Niemi *et al.*, 2003:239). A sub-clinical prodromal phase usually precedes full-

blown disease expression, with signs such as altered mood, occupational withdrawal, loss of interest in activities, change in personality and altered social nature / social withdrawal (Meyer & MacCabe, 2012:586). Explicit symptoms of schizophrenia tend to manifest itself only after the beginning of puberty (Maynard *et al.*, 2001:457), usually in the form of delusions and hallucinations (defined below) (Haas & Sweeney, 1992:373). However, in some cases it may manifest itself at varying ages ranging from early childhood to old age (Ross *et al.*, 2006:139). Schizophrenia is regarded as a neurodegenerative disorder, implicating that it is progressive in nature. Illness deterioration is usually evident by an increasing number and severity of psychotic, as well as negative symptoms (description to follow), cognitive impairment, and reduced social and functional ability (Lieberman, 1999:729). The probability that a patient will experience disease progression and psychological decline may be directly correlated to the frequency of early psychotic symptoms (Wyatt, 1991:325).

Schizophrenia is a heterogeneous syndrome, lacking a single defining symptom or sign, or a clear-cut diagnostic laboratory test (Ross *et al.*, 2006:139). The clinical manifestation of schizophrenia is characterized by three distinct collections of symptoms, referred to as positive, negative and cognitive symptoms (Fig. 2) (Meyer, 2013:20).

2.1.2. a) *Positive symptoms:*

Positive symptoms (as indicated in Fig. 2) are usually the first indication of schizophrenia and refers to psychotic manifestations, such as auditory hallucinations, delusions and changes in psychomotor activity (Gonzalez-Burgos *et al.*, 2011; Lieberman, 1999:729; Piotrowski & Tischauser, 2013). A hallucination is defined as a perception that comes about without an appropriate stimulus, having the impact of matching real perception, but is out of the direct control of the person who is experiencing it (Slade & Bentall, 1988). Auditory hallucinations usually occur as voices making remarks about the patient, speaking among themselves or, most commonly, being hostile and critical towards the patient (Tandon *et al.*, 2009:1). A delusion, on the other hand, is a true perception to which a person attributes an untrue meaning (Schneider, 1959). These symptoms are usually witnessed during the acute psychotic state, and include formal thought disorder (disintegration of logical, goal-orientated thought processes), paranoia and loss of control over normal actions, that may accompany the above mentioned manifestations (Andreasen, 1979:1315; Meyer & MacCabe, 2012:586).

2.1.2. b) *Negative symptoms:*

The term “negative symptoms” is ascribed to symptoms that are regarded as a loss or absence of normal functions (Andreasen, 1995:477). These symptoms include reduced

sociability, poverty of speech, lack of drive / motivation (apathy) and a reduced ability to experience pleasure (anhedonia) (Brisch *et al.*, 2014:47; Gonzalez-Burgos *et al.*, 2011), as indicated in Figure 2. Patients may furthermore reveal reduced emotional expression and response, withdrawal from interpersonal relationships, and lack of facial expression (Piotrowski & Tischauser, 2013; Ross *et al.*, 2006:139). Initially these symptoms tend to be less common and more challenging to observe. However, with disease progression, negative symptoms tend to increase in prevalence and severity, usually presenting itself as a chronic phase during and in-between acute psychotic episodes (Lieberman, 1999:729; Meyer & MacCabe, 2012:586; Piotrowski & Tischauser, 2013). Although negative symptoms may be less readily observed than positive manifestations, it commonly proves to be more impairing and resistant to treatment (Ross *et al.*, 2006:139).

2.1.2. c) *Cognitive symptoms:*

The third sphere of symptoms (indicated in Fig. 2), viz. cognitive impairments, is also less explicit but can play a major role in the disablement of the schizophrenia patient (Meyer & MacCabe, 2012:586). Symptoms such as deficits in working memory, attention, sensory processing, executive and language functioning and social emotional processing are usually chronic and independent from positive symptoms (Gonzalez-Burgos *et al.*, 2011; Meyer & MacCabe, 2012:586). Loss of executive function leads to an absence in planning abilities, inhibition, mental flexibility and the commencement and monitoring of activities (Chan *et al.*, 2008:201). Cognitive symptoms, which remain difficult to treat with customary antipsychotics, ultimately lead to an inability to exercise control over one's life, together with the incapacity to work effectively and consecutively (Simpson *et al.*, 2010:585), drastically affecting the quality of life of the patient and his/her contribution to society.

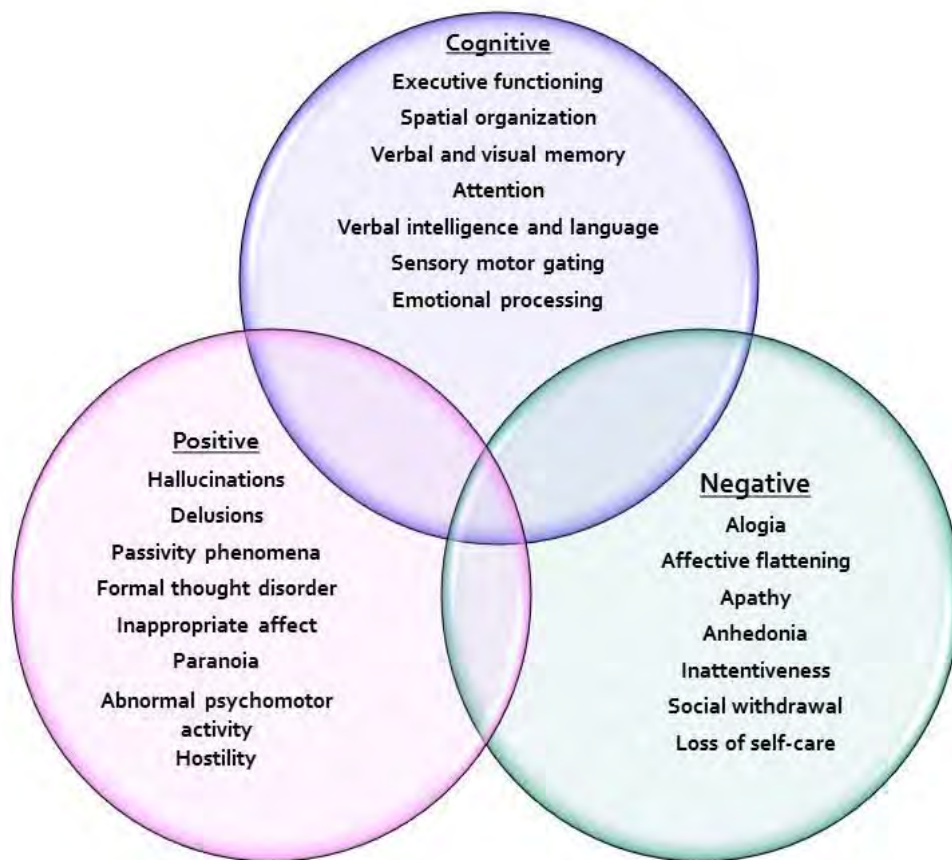


Figure 2: The three clusters of schizophrenia symptoms

Other manifestations observed in patients who suffer from schizophrenia include:

- Increased suicidal behaviour (Tandon *et al.*, 2009:1)
- Co-morbid depression (Gonzalez-Burgos *et al.*, 2011)
- Aggressiveness and increased violent behaviour (Fleischman *et al.*, 2014:3051; Soyka, 2011:913)
- Higher prevalence of substance abuse, such as nicotine, cannabis and alcohol (Gonzalez-Burgos *et al.*, 2011; Tandon *et al.*, 2009:1)
- Poor social outcomes such as unemployment, financial dependence and homelessness (Tandon *et al.*, 2009:1)

- Higher prevalence of obesity and metabolic abnormalities, such as insulin resistance (may be related to antipsychotic treatment) (Harris *et al.*, 2013:752; Tandon *et al.*, 2009:1)
- Higher prevalence of cardiovascular disease (may be related to antipsychotic treatment) (Gonzalez-Burgos *et al.*, 2011; Tandon *et al.*, 2009:1)
- Increased anxiety-related conditions (Cosoff & Hafner, 1998:67; Pallanti *et al.*, 2004:53)

2.1.3. Diagnosis of schizophrenia

The Diagnostic and Statistical Manual of Mental disorders (DSM), in various editions, have over the years been successfully implemented in the diagnosis of schizophrenia, with the DSM-IV contributing to a 90% positive diagnosis rate (Bromet *et al.*, 2011:1186). The newer edition, the DSM-V, retains most of the criteria as described in the DSM-IV with limited changes only in *Criterion A* (Tandon *et al.*, 2013:3). *Criterion A* refers to the five characteristic symptoms of schizophrenia, of which at least two must be present for a minimum of one month to make a positive diagnosis (American Psychiatric Association, 1994). These include (Tandon *et al.* 2013):

- a. Delusions
- b. Hallucinations
- c. Disorganized speech
- d. Grossly disorganized or catatonic behaviour
- e. Negative symptoms, i.e. affective flattening, alogia or avolition

Modest changes in the DSM-V include the removal of the special treatment of Schneiderian “first-rank” hallucinations and bizarre delusions, a clarified definition of negative symptoms and the addition of a requirement that at least one of the minimum two requisite characteristic symptoms must be hallucinations, delusions, or disorganized speech (Tandon *et al.*, 2013:3). If a patient’s symptoms do not apply to the above mentioned list, a diagnosis for schizophrenia can be made according to *Criterion B* (social / occupational dysfunction), *C* (duration of 6 months), *D* (schizoaffective and mood disorder exclusion), *E* (substance / general mood condition exclusion) or *F* (relationship to Global Developmental Delay or Autism Spectrum Disorder) (American Psychiatric Association, 1994).

2.1.4. The pathophysiology of schizophrenia

In recent years our understanding of the pathophysiology of schizophrenia has dramatically increased owing to sophisticated new neuroimaging and laboratory techniques (van Os & Kapur, 2009:635). Various neurochemical alterations, anatomical abnormalities, neuropsychological anomalies and oxidative mechanisms have all been associated with the pathophysiology of schizophrenia (Bitanirwe & Woo, 2011:878; Ross *et al.*, 2006:139). These will be discussed individually below.

2.1.4. a) Neuropsychology:

The cognitive symptoms of schizophrenia are steadily receiving more attention as a central aspect of the disease (Ross *et al.*, 2006:139). One of the prominent features of schizophrenia is intellectual decline, usually beginning during childhood (Seidman *et al.*, 2006:225). Moreover, 85% of schizophrenia patients show sub-standard performance in a neuropsychological study, compared to only 5% in a control group (Palmer *et al.*, 1997:437). This leads to the assumption that a greater vulnerability to psychosis and disease onset may be present when there are pre-existing neurological and neurophysiological abnormalities (Mortimer, 2005:26). Conversely, it is possible that antipsychotic treatment may influence neuropsychology; however Hill *et al.* (2004:49) found that even medication-naïve schizophrenia patients have impairments in cognitive domains. It is evident that although neuropsychological impairments exist before schizophrenia presentation, an even greater decline originates after the first episode (Mortimer, 2005:26). An early study suggested that abnormalities in the left-temporal hippocampus could be responsible for poor performance in verbal memory tasks (Saykin *et al.*, 1994:124). In addition, Mortimer (2005:26) also suggests an integral role for the hippocampus in memory performance, while positron emission tomography (PET) studies during challenging working memory tasks show that regional cerebral blood flow (rCBF) in the prefrontal cortices (PFC's) significantly decreased (Mortimer, 2005:26). Moreover, schizophrenia patients subjected to the Wisconsin Card Sorting Test (WCST), used to evaluate executive function, show weak performance that is comparable to that observed in patients with frontal lobe lesions (Mortimer, 2005:26). A role for dopamine (DA) in the regulation and integration of cognitive functions is noteworthy (Nieoullon & Coquerel, 2003:S3), and will be discussed later. The above studies make it clear that our knowledge of the pathophysiological markers of the neuropsychological impairments associated with schizophrenia is still in its infancy and that future research in this area is of immense importance.

2.1.4. b) Neuroanatomy:

The first discovery of structural brain abnormalities in schizophrenia patients occurred in 1976 when computed tomography (CT) scans revealed abnormally enlarged cerebral ventricles in these subjects (Johnstone *et al.*, 1976:924). Since the introduction of more sophisticated detection techniques, such as magnetic resonance imaging (MRI), researchers have found that schizophrenia patients have abnormalities in almost every cortical and subcortical region of the brain (Antonova *et al.*, 2004:117). More importantly, studies have been able to pinpoint the occurrence of progressive brain changes, suggesting a role for neurodevelopmental insults in schizophrenia pathology (Meyer & Feldon, 2010:285).

Although studies proving reduced total brain volume are rare (McCarley *et al.*, 1999:1099), researchers have observed overall smaller brains in schizophrenia patients (Gur *et al.*, 1994:343; Harrison *et al.*, 2003:25; Shenton *et al.*, 1992:604). Comparing the brains of 540 schizophrenia patients to that of 794 controls, Harrison *et al.* (2003:25) found a 2% reduction in the total brain weight of schizophrenia patients. Reductions in both the total grey (Fig. 3) (Gur *et al.*, 1994:343; Krull *et al.*, 1991:651) and white matter (Cheung *et al.*, 2011:1709; Hao *et al.*, 2009:128; Rowland *et al.*, 2009:1514) have also been detected in schizophrenia brains, while DeLisi, *et al.* (1997:129) observed a reduction in the hemispheric volumes of such patients. Alterations in various specific brain regions also contribute to the pathological markers of schizophrenia (Table 1). These include reductions in the frontal (Ho *et al.*, 2003:585), temporal (Wright *et al.*, 2000:16), parietal (Job *et al.*, 2005:1023) and occipital lobes (Davatzikos *et al.*, 2005:1218). Researchers furthermore observed a loss of the normal cerebral asymmetries in schizophrenia (Szeszko *et al.*, 2008:976), as well as reduced cerebral volumes. Sub-cortically, the structures of the corpus callosum (DeLisi *et al.*, 1997:129), basal ganglia (Keshavan *et al.*, 1995:87), thalamus (Buchsbaum *et al.*, 1996:191) and cavum septi pellucidi (Nopoulos *et al.*, 1997:1102) have all revealed alterations in persons affected with schizophrenia.

These structural brain abnormalities have been shown to occur gradually in people with prodromal symptoms, who are only at risk to develop schizophrenia but do not present with its full symptomology (Pantelis *et al.*, 2003:281). This highlights the causal role of neurodevelopmental insults in progressive changes in the brains of people who are vulnerable to developing the disease and who may later go on to develop the full-blown illness (Meyer & Feldon, 2010:285). Increased ventricle size is also evident during early stages of schizophrenia development, suggesting that mechanisms during early brain development play a role in the pathological features of the disease (Pagsberg *et al.*, 2007:489). It has been suggested that anatomical abnormalities may be caused by faulty

neuronal migration during prenatal periods, especially the second trimester (Meyer & Feldon, 2010:285). Further evidence for the significant role of maternal developmental insults was provided by Nopoulos, *et al.* (1997:1102) who observed a higher occurrence of cavum septum pellucidum (CSP) in schizophrenia patients.

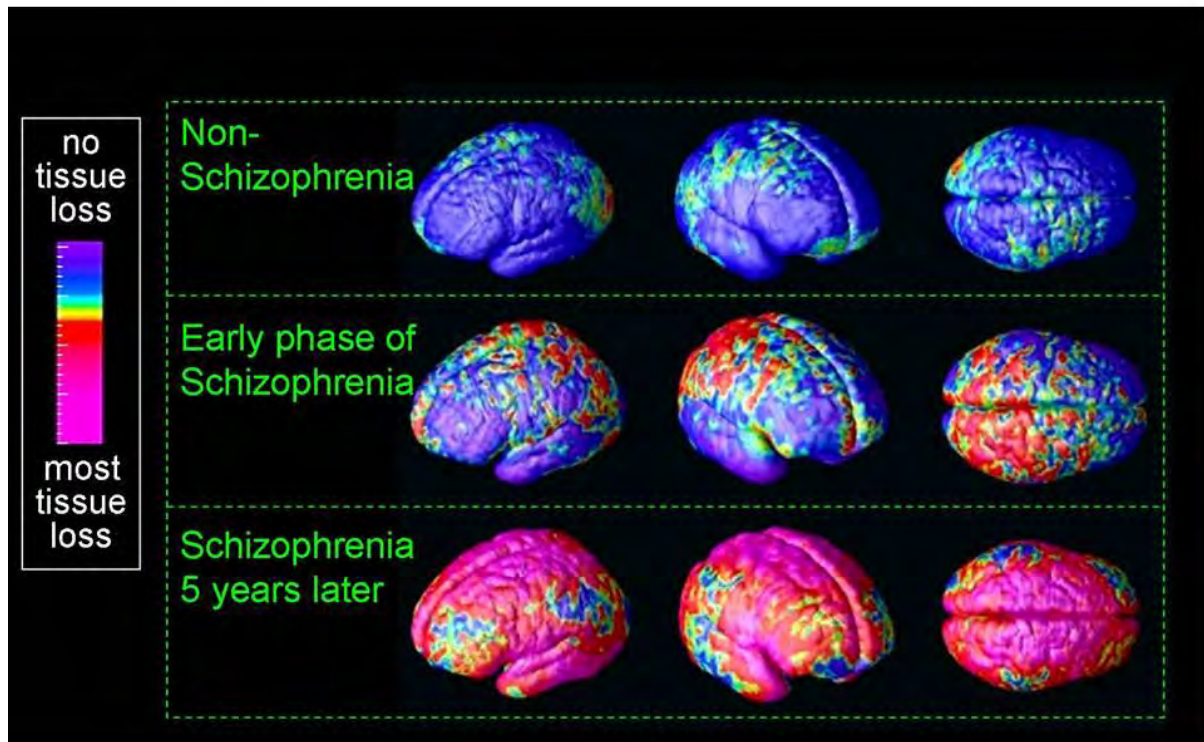


Figure 3: Progressive gray matter loss in schizophrenia (Thompson *et al.*, 2001:11650)

Table 1: Common anatomical changes in different brain areas of schizophrenia patients.
Adapted from McCarley, et al. (1999:1099) and Pearlson, et al. (1999:627).

Structural abnormalities	Researchers
Reduction in total brain volume	Andreasen <i>et al.</i> (1994:1763), Gur <i>et al.</i> (1994:343), Harrison, <i>et al.</i> (2003:25), Nasrallah <i>et al.</i> (1990:205)
Increased ventricle size	Gur, <i>et al.</i> (1999:905), Harvey <i>et al.</i> (1993:591), Ho <i>et al.</i> (2003:585), Kasai <i>et al.</i> (2003:766), Thompson <i>et al.</i> (2001:11650), Zipursky <i>et al.</i> (1992:195)
Decreased grey matter	Gur <i>et al.</i> (1994:343), Johnstone <i>et al.</i> (1976:924), Krull <i>et al.</i> (1991:651), Nasrallah <i>et al.</i> (1990:205), Pearlson <i>et al.</i> (1989:690), Rabins <i>et al.</i> (1987:1216)
Decreased white matter	Cheung <i>et al.</i> (2011:1709), Hao, <i>et al.</i> (2009:128), Rowland, <i>et al.</i> (2009:1514), Szeszko, <i>et al.</i> (2008:976)
Frontal lobe volume reductions	Gur <i>et al.</i> (1998:145), Ho <i>et al.</i> (2003:585), Sanfilipo <i>et al.</i> (2000:471), Sullivan <i>et al.</i> (1998:118)
Temporal lobe volume reductions	Marsh, <i>et al.</i> (1997:1104), Sanfilipo <i>et al.</i> (2000:471), Shenton <i>et al.</i> (1992:604), Wright <i>et al.</i> (2000:16)
Occipital lobe volume reductions	Davatziko, <i>et al.</i> (2005:1218), Onitsuka, <i>et al.</i> (2007:197), Sullivan <i>et al.</i> (1998:118), Zipursky, <i>et al.</i> (1992:195)
Parietal lobe volume reductions	Buchanan, <i>et al.</i> (2004:322), Frederikse <i>et al.</i> (2000:422), Job <i>et al.</i> (2005:1023), Schlaepfer, <i>et al.</i> (1994:842), Zhou, <i>et al.</i> (2007:35)

2.1.4. c) Neurochemistry

The DA hypothesis is perhaps the most persistent theory to explain neurochemical abnormalities in schizophrenia (Howes & Kapur, 2009:549). This hypothesis originated when researchers observed that the administration of DA D₂ receptor antagonists have an antipsychotic effect, while agonists at this receptor lead to psychotic manifestations (Meltzer & Stahl, 1976:19). Since then, the narrow interaction between different neurotransmitters have led to a more multifactorial view wherein serotonin (5-HT), noradrenaline (NA), glutamate and gamma-aminobutyric acid (GABA) all play a significant role in schizophrenia (Carlsson *et al.*, 2001:237). Each of these neurochemicals will henceforth be discussed individually.

Dopamine (DA):

After the discovery of the antipsychotic effects of dopamine D₂ antagonists in the 1950's, it was postulated that an overall increase in DA receptor activity underlies the neurochemical mechanism of schizophrenia (Howes & Kapur, 2009:549). This, however, did not explain the positive and negative dimensions of the disease until it was found that DA hyperactivity exists predominantly in the striatum, while DA function in the PFC is diminished in schizophrenia (giving way to negative symptom expression) (Meyer-Lindenberg *et al.*, 2002:267). It has since been suggested that the DA hypothesis is not so much grounded on receptor alterations, but rather on heightened dopaminergic transmission, resulting from increased DA production, increased DA release and elevated levels of synaptic DA (reviewed in Kapur 2003:13). DA, a catecholamine, exerts its function by binding to five related, but separate G protein-coupled receptors, grouped as D₁-like (includes D₁ and D₅) and D₂-like (includes D₂, D₃, D₄) receptors, based on their biochemical properties (Vallone *et al.*, 2000:125). After DA is synthesized in the brain stem ventral tegmental nuclei, it projects into 4 different pathways, namely the nigrostriatal, mesocortical, mesolimbic and the tuberoinfundibular pathways (Vallone *et al.*, 2000:125). These pathways can be related to various schizophrenia symptoms, as well as the therapeutic and adverse effects of antipsychotic agents.

The mesocortical pathway, starting from the ventral tegmental area (VTA), innervates various areas of the PFC to regulate certain aspects of memory and learning (Fig. 3) (Le Moal & Simon, 1991:155). The levels of homovanillic acid (HVA), a DA metabolite, have been observed to be decreased in the PFC of schizophrenics, which in turn could be positively correlated to poor working memory (Kahn *et al.*, 1994:217). Other studies have also associated abnormalities in PFC DA function to memory-related deficits in schizophrenia (Barch *et al.*, 2001:280; Menon *et al.*, 2001:433). A study implementing the WCST observed decreased DA activity in the PFC, while a significant rise in DA activity in the striatum (mesolimbic pathway) was detected, suggesting a link between DA function in these two brain areas (Meyer-Lindenberg *et al.*, 2002:267). In fact, it is hypothesized that poor control of dopaminergic function by the PFC may lead to dysfunction of DA systems in the striatum (Kegeles *et al.*, 2000:627).

The mesolimbic pathway originates from the VTA, projecting to the nucleus accumbens, striatum and olfactory tubercle (Fig. 3) (Meltzer & Stahl, 1976:19). The limbic striatum flows out to various brain regions, including the frontal lobe, hypothalamus, and other cortical

areas (Meltzer & Stahl, 1976:19). Several researchers found increased DA neurotransmission in the striatum of schizophrenics, which was connected with prodromal and psychotic symptoms (Abi-Dargham *et al.*, 2000:8104; Fusar-Poli & Meyer-Lindenberg, 2013:33; Laruelle *et al.*, 1996:9235). DA is well-known to play a vital role in reward learning and this effect has been proposed to be relevant in psychosis relating to aberrant salience (Kapur, 2003:13; Roiser *et al.*, 2009:199). According to this theory, psychotic symptoms are a result of the development of abnormal stimulus-reinforcement associations, in other words: elevated DA stimulation results in out of line aberrant assignment of salience to external stimuli, eventually giving way to psychotic symptoms (Kapur, 2003:13; Roiser *et al.*, 2009:199). Schizophrenia patients have also been observed to have significantly lower levels of HVA in their cerebrospinal fluid (CSF) than the control group, supporting the premise of DA hyperactivity in schizophrenia (Wieselgren & Lindström, 1998:101). Although the DA hypothesis contributes greatly to our understanding of schizophrenia pathophysiology, a recent meta-analysis failed to demonstrate any abnormalities in DA transmission in schizophrenia patients (Kambeitz *et al.*, 2014:420). Moreover, pharmacological agents targeting the DA pathways tend to be unsuccessful in treating negative and cognitive symptoms (Miyamoto *et al.*, 2005:79), proposing that other biological systems also play a role in schizophrenia.

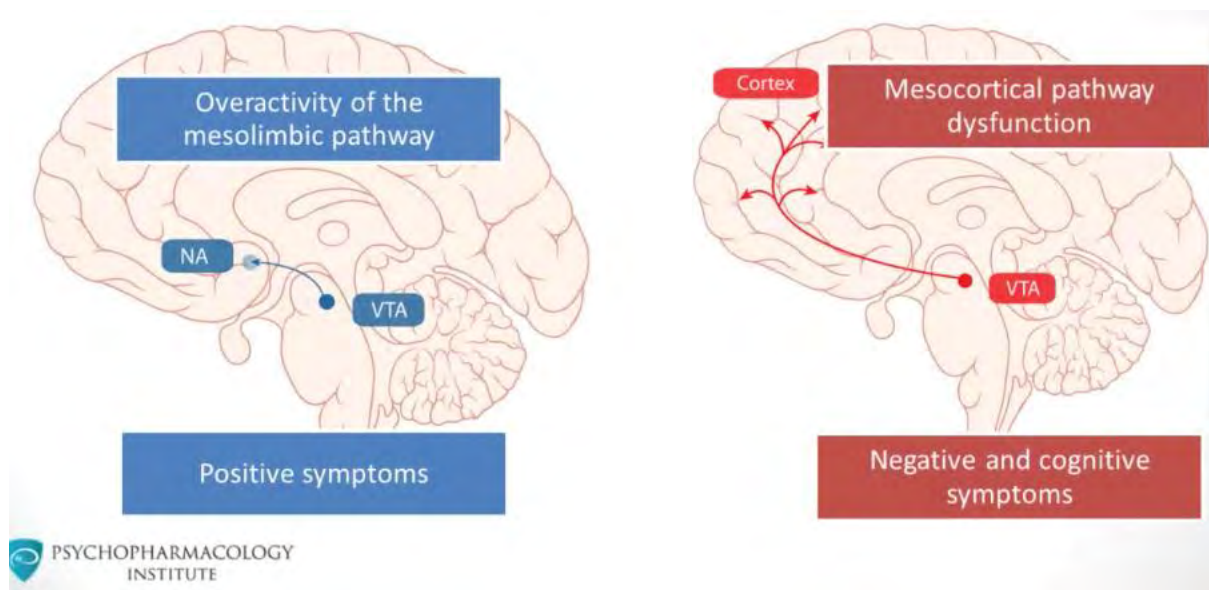


Figure 4: The brain DA pathways linked to schizophrenia pathophysiology. Figure from Psychopharmacology Institute (Available from www.psychopharmacologyinstitute.com, date of access: 24 June 2015)

Serotonin:

5-hydroxytryptamine (5-HT), or serotonin, is a monoamine neurotransmitter derived from tryptophan (Côté *et al.*, 2003:13525). In the human body 95% of 5-HT can be found in the gastro-intestinal tract (GIT), with the rest functioning in blood vessels, the pineal gland, and in the CNS where it is a key regulator of affect (Côté *et al.*, 2003:13525; Robinson & Sahakian, 2009:89). The 5-HT hypothesis of schizophrenia originated after it was discovered that the hallucinatory drug, lysergic acid diethylamide (LSD) is a 5-HT antagonist (Bleich *et al.*, 1988:297). Impairments in affect, especially regarding negative symptoms, are frequently observed in schizophrenia, suggesting an integral role for 5-HT (Selvaraj *et al.*, 2014:233). To confirm this theory, several studies found amplified 5-HT_{1A} receptor binding in the PFC of patients with schizophrenia when compared to controls (Gurevich & Joyce, 1997:529; Hashimoto *et al.*, 1991:355; Simpson *et al.*, 1996:919). Studies on 5-HT_{2A} is somewhat contradictory, but some researchers have observed decreased binding at this receptor in cortical and subcortical brain regions of schizophrenia patients (Arora & Meltzer, 1991:19; Gurevich & Joyce, 1997:529). The serotonin transporter (5-HTT) has also been found to have decreased function in schizophrenia in the PFC (Laruelle *et al.*, 1993:810) and the hippocampus (Naylor *et al.*, 1996:749). Finally, the ability of various atypical antipsychotics

to bind to an array of 5-HT receptors serves as additional confirmation for a role of 5-HT in schizophrenia (Meltzer, 2012:87). In this regard, the 5HT_{2c} receptor has taken centre stage where activation of 5HT_{2c} heteroreceptors acts to modify DA release in several brain regions, which has been suggested to play a role in the alleviation of the cognitive and affective symptoms of schizophrenia (Alex & Pehek, 2007:296).

Noradrenaline (NA):

An involvement for noradrenaline (NA) in schizophrenia was suggested more than forty years ago (Stein & Wise, 1971:1032). NA is a catecholamine neurotransmitter that is widely distributed throughout the brain, rendering it important in “higher brain functions” (Yamamoto & Hornykiewicz, 2004:913). NA plays a role in attentiveness, memory, learning, stress, anxiety and the sleep-wake cycle, to name a few (Klimek *et al.*, 1999:69; Yamamoto & Hornykiewicz, 2004:913). In post-mortem studies, several researchers found increased levels of NA in the brains of schizophrenia patients (Bird *et al.*, 1980:63; Bridge *et al.*, 1985:57; Farley *et al.*, 1978:456). This monoamine was also found to be elevated in the CSF of schizophrenia patients, and this was correlated with increased positive symptoms (Gomes *et al.*, 1980:346; Kemali *et al.*, 1990:49; Sternberg *et al.*, 1981:1045). Furthermore, studies in laboratory animals indicate that NA regulates DA-induced behaviours, suggesting an important link between NA and DA dysfunction, and thus in schizophrenia (Klimek *et al.*, 1999:69; Plaznik *et al.*, 1982:619). In fact, Grenhoff, *et al.* (1993:79) found that systemic prazosin (an α_1 -receptor antagonist) has an inhibitory effect on DA neuronal activity. What's more, atypical antipsychotics have also been shown to exercise an important effect on adrenergic receptors, especially the α_1 receptors (Svensson, 2003:1145). Because atypical antipsychotics tend to demonstrate increased effectiveness when compared to traditional typical antipsychotics (Svensson, 2003:1145), their function on adrenergic function highlights the importance of NA in the pathophysiology of schizophrenia. Recent work has noted that the α_{2c} subtype plays a deciding role in how the adrenergic system mediates or regulates cognition, as well as anxious or fear-related behaviour and has channelled new hope into investigating these agents as novel antipsychotic agents (Erasmus *et al.*, 2015:in preparation).

Glutamate and Gamma-amino butyric acid (GABA):

The feedback that exists between PFC and striatal dopaminergic function is predominantly controlled by glutamatergic (and GABA-ergic) effects from the PFC (Meyer-Lindenberg *et al.*, 2002:267). Glutamate, an abundant excitatory neurotransmitter in the mammalian brain,

exerts its effects by binding to one of two classes of glutamate receptors in subcortical and cortical brain regions: ionotropic and metabotropic receptors (Javitt, 2012:911; Lin *et al.*, 2012a:665). Ionotropic glutamate receptors (iGluRs) are ligand-gated cation channels and include three main classes of receptors, namely NMDA receptors, α -amino-3-hydroxy-5-methyl-4-isoazolepropionic acid (AMPA) and 2-carboxy-3-carboxymethyl-4-isopropenylpyrrolidine (kainate) receptors (Kew & Kemp, 2005:4). Although it has been shown that NMDA receptors interact with AMPA receptors (Yu *et al.*, 2008:889), most research remain focussed on the role of NMDA receptors independently in schizophrenia pathology (Lin *et al.*, 2012a:665). This is rightly so, seeing as NMDA receptors have shown to be the most relevant glutamate receptor in schizophrenia neurochemistry, especially when taking into consideration that it plays a vital role in cognition and neurotoxicity (Kalia *et al.*, 2008:742; Lipton & Rosenberg, 1994:613). An important feature of the NMDA receptor is that it is not only modulated by its agonists / co-agonists, but also by several other molecules, such as zinc, magnesium and polyamines, implying that the NMDA receptor can adjust to endogenous and exogenous signals to support numerous important brain functions (Lin *et al.*, 2012a:665). On the other hand, metabotropic glutamate receptors (mGluRs) are G-protein coupled receptors, divided into three distinct groups, with a total of 8 different receptors. These are group I (mGluR1 and 5), group II (mGluR2 and 3) and group III (mGluR4, 6, 7, 8) metabotropic receptors (Kew & Kemp, 2005:4). mGluRs are also important in schizophrenia research, seeing that they have been shown to play a role in regulating NMDAR-mediated neurotransmission (Lin *et al.*, 2012a:665).

Glutamate pyramidal neurons located in the frontal cortex project to the midbrain, limbic areas and brainstem (Schwartz *et al.*, 2012:195). The cortical brainstem glutamate projection is linked to the positive symptoms of schizophrenia, as follows. Starting from the frontal cortex, this neuron projects to the deeper mesolimbic DA pathway. In the normal brain, (1) a full-functioning pyramidal glutamate neuron fires upon (2) a smaller GABA interneuron, which then releases inhibitory GABA onto (3) a secondary glutamate pyramidal neuron causing it to lower its firing rates (normal state / homeostasis). The fourth neuron in the loop is (4) DAergic which fires at a normal rate, completing the glutamate-GABA-glutamate-DA neurocircuit responsible for maintaining a normal, non-psychotic state (Fig. 5). However, in the schizophrenia brain, sub-optimal NMDA receptor function gives way to a compromised cortical brainstem glutamate projection. The glutamate neuron still fires upon a GABA interneuron, but this now has reduced functioning NMDA receptors located on it, leading to hypofunctioning of the GABA interneuron. The subsequent loss of GABA function leads to abnormally increased firing rates of the secondary glutamate neuron, which fires on the DA neuron, causing excessive DA activity. The end result of this malfunctioning is the

generation of psychotic symptoms (Fig. 5). When discussing the NMDA receptor hypofunction hypothesis for negative symptoms, a more complex glutamate neurocircuitry comes into play. A similar pathway as discussed above exists up to the third glutamate neuron, which then leads to a deeper midbrain GABA interneuron, impacting on DA projections that lead back to the frontal cortex, thus forming the glutamate-GABA-glutamate-GABA-DA neurocircuit. In the schizophrenia brain, the same malfunctioning pathway as discussed above takes place, leading to hyperactivity of the secondary glutamate neuron. This increased glutamate tone now (5) stimulates an extra GABA interneuron, which then releases elevated levels of GABA. This causes (6) inhibition of the DA neurons projecting to the frontal cortex, leading to poor DA activity and the subsequent presentation of negative symptoms (Fig. 5) (reviewed in Schwartz et al., 2012:195).

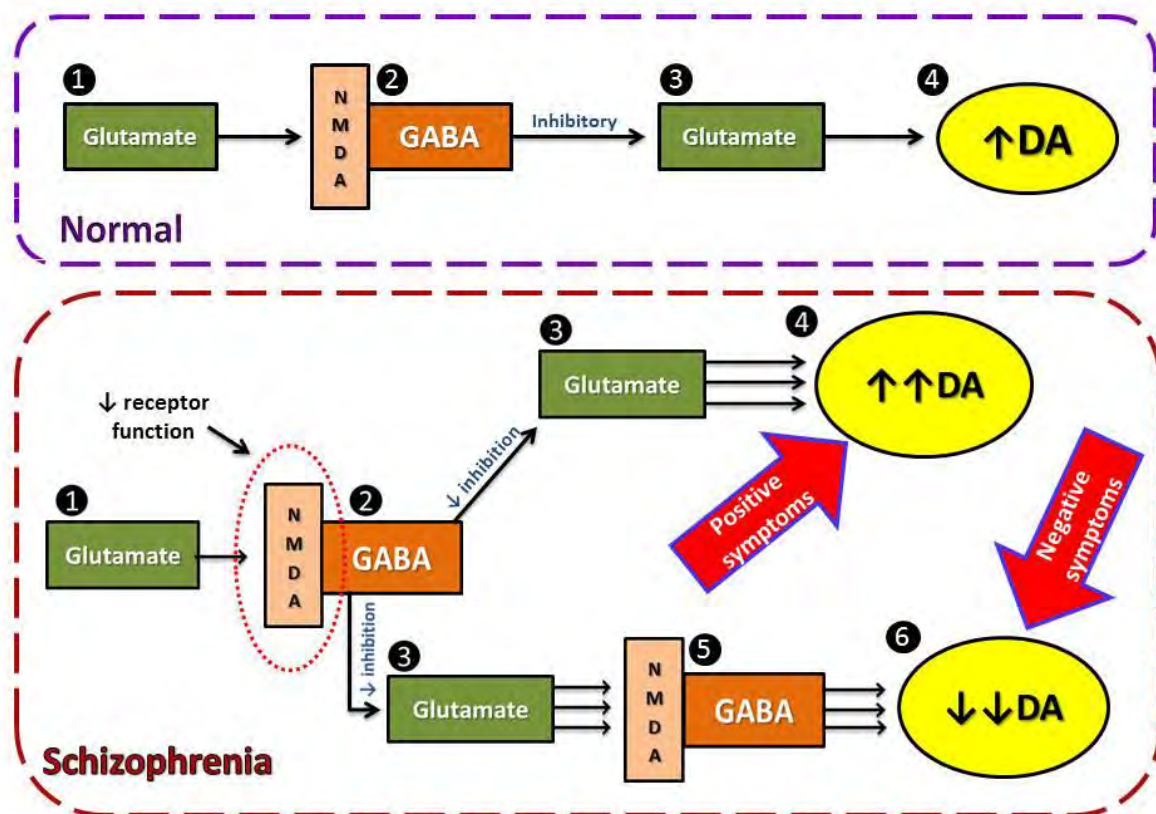


Figure 5: The normal glutamate-GABA-glutamate-DA neurocircuit compared to abnormal glutamate-GABA-glutamate-DA neurocircuitry in schizophrenia.

As evidence for this hypothesis, Kegeles, *et al.* (2000:627) found that the disruption of glutamatergic NMDA neurotransmission via ketamine administration gave way to increased

amphetamine-induced DA release in the striatum. Additionally, it has been found that chronic administration of NMDA receptor antagonists to non-human primates leads to depleted DA levels in the PFC in a similar manner as in schizophrenia (Tsukada *et al.*, 2005:1861). Other researchers also observed that blocking NMDA receptors leads to psychosis similar to the symptomology of schizophrenia (Kalia *et al.*, 2008:742; Lin *et al.*, 2012a:665). Furthermore, Kargieman, *et al.* (2008:129) showed that phencyclidine (PCP) administration, another NMDA antagonist, led to disrupted neuronal activity in the PFC and this may be associated with cognitive dysfunction in schizophrenia. Post-mortem studies revealed altered NMDA receptor binding in the hippocampus, cortex and thalamus of schizophrenia patients (Kristiansen *et al.*, 2007:48), while a role for NMDA receptor function in the action of antipsychotics has been suggested (Riva *et al.*, 1997:136). In addition, other studies have found evidence of reduced glutamate function in various brain areas of patients with schizophrenia (reviewed in Bauer *et al.*, 2012:1568). In contrast, Poels *et al.* (2014:325) found elevated levels of glutamate in the PFC and striatum of patients with schizophrenia. Importantly, the subjects in the latter study were medication-free, thus deviating from the hypo-glutamatergic hypothesis of schizophrenia. However, this phenomenon could also be explained by the occurrence of both hyper- and hypoglutamate states at different neurons, as discussed above. Finally, deficits in hippocampal GABA-ergic uptake sites, as well as GABA-parvalbumin (PV) containing neurons have been detected in schizophrenia patients (Reynolds *et al.*, 1990:1038). Researchers also observed decreased levels of glutamic acid decarboxylase (GAD) 67, an important enzyme in the synthesis of GABA, in the cortex of patients with schizophrenia (Gonzalez-Burgos *et al.*, 2010:335; Guidotti *et al.*, 2000:1061). Together with this, GABAA receptor density and binding is altered in the cerebral cortex of schizophrenia patients (Benes *et al.*, 1996:1021). Interestingly, Yang, *et al.* (2011:63) found an elevated number of GABAergic interstitial white matter neurons in the PFC of schizophrenics, while GABAergic interneuron markers were reduced in the overlying grey matter, suggesting faulty migration of interneurons from the white matter to the cortex (Yang *et al.*, 2011:63). Reelin (RELN) is an important signalling protein in neurodevelopment secreted by GABA interneurons (Rice & Curran, 2001:1005). Further evidence in support of the GABA hypothesis is the observation that RELN and its mRNA are decreased in the post-mortem brains of schizophrenia patients (Impagnatiello *et al.*, 1998:15718). Antipsychotics have also been suggested to exert an effect on the GABA system, with several researchers reporting increased expression of GAD67 and GAD67 mRNA in the basal ganglia after typical antipsychotic treatment (Johnson *et al.*, 1994:1003; Sakai *et al.*, 2001:152). Although an important mechanism, our understanding of GABA-glutamatergic function remain modest and future studies in this direction are warranted.

Acetylcholine (ACh):

The involvement of the neurotransmitter acetylcholine (ACh) (a potent regulator of neuronal activity in the CNS) in schizophrenia pathology has recently gained more attention (Higley & Picciotto, 2014:88) when researchers noticed a pattern of extreme tobacco use in schizophrenia patients (Aubin *et al.*, 2012:271). The authors suggest that tobacco use is a form of “self-medication”, proposing a role for increasing nicotinic neurotransmission and thus benefiting cognition as a basis for its clinical value (Poorthuis *et al.*, 2009:668; Winterer *et al.*, 2013:197). ACh exerts its function by binding to two main classes of receptors: metabotropic muscarinic receptors (mAChR) or the ionotropic nicotinic receptors (nAChR) (Picciotto *et al.*, 2000:451; Wess, 2003:414). Seeing that ACh signalling plays a vital role in the functioning of various brain areas, most notably cortical function, disruptions in this system may contribute to a number of neuropsychiatric illnesses (Higley & Picciotto, 2014:88). Indeed, post-mortem findings have observed decreased expression of low-affinity nicotinic $\alpha 7$ acetylcholine receptor ($\alpha 7$ nAChR) in several brain areas of schizophrenics (Freedman *et al.*, 1995:22; Guan *et al.*, 1999:1779; Leonard *et al.*, 2000:237). Studies have also shown that mAChR and nAChR agonists are able to correct certain features of cognitive symptoms in schizophrenia (Harris *et al.*, 2004:1378; Shekhar *et al.*, 2008:1033). Additionally, researchers have also observed that the administration of ACh antagonists can aggravate existing positive symptoms in patients with schizophrenia (Tandon *et al.*, 1991:23), while scopolamine, an anticholinergic, induces psychosis in normal children (Hamborg-Petersen *et al.*, 1984:485). Genetic studies have also implicated the ACh system by observing a dinucleotide polymorphism at chromosome 15q13e14, the site of the nicotinic $\alpha 7$ nAChR CHRNA7 gene, in schizophrenia patients (Freedman *et al.*, 1997:587; Leonard & Freedman, 2006:115). These findings underline the importance of ACh in the pathophysiology of schizophrenia and may contribute to the development of new therapeutic options.

2.1.4. d) Mitochondrial function

A role for mitochondrial dysfunction in the pathology of schizophrenia has been suggested (reviewed in Clay *et al.*, 2011:311 and Rajasekaran *et al.*, 2015:10). Mitochondria are cell organelles enclosed by two phospholipid bilayer membranes, with an intermembrane space in between and the mitochondrial matrix as the innermost area of the organelle (Clay *et al.*, 2011:311). Mitochondria are the primary generators of high energy intermediates (Valvassori *et al.*, 2010:903), with various other important functions such as calcium homeostasis, redox

signalling, and apoptotic cell death (Rajasekaran *et al.*, 2015:10). It also plays an important role in neurodevelopment and the modulation of neuronal activity (Rajasekaran *et al.*, 2015:10; Robicsek *et al.*, 2013:1067). Mitochondria produce energy to cells by means of a complex process which could simply be described as the conversion of chemical energy stored in sugars to high-energy phosphates (ATP) via oxidative phosphorylation (Clay *et al.*, 2011:311). From an evolutionary perspective it has been proposed that mitochondrial function “failed” to keep up with the fast progression of the human brain, leaving the brain more vulnerable to conditions where excessive energy requirements will compromise neuronal function and survival. The reduced energy production in turn will lead to the development of neurodegenerative diseases, one being schizophrenia (Gonçalves *et al.*, 2015:13). Furthermore, mitochondria play an important role in inflammatory, oxidative and nitrosative stress (Rajasekaran *et al.*, 2015:10), suggesting different mechanisms by which these organelles could play a role in disease pathology, apart from energy production. Indeed, defects in the mitochondrial pathway can lead to the increased formation of ROS (Rajasekaran *et al.*, 2015:10), which may give way to DNA, lipid and protein damage. Mitochondrial DNA (mtDNA) is particularly vulnerable to oxidative stress due to it being located at the main site of ROS production, and also since mtDNA lacks protective histones (Wallace, 2005:359). A third mechanism for mitochondrial dysfunction in schizophrenia stems from its involvement in neurotransmission (Rosenfeld *et al.*, 2011:980). For instance, Ben-Schachar, *et al.* (1995:718) observed that catecholamines such as DA can lead to neurotoxicity through direct interaction with the mitochondrial electron transport system (Ben-Shachar *et al.*, 1995:718). Indeed, factors involved in cellular oxidative stress are known to evoke monoaminergic changes that in turn may mediate psychiatric manifestations (Garcia-Cazorla *et al.* 2008:273, Möller *et al.* 2013a:687). In many instances, oxidative stress can be causally related to increased glutamate activity evident in both the striatum and frontal cortex (Haroutunian *et al.*, 2003:67), while it has been noted earlier that schizophrenia is closely linked to altered glutamatergic activity.

Several studies have reported of decreased energy metabolism in schizophrenia, especially relating to the theory of hypofrontality (Buchsbaum & Hazlett, 1998:343; Gur *et al.*, 1987:126). A direct link between evidence for reduced energy metabolism and mitochondrial dysfunction has been provided by Volz *et al.* (2000:954) who observed reduced ATP in the frontal and left temporal lobes of schizophrenia patients (Volz *et al.*, 2000:954). Additionally, post-mortem studies found evidence of abnormal brain mitochondria metabolism in schizophrenia patients (Prabakaran *et al.*, 2004:684), while a CSF study found elevated levels of lactate in these patients, indicating increased extra-mitochondrial and anaerobic glucose metabolism, consistent with impaired mitochondrial metabolism (Regenold *et al.*,

2009:489). Reductions in the quantity of mitochondria have also been observed in the brains of schizophrenics (Kung & Roberts, 1999:67; Uranova *et al.*, 2001:597). Karry *et al.* (2004:676) found that the expression of mitochondrial complex I subunits was reduced in the PFC of patients with schizophrenia, also in line with hypofrontality. Likewise, reduced levels of complex III and IV have been observed in schizophrenia (Maurer *et al.*, 2001:125). Finally, several genetic studies also linked alterations in mitochondrial function to schizophrenia pathology (Altar *et al.*, 2005:85; Kvajo *et al.*, 2008:685; Munakata *et al.*, 2005:525), confirming the mechanistic role of mitochondria in schizophrenia pathology.

2.1.4. e) Oxidative stress

Oxidative damage to proteins, lipids and DNA have been observed in schizophrenia, correlating to the deteriorating progression of the illness and suggesting a role for oxidative stress in schizophrenia pathology (Bitanhirwe & Woo, 2011:878). Simply stated, oxidative stress involves a discrepancy between the overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS), and a deficiency in enzymatic and non-enzymatic antioxidant systems (Fig. 6) (Do *et al.*, 2009:220). The “oxygen paradox”, as termed by Davies (1995:1), states that oxygen plays conflicting roles in the human body: one as a molecule vital for life, and the other as an inherently dangerous element. The negative role of oxygen is revealed during its chemical reduction to water, seeing that this process results in the development of reactive intermediates. These reactive intermediates include ROS, the collective term for superoxide, hydrogen peroxide, peroxy radical and hydroxyl radical, as well as RNS, which comprise nitric oxide (NO) and peroxynitrite (Do *et al.*, 2009:220). Although able to have a destructive effect, normal levels of these reactive species also play a functional role in influencing cellular target gene expression and modulating signalling pathways (Sun & Oberley, 1996:335). Indeed, the RNS NO is known to have important neuromodulatory and neurotransmitter properties in the cardiovascular and central nervous systems as well as other processes such as immunity (Garthwaite, 2010:221; Nasyrova *et al.*, 2015:139; Prast & Philippu, 2001:51; Serafim *et al.*, 2012:386). However, “normal levels” is key and this is controlled by anti-oxidant systems which include enzymes such as superoxide dismutase (SOD), glutathione peroxidases (GPx) and catalase, as well as the non-enzymatic anti-oxidants glutathione (GSH), ascorbic acid (vitamin C), α -tocopherol (vitamin E), carotenoids, and flavonoids (Do *et al.*, 2009:220). These systems function in different ways to protect an organism against oxidative damage, including the removal of ROS/RNS or the inhibition of ROS/RNS formation (Bitanhirwe & Woo, 2011:878). However, when anti-oxidant systems fail to provide adequate protection, or ROS and RNS are present

in abnormally high levels, important macromolecules such as DNA, proteins, and lipids are at risk (Kohen & Nyska, 2002:620).

If we take into account that the human brain utilizes more than 20% of the oxygen expended by the body, and that it comprises limited anti-oxidant systems together with high contents of both polyunsaturated fatty acids (PUFA's) and metals (iron, zinc, copper, etc.) (Bitanirwe & Woo, 2011:878), the extent to which oxidative stress could contribute to the development of mental disease is clear. Diverse alterations in anti-oxidant systems have been reported in schizophrenia. Some studies have observed higher levels of anti-oxidant systems in patients with schizophrenia (Dakhale *et al.*, 2004:205; Kunz *et al.*, 2008:1677), indicative of a reactive up-regulation of these mechanisms in the face of redox challenge, while others have found reduced levels thereof (Raffa *et al.*, 2009:1178; Singh *et al.*, 2008:171). Data for specific anti-oxidant systems is similarly inconsistent. Numerous studies have detected higher levels of SOD in the serum and plasma of schizophrenia patients (Gama *et al.*, 2006:512; Padurariu *et al.*, 2010:317; Sarandol *et al.*, 2007:1164; Wu *et al.*, 2012:34), while Zhang, *et al.* (2010:112) found the opposite. This contradictory trend can also be seen for GPx (Gawryluk *et al.*, 2011:123; Herken *et al.*, 2001:66) and for catalase (Herken *et al.*, 2001:66; Reddy *et al.*, 1991:409). Although diminished levels of anti-oxidant systems in schizophrenia would normally be expected according to the oxidative stress hypothesis, the phenomenon of inconsistent results could be explained by a physiological attempt to repair this unfavourable imbalance. Indeed, increased SOD levels have been observed in chronic schizophrenia patients (Reddy *et al.*, 1991:409), while first-episode antipsychotic-naïve schizophrenia patients revealed decreased levels of this anti-oxidant (Raffa *et al.*, 2009:1178). Hence, the anti-oxidant status may vary according to time of measurement (first episode or progressed disease status) and antipsychotic treatment (Dakhale *et al.*, 2004:205). For instance, Zhang and colleagues found that elevated levels of SOD in schizophrenia patients were successfully reduced by both typical and atypical antipsychotic treatment (Zhang *et al.*, 2012a:1928). In an attempt to explain the unpredictable nature of antioxidant therapy, Möller and colleagues suggest that antioxidants such as α -lipoic acid, NAC and others may perform differently depending on the cellular milieu, acting either as an antioxidant or as a pro-oxidant depending on the redox status of the organism (Möller *et al.*, 2015:987). Moreover, such redox status very likely relates to illness severity and its progression overtime that in turn will predetermine how an antioxidant will perform and at what dose. Thus, conditions such as the applied dose of the antioxidant as well as illness state / severity will have a role in determining the clinical outcome and response to an antioxidant.

Apart from anti-oxidant systems, there is also evidence for changes in peroxidation products in schizophrenia patients. Malondialdehyde (MDA) is the end-product of lipid peroxidation and increased levels of this marker have been reported in schizophrenia (Dietrich-Muszalska & Olas, 2009:27; Zhang *et al.*, 2006:291). Previous studies also indicated that RNS and NO play various roles in brain functioning (reviewed in Bitanirwe & Woo 2011:878). Importantly, NO acts as a second messenger following NMDA receptor activation, the latter being involved in both dopaminergic and serotonergic pathways (Brenman & Bredt, 1997:374; Lorrain & Hull, 1993:87). Numerous studies have reported elevated NO levels in the blood of patients with schizophrenia (Suzuki *et al.*, 2003:288; Yilmaz *et al.*, 2007:137). On the other hand, Ramirez *et al.*, (2004:357) found reduced levels of the NO metabolites, nitrite and nitrate, correlating directly to lessened NO production. Although oxidative stress data remain inconsistent, its role in schizophrenia is evidently very important.

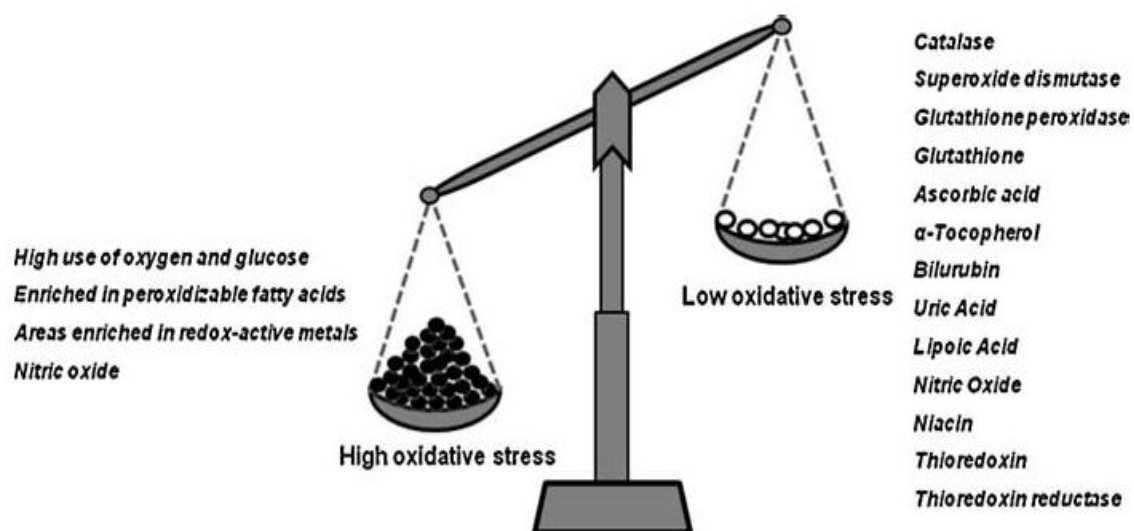


Figure 6: The oxidative balance. Figure from: Bitanirwe, Woo (2011:878)

2.1.4. f) Inflammation

Numerous immune alterations and inflammatory co-morbidities have been reported in schizophrenia and other mood disorders (Benros, 2013:170). Subsequently, an important role for neuro-inflammation has been suggested in the development of schizophrenia (Drexhage *et al.*, 2011:169). The immune system consists of (1) the non-antigen specific innate immune response and (2) the antigen specific adaptive immune reaction; both need

to be closely regulated after activation to ensure that any unwarranted damage to surrounding tissue is minimised (Gibney & Drexhage, 2013:1). An immune response is elicited by a class of different polypeptides, termed cytokines, which include interleukins, interferons, tumour necrosis factors, and certain growth factors (Gibney & Drexhage, 2013:1; Hopkins & Rothwell, 1995:83). Upon activation of the immune system, these molecules are released to bind to receptors on glial cells and neurons, which will then lead to the production of more cytokines, prostaglandins, chemokines and free radicals, further increasing the neuro-inflammatory response and ultimately giving way to a cascade of cytokine activation in the brain (Hopkins & Rothwell, 1995:83; Sawada *et al.*, 1993:131). Subsequently, dysregulation of the immune system may negatively influence neurobiological systems and may therefore contribute to the development of neurodegenerative diseases (Gibney & Drexhage, 2013:1).

Cytokines can be divided into either pro- and anti-inflammatory cytokines. Pro-inflammatory cytokines such as tumor necrosis factor (TNF)- α , interferon (IFN)- γ , interleukin (IL)-1 ($\alpha + \beta$), IL-12 and IL-6, are responsible for the induction of an inflammatory process to fight infection (Gogos *et al.*, 2000:176). On the other hand, anti-inflammatory cytokines such as transforming growth factor (TGF)- β , IL-10, IL-4 and IL-13 are important to control the inflammatory process and to prevent excessive inflammatory damage (Gogos *et al.*, 2000:176). A meta-analysis studying more than 80 scientific papers has provided evidence for this theory. Various cytokines are increased in patients with schizophrenia, while some of these alterations can be reversed by antipsychotic treatment (Miller *et al.*, 2011:127). Specific cytokine reductions following antipsychotic treatment include: TNF- α , IFN- γ , IL-1, IL-2, IL-6 and IL-10 (Monji *et al.*, 2013:115). Kim and colleagues attempted to relate these changes to specific clinical features of schizophrenia and found that increased plasma levels of the pro-inflammatory IL-2 could be indirectly correlated to the positive symptoms of the disease (Kim *et al.*, 2000:165). This association makes sense when considering that the administration of recombinant IL-2 to cancer patients have been linked to psychotic symptoms, such as hallucinations and delusions (Denicoff *et al.*, 1987:293). IL-6, on the other hand, has been proposed to play a role in both the severity of negative symptoms, as well as the duration of the disease (Kim *et al.*, 2000:165). A possible explanation for these phenomena may lie in the involvement of IL-6 in anatomical brain changes, which have been linked to both the above-stated disease factors (Crow, 1980:66; Waddington, 1993:55). Another study administered the bacterial endotoxin, lipopolysaccharide (LPS), to schizophrenia patients and found that those who in return exhibited a heightened IFN response showed predominantly positive symptoms, while those with a decreased IFN response were more likely to display dominant negative symptoms (Inglot *et al.*, 1994:464).

It is thus clear that various cytokines fulfil diverse roles in the development and symptomology of schizophrenia.

The resident macrophage of the CNS and main producers of cytokines, microglia, have also been implicated in schizophrenia (Fig. 7) (Gibney & Drexhage, 2013:1). Apart from their vital role in immune processes, microglia are proposed to be involved in synaptogenesis, synaptic pruning, neuronal apoptosis, as well the expression of receptors for dopamine, glutamate, GABA, and glucocorticoids (reviewed in Gibney & Drexhage 2013:1). Indeed, researchers have observed microglial activation or increased microglial cellular density in the post-mortem brains of schizophrenia patients (Bayer *et al.*, 1999a:126). The effects of this hyper-functioning microglia are wide-spread. Microglia-derived TNF- α and NO have shown detrimental effects on neurogenesis, together with increased apoptosis, both pathological processes frequently detected in schizophrenia (Fig. 7) (Ekdahl *et al.*, 2003:13632; Hu *et al.*, 1997:427). Furthermore, a possible link between microglia activation and suicidal behaviour in schizophrenia has been suggested, considering that the cytokines and free radicals released by microglia are able to modulate 5-HTergic and noradrenergic neurotransmission (Steiner *et al.*, 2008:151). It is clearly undeniable that microglia activation and inflammatory cytokines play a very important role in schizophrenia development and that these factors could give way to life threatening consequences.

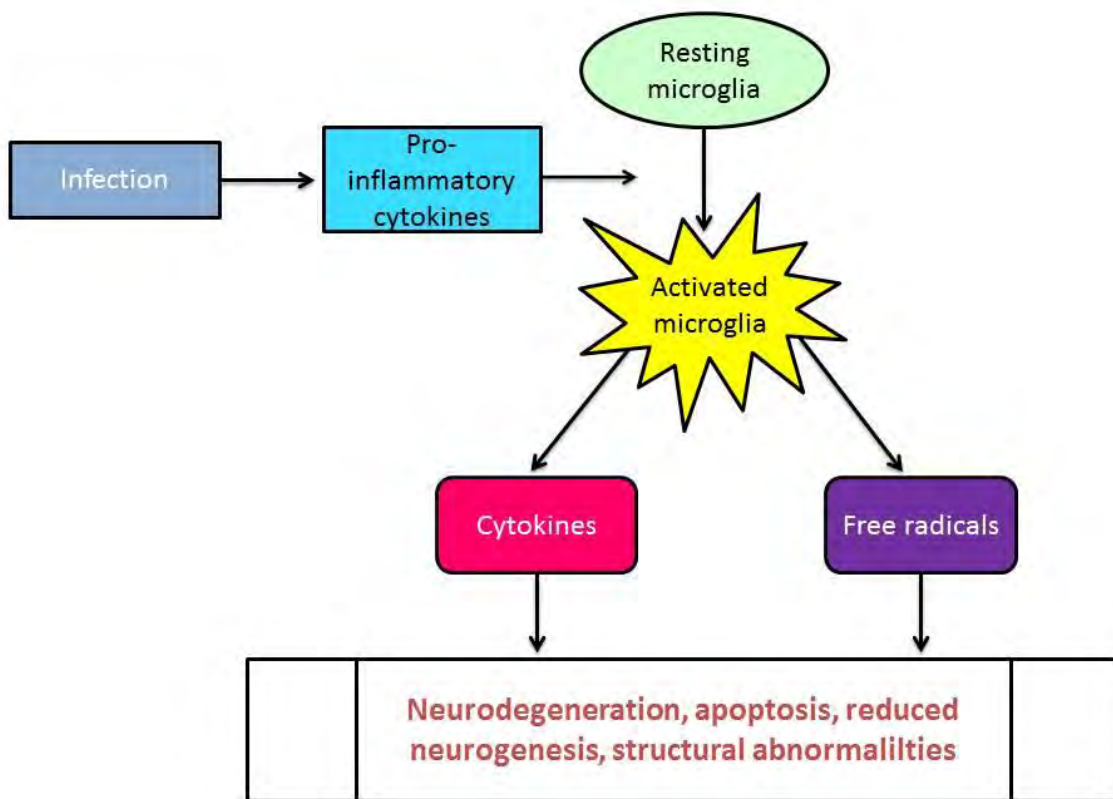


Figure 7: The role of microglia in schizophrenia pathology

2.1.4. g) Kynurenine pathway

The kynurenine pathway is the chief route for the metabolism of tryptophan, an essential amino-acid required for 5-HT production (Myint & Kim, 2014:304; Stone & Darlington, 2002:609). Tissue-specific enzymes are responsible for the first step in tryptophan degradation, namely (1) tryptophan 2,3-dioxygenase (TDO) which functions almost entirely in the liver and (2) indoleamine 2,3-dioxygenase (IDO) found in most other tissues, including the CNS (refer to Fig. 8) (Stone & Darlington, 2002:609). Tryptophan catabolism in the brain occurs in microglia and astrocytes, although up to 60% of cerebral kynurenine derives from the periphery (Gal & Sherman, 1980:223; Heyes *et al.*, 1996:595). Up regulation of these two enzymes is predominantly immune regulated for IDO, while TDO is activated by downstream product depletion and corticosteroids (reviewed in Barry *et al.*, 2009:287). After the first metabolizing step, kynurenine (KYN) is formed which then follows down one of three routes, as indicated in fig 6: (1) enzymatic degradation of KYN via kynurenine-3-monooxygenase (KMO) to 3-hydroxy-kynurenine (3HK), or (2) kynurenine aminotransferases I & II can form kynurenic acid (KYNA) (final product), or (3) kynureninase

may lead to the formation of anthranilic acid (final product) (Erhardt *et al.*, 2007:203; Myint & Kim, 2014:304). In the first route described above, kynureninase now degrades 3HK to 3-hydroxyanthranilic acid (3-OHAA), leading to the formation of adenosine triphosphate (ATP), or to the production of quinolinic acid (QUIN), the latter used to eventually form nicotinamide adenine dinucleotide (NAD) (Erhardt *et al.*, 2007:203; Myint & Kim, 2014:304). This process leads to the formation of free radicals, while both KYNA and QUIN are able to function at ionotropic glutamate receptors (Barry *et al.*, 2009:287).

The neuroprotective KYNA is a non-selective antagonist of the excitatory NMDA receptor with a high affinity for the glycine co-agonist site on this receptor (Schwarcz, 2004:12). It is also able to antagonize the $\alpha 7$ nACh receptor, giving way to decreased glutamate, DA, ACh, and GABA release (Alexander *et al.*, 2012:627) and affecting memory and learning (Chess *et al.*, 2007:797). QUIN, on the other hand, is neurotoxic and functions as a NMDA receptor agonist (Barry *et al.*, 2009:287). Pro-inflammatory cytokines and reactive oxygen species are both able to induce IDO activity in the extrahepatic tissue, such as the brain, leading the process away from liver metabolism (Heyes *et al.*, 1993:1425). Moreover, inflammatory cytokines can also enhance the activity of KMO, favouring QUIN formation, which may result in excitotoxicity to neurons and apoptosis to astrocytes (Mellor & Munn, 1999:469; Myint & Kim, 2014:304). Subsequently, when a pro-inflammatory state exists in schizophrenia (as discussed in section 1.4.4) it may lead to increased activation of IDO and KMO, causing neuronal damage, further contributing to the degenerative pathology of schizophrenia. Additionally, the kynurenine pathway has been proposed to alter glutamatergic, 5-HTergic and DAergic pathways, all involved in schizophrenia pathology (reviewed in Myint & Kim 2014:304). Elevated levels of both KYN and KYNA have been found in the PFC and CSF of patients with schizophrenia (Erhardt *et al.*, 2003:155; Schwarcz *et al.*, 2001:521), evidently pointing to abnormal kynurenine metabolism in schizophrenia. In addition, post-mortem studies discovered increased levels of tryptophan, 3-OHAA, kynurenine and QUIN in a variety of brain regions in schizophrenia subjects (Torrey *et al.*, 1998:91). Specific analysis of QUIN has also revealed increased levels of this neurotoxic metabolite in the CSF of patients with schizophrenia and that could be correlated with recent exacerbation of illness (Torrey *et al.*, 1998:91). In addition, increased levels of TDO have been reported in the frontal cortex of post-mortem schizophrenia patients, confirming heightened enzyme activity in the kynurenine pathway (Miller *et al.*, 2004:618). While one study confirmed a decrease in plasma KYNA levels and the neuroprotective ratio in medication-free schizophrenia patients (Myint *et al.*, 2011:1576), another found elevated CSF KYNA levels in medicated patients (Linderholm *et al.*, 2012:426). This suggests that antipsychotics may play a role in normalizing kynurenine pathway deficits.

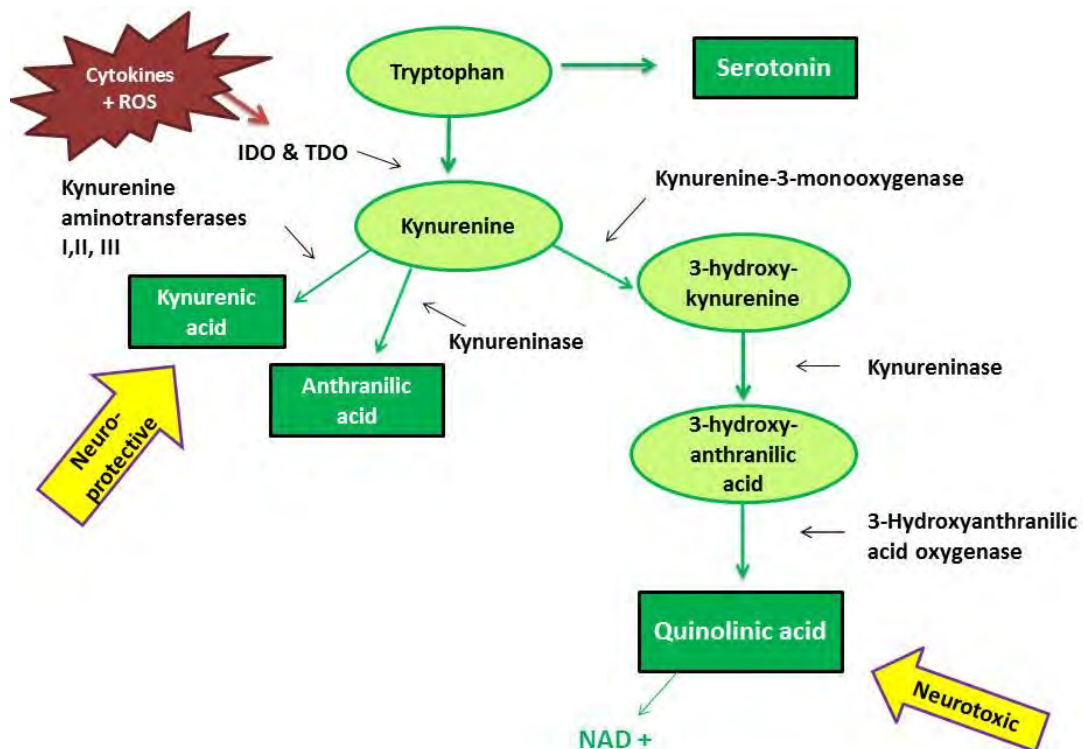


Figure 8: Tryptophan metabolism via the kynurenine pathway (ROS: Reactive oxygen species; IDO: Indoleamine 2,3-dioxygenase; TDO: Tryptophan 2,3-dioxygenase; NAD: Nicotinamide adenine dinucleotide).

2.1.4. h) Neurodevelopmental insults

The neurodevelopmental model of schizophrenia theorizes that the disease is the outcome of an anomaly in neurodevelopmental processes that begins prior to the onset of clinical symptoms, as early as *in utero* (Rapoport *et al.*, 2005:434; Singh *et al.*, 2004:435). The manifestation of behavioural and cognitive abnormalities during childhood, long before the onset of psychosis, suggests insults during early years of development and serves as evidence for the neurodevelopmental hypothesis (Lewis & Levitt, 2002:409). Causative factors include genetic influences and environmental insults, which may play a role in early (pre- or perinatal) brain development (Gilmore *et al.*, 1998:133) or during late adolescent brain maturation (Feinberg, 1982:319). The theories and observations on the exact period of

vulnerability are inconsistent (Rapoport *et al.*, 2005:434), and there is no clear evidence that the underlying pathophysiological process operates during maternal development or adolescence, respectively (Marenco & Weinberger, 2000:501). Consequently this literature review and subsequent experimental study will focus on both time periods as possible windows for disease development, but omitting genetic factors which is out of the scope of this dissertation.

Pre- and perinatal insults:

Various prenatal insults have been linked to subsequent schizophrenia development in later life. These insults include nutritional deficiencies (Susser *et al.*, 1996:25; Xu *et al.*, 2009:568), maternal stress exposure (Van Os & Selten, 1998:324), obstetric complications (Geddes & Lawrie, 1995:786) and prenatal infection / immune activation (Meyer, 2013:20).

i. Maternal infections and immune activation:

Various viruses and other infections are capable of interfering with foetal neurodevelopment, giving way to brain abnormalities such as structural changes as well as behavioural alterations like learning disabilities and mental retardation (Remington *et al.*, 2006). Infection with the *Influenza* virus during pregnancy was the first to be implicated in schizophrenia development in offspring (reviewed in Brown 2011:23). Subsequently, Brown and colleagues examined serological evidence of prenatal influenza infection and found that the risk of schizophrenia was amplified 7-fold for influenza exposure during the first trimester (Brown *et al.*, 2004:774). However, it was soon discovered that other pathogens may also contribute to the risk of schizophrenia (Krause *et al.*, 2010:739), suggesting that the underlying mechanisms in schizophrenia development may not be caused by a pathogen itself. Indeed, upon this discovery, Krause *et al.* (2010:739) theorized that an altered immune response may be the main culprit. It is important to note that cytokines play an essential role in brain development (Meyer, 2013:20), highlighting the potential influence that changes in the immune system may have on the evolving brain. Indeed, a study using in situ hybridization and immunohistochemistry showed more than 100% cytokine gene expression in normal human embryonic forebrain cells by week 19 (Mousa *et al.*, 1999:55). Further evidence for this hypothesis rests on observations of elevated pro-inflammatory cytokine levels in patients with schizophrenia (Miller *et al.*, 2011:127), pointing to existing immune irregularities in these individuals. Figure 9 describes a pathway from prenatal infection to brain pathology: when the foetus is exposed to infectious pathogens and/or inflammation, it may give way to an enhanced pro-inflammatory state in the foetal brain. This in turn causes microglia activation, which is (as discussed earlier) accompanied by oxidative stress. This early neuro-inflammatory state may result in altered neurodevelopment, priming an individual for long-

standing brain pathology. Altered microglia and astrocyte function as a consequence of early neuro-inflammation may also exert long-term postnatal effects on the brain, contributing to the progressive brain and behavioural pathology observed in schizophrenia (Meyer, 2013:20).

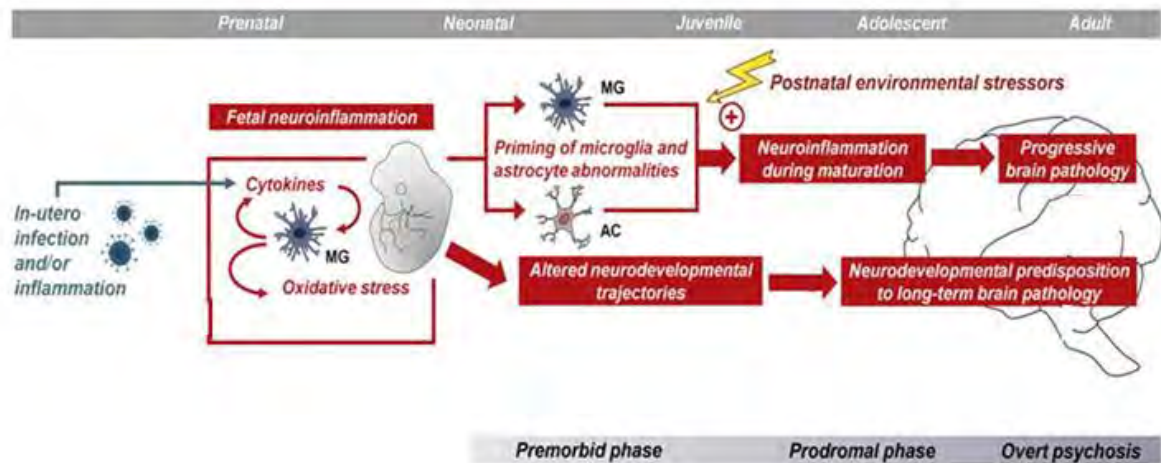


Figure 9: Maternal immune activation and the consequent postnatal cascade contributing to schizophrenia development. Figure adapted from Meyer (2013:20).

Postnatal insults:

Postnatal life events have also been implicated in the neurodevelopment of schizophrenia. These include childhood trauma (Rapoport *et al.*, 2012:1228), socioeconomic status (Brown 2011:23), infections during adolescence and adulthood (Brown 2011:23), stressful life events, as well as urban rearing (social isolation-relating factors) (McDonald & Murray 2000:130). However, for the purposes of this study, only drug use will be discussed as a postnatal insult.

ii. Drug-use:

Up to 55% of schizophrenia patients have been reported to have abused psychoactive drugs (Swofford *et al.*, 2000:343). This high incidence could be explained by two different theories: (a) drug abuse causes schizophrenia or gives way to schizophrenia manifestation in susceptible individuals, or (b) drug abuse is a form of self-medication in patients who already present with schizophrenia (Dixon *et al.*, 1991:224). Apart from drug abuse elevating the risk of schizophrenia, it has also been observed that substance abusing patients develop the

disease earlier and present with better premorbid adjustment, suggesting a possible initiating role for drug abuse in schizophrenia manifestation (reviewed in Dixon *et al.*, 1991:224).

One important drug of abuse in the 21st century is methamphetamine (MA), a potent releaser of DA, NA and 5-HT (Kirkpatrick *et al.*, 2012:109; Yamamoto *et al.*, 2010:101). MA was first used by soldiers in Japan during World War II to reduce fatigue (Machiyama, 1992:107), with psychiatrists later discovering both clinical and neurochemical parallels between MA psychosis and schizophrenia in these populations (Machiyama, 1992:107). Indeed, other researchers have also reported that the hallucinations and delusions experienced by MA abusers corresponds narrowly to the symptoms of schizophrenia, and that it is almost indistinguishable from paranoid schizophrenia (Schuckit, 2000). Bell and colleagues administered MA to psychiatric patients who were previously addicted to amphetamine, and noted that 12 of the 14 volunteers experienced psychosis lasting for up to 2 days, with 2 patients remaining in a psychotic state for up to 6 days (Bell, 1973:35). Researchers have identified “3 types” of MA psychosis: the transient type, prolonged type, and persistent type, with the last two implying persistent brain changes (discussed in greater detail in section 2) (Sato *et al.*, 1992:115). This suggests that permanent changes takes place that could ultimately lead to schizophrenia development.

Apart from MA, cannabis-use is also regularly implicated in schizophrenia development (Thorncroft, 1990:25). Cannabis exerts a very potent agonistic effect on the cannabinoid receptor 1 (CB1) and excessive stimulation of these receptors may affect the regulation of DAergic projections in certain brain areas (Brown, 2011:23). Robust evidence for a link between cannabis abuse and schizophrenia have been provided by Andreassen and colleagues, who found that 18 year old, heavy cannabis users had a 6-fold greater risk of developing schizophrenia later in life (Andreasson, 1988:1483). Another study found that 10% of cannabis users at the age of 15 developed schizophrenia by the age of 26 (Arseneault *et al.*, 2002:1212). Moore *et al.* (2007:219) also observed an increased risk to develop psychosis with cannabis use, with a greater risk in frequent users. It has also been noted that cannabis use is associated with earlier onset of psychotic disorders compared to non-users (Large *et al.*, 2011:555). Although the exact mechanisms remain elusive, the role of substance abuse in schizophrenia cannot be ignored, and is indeed a very important risk factor to take into consideration.

2.1.5. The two-hit hypothesis of schizophrenia

Seeing that schizophrenia presents with complex genetics, progressive pathology and late-life onset of the clinical syndrome, the “two-hit” hypothesis of schizophrenia has recently grown more important in an attempt at understanding the aetiology of this complex disorder (Maynard *et al.*, 2001:457). The two-hit hypothesis of schizophrenia theorizes that a genetic susceptibility combined with a particular developmental insult can prime a person for a later event, eventually giving way to schizophrenia presentation (Bayer *et al.*, 1999b:543). Genetic studies have failed to identify specific pathogenic genes (discussed in section 1.1), while twin studies generated probandwise concordance rates of only 41-65% in monozygotic siblings (Cardno *et al.*, 1999:162), proposing that other factors apart from genes alone contribute to schizophrenia development. Abnormal brain development, as observed in schizophrenia, is another aspect that has contributed to the development of this hypothesis (Bayer *et al.*, 1999b:543). Various structural abnormalities have been observed in schizophrenia (discussed in section 1.4.2) and several researchers have suggested dysfunctions in early brain development (either pre- or postnatal) as the cause of this pathological manifestation (Feinberg, 1982:319; Weinberger, 1995:552). Remarkably, a study has found that between monozygotic twins, the twin that developed schizophrenia later in life was found to present with either neurological or behavioural differences compared to his/her sibling by age 5 years (Torrey *et al.*, 1994:423). The fact that schizophrenia usually manifests during late adolescence / early adulthood when brain development ceases, is another contributor in support of the two-hit hypothesis (Bayer *et al.*, 1999b:543).

Although Bayer *et al.* (1999b:543) suggested genetic susceptibility to be the “first hit”, environmental influences during the prenatal period may similarly present the first hit for neurodevelopmental disturbances in offspring (Meyer & Feldon, 2010:285). This is indeed a critical period for brain development and the occurrence of a pathological event during this time could lead to abnormal brain maturation (Weinberger, 1995:552). One such event could very well be infection, considering the evidence that the immune system plays a role in CNS development (reviewed in Feigenson *et al.* 2014:72). Indeed, as mentioned above, Mousa *et al.* (1999:55) reported that over 100% cytokine gene expression has been observed in human embryonic forebrain cells by week 19. Furthermore, evidence for immune abnormalities in schizophrenia (section 1.4.4 & 1.4.8) confirms the possible role of cytokines and inflammation as first hit. Regarding the second hit, various environmental factors may represent this final influence including exposure to stress or psychotropic drugs during adolescence (Meyer & Feldon, 2010:285). Adequate evidence for the role of drug abuse in schizophrenia development is discussed in section 1.4.8, implying that it must be considered a strong candidate as a “second hit”. Additionally, Chen and colleagues found that MA

abusers with a pre-morbid schizoid / schizotypal personality were more likely to manifest with psychosis and that it tended to be more persistent (Chen *et al.*, 2005:87). When thinking of the two-hit hypothesis of schizophrenia, it could be proposed that these patients' pre-morbid state was caused by a "first hit" (genetic / environmental factors) and that subsequent MA-abuse was the "second hit", leading to schizophrenia symptomology. It is clear that the two-hit hypothesis provide a better explanation for the developing nature of schizophrenia, while also having the potential to clarify its pathology and aetiology, rendering this theory a valuable angle for future research, as has recently been presented by Möller and colleagues (Möller *et al.*, 2015:987).

2.1.6. Treatment

Not long ago, the pharmacological treatment of schizophrenia was based primarily on the modulation of DAergic systems, specifically antagonism of D₂ receptors with typical antipsychotics (Lewis & Levitt, 2002:409). These first generation drugs (refer to table 2 for examples) evolved from the discovery during the 1950's that the D₂ antagonist, chlorpromazine, revealed antipsychotic effects in schizophrenia patients (Anderson & Maes, 2013:5). These drugs are usually accompanied by prominent side effects such as extrapyramidal syndrome, tardive dyskinesia, and neuroleptic malignant syndrome (reviewed in Beaulieu & Gainetdinov 2011:182). Upon the more recent discovery of the newer atypical antipsychotics (refer to table 2 for examples), the underlying mechanisms broadened to include other neurotransmitter pathways, such as 5-HT, Ach, noradrenaline and glutamate (Kinon & Lieberman, 1996:2). These drugs either possess low ratios of affinities for D₂ vs. 5-HT_{2A} receptors, or for different combinations of multiple receptors and reuptake sites (Lewis & Levitt, 2002:409). Serotonin specifically plays a major role in the mechanisms of various atypical antipsychotics, such as 5-HT_{2A} receptor antagonists, 5-HT_{1A} receptor agonists or antagonists, 5-HT_{2C} receptor inverse / partial agonists or neutral antagonists, 5-HT₆ receptor antagonists, and 5-HT₇ receptor antagonists (Meltzer *et al.*, 2011:289). These compounds tend to be more effective (especially against negative or cognitive symptoms) or better tolerated (fewer side effects) (Geddes *et al.*, 2000:1371). However, this is not always the case and these drugs possess their own array of serious side-effects such as weight gain and impaired glucose tolerance (Kapur & Remington, 2001:873). Apart from the numerous side-effects, different psychotic symptoms respond diversely to pharmacotherapy, with positive symptoms usually responding successfully to both typical and atypical antipsychotics, whereas negative and cognitive symptoms do not always fare so well (Kapur & Remington, 2001:873). Moreover, it is predicted that up to 25% of patients are treatment-

resistant with regards to current antipsychotic drugs (Remington *et al.*, 2005:843). Clearly, the discovery of new treatment strategies is essential; especially routes that take into account the other equally important pathological mechanisms of schizophrenia, such as redox-inflammatory pathways.

Table 2: List of typical and atypical antipsychotics, respectively. Adapted from Beaulieu & Gainetdinov (2011:182) and Seeman (2002:27)

Typical antipsychotics	Atypical antipsychotics
Chlorpromazine	Clozapine
Prochlorperazine	Risperidone
Haloperidol	Sulpiride
Promazine	Amisulpride
Flupentixol	Ziprasidone
Pimozide	Aripiprazole
Droperidol	Olanzapine
Fluphenazine	Quetiapine
Perphenazine	Remoxipride,
Triflupromazine	Sertindole
Perazine	Melperone
Trifluoperazine	Perlapine
Sultopride	Amoxapine
Clopendixol	lloperidone
Thiothixene	Asenapine
Tiapride	Lurisadone
Thioridazine	
Fluspirilene	
Benperidol	
Trifluoperidol	
Penfluridol	
Loxapine	

2.2. Methamphetamine (MA)

In 1887, MA, a derivative of amphetamine, was synthesized for the first time by a German chemist (Anglin *et al.*, 2000:137). Its first medical use was as a bronchodilator as a nasal spray for asthma during the early 1930's and its later indications included low blood pressure, morphine addiction and even schizophrenia (Anglin *et al.*, 2000:137). However, MA was not widely used until World War II when the United States, Germany and Japan supplied the drug to military workers as an endurance-enhancer (Anglin *et al.*, 2000:137; Machiyama, 1992:107). Today amphetamine derivatives (in particular MA and 3,4 methylenedioxymethamphetamine (MDMA), or ecstasy) are regarded as the most

universally abused drugs (Iacovelli *et al.*, 2006:24). This upsurge in abuse is mostly due to the drugs' ability to induce euphoria; however the long-term negative consequences of this life-altering addiction are rapidly coming more to light (Halpin *et al.*, 2014:37). MA-abuse is in particular a growing problem, especially in the Cape Province of South Africa (Plüddemann *et al.*, 2008:185). Henceforth, this literature review will focus on the effects and toxicities of MA in particular.

2.2.1. Chemistry and physical properties

"Amphetamines", initially synthesized as alternatives to ephedra (*Ephedra sinica*), refer to chemical compounds that contain the amphetamine structure, an α -methyl-phenethyl-amine, illustrated in Figure 10 (Sulzer *et al.*, 2005:406). The physical features of their key structure are defined as (1) an unsubstituted phenyl ring, (2) a two-carbon side chain between the phenyl ring and nitrogen, (3) an α -methyl group, and (4) a primary amino group (Biel & Bopp, 1978). MA (chemical name: N-methyl-1-phenylpropan-2-amine) is the N-methyl derivative of amphetamine, although it does not contain a primary amino group as described by the fourth rule above (see Figure 10) (Cruickshank & Dyer, 2009:1085; Sulzer *et al.*, 2005:406). MA is highly lipid soluble because of the N-methyl group present in its structure, leading to rapid distribution in the CNS after administration. Furthermore, MA consists of a mixture of stereoisomers, namely *d*- and *l*-stereoisomers, of which *d*-methamphetamine is the more biologically active (Cho, 1990:631; Sulzer *et al.*, 2005:406).

In the 1800's Japanese scientist Nagai Nagayoshi synthesized MA for the first time using ephedrine, the active alkaloid in ephedra, as precursor (Panenka *et al.*, 2012:1). Later, in 1919 Akira Ogata established the method of producing crystallized MA by reducing ephedrine via red phosphorous and iodine, providing the basis for its future production (Panenka *et al.*, 2012:1; Vearrier *et al.*, 2012:38). This ephedrine reduction method is simple, inexpensive and more efficient than its predecessor, resulting in purer yields of the intoxicating and addictive D-isomer of MA (Meredith *et al.*, 2005:141). Crystal MA (*d*-methamphetamine hydrochloride) presents as white or translucent crystals for vapour inhalation (Cruickshank & Dyer, 2009:1085).

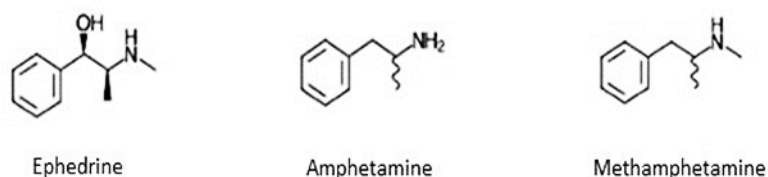


Figure 10: The chemical structures of ephedrine, amphetamine and MA respectively (Vearrier *et al.*, 2012:28).

2.2.2. Pharmacology of MA

After years of extensive study, researchers have established that MA exerts its main effect at the presynaptic terminal of catecholamine neurons (Cho, 1990:631). MA stimulates the release of catecholamines in the CNS and blocks, to some degree, their presynaptic reuptake, resulting in postsynaptic receptor stimulation (Cho, 1990:631; Meredith *et al.*, 2005:141). Thus, apart from disturbing vesicular storage systems, MA also interacts with monoamine oxidase (MAO) and the neuronal transporter (uptake I transporter) (Cho, 1990:631). Furthermore, it has been discovered that MA more potently increases NA levels, followed by DA and lastly 5-HT (Rothman *et al.*, 2001:32).

Although DA is not responsible for all the amphetamine-induced effects of MA in humans (Rothman *et al.*, 2001:32), it has been the neurotransmitter most studied, primarily due to its essential role in reward and reinforcement processes (Panenka *et al.*, 2012:1). Indeed, the mesolimbic dopamine system has been suggested to play a key role in the process of salience, as discussed in Section 1.4.3 (Kapur, 2003:13). MA's main mechanism of action at DA terminals is its interaction with the vesicular monoamine transporter-2 (VMAT-2) and the plasmalemmal dopamine transporter (DAT) (Panenka *et al.*, 2012:1). MA is able to compete with synaptic DA at the extracellular site on DAT, causing DA to be reverse transported outside the cell (refer to Fig. 11) (Panenka *et al.*, 2012:1). VMAT-2 is an important membrane protein which acts as a transporter of monoamines from the intracellular cytosol into synaptic vesicles (Fleckenstein *et al.*, 2009:133). VMAT-2 is linked to a vacuolartype H⁺-pumping ATPase, generating a pH gradient across the vesicle membrane, with the vesicular internal environment being somewhat acidic (reviewed in Panenka *et al.*, 2012:1). MA, a weak basis, disrupts the pH gradient at high concentrations and causes synaptic vesicles to leak monoamines into the surrounding cytosol (refer to Fig. 11) (Schwartz *et al.*, 2006:1347). In addition, MA is a substrate for MAO so that it can be transported into presynaptic

terminals where it inhibits both DA storage, as well as its degradation by MAO, resulting in increased cytoplasmic DA levels (Fig. 11) (Cho, 1990:631). In addition to causing changes in presynaptic DA markers, MA has also been shown to reduce postsynaptic D₁ and D₂ receptors in preclinical and clinical studies (Cadet *et al.*, 1998:240; Volkow *et al.*, 2001:2015).

Despite the above knowledge, our understanding of the stimulating effects of MA on monoamines remains limited for various reasons (discussed in Cruickshank & Dyer 2009:1085). For instance, baseline DA function seems to influence the response to MA, with low baseline D₂ density linked to a pleasant response, while high baseline D₂ density may produce unpleasant responses after MA administration (Volkow *et al.*, 1999:1440). Subsequently, our understanding of MA effects on NA and 5-HT systems requires further study. Moreover, MA has been reported to influence these systems via action on the noradrenaline transporter (NET) and serotonin transporter (SERT) (Cruickshank & Dyer, 2009:1085).

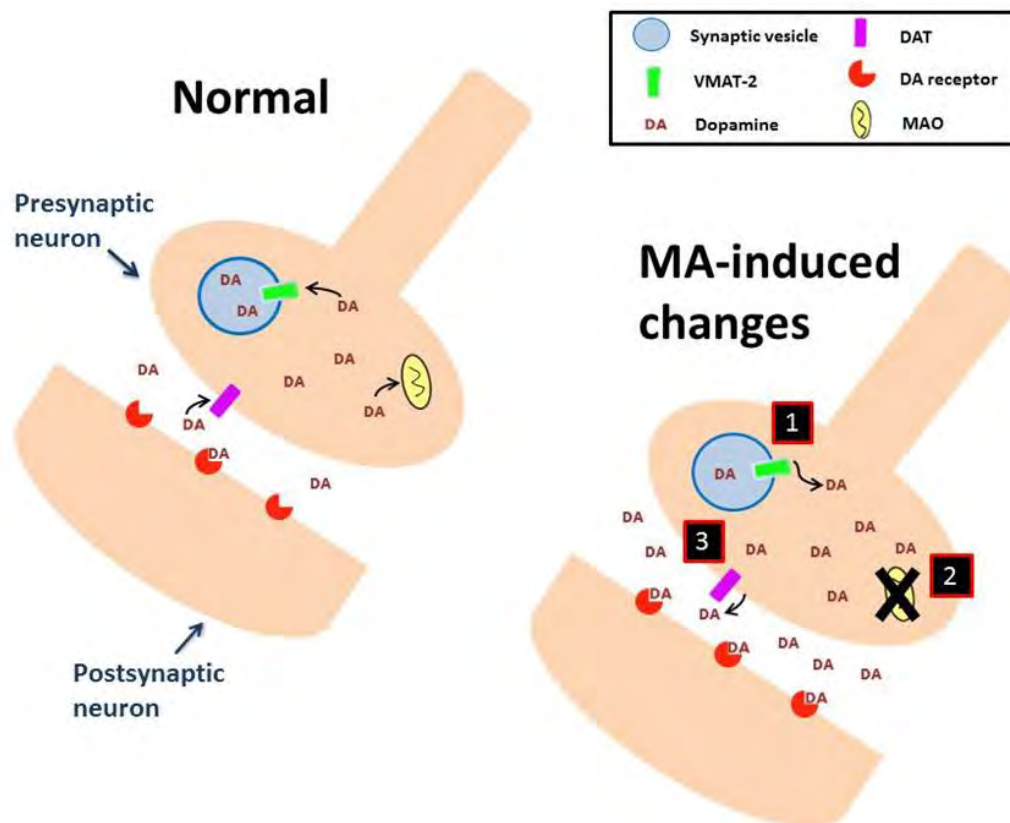


Figure 11: Dopamine changes induced by MA at the synaptic level: 1) MA disrupts pH gradient of VMAT-2 coupled H^+ -pumping ATPase, causing DA to leak out of synaptic vesicles, 2) MA inhibits the activity of MAO, thereby increasing cytosolic DA levels, 3) MA blocks DAT and causes reverse transportation of DA to the outside of the postsynaptic cytosol (Cho, 1990:631; Kish, 2008:1679; Vearrier et al., 2012:38).

2.2.3. Pharmacokinetics of MA

The absorption and elimination of MA is important within the context of its abuse (Cho, 1990:631). As stated before, MA is highly lipophilic, ensuring its rapid penetration into the brain. MA can be administered intravenously (IV), producing euphoric effects within seconds, or it can be inhaled; a route by which it is also rapidly absorbed (Cho, 1990:631). Apart from brain penetration, researchers have also observed rapid distribution of MA in most organs after administration, especially in the lungs, liver and kidneys (Volkow et al., 2010:1). MA has a half-life of 12 hours, hence users present promptly with euphoria, followed by prolonged psychoactive effects (Meredith et al., 2005:141). The metabolism of MA takes place predominantly in the liver via cytochrome P450 2D6 (Fig.12), with numerous metabolites being produced along these pathways (Caldwell et al., 1972:11).

These metabolites are not thought to contribute significantly towards the acute effects of MA. In fact, amphetamine concentrations originating from MA metabolism have been found to be low to insignificant (Cruickshank & Dyer, 2009:1085). However, some of these metabolites are indeed pharmacologically active and with repeated MA administration may accumulate in the body, resulting in some of the pathological ramifications observed in MA abusers (Cho, 1990:631). Within 24 hours, up to 70% of the ingested MA dose is excreted in the urine: 30-50% as MA, 15% as 4-hydroxymethamphetamine and 10% as amphetamine (Cook *et al.*, 1993:717; Kim *et al.*, 2004:664).

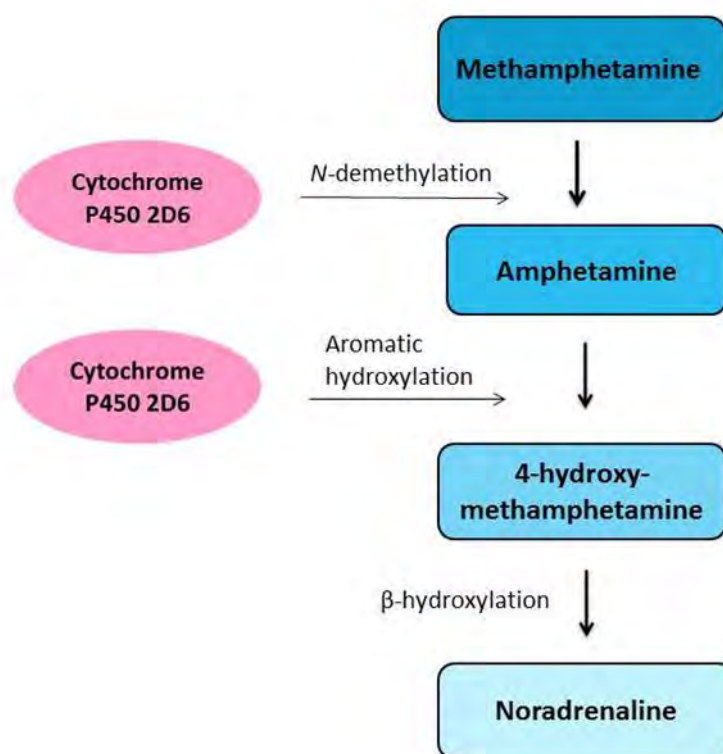


Figure 12: Metabolic cascade of MA in the liver: 1) via Cytochrome P450 2D6, N-demethylation of MA takes place, producing amphetamine, 2) followed by aromatic hydroxylation via Cytochrome P450 2D6 that reduces amphetamine to 4-hydroxy-methamphetamine, and 3) as a final step, β -hydroxylation produces NA as the end-metabolite (Cruickshank & Dyer, 2009:1085).

2.2.4. Effects and abuse of MA

Like most abused substances, such as opiates, marijuana and nicotine, plant products of amphetamines have been used for ages before it was first chemically synthesized (Sulzer *et al.*, 2005:406). Historic use of the *Ephedra* genus and the *Catha edulis* tree extracts were fuelled by their ability to create feelings of contentment and sociability, suppression of fatigue, and appetite loss (Sulzer *et al.*, 2005:406). Today, MA, a potent psychomotor stimulant drug with strong physiological effects on peripheral and central systems, is widely abused because of effects such as euphoria, increased energy and attention, initial hypersexuality and appetite suppression (Nordahl *et al.*, 2003:317; Panenka *et al.*, 2012:1). In addition to these desired effects, MA also causes increased heart function (elevated pulse rate and blood pressure), bronchodilation, vasoconstriction and inhibited intestinal function (Büttner, 2011:118; Panenka *et al.*, 2012:1). The conventional dose of MA for CNS stimulation is approximately 10 mg (Cho, 1990:631); however abusers have reported patterns of repeated dosing of about 20-40 mg every 2-3 hours, which can result in up to 1 g being consumed during a 24-hour binge (Meredith *et al.*, 2005:141). With eventual excretion of MA, the withdrawal syndrome commences with dysphoria and depression, anxiety, irritability, aggression and intense fatigue coupled with hypersomnia (reviewed in Meredith *et al.*, 2005:141). This state, together with intense drug cravings, represents a strong motivation for repeated drug use. Repetitive use eventually results in MA tolerance, driving the user to increase the dose or to seek more potent forms of MA, hereby setting off a damaging cycle of abuse (Darke *et al.*, 2008:253). Currently, MA is regarded as the second most abused drug, after cannabis, world-wide (Cruickshank & Dyer, 2009:1085), while MA abusers represents up to 12.8% of admissions to drug addiction treatment centres in South Africa (Peltzer *et al.*, 2010:2221).

2.2.5. Adverse effects of MA abuse

From as early as 1935, serious health effects have been linked to the use of MA (Vearrier *et al.*, 2012:38). These early studies reported pallor, flushing, palpitations, elevated pulse rate and high blood pressure, later followed by more serious complaints such as troubled breathing, convulsions, coma, psychosis, and cyanosis (Vearrier *et al.*, 2012:38). This literature review will focus primarily on the neurobiological adverse effects of MA, however a short description of peripheral negative effects will be covered in section 2.5.2.

2.2.5. a) Neurotoxic effects and CNS-related toxicity

As previously discussed, MA leads to increased neurotransmitter release in the CNS. This potent effect consequently gives way to prolonged damage to dopaminergic and serotonergic axon terminals in various brain regions, including the striatum, hippocampus and PFC (reviewed in Halpin *et al.* 2014:37). Although the precise mechanisms of these harmful effects are not exactly understood, it is proposed that the oxidation of cytosolic DA and 5-HT to 6-hydroxydopamine and 5,6-dihydroxytryptamine, respectively, may oxidize proteins and lipids in DA and 5-HT-rich neurones (Cruickshank & Dyer, 2009:1085). Subsequent neurochemical markers of MA toxicity include decreased DAT and SERT expression, as well as reduced DA and 5HT levels in tissue (reviewed in Halpin *et al.* 2014:37). Together with diminished DAT and DA, a post-mortem study also revealed decreased TH in the caudate and putamen (Wilson *et al.*, 1996:699). In addition, it has been observed that DAT binding in previous MA abusers was reduced for up to 3 years of abstinence (McCann *et al.*, 1998:8417). Impaired DAT results in the inhibition of DA reuptake and this in turn leads to the oxidation of DA and the formation of ROS (Riddle *et al.*, 2006:E413; Vearrier *et al.*, 2012:38). Various brain regions also present with MA-induced serotonergic damage, including the hippocampus, perirhinal cortex, anterior cingulate cortex, nucleus accumbens, caudate nucleus and amygdala (reviewed in Panenka *et al.*, 2012:1).

In addition to dopaminergic and serotonergic pathways, MA-abuse has also been linked to cell-death in different subpopulations of GABA-interneurons (Halpin *et al.*, 2014:37). The suggested mechanisms of MA-induced apoptosis include: (1) DNA damage, (2) alterations in gene expression and (3) up-regulation of caspase-3 activity and the Fas ligand / Fas death pathway (Jayanthi *et al.*, 2001:1745; Jayanthi *et al.*, 2005:868; Jeng *et al.*, 2006:638). Indeed, cell loss by means of apoptosis has been observed in the striatum after MA administration (Tulloch *et al.*, 2011:162). Excitotoxic damage of neurons as a result of increased glutamate release has also been reported after MA exposure (Yamamoto & Bankson, 2005:87). Distorted, swollen nerve terminals and oedematous and degenerative changes have been described in preclinical studies, suggesting morphological changes to axon terminals caused by MA (reviewed in Halpin *et al.* 2014:37). MA has also been shown to cause reductions in hippocampal volume and grey matter, as well as hypertrophy of white matter in prefrontal cortical regions (Daumann *et al.*, 2011:794; Thompson *et al.*, 2004:6028). In addition, enlarged striatal volumes have been detected in regular MA users after abstinence (Chang *et al.*, 2005:967).

Other frequent and serious CNS complications linked to MA abuse include seizures and intracranial haemorrhage (Alldredge *et al.*, 1989:1037, Vearrier *et al.*, 2012:38).

Hyperpyrexia, resulting from elevated catecholamine levels, has been suggested to be the contributing factor to these conditions (Meredith *et al.*, 2005:141). Hypertension and tachycardia may also contribute to the development of intracranial haemorrhage (Inamasu *et al.*, 2003:82). Ruptured berry aneurysms have also been observed in MA-abusers, usually followed by death (Davis & Swallow 1994:1481). In addition, fatal intraparenchymal bleeds in numerous brain regions, including the cerebrum, cerebellum, corpus callosum, basal ganglia, and brainstem, have been linked to MA abuse (reviewed in Vearrier *et al.*, 2012:38). Ischemic or haemorrhagic stroke is another dangerous complication associated with MA use (Perez Jr. *et al.*, 1999:469). MA-induced cerebral vasculitis has been suggested as an important contributing pathophysiologic mechanism of these fatal occurrences (Perez Jr. *et al.*, 1999:469). Finally, several lines of evidence suggest blood-brain barrier (BBB) dysfunction following MA abuse (Cadet & Krasnova, 2009:101).

2. 2.5. b) *Peripheral adverse effects*

Cardiovascular adverse effects are common complaints amongst MA abusers (Vearrier *et al.*, 2012:38). Chest pain is one of the most prevalent symptoms (Richards *et al.*, 1999:198), usually as a result of MA-induced hypertension or tachycardia (Wijetunga *et al.*, 2004:8). However, researchers have reported more serious cardiovascular conditions in MA-abusers, viz. unstable angina, acute myocardial infarction or acute aortic dissection (Swallow & Davis, 1999:23; Wijetunga *et al.*, 2004:8). The excessive discharge of catecholamines by MA may lead to sensitization of the abuser's myocardium to ectopic cardiac stimuli, which can develop into lethal cardiac arrhythmias (Meredith *et al.*, 2005:141). Other cardiac complications include accelerated atherosclerosis, ventricular fibrillation, ventricular tachycardia, supraventricular tachycardia, hyper-coagulability and acute dilated cardiomyopathy with global ventricular dysfunction (Chen, 2007:E89; Turnipseed *et al.*, 2003:369; Vearrier *et al.*, 2012:38). Finally, as with most IV abused drugs, MA is also associated with right-sided infective endocarditis that can be fatal (Takasaki *et al.*, 2003:65).

Other dangerous adverse effects include renal failure, hepatotoxicity and rhabdomyolysis (Bingham *et al.*, 1998:2654; Kamijo *et al.*, 2002:216; Shaw, 1999:27). A study consisting of 367 patients diagnosed with rhabdomyolysis found that 43% of these subjects tested positive for MA (Richards *et al.*, 1999:198). Decreased calorie and fluid intake, together with increased ATP demand via the sympathomimetic effects of MA, have been suggested as a contributing cause to rhabdomyolysis development (Richards *et al.*, 1999:198). Hypertension, myoglobinuria and necrotizing angitis caused by MA have all been implicated in the development of renal damage (Brannan *et al.*, 2004:8). MA-induced acute interstitial

nephritis, giving way to renal failure, has also been described previously (Foley *et al.*, 1984:258). Apart from having a higher risk of hepatitis C infection via contaminated needles and irresponsible sexual behaviours, MA-abusers have also been reported to develop acute liver injury with hepatic necrosis and centrilobular degeneration, possibly as a direct detrimental effect of MA (Kamijo *et al.*, 2002:216). In addition, MA may also enhance the hepatotoxic effect of other substances (Vearrier *et al.*, 2012:38).

Other somewhat less dangerous effects of MA include dental, dermatological and ophthalmologic effects (Vearrier *et al.*, 2012:38). Dental side-effects also referred to as “meth-mouth”, comprise general drug-induced hyposalivation, which leads to tooth decay, as well as MA-specific effects including more extensive decay, bruxism (repetitive grinding / clenching of the teeth) and almost non-existent oral hygiene (Donaldson & Goodchild, 2006:2078). Xerostomia is also frequently observed, developing from sympathetic overstimulation and bruxism (Goodchild *et al.*, 2007:124). Factors contributing to the development of xerostomia include dehydration as a result of poor fluid intake, as well as a result of the inhibitory effect of MA on α_2 -receptors, leading to decreased saliva production (Curtis, 2006:125; Donaldson & Goodchild, 2006:2078). In the absence of protective saliva, MA-users also tend to crave sugary drinks, eventually contributing to widespread decay involving the facial and cervical areas of the maxillary and mandibular teeth, with subsequent progression to frank coronal involvement (Donaldson & Goodchild, 2006:2078). The main dermatological effects of MA are skin and soft-tissue infections (Vearrier *et al.*, 2012:38). MA-users have a higher risk of contracting such infections because of the use of non-sterile needles, as well as skin-picking practices induced by MA-related formication or delusions (Marschall *et al.*, 1991:328; Vearrier *et al.*, 2012:38). Ischemic optic neuropathy secondary to MA-induced vasospasm and MA-related vasculitis has been reported to lead to acute unilateral vision loss (Wijaya *et al.*, 1999:19). Such permanent vision loss has been described after only a single nasal administration of MA, highlighting the dangers of MA-abuse (Wijaya *et al.*, 1999:19).

2.2.5. c) Overdose

Toxic plasma levels of MA ranges between 200 - 5 000 mg/L, with fatal levels estimated at a concentration of more than 10 000 mg/L (Inoue *et al.*, 2006:150). A range of acute psychological and physical symptoms are associated with MA overdose (Darke *et al.*, 2008:253). Psychological symptoms may comprise extreme agitation and anxiety, paranoia, hallucinations and excited delirium, while physical symptoms usually include an elevated body temperature, dilated pupils, tachycardia, chest pain, vomiting, breathing irregularities and seizures (Cruickshank & Dyer, 2009:1085; Darke *et al.*, 2008:253). Hepatic and renal

failure can also occur (Cruickshank & Dyer, 2009:1085). MA overdose may lead to death, usually on account of preceding cerebrovascular haemorrhage, pulmonary oedema / congestion, congestion of multiple organs, ventricular fibrillation or acute cardiac failure (Inoue *et al.*, 2006:150; Kalant & Kalant, 1975:299). Asphyxia by the aspiration of vomit may also give way to fatalities (Kalant & Kalant, 1975:299). Behavioural disturbances after toxic doses of MA may sometimes account for MA-associated deaths, usually in the form of suicide, crime activities and accidents (Logan *et al.*, 1998:28; Zhu *et al.*, 2000:443).

2.2.6. The neurobiological basis of MA toxicity

A number of molecular mechanisms have been implicated as the underlying causes of MA-induced neurotoxicity, and include microglial activation, oxidative stress, excitotoxicity, apoptotic pathways and DNA damage (Cadet & Krasnova, 2009:101). Although our understanding of these mechanisms in relation to MA is still somewhat limited, especially in humans, I will henceforth discuss these factors as extensively as possible. Importantly, knowledge of these mechanisms may provide clues to treating MA-associated toxicity.

2.2.6. a) Inflammation

It has been reported that MA administration leads to microglia activation (Sekine *et al.*, 2008:5756; Thomas *et al.*, 2004:349). This causes the release of pro-inflammatory cytokines and prostaglandins, as well as oxidative species, such as superoxide and NO (Hanisch, 2002:140). MA is regarded as a neurotoxin due to its widespread damage to various nerve terminals (discussed in section 2.5.1). An important role for microglia function in the toxicity of MA was confirmed when Thomas and colleagues concluded that only the *neurotoxic* amphetamines activated microglia (Thomas *et al.*, 2004:349). Activated microglia excretes pro-inflammatory cytokines such as IL-1 α , IL-1 β , IL-6, and TNF- α , which have been implicated in the neurotoxic effects of MA (reviewed in Yamamoto & Raudensky 2008:203). Indeed, it has been suggested that the inflammation caused by MA may be directly linked to DA and 5-HT terminal degeneration (Yamamoto *et al.*, 2010:101). Furthermore, MA produces microglial activation in various brain areas, such as the striatum, cortex and hippocampus, but not in areas where DA levels remain unaltered (Yamamoto *et al.*, 2010:101). MA-induced increased glutamate release has also been implicated as a mechanism by which microglia activation and subsequent neuro-inflammation take place (Yamamoto *et al.*, 2010:101). A neurotoxic cycle forms: the stimulation of glutamate receptors leads to microglia activation, while the cytokines produced by microglia may in turn increase extracellular glutamate levels (Yamamoto *et al.*, 2010:101).

2.2.6. b) Oxidative stress

Several human studies have detected signs of oxidative stress in MA-abusers (Fitzmaurice *et al.*, 2006:703; Mirecki *et al.*, 2004:1396). MA has been shown to produce ROS, RNS and products of lipid peroxidation in rat brain tissue (Yamamoto *et al.*, 2010:101). Similarly, increased striatal formation of hydroxylated metabolites of salicylate (2, 3 dihydroxybenzoic acid) and d-phenylalanine (p-tyrosine) have been observed, suggesting elevated production of free radicals following MA consumption (Yamamoto & Zhu, 1998:107). Additionally, MA heightens microglia activation, which is likewise associated with free radical production (Hanisch, 2002:140; Thomas *et al.*, 2004:349). Subsequent elevated free radical production gives way to amplified lipid peroxidation and protein oxidation, measured by raised levels of malonyldialdehyde and protein carbonyl groups, respectively (Gluck *et al.*, 2001:152; Yamamoto & Zhu, 1998:107). Moreover, it has been reported that MA reduces the levels of antioxidants in both DAergic and 5-HTergic terminals (Yamamoto *et al.*, 2010:101), rendering nerve terminals susceptible to damage from oxidative species.

NO, an important neuromodulator, has been shown to be increased after MA administration (Friend *et al.*, 2014:153). Although NO plays an integral part in normal physiological processes, overproduction of this molecule can have detrimental effects (Friend *et al.*, 2014:153). For instance, NO-induced nitrosylation of proteins gives way to malfunctioning proteins and enzymes (Blanchard-Fillion *et al.*, 2001:46017). In addition, NO can form peroxynitrate by interacting with superoxide (Beckman *et al.*, 1990:1620), with the former inhibiting components of the mitochondrial electron transport chain as well as causing lipid peroxidation, protein nitration and DNA damage (reviewed in Friend *et al.*, 2014:153). Although the precise role of NO in MA-induced toxicity is vague, it has been suggested to be a necessary co-factor in the degenerative processes that follows MA exposure (Friend *et al.*, 2014:153).

Oxidative stress resulting from elevated DA and glutamate also contributes to the neurotoxic ramifications of MA abuse (Tata & Yamamoto, 2007:49). Heightened levels of intracellular DA and 5-HT after MA administration lead to an overproduction of oxidized DA and 5-HT metabolites, i.e. 6-hydroxydopamine and 5,6-dihydroxytryptamine (Wrona *et al.*, 1997:146). These metabolites are able to induce the oxidation of proteins and lipids in DA- and 5-HT-rich neurons (Cruickshank & Dyer, 2009:1085). DA that is not rapidly metabolized autoxidizes to form quinones which are able to attack lipids and proteins, as well as boost free radical production (Brown & Yamamoto, 2003:45). In addition, DA quinones are able to bind to cysteinyl residues, forming protein-bound cysteinyl catechols that are selectively toxic to DA terminals (LaVoie & Hastings, 1999:1484).

2.2.6. c) *Glutamatergic system*

As noted above, MA increases glutamate levels (Abekawa *et al.*, 1994:276; Nash & Yamamoto, 1992:237). Glutamate, a major excitatory neurotransmitter, plays a vital role in the modulation of excitatory neurotransmission in the CNS (Tata & Yamamoto, 2007:49). Excitotoxicity is the overstimulation of excitatory amino acid receptors (ionotropic receptors), which leads to cell damage and apoptosis (Thrash *et al.*, 2009:966). Prolonged elevations in glutamate levels also leads to nitric oxide synthase (NOS) activation and the subsequent production of NO and superoxide (reviewed in Tata & Yamamoto, 2007:49). As discussed in section 2.6.2, NO reacts with superoxide to form highly reactive peroxynitrite. Seeing that MA has been reported to increase extracellular concentrations of glutamate in vital brain areas, such as the hippocampus and striatum (Tata & Yamamoto, 2007:49), it is evident that glutamate-induced oxidative stress and excitotoxicity plays a major role in the neurotoxicity of MA.

2.2.6. d) *Mitochondrial function*

Mitochondria are the primary source of cellular ATP (covered in section 1.4.7). MA has been reported to inhibit enzyme complexes in the electron transport chain (ETC) involved in the functioning of mitochondria (Burrows *et al.*, 2000:11). ATP production produces ROS under normal conditions and further inhibition of the ETC leads to heightened levels of these reactive species (Brown & Yamamoto, 2003:45). MA has also been implicated in diminishing the function of specifically complex II and IV of the mitochondrial chain (Brown *et al.*, 2005:429; Burrows *et al.*, 2000:11). Subsequent altered energy metabolism has been casually associated with MA-induced damage to DA nerve terminals (Tata & Yamamoto, 2007:49). Indeed, reduced ATP production can damage cells by means of necrosis and apoptosis (Thrash *et al.*, 2009:966). Although it is not clear how MA inhibits mitochondrial function, elevations in glutamate noted above have been suggested as a causative factor (Tata & Yamamoto, 2007:49). Glutamate-associated excitotoxicity is mediated by mitochondrial damage through stimulation of NMDA receptors. The subsequent increase in Ca^{2+} influx results in a decrease in mitochondrial membrane potential and amplified permeability of the transition pore, resulting in cell death (Schinder *et al.*, 1996:6125). The lipid solubility of MA also enables it to penetrate mitochondria cells leading to the prolongation of its damaging effects (Thrash *et al.*, 2009:966).

2.2.7 MA and psychiatric illness

Several psychiatric symptoms are manifest during and after MA abuse, including paranoia, hallucinations, disturbed consciousness, irritability, poor concentration and depression (Nakatani & Hara, 1998:131; Newton *et al.*, 2004:248). These symptoms are usually only temporary, although MA users tend to develop more lasting psychiatric illnesses (Copeland & Sorensen, 2001:91). Indeed, up to a quarter of MA users present with psychiatric symptoms of such severity as to warrant hospitalisation, while a third have a history of being prescribed one or other psychiatric medication (Zweben *et al.*, 2004:181). Psychosis is one of the most frequent psychiatric presentations seen in psychostimulant abuse (Darke *et al.*, 2008:253). The amphetamines have been reported to aggravate psychotic symptoms in schizophrenia patients, but may also induce psychosis in users devoid of a history of psychiatric disorders (reviewed in Grant *et al.*, 2012:113). A study found that up to 23% of MA users presented with at least one psychotic symptom, and that MA abusers are eleven times more likely to develop psychosis than the average person (McKetin *et al.*, 2006:1473). Psychotic symptoms induced by MA usually include hallucinations (commonly auditory), delusions and pathologic hostility, which in some cases is accompanied by disorganized behaviour and speech (Darke *et al.*, 2008:253; McKetin *et al.*, 2008:235). Generally, MA psychosis mimics paranoid schizophrenia, even presenting with similar deficits in neurocognitive functioning (Jacobs *et al.*, 2008:98). Psychotic symptoms in MA-abusers can last several days and in serious cases may require hospitalisation and antipsychotic treatment (Darke *et al.*, 2008:253). Although a psychotic state following MA abuse is usually temporary, chronic cases have been reported; generally amongst users who presented with premorbid schizotypal signs or a family history of schizophrenia (Chen *et al.*, 2003:1407; Chen *et al.*, 2005:87; Sato *et al.*, 1992:115).

Depressive symptoms are also common among MA users, with up to a third being diagnosed with depression at some point in their lives (Zweben *et al.*, 2004:181). This can be fatal, seeing as MA-induced depression is frequently accompanied by suicidal ideation (Yen & Shieh, 2005:444). Suicidal behaviour is specifically observed with IV MA abuse, and is also more common among women and users with a history of psychotic disorders (Glasner-Edwards *et al.*, 2008:24). Strikingly, up to a quarter of MA users attempt suicide, while only 5% of the general population report a similar history (reviewed in Darke *et al.*, 2008:253). Apart from self-inflicted damage with suicidal intention, MA users also tend to exhibit more self-mutilation (Vearrier *et al.*, 2012:38). These include genital self-mutilation, self-inflicted stabbings and eye enucleating, usually driven by sexual, religious or neurotic themes under drug influence (Kratofil *et al.*, 1996:117).

MA use has also been linked with attention deficit hyperactivity disorder (ADHD), anxiety, impaired neurocognitive and/or motor functioning and manic / hypomanic episodes (Meredith *et al.*, 2005:141).

2.2.8 Treatment

MA dependence is primarily treated by means of behavioural therapies (Meredith *et al.*, 2005:141). The most tried and tested treatment approach is known as the Matrix Model: an intensive 4 month outpatient therapy which combines cognitive, behavioural, and psychological approaches (Meredith *et al.*, 2005:141). However, to enhance motivation for abstinence, this program relies on the patient's attendance, intellectual capacity, and effective memory functioning (Schuckit, 1994:1559), rendering this program ineffective in patients suffering from MA-induced cognitive impairments (Rose & Grant, 2008:145). Consequently, various pharmacological approaches have also been tested in the treatment of MA addiction, and these usually include antipsychotics, antidepressants and replacement therapies (e.g. dextroamphetamine) (Rose & Grant, 2008:145). Specific pharmacotherapies comprise DA agonists and antagonists, 5-HT agonists, mixed monoamine agonist / antagonists and GABA receptor agonists (reviewed in Rose & Grant 2008:145).

Seeing that DA plays a significant role in the mechanism of action and neurotoxicity of MA, antipsychotics functioning as DA receptor antagonists have become a potential treatment option in MA addiction (Panenka *et al.*, 2012:1). These drugs also represent the first line of pharmacotherapy for MA-induced psychosis (Panenka *et al.*, 2012:1). Low dose aripiprazole, a second generation antipsychotic with partial D₂ receptor agonistic activity, has been found to attenuate certain abuse-related behaviours associated with *d*-amphetamine (Stoops *et al.*, 2006:206). However, another study found that higher doses of aripiprazole *increased* IV amphetamine use (Tiihonen *et al.*, 2007:160). A different study showed that neither haloperidol nor risperidone were able to decrease the stimulant-like effects of MA (Wachtel *et al.*, 2002:23). MA also exerts an effect on 5-HT receptors and as such, antidepressants have been tried as possible treatment options (Rose & Grant, 2008:145). However, most clinical trials have failed to observe positive clinical outcomes after administering 5-HT-stimulating drugs, such as paroxetine, sertraline or imipramine to MA abusers (reviewed in Brackins *et al.*, 2011:541 and Rose & Grant 2008:145). On the other hand, bupropion, a DA agonist antidepressant has shown positive outcomes by reducing drug cravings in MA users not seeking treatment (Newton *et al.*, 2006:1537).

Other diverse treatments noted in the literature include the centrally-acting sympathomimetics, modafinil and methylphenidate; ondansetron (5HT₃ antagonist); baclofen (central-acting muscle relaxant) and the anti-epileptics, gabapentin and topiramate (reviewed in Brackins *et al.*, 2011:541 and Rose & Grant 2008:145). As substitution therapy, dextroamphetamine (a CNS stimulant) has been tested, producing mixed results (Longo *et al.*, 2010:146; Shearer *et al.*, 2001:1289). In conclusion, no standard therapy exists for MA abuse and current treatments produce varied results, emphasizing the importance of discovering different drug pathways for this prevalent problem.

Consequently, alternative experimental pathways are now being considered that target the pathways suggested to be involved in MA-associated neurotoxicity and inflammation. This includes immuno-pharmacotherapy and redox modulation using antioxidants such as N-acetyl cysteine (NAC) (Grant *et al.*, 2010:823; Meijler *et al.*, 2004:77). With the NAC being the focus of this study, I will now discuss this compound in more detail below.

2.3. N-acetyl cysteine (NAC)

For several decades NAC has been used in clinical practice, mainly as a mucolytic agent and as an antidote for paracetamol toxicity (Arakawa & Ito, 2007:308). More recent applications include acute respiratory distress syndrome, bronchitis, several cardiac conditions, chemotherapy toxicity, heavy metal toxicity, HIV/AIDS and psychiatric conditions (Samuni *et al.*, 2013:4117). NAC, a thiol, is a membrane-permeable cysteine precursor which unlike free occurring cysteine does not require active transport mechanisms to penetrate cells (Sen, 1997:660; Zafarullah *et al.*, 2003:6). After cell penetration, NAC is quickly hydrolyzed to release cysteine, a vital rate-limiting precursor in the synthesis of GSH (Arakawa & Ito, 2007:308; Meister, 1995:3). Essentially, NAC bolsters antioxidant potential by encouraging intracellular GSH formation (Arakawa & Ito, 2007:308). However, NAC itself is also an intracellular source of sulfhydryl groups and a free radical scavenger, interacting with ROS (Zafarullah *et al.*, 2003:6). The GSH-independent mechanisms involved in NAC's activity are presently not well understood, but NAC has been shown to also interact with other biological pathways, such as cell-cycle regulation and apoptosis, gene expression, mutagenesis, carcinogenesis, immune-modulation and mitochondrial function (Samuni *et al.*, 2013:4117; Zafarullah *et al.*, 2003:6). In addition, by the provision of additional cystine, NAC has also been shown to regulate glutamate neurotransmission via the cystine–glutamate antiporter (Baker *et al.*, 2002:9134).

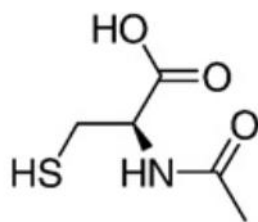


Figure 13: Molecular structure of N-acetyl cysteine (Hashimoto, 2009:105).

Several studies have revealed that NAC is able to reverse GSH deficits, as well as oxidative stress in rodents (Choy *et al.*, 2010:224; Dean *et al.*, 2004:S262; Otte *et al.*, 2011:2233). Additionally, another preclinical study showed that redox dysregulation in mice with impaired GSH synthesis (Gclm knockout [KO] mice), rendered PV interneurons vulnerable and more susceptible to exhibit oxidative stress, which could then be reversed by NAC administration (Cabungcal *et al.*, 2013:574). Importantly, NAC amide, a more membrane permeable form of NAC (Ates *et al.*, 2008:372), has shown the ability to decrease oxidative damage in mice treated with MA (Zhang *et al.*, 2012b:931). NAC has also been shown to possess anti-inflammatory properties by being able to inhibit the activation of microglia and macrophages and preventing the subsequent production of cytokines and oxidative species (Tsai *et al.*, 2009:223). Of note, foetal brains of rats prenatally treated with LPS showing a marked increase in IL-6 and IL-10 levels, which could be reversed by NAC administration (Beloosesky *et al.*, 2012:1324). These results suggest that NAC may be able to provide protection against prenatal inflammation-associated brain damage and possible long term pathology (Beloosesky *et al.*, 2012:1324). Apart from NAC's potential to modify GSH, oxidative and inflammatory pathways, its capacity to regulate the cystine–glutamate antiporter, as mentioned above, contributes to the control of extracellular glutamate and feedback regulation of glutamate release (Baker *et al.*, 2002:9134). Evidence for NAC's beneficial effects on altered glutamate systems has been provided in a psychotomimetic (phencyclidine) model of schizophrenia (Baker *et al.*, 2008:1760). Taking into account NAC's ability to regulate the cystine–glutamate antiporter and metabotropic glutamate receptors (via extracellular glutamate), as well as its involvement in antioxidants systems, it can also affect DA release from presynaptic terminals (Baker *et al.*, 2002:9134; Gere-Pászti & Jakus, 2009:658). Indeed, a preclinical study implementing social isolation as a schizophrenia model found that NAC was successful in reversing cortico-striatal monoamine changes,

especially with regards to DA, 5-HT and NA (Möller *et al.* 2013a:687), as well as reverse certain psychosis-related behaviours in these animals (Möller *et al.* 2013b:156). Furthermore, Shivalingappa *et al.* (2012) found that NAC was successful in protecting DAergic cell death induced by MA.

In clinical studies of schizophrenia, NAC as adjunctive therapy was able to improve scores on the Positive and Negative Symptoms Scale (PANSS) (Berk *et al.*, 2008a:361). In addition, Carmeli *et al.* (2012) observed that NAC treatment was able to affect EEG synchronization in several brain areas of schizophrenia patients, hinting at the possible improvement of abnormal functional connectivity. Regarding other psychiatric disorders, NAC has also shown beneficial effects in clinical studies of bipolar disorder (Berk *et al.*, 2011:389; Berk *et al.*, 2008b:468; Magalhães *et al.*, 2011:317). Taking into account its influences on various biological systems, especially those relating to MA-abuse and schizophrenia pathology, NAC may prove to be a beneficial alternative treatment option, either as mono- or adjunctive therapy.

2.4. Modelling schizophrenia in animals

An animal model is defined as an experimental design developed to study certain human conditions in animals in order to identify the underlying biological mechanisms and to develop novel therapeutic agents (Lipska, 2004:282; Young *et al.*, 2010:391). To assure that these models are effective in their given purpose, certain criteria must be met to establish its validity, namely (1) face, (2) construct and (3) predictive validity (Fig.14) (Jones *et al.*, 2011:1162).

Face validity refers to behavioural features indicative of the human disorder that is modelled in the animal (Nestler & Hyman, 2010:1161). In other words, the behaviour of the animal must share a strong resemblance to specific symptoms observed in the human illness (Young *et al.*, 2010:391). Thus, animal models of schizophrenia must mimic one or more symptoms of the three domains of schizophrenia in order to have face validity (Fig.14). Construct validity states that the animal model must be able to reproduce the biological processes of the human disease, such as structural or neurochemical defects (Fig. 14) (Jones *et al.*, 2011:1162). In the perfect situation, construct validity would be achieved by reconstructing the specific etiologic processes that cause a disease in humans, and thus replicate pathological features of the illness in the animals (Chadman *et al.*, 2009:1). However, this is rarely achievable, especially with regards to schizophrenia in which the exact pathological basis remains unknown. The third criterion, predictive validity, refers to

the model's ability to respond to pharmacological treatments in a manner that predicts the effects of those treatments in humans (Nestler & Hyman, 2010:1161). In schizophrenia research, animal models should ideally respond positively to treatment with established therapeutic agents (i.e. antipsychotics), and also shed light on the effects of newer therapeutics (Fig. 14). Evidently, the more types of validity an animal model fulfils, the higher its relevance to the human condition. Although this is seldom attainable, models formed on the basis of aetiological and pathophysiological mechanisms (construct validity) typically possess some degree of face and predictive validity, suggesting this method to be the most reliable for preclinical research (Lipska & Weinberger, 2000:223).

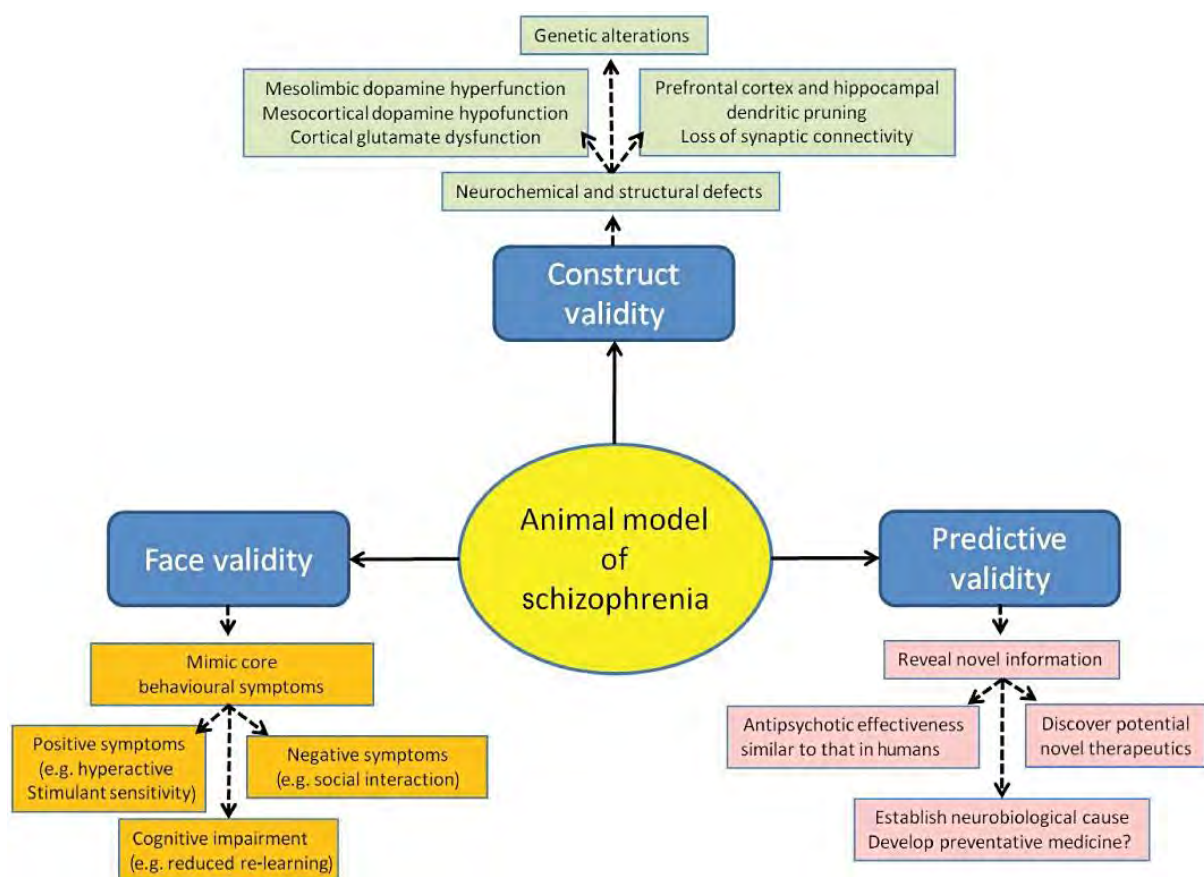


Figure 14: The three main criteria for the establishment of relevant animal models (Jones et al., 2011:1162).

2.4.1 Neurodevelopmental models

In recent years epidemiology driven neurodevelopmental animal models of schizophrenia have gained more attention as they account for the evolving nature of schizophrenia and

also incorporate important etiological factors of the disorder (Meyer & Feldon, 2010:285). Neurodevelopmental models can be based on either prenatal or postnatal exposure to environmental influences, which are suggested to cause long-term changes in neurodevelopment (Meyer & Feldon, 2010:285). Prenatal insults include infection / immune activation, nutritional deficiencies, maternal exposure to stress, obstetric complications and models based on disrupted neurogenesis (Jones *et al.*, 2011:1162; Lipska & Weinberger, 2000:223; Meyer & Feldon, 2010:285). Examples of postnatal insults include maternal separation, post-weaning social isolation and neonatal lesion models (Geyer & Moghaddam, 2002:689; Jones *et al.*, 2011:1162; Lipska & Weinberger, 2000:223). For the purpose of this dissertation, only prenatal infection / inflammation will be discussed in detail.

2.4.1. a) Animal models of prenatal inflammation

As discussed in section 1.4.8, there is compelling evidence for the role of environmental insults during the prenatal period in schizophrenia development. In particular, mention was made of maternal infection and the role of early-life inflammation in laying the foundation for the later development of schizophrenia. Developmental animal models of schizophrenia incorporate environmental influences during the sensitive pre-/perinatal period, to create permanent changes in CNS development (Jones *et al.*, 2011:1162). Regarding prenatal inflammation, a number of approaches have been established for inducing an immune response in animals, including polyinosinic:polycytidylic acid (Poly I:C) (Missault *et al.*, 2014:138; Ozawa *et al.*, 2006:546; Van Den Eynde *et al.*, 2014:179; Wolff & Bilkey, 2010:323), lipopolysaccharide (LPS) (Graciarena *et al.*, 2010:1301; Kirsten *et al.*, 2010:77; Lin *et al.*, 2012b:459) and the direct administration of cytokines (Tohmi *et al.*, 2004:67).

LPS is the bacterial cell wall component of Gram negative bacteria that results in enhanced release of cytokines, together with accompanying fever and inflammation (Jenkins, 2013:110). LPS binds to toll-like receptor-4 (TLR-4) on macrophages and other immune cells to trigger a signal transduction cascade leading to the activation of transcription factors such as nuclear factor kappa B (NFkB) as well as genes coding for pro- and anti-inflammatory mediators such as cytokines, chemokines and proteins of the complement system (Aderem & Ulevitch, 2000:782). This then leads to the release of a range of pro-inflammatory cytokines, such as IL-1, IL-6 and TNF- α (Boks, 2010:881). Prenatal LPS models have revealed various alterations akin to schizophrenia symptomology and neurobiology in rodents. For a more comprehensive overview of data obtained from prenatal inflammation models of LPS (and other immunogens) please refer to Chapter 4 (Review: Neurodevelopmental Animal Models Reveal the Convergent Role of Neurotransmitter

Systems, Inflammation, and Oxidative Stress as Biomarkers of Schizophrenia: Implications for Novel Drug Development).

2.4.2 Pharmacological models

Pharmacological models of schizophrenia rely on our knowledge of neurotransmitter alterations witnessed in the disease (Marcotte *et al.*, 2001:395). Seeing that the DA hypothesis forms the basis of our understanding of the neurochemical alterations in schizophrenia, amphetamine models were the first to be developed in the 1950's since their clinical presentation in humans included delusions and hallucinations similar to the positive symptoms of schizophrenia (Jones *et al.*, 2011:1162). Administration of amphetamine to rats has been shown to cause behaviours such as constant grooming, gnawing, sniffing and dyskinetic motor activities which can be inhibited by antipsychotic drugs, suggesting that these stereotypical behaviours may be comparable to human psychotic disorders (reviewed in Kokkinidis & Anisman 1980:551). Amphetamine models have also revealed deficits in cognitive tasks, such as alterations in attention (Fletcher *et al.*, 2007:1122; Martinez & Sarter, 2008:2635), which are also reversed by certain antipsychotics (Martinez & Sarter, 2008:2635). However, the neurochemical alterations of schizophrenia are far more complex than just a DA imbalance and this model fails to take into account the intricate connections between other neurotransmitter systems and DA function (Kokkinidis & Anisman, 1980:551; Marcotte *et al.*, 2001:395). In addition, Sams-Dodd (1995:55) failed to observe changes in social interaction in an amphetamine model, proposing that this model does not imitate the negative symptoms of schizophrenia. That being said, the amphetamine model has for many years enabled researchers to explore schizophrenia pathology and treatment, especially with regards to psychosis.

Other pharmacological models of schizophrenia include agents functioning on:

- Glutamate: Pharmacological agents include the NMDA receptor antagonists, such as PCP, ketamine and dizocilpine (MK-801). Preclinical models using these drugs observed hyperactivity, changes in social behaviour, sensorimotor gating deficits and cognitive alterations in experimental animals (Moghaddam & Jackson, 2003:131).
- Serotonin: Pharmacological agents include LSD and mescaline, which are believed to function mainly on 5-HT_{2A} receptors (Aghajanian & Marek, 2000:302). Laboratory animals treated with these hallucinogens have shown disrupted startle habituation and deficits in PPI, as well as disrupted latent inhibition similar to clinical observations in acutely ill human subjects (Geyer & Braff, 1987:643; Hitchcock *et al.*, 1997:43).

- GABA: The GABA_A receptor antagonist, picrotoxin, has been found to induce deficits in PPI in experimental animals (Japha & Koch, 1999:347).

2.4.3 Genetic models

As discussed previously, schizophrenia is a complex disease influenced by genetic as well as environmental factors (Maier *et al.*, 2005:159). Seeing as schizophrenia is not associated with a single phenotype, genetic linkage studies have to date not been thoroughly successful (Geyer & Moghaddam, 2002:689). The current methods used in clinical practice include:

- Genetically modified animals: Transgenic and knockout animals (Geyer & Moghaddam, 2002:689). Examples include DISC-1 transgenic mice, which present with decreased brain volume, enlarged lateral ventricles, reduced cortical thickness (Jaaro-Peled *et al.*, 2010:301), deficits in PPI and altered social behaviour (Clapcote *et al.*, 2007:387); DTNBP1 mutant animals, which revealed altered brain anatomy (Jaaro-Peled *et al.*, 2010:301), changes in working memory and elevated locomotor activity (Papaleo *et al.*, 2012:85); etc.
- Strain differences: Several strains of rodents have been found to exhibit deficits in sensorimotor gating and latent inhibition (reviewed in Geyer & Moghaddam 2002:689).

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Chapter 3: Concept article

This chapter presents the full-length manuscript for submission to International Journal of Neuropsychopharmacology. The manuscript is presented in the required format prescribed by Instructions to the Authors, as described on the journal website:

http://ijnp.oxfordjournals.org/for_authors/index.html (Accessed: 11/11/2015)

Co-authors' contribution:

- Twanette Swanepoel contributed to the planning of the project; carried out all drug administrations to experimental animals; performed all the behavioural analyses; in assistance with the NWU Vivarium personnel, sacrificed animals and collected brain tissue and plasma; undertook the neurochemical analyses with assistance from Marisa Möller, Francois Viljoen and Walter Dreyer; performed all statistical analyses with assistance from Marisa Möller; developed the lay-out of the current article; wrote the first article draft and completed the final version for submission.
- Marisa Möller contributed to the planning of the project, helped with the plasma reactive oxygen species analysis, assisted in statistical analyses, aided in the layout of the current article and contributed to the writing of the final draft.
- Walter Dreyer assisted in all steps of the regional brain lipid peroxidation measurement and the analysis of plasma cytokine levels, as well as data calculation of all aforementioned analyses.
- Francois Viljoen assisted in all steps of the regional brain monoamine analysis, as well as subsequent data calculation.
- Brian Harvey served as study leader for the current project and contributed greatly to the planning of the entire project, the layout of the current article and the writing of the final draft.

Furthering the two-hit model of schizophrenia with lipopolysaccharide and methamphetamine: Investigating the nexus between immune-inflammatory and stress modelling in schizophrenia using N-acetyl cysteine (NAC) as a therapeutic intervention.

Research article

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ABSTRACT

Background: Schizophrenia is linked to prenatal inflammation, early life stress and oxidative stress. We investigated whether maternal immune activation (MIA) with / without early-adolescent methamphetamine (MA) exposure elicits similar ensuing behavioural and biochemical changes and whether these can be reversed with the antioxidant, N-acetylcysteine (NAC).

Methods: Pregnant Sprague-Dawley rats received either saline or 100 µg/kg lipopolysaccharide (LPS) on gestational days (GD) 15-16. Offspring were divided into: LPS, MA alone and LPS+MA. MA was administered from postnatal day (PND) 53-50 (0.2 mg/kg twice daily escalating). Vehicle or 150 mg/kg NAC were given from PND 51-64. Social interaction, novel object recognition memory and prepulse inhibition of acoustic startle, as well as regional brain monoamines, lipid peroxidation, plasma reactive oxygen species and pro- and anti-inflammatory cytokines (tumor necrosis factor- α (TNF- α); interleukin-10 (IL-10)), were accessed.

Results: LPS and LPS+MA significantly increased social behaviours, with complete reversal by NAC. LPS+MA significantly impaired memory, with NAC tending to reverse these changes. Sensorimotor-gating deficits (PPI) were evident in MA, LPS and LPS+MA models, with reversal of the deficit by NAC administration only in LPS+MA rats. Lipid peroxidation (frontal cortex and striatum), was significantly increased in both LPS and LPS +MA animals, with NAC partially reversing these changes. Reactive oxygen species were significantly increased in LPS and LPS +MA animals, with NAC administration attenuating peroxidation in LPS animals only. IL-10 was significantly decreased in LPS and LPS+MA animals, with TNF- α increased in LPS animals and NAC restoring both TNF-alpha and IL-10 levels.

Conclusions: LPS and LPS+MA contribute to redox and inflammatory pathways that impact schizophrenia development and NAC effectively reverses the subsequent behavioural and redox-inflammatory manifestations.

Keywords: schizophrenia, prenatal infection, two-hit hypothesis, methamphetamine, N-acetyl cysteine.

1. Introduction:

Schizophrenia is among the world's top ten causes of long-term disability, affecting up to 1% of the global population (Harris et al., 2013; Laruelle, 2014). The disease is characterized by early adult-life onset, usually occurring at the age of 20-30 years (Ross et al., 2006). The clinical features of schizophrenia are subdivided into positive (e.g. hallucinations and delusions), negative (e.g. apathy and social withdrawal) and cognitive symptoms (e.g. reduced memory function, attention disorders and altered sensory processing) (Tandon et al., 2009; Gonzalez-Burgos et al., 2011; Meyer and MacCabe, 2012). Despite substantial research effort, the aetiology and biological mechanisms involved in schizophrenia remain unclear and treatments are suboptimal (Meyer, 2013).

The neurodevelopmental hypothesis of schizophrenia proposes that early life environmental insults result in pathological brain changes (Lieberman, 1999), one factor being maternal immune activation (MIA) (Brown and Derkits, 2010) with, for example the influenza virus (Mednick et al., 1988; Brown et al., 2004) as well as a host of other prenatally acquired pathogens (Brown et al., 2001; Brown et al., 2005; Mortensen et al., 2007; Buka et al., 2008). Although certain pathogens can cross the placenta, maternal and/or foetal inflammation may be the chief malefactor (Meyer et al., 2009). Lipopolysaccharide (LPS), an endotoxin of gram negative bacteria that binds to the Toll-like receptor 4 (TLR-4) on immune cells to trigger pro-inflammatory cytokine release (Aderem and Ulevitch, 2000; Boksa, 2010), is widely recognized to lead to several schizophrenia-related behavioural, neurochemical and inflammatory changes in rodents (reviewed in Bayer et al., 1999). Other environmental factors additionally contribute to maternal immune activation including trauma and stress, poor diet, smoking and substance abuse (Debnath et al., 2015).

Schizophrenia is characterized by delayed symptom development (Lieberman, 1999), thus early neurodevelopmental insults combined with postnatal environmental influences (such as

stress or drug abuse) may be required for disease onset (Lieberman, 1999; Meyer, 2013). This reasoning has consequently given rise to the two-hit hypothesis, which suggests that a genetic susceptibility with developmental insults can prime a person for schizophrenia (Bayer et al., 1999). However, prenatal insults may also play the role of “first hit” which if combined with a later-life insult, may predispose to schizophrenia (Feigenson et al., 2014).

Methamphetamine (MA) is a potent central nervous system stimulant, currently regarded as the second most abused substance globally (Cruickshank and Dyer, 2009; Panenka et al., 2012). MA affects monoamine release and reuptake through various mechanisms, causing euphoria, alertness and loss of appetite (Yamamoto et al., 2010; Kirkpatrick et al., 2012; Panenka et al., 2012). Chronic MA-use tends to deplete dopamine (DA) (Grace et al., 2010), and is also a powerful neurotoxin that induces apoptosis and oxidative stress (Darke et al., 2008). MA often leads to psychosis, with symptoms reminiscent of schizophrenia (Darke et al., 2008; Granado et al., 2013). Although these effects can usually be reversed by stopping the drug, a persistent psychotic profile after cessation has been reported in some individuals (Chen et al., 2003). It has been noted that abusers who experience such prolonged psychosis also exhibited schizophrenia-like symptoms during childhood, prior to drug abuse (Chen et al., 2005), supporting a two-hit gene-environment interaction.

Oxidative stress has been implicated as an important factor in both the development of schizophrenia (Zhang et al., 2006; Dietrich-Muszalska and Olas, 2009; Bitanhirwe and Woo, 2011) and MA-abuse (Mirecki et al., 2004; Fitzmaurice et al., 2006; Yamamoto et al., 2010). Excessive DA, as seen in schizophrenia (Meyer-Lindenberg et al., 2002) and acute MA abuse (Panenka et al., 2012), may autoxidize to form quinones which are able to attack lipids and proteins, as well as boost free radical production (Brown and Yamamoto, 2003). Clinical studies have found that anti-oxidant treatment with N-acetyl cysteine (NAC) is beneficial as an adjunctive treatment in schizophrenia (Berk et al., 2008; Carmeli et al., 2012), recently corroborated in a translational animal model (Möller et al., 2013a), while

there is preclinical (Shivalingappa et al., 2012; Zhang et al., 2012) and early clinical evidence of its use in MA abuse and dependence (Mousavi et al., 2015).

The aim of this study was to assess whether LPS, and LPS+MA are equally effective in inducing schizophrenia-like bio-behavioural changes in rodents, and whether chronic NAC administration can reverse these changes. Several behavioural markers were analysed, as well as regional brain monoamines and immune-inflammatory-redox pathways, to shed light on the aetio-pathological processes involved in disease development and their potential reversal by NAC.

2. Materials and methods

2.1. Animals:

Pregnant Sprague-Dawley rats were used for maternal immune activation (MIA). After weaning, male offspring from these dams (± 4.4 per dam) were randomly divided into different treatment groups. All experimental animals were provided by, and housed in the vivarium of the Preclinical Drug Development Platform Research Centre at the North West University in Potchefstroom, South Africa. The rats were kept in cages (230(h) $\times$ 380(w) $\times$ 380(l) mm) with corn cob (replaced weekly). Housing conditions included constant temperature (21 ± 0.5 °C) and humidity ($50\pm 10\%$) with an artificial 12 hour light/dark cycle, and free access to food (standard rat chow) and water. Behavioural analyses were conducted between 15:00-21:00. The animals were handled according to the code of ethics in research, training and testing of drugs in South Africa (Ethical approval number: NWU-00147-14-A5).

2.2. Study design:

Pregnant Sprague-Dawley dams (n = 32) used for the prenatal study were randomly divided into either a control group (n = 8) or LPS group (n = 24) (Fig. 1). LPS or saline were administered on gestation day (GD) 15-16. Offspring born from these groups were randomly divided into either MA or saline groups (n = 12/group) from PND 35-50. LPS offspring with or without MA administration were further divided into either NAC or saline receiving groups from PND 51-64. Control groups received equivalent doses of saline on the coinciding days. Seeing that this study did not focus specifically on the mechanisms and treatment of MA-abuse, but rather on schizophrenia and MA's role as a second hit in its development, the MA alone group was excluded from the NAC treatment leg of the study. Eventually, we found that MA failed to alter markers of oxidative stress, which provided additional support for our decision to omit a treatment group for MA-alone. Behavioural analyses began on PND 62 with the social interaction (SI) test, the novel object recognition test (nORT) on PND 63 and assessment of sensorimotor gating (%PPI) on PND 64. The animals were decapitated 24 hours later, and brain tissue (frontal cortex and striatum) and trunk blood were collected for bio-analysis. All samples were stored at -80°C until the day of analyses.

2.3. Drugs and treatment protocol:

LPS from *E.coli* (Sigma-Aldrich, Johannesburg, South Africa) was dissolved in saline and administered subcutaneously (SC) to pregnant dams at a dose of 100 µg/kg at GD 15-16 (Fortier et al., 2007; Baharnoori et al., 2013). MA hydrochloride (Sigma-Aldrich, Johannesburg, South Africa) was dissolved in saline and administered in escalating doses, as previously determined (Strauss et al., 2014), of 0.2mg/kg SC (starting at 0.2 mg/kg up to 6 mg/kg) twice daily (09:00 and 15:00) for 16 days. NAC (Sigma-Aldrich, Johannesburg, South Africa), dissolved in saline and buffered with 1 M glacial acetic acid and NaOH

(pH=6.0), was administered at a dose of 150 mg/kg SC (Möller et al., 2013a). All control animals received an equivalent volume of saline at coinciding times.

2.4. Behavioural analyses:

2.4.1. Social interaction (SI) test

Total social behaviours were measured according to the protocol of File et al. (2005) in a low-light (40 lux red light), unfamiliar arena. Testing took place in an open field arena (70 cm×70 cm×40 cm) and behaviour monitored 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. The behaviours included: sniffing, nipping, grooming, following, mounting, kicking, boxing, wrestling, jumping on, crawling under/over the partner (File and Hyde, 1978) and presented as the time spent in total social behaviour. The testing arena was cleaned before and after each session with a 10% ethanol solution.

2.4.2. Novel object recognition test (nORT)

Analysis of memory was carried out in the dark phase from 19:00 as previously described (Möller et al., 2013a). The rats were placed in a Plexiglas box (70×70×40 cm) with two similar objects for a 5 minute acquisition trial and then returned to their home cages for a 1.5 hour inter-trial interval. One of the objects was then replaced with a novel object. For the retention trial, the rats were returned to the testing area and allowed to explore the objects (familiar and novel) for a total period of 5 minutes. Experiments were digitally recorded and sniffing, licking and touching the objects with the forepaws were defined as exploration (Grayson et al., 2007). Essentially, more exploration time at the novel object reflects accurate recollection that the familiar object has been presented before, thus prompting greater exploration of the

novel object. nORT scores were defined as the percentage of the discrimination index (DI), calculated with the following formula: $[DI = (\text{time spent at novel object} - \text{time spent at familiar object}) / (\text{time spent at novel object} + \text{time spent at familiar object})] \times 100$ (Ennaceur and Delacour, 1988; Antunes and Biala, 2012)

2.4.3. Prepulse inhibition (PPI)

Prepulse inhibition of sensorimotor gating refers to the ability of an individual to effectively filter insignificant and excessive information (Geyer et al., 2001). PPI was measured as described previously (Swerdlow and Geyer, 1998; Wang et al., 2003; Möller et al., 2011). The SR LAB (San Diego Instruments, San Diego, USA) was used for PPI analysis. PPI testing began with a 5-min acclimatization period and a 68-dB background noise level that remained constant throughout the test session. The basal startle response was first evaluated by delivering 10 trials consisting of a single 40 ms 120 dB white-noise startle. The test session followed with 80 trials consisting of random deliveries of twenty 120 dB pulse-alone trials, 50 prepulse trials, and 10 no-stimuli trials. Prepulse trials included a single 120 dB pulse preceded by a 20 ms non-startling prepulse stimulus at four different intensities, viz. 72, 76, 80 or 84 dB. The last 10 trials were single 40 ms 120 dB pulse-alone startle stimuli. The first and last 10 pulse-alone stimuli (Block 1 and Block 4, respectively) and the 20 pulse-alone stimuli included in the PPI block itself (Block 2 and Block 3) were used to obtain a measure of mean startle amplitude indicative of habituation in response to repeated delivery of startling stimuli (van den Buuse and Eikelis, 2001; Wang et al., 2003). Percentage PPI (% PPI) for the four prepulse intensities was calculated according to the following formula: $\% \text{ PPI} = [100 - (\text{startle response for PREPULSE+PULSE trial}) / (\text{startle response for PULSE ALONE trial}) \times 100]$ (van den Buuse and Eikelis, 2001).

2.5. Neurochemical and redox-immune-inflammatory analyses:

2.5.1. Preparation of brain tissue

The rats were decapitated and the frontal cortex and striatum immediately dissected out on an ice-cooled glass slab. After removing the olfactory bulb from the cortex and then cutting around the anterior tip of the corpus callosum, the frontal cortical tissue was dissected (Toua et al., 2010; Möller et al., 2011). When dissecting the striatum, the dorsal side of the brain was placed side up. The two cerebral hemispheres were split and the striatum dissected with the corpus callosum as external limits and the external walls of the lateral ventricles as internal limits. The brain regions were then fixed in liquid nitrogen and stored at -80°C until the day of monoamine analysis. On the day of assay, the brain regions were removed from the freezer, weighed and allowed to thaw on ice.

2.5.2. Monoamine analysis:

The quantification of regional brain DA, serotonin (5-HT) and noradrenaline (NA) were performed using a High performance liquid chromatography (HPLC) system with electrochemical detection (HPLCEC), as previously described (Harvey et al., 2006) (full method described in Addendum B). The monoamine concentrations were calculated by comparing the area under the peak of each monoamine to that of the internal standard, isoprenaline (range 5–50 ng/ml; Agilent 1200 series HPLC, equipped with an isocratic pump, autosampler, coupled to an ESA Coulochem Electrochemical detector and Chromeleon® Chromatography Management System version 6.8). Linear standard curves (regression coefficient greater than 0.99) were found in this specific range. Monoamine concentrations were expressed as ng/g wet weight of brain tissue.

2.5.3. Lipid peroxidation measurement:

Lipid peroxidation of membranes leads to the accumulation of malondialdehyde (MDA) in tissues, which can be used as a measure of oxidative stress induced cellular damage. The Parameter™ TBARS assay from R&D Systems (Minneapolis, USA; catalogue number KGE013) was used to analyse lipid peroxidation in brain tissue (full method described in Addendum B). MDA is measured as thiobarbituric acid reactive substances (TBARS) and quantified using a microplate reader measuring at 530-532 nm.

2.5.4. Analysis of total reactive oxygen species (ROS):

Serum ROS was determined based on the principle of the derivatives of reactive oxygen metabolites test (full method described in Addendum B). In an acidic medium, ROS will react with transition metals (e.g. iron) to form alkoxy and peroxy radicals. These radicals are able to oxidize a chromogenic substrate, such as N,N-diethyl-para-phenyldiamine (DEPPD) to its corresponding radical cation. The concentration of the cation can then be measured at 546 nm using a microplate reader. The Bio-Tek FL600 Microplate Fluorescence Reader (Bio-Tek, Instruments, Inc., Highland Park, Winooski, VT, USA) was used to measure ROS levels, where 1.0mg l^{-1} H_2O_2 represents one unit of ROS (Hayashi et al., 2007).

2.5.5. Cytokine measurement:

Plasma levels of a pro- and an anti-inflammatory cytokines were determined by enzyme-linked immunosorbent assay (ELISA) (full methods described in Addendum B). Plasma tumour necrosis factor- α (TNF- α ; pro-inflammatory) was measured by means of the Rat TNF- α ELISA MAX™ Deluxe Set (catalogue number 438204) from Bio Legend (San Diego, USA), with interleukin-10 (IL-10; anti-inflammatory) measured using the Rat IL-10 ELISA Kit

(catalogue number RAB0246-1KT) from Sigma-Aldrich (Johannesburg, South Africa). In both cases the above a microplate reader was used, measuring at 450 nm.

2.6. Statistical analyses:

One-way factorial analysis of variance (ANOVA) and Bonferroni post-hoc tests were used for the statistical analyses of SI scores, nORT percentages, brain lipid peroxidation levels, total ROS and the cytokine analyses. For analysis of %PPI data, two-way ANOVA (prepulse intensity*treatment and %PPI*treatment) was used with Bonferroni post-hoc tests. Data are expressed as the mean $\pm$ standard error of the mean (SEM), with a p-value of <0.05 considered statistically significant (Graphpad Prism 6, San Diego California, U.S.A.)

3. Results

3.1. Social interaction

One-way ANOVA indicated a significant treatment*social behaviour interaction ($F(5,18) = 13.92, p < 0.0001$) (Fig. 2). Bonferroni post-hoc analysis revealed that the LPS-S-S group (MIA animals) and the LPS-MA-S group (dual-hit model) both showed significantly increased total social behaviours vs. controls (S-S-S) ($p < 0.05$) (Fig. 2). MA alone (S-MA-S) didn't alter social activities. NAC treatment significantly reversed SI changes in both LPS (LPS-S-NAC) ($p < 0.001$) and the dual-hit animals (LPS-MA-NAC) ($p < 0.001$) (Fig. 2).

3.2. Novel object recognition memory

One-way ANOVA indicated a significant treatment*novel object exploration interaction ($F(5, 66) = 2.819, p = 0.02$) (Fig. 3). Bonferroni post hoc test revealed that the dual-hit model (LPS-MA-S) significantly decreased the time spent exploring the novel object vs. the control group ($p < 0.05$) (Fig. 3). LPS alone (LPS-S-S) and MA alone (S-MA-S) failed to alter time spent exploring the novel object. NAC treatment showed a trend toward reversal of memory deficits in the dual-hit group (LPS-MA-NAC), that was not statistically significant ($p = 0.17$) (Fig. 3).

3.3. Prepulse inhibition of acoustic startle

Basal startle analysis (data not shown) showed a significant decrease in startle amplitudes from blocks one to two in the S-S-S (controls), S-MA-S and LPS-MA-NAC groups, indicating towards habituation in these groups. There were no changes in any of the groups from block two to four.

Two-way ANOVA showed no significant prepulse intensity*treatment interaction ($F(15, 165) = 1.63, p = 0.07$), although a significant %PPI*treatment interaction was observed ($F(5, 55) = 4.13, p < 0.003$) (Fig. 4). Bonferroni post hoc test revealed deficits in %PPI in the LPS-S-S group at two of the four prepulses [74 dB ($p < 0.01$) and 84 dB ($p < 0.001$)] and a trend towards reduced %PPI at 72 and 80 dB although not significant, vs. the control group (S-S-S) (Fig. 4). In the LPS-MA-S group, significant deficits in %PPI were seen at 72 dB ($p < 0.01$), 74 dB ($p < 0.001$) and 84 dB ($p < 0.01$) and a trend towards reduced %PPI at 80 dB although not significant, vs. the control group (S-S-S) (Fig. 4). NAC successfully reversed %PPI deficits in the dual-hit model at a prepulse of 72 dB ($p < 0.05$) (Fig. 4). For both the MIA model and the two-hit model, NAC treatment had no significant effects in reversing the %PPI deficits at the other prepulses, although a trend towards reversal is visible at 84 dB (Fig. 4). MA alone (S-MA-S) also induced a significant reduction in %PPI at two prepulses [72 dB ($p < 0.0001$) and 74 dB ($p < 0.01$)], vs. the control group (S-S-S) (Fig. 4).

3.4. Monoamine analyses

Dopamine: One-way ANOVA revealed a significant treatment*frontal cortical DA interaction ($F(5, 30) = 7.13, p < 0.0002$) and a significant treatment*striatal DA interaction ($F(5, 66) = 2.47, p = 0.04$) (Fig 5a and b respectively). Bonferroni post-hoc analysis revealed a significant increase in frontal cortical DA levels in the LPS-S-S group ($p < 0.05$) (Fig. 5a), with MA alone (S-MA-S) and LPS+MA (LPS+MA+S) evoking a slight but insignificant decrease in DA (Fig. 5a) all in comparison to the control group. NAC successfully and fully reversed the LPS-induced DA changes in the frontal cortex (LPS-S-NAC) (Fig. 5a).

Serotonin: One-way ANOVA revealed a significant treatment* serotonin frontal cortex interaction ($F(5, 66) = 16.61, p < 0.0001$) and a significant treatment* serotonin striatal interaction ($F(5, 66) = 3.03, p = 0.02$) (Fig. 6a and b respectively). Bonferroni post-hoc analysis of the frontal cortical data revealed that both the LPS-S-S and LPS-MA-S groups

had significantly decreased levels of serotonin ($p < 0.0001$ in both instances), while MA alone (S-MA-S) was without any significant effect (Fig. 6a) in comparison to the control group. NAC normalized altered frontal cortical serotonin levels in the LPS alone model (LPS-S-NAC) ($p < 0.0001$) and the dual-hit model (LPS-MA-NAC) ($p < 0.001$) (Fig. 6a). Bonferroni showed no significant effects with respect to serotonin changes in the striatum (Fig. 6b).

Noradrenaline: One-way ANOVA revealed a significant treatment *frontal cortex noradrenaline interaction ($F(3.13, 34.42) = 58.01, p < 0.0001$) and a significant treatment*striatal noradrenaline interaction ($F(5, 66) = 16.19, p < 0.0001$) (Fig 7a and b respectively). In the frontal cortex, Bonferroni post-hoc analysis revealed significant reductions in NA levels in the MA alone (S-MA-S) ($p < 0.05$), LPS alone (LPS-S-S) ($p < 0.0001$) and LPS+MA (LPS-MA-S) groups ($p < 0.0001$) in comparison to the control group (Fig. 7a). NAC treatment reversed these changes in both the LPS (LPS-S-NAC) and LPS+MA (LPS-MA-NAC) groups ($p < 0.0001$ in both instances) (Fig. 7a). In the striatum, significantly reduced NA levels were observed in the MA alone (S-MA-S) ($p < 0.05$), LPS alone (LPS-S-S) ($p < 0.0001$) and LPS+MA (LPS-MA-S) groups ($p < 0.0001$) in comparison to the control group (Fig. 7b). Again, NAC treatment reversed these changes in both the LPS group (LPS-S-NAC) and LPS+MA (LPS-MA-NAC) groups ($p < 0.0001$ in both instances) (Fig. 7b).

3.5. Lipid peroxidation and total reactive oxygen species

Lipid peroxidation: One-way ANOVA revealed a significant treatment*lipid peroxidation interaction in the frontal cortex ($F(5, 66) = 11.80, p < 0.0001$) (Fig 8a) and the striatum ($F(5, 66) = 13.32, p < 0.0001$) (Fig. 8b). In the frontal cortex (Fig. 8a), Bonferroni post hoc test indicated that MDA levels were significantly increased in the LPS alone (LPS-S-S) and LPS+MA (LPS-MA-S) animals, when compared to the control group (S-S-S) ($p < 0.001$) (Fig. 8a). NAC partially but not significantly reversed these effects (Fig. 8a). MA-alone did not alter lipid peroxidation in this brain region (Fig.8a).

In the striatum (Fig. 8b), Bonferroni post-hoc analysis showed a significant elevation in MDA levels in the LPS alone (LPS-S-S) and LPS+MA (LPS-MA-S) groups, when compared to controls (S-S-S) ($p < 0.0001$) (Fig. 8b). NAC successfully and significantly reversed these changes in both groups ($p < 0.01$ in both instances), however, MA alone (S-MA-S) was unsuccessful in altering lipid peroxidation (Fig. 8b).

Plasma reactive oxygen species (ROS): One-way ANOVA revealed a significant treatment*total ROS interaction ($F(5, 66) = 51.23$, $p < 0.0001$) (Fig. 9). The LPS alone (LPS-S-S) and the LPS+MA (LPS-MA-S) groups both revealed significant elevations in plasma ROS levels vs. control (S-S-S) ($p < 0.0001$ in both instances) (Fig. 9). NAC successfully reversed this increased oxidative state in the prenatal LPS group (LPS-S-NAC) ($p < 0.0001$), but did not do so significantly in the dual-hit model (LPS-MA-NAC) (Fig. 9). The S-MA-S group did not show any changes in ROS levels (Fig. 9).

3.6. Cytokines

IL-10: One-way ANOVA revealed a significant treatment*IL-10 interaction ($F(5, 66) = 22.28$, $p < 0.0001$) (Fig. 10a). Bonferroni post-hoc analyses noted significantly decreased levels of IL-10 in the LPS alone (LPS-S-S) and the LPS+MA (LPS-MA-S) groups vs. control (S-S-S) ($p < 0.0001$ in both instances) (Fig. 10a). MA alone (S-MA-S) did not significantly affect IL-10 levels, although a trend towards increased levels was observed ($p = 0.69$) (Fig. 10a). In both the LPS alone (LPS-S-NAC) and the dual-hit model (LPS-MA-NAC) NAC significantly reversed the reduced IL-10 levels ($p < 0.001$ in both instances) (Fig. 10a).

TNF- α : One-way ANOVA revealed a significant treatment*TNF- α interaction $F(5, 66) = 18.79$, $p < 0.0001$) (Fig 10b). Bonferroni post hoc analysis indicated that levels of TNF- α were significantly increased in the LPS alone (LPS-S-S) animals ($p < 0.0001$), when compared to the control group (S-S-S) (Fig. 10b). Interestingly, LPS+MA (LPS-MA-S) and MA-alone (S-

MA-S) groups did not show any significant changes with regards to TNF- α levels (Fig. 10*b*). NAC successfully reversed the elevated levels of TNF- α levels in the LPS alone group (LPS-S-NAC) ($p<0.0001$) (Fig. 10*b*).

4. Discussion

The most important findings of this study are that prenatal inflammatory insults, as well as combined pre- and post-natal insults (LPS+MA exposure) similarly promote schizophrenia-like bio-behavioural changes. MA alone, however, is less inclined to evoke this response. Furthermore, the bio-behavioural changes engendered by prenatal MIA, with/without post-natal MA exposure, are reversible by NAC treatment. NAC has multiple complex and interacting actions that include antioxidant and anti-inflammatory properties and effects on glutamate, glutathione, mitochondrial biogenesis, apoptosis and neurogenesis (Samuni et al., 2013). The individual behavioural and biological findings will firstly be discussed separately, followed by a drawing together of these entities and a final unifying conclusion.

Social interaction:

In line with a previous study (Harvey and Boksa, 2014) we observed that prenatal LPS administration and LPS+MA cause an *increase* in social activities in offspring (Fig. 2). This is unexpected considering that social withdrawal is one of the key negative symptoms in schizophrenia (Meyer and MacCabe, 2012), but may have implications for other disorders such as Attention Deficit Hyperactivity Disorder. Other prenatal inflammation studies using LPS found *decreased* social interaction in offspring (Kirsten et al., 2010; Oskvig et al., 2012; Basta-Kaim et al., 2015), while Foley et al. (2014) observed no changes. It has been suggested that differences in dose and timing of LPS administration, and rat-strain differences may influence SI (Foley et al., 2014; Harvey and Boksa, 2014). As we administered LPS at the same time and used the same rat strain as Harvey and Boksa, we suggest that these findings may be peculiar to this method and the animals used. Furthermore, increased noradrenaline release in several brain areas has been connected to anxiety (Tanaka et al., 2000). We observed significantly *decreased* levels of NA in both brain regions studied (Fig. 7a, b) suggesting that this may be driving increased social behaviour in these animals through an anxiolytic-like effect (File and Hyde, 1978; File et al., 2005).

Decreased NA levels (Fig. 7a and b) and elevated social behaviours (albeit insignificant) are also evident in the MA alone group. Thus, the severity of noradrenergic suppression correlates with increased social behaviours.

nORT:

Memory impairments are well described in schizophrenia (Lee and Park, 2005) as well as in animals following prenatal LPS exposure (Graciarena et al., 2010; Wischhof et al., 2015). However, we (Fig. 3) and other authors (Rico et al., 2010) show that LPS alone does not significantly alter memory function. Similarly, MA alone was without effect, in line with the findings of Clark et al. (2007). However, LPS+MA significantly impaired visual recognition memory (Fig. 3). It can therefore be argued that multiple hits during development more severely compromise memory function.

PPI:

Changes in PPI are known to influence attention and other cognitive abilities (Geyer et al., 2001). Here we show (Fig. 4), as have others (Borrell et al., 2002; Romero et al., 2007; Basta-Kaim et al., 2011), that LPS alone can significantly compromise %PPI. While MA alone also decreased %PPI, combined LPS+MA caused the most robust deficits in %PPI. Importantly, altered PPI can be correlated to disorganised cortico-striatal monoamines such as reduced NA and 5-HT (described below) that are well-known to fragment sensorimotor gating (Sipes and Geyer, 1994; Alsene et al., 2011) and is typical of schizophrenia (Braff and Geyer, 1990; Vollenweider et al., 2007).

Monoamines:

The revised DA hypothesis of schizophrenia suggests there is decreased frontal and increased subcortical dopamine activity (Davis et al., 2014; Meyer-Lindenberg et al., 2002), we observed *increased* levels of DA in the frontal cortex of prenatal LPS treated animals (Fig. 5a). Interestingly, this did not occur in the dual-hit animals or animals receiving MA

alone (Fig. 5a). With reduced frontal cortical DA suggested to be characteristic of schizophrenia (Brand et al., 2015), the levels of DA were indeed reduced (albeit non-significantly) in the MA alone and MIA+MA groups. Nevertheless a clinical study has linked cortical hyper-dopaminergia to schizophrenia (Lindstrom et al., 1999), in line with our findings. No changes were noted for striatal DA in any of the groups (Fig. 5b), while schizophrenia is suggested to involve striatal hyper-dopaminergia (Möller et al., 2015). Nevertheless, a subtype of schizophrenia where dopaminergic alterations are *not* present has been proposed (Howes and Kapur, 2014).

Serotonin levels were greatly reduced in the frontal cortex of LPS alone and dual-hit animals (Fig. 6a), concordant with decreased frontal serotonergic function associated with the negative symptoms of schizophrenia (reviewed in Howes and Kapur, 2014). Translational models, such as social isolation rearing, show decreased frontal cortical and increased striatal levels of DA and serotonin (Möller et al., 2013b), while neurobiological studies have confirmed the close inter-relationship between DA and 5-HT in mediating psychotic behaviour (Möller et al., 2015). The absence of any change in 5-HT in the striatum suggests a predominantly serotonergic dysfunction in the frontal cortex, at least following LPS and/or LPS+MA challenge. MA neurotoxicity is strongly dose- and duration dependent, with lower doses producing neurotoxic effects only when administered continuously or over short time intervals (i.e. 2 hourly) (Villemagne et al., 1998), so that our MA and LPS+MA protocols are possibly sub-optimal in their ability to induce enduring DA (and serotonin) changes.

Noradrenergic data relating to schizophrenia pathology are somewhat inconsistent (Möller et al., 2015). Prenatal LPS alone, MA alone and the combination elicit significantly reduced levels of NA in the frontal cortex and striatum (Fig. 6a and b). The positive and negative symptoms of schizophrenia may be related to some form of hyper - and hypo-noradrenergia (Yamamoto and Hornykiewicz, 2004), while anxiety in schizophrenia may also have its origin in altered NA (Van Kammen et al., 1994; Yamamoto and Hornykiewicz, 2004). Our findings

convincingly indicate that a hypo-noradrenergic state is expressed in two separate animal models of schizophrenia. Since NA plays a vital role in anxiety, learning, attention, memory and sensorimotor gating (Tanaka et al., 2000; Sara, 2009; Alsene et al., 2011), and that schizophrenia is strongly correlated with dysfunction of these processes, reduced fronto-striatal noradrenergic function may indeed contribute to the behavioural changes described here and in schizophrenia.

Oxidative markers:

The role of oxidative stress in schizophrenia is compelling (Prabakaran et al., 2004; Brand et al., 2015; Möller et al., 2015), being implicated in damage to DNA, proteins and lipids that in turn can be correlated to illness progression (Bitanirwe and Woo, 2011). LPS alone (LPS-S-S) significantly increased lipid peroxidation in both brain regions studied (Fig. 8a, b, c), in agreement with previous studies (Zhu et al., 2007). The dual-hit model (LPS-MA-S) also caused a significant increase in lipid peroxidation in the frontal cortex and striatum (Fig. 8a, b), while MA alone (S-MA-S) failed in this regard (Fig. 8a, b). Similarly, plasma ROS was significantly increased in LPS alone and LPS+MA animals (Fig. 9), with MA again not able to induce changes in ROS levels. The lack of an effect following MA is intriguing, but may be related to sub-optimal exposure. In fact, such a response is remarkably consistent across most of the behavioural and neurochemical parameters described here, except on %PPI and cortico-striatal NA levels. LPS represents the most reliable inducer of lipid peroxidation in brain tissue, with the effect of LPS+MA likely to represent a dominant effect from LPS. Of interest is the apparent lack of an added effect by LPS+MA across most parameters.

Immune markers:

Redox disturbances and altered immune state often go hand in hand. Indeed, altered immunity is implicated in schizophrenia (Kneeland and Fatemi, 2012). Both LPS alone (LPS-S-S) and LPS+MA (LPS-MA-S) significantly reduced the levels of the anti-inflammatory cytokine, IL-10, although MA alone (S-MA-S) engendered a slight but insignificant increase

(Fig. 10a). Thus prenatal LPS, with or without MA, adversely affects the anti-inflammatory capacity of offspring later in life. On the other hand, LPS alone, but not LPS+MA or MA alone, significantly increased the levels of the pro-inflammatory cytokine, TNF- α (Fig. 10b). These findings are in agreement with a previous study where methylenedioxymethamphetamine (MDMA or “Ecstasy”) plus LPS resulted in suppression of LPS-induced elevations in TNF- α (Boyle and Connor, 2007). Also, pre-treating LPS-treated animals with an anti-IL-10-antibody blocked the suppressive effect of MDMA on TNF- α (Boyle and Connor, 2007). Earlier work has shown that MIA increases inflammatory cytokines, e.g. TNF- α , by maternal cells that cross the placenta to induce foetal inflammation (Ohyama et al., 2004). This in turn leads to free radical production, and nuclear and mitochondrial DNA damage (Bitanirwe and Woo, 2011).

NAC treatment:

NAC normalized altered social behaviour in both LPS and LPS+MA models (Fig. 2). In addition, NAC reversed cortico-striatal hypo-noradrenergia in these groups (Fig. 7a, b), as well as reversed cortical hypo-serotonergia (Fig. 6a), reaffirming that these alterations may have their origin in oxidative stress. In animal models of schizophrenia, anti-oxidant therapies have been shown to improve performance in the nORT (Goyarzu et al., 2004; Pardo Andreu et al., 2010; Möller et al., 2013a) as well as to reverse %PPI deficits (Möller et al., 2013a). Although NAC tended to reverse the dual-hit induced memory impairment (Fig. 3), this missed significance ($p = 0.167$). NAC was successful in reversing the %PPI deficits induced by the dual-hit model at a prepulse of 72 dB with a trend at the other prepulses (Fig. 4), thus supporting an earlier animal study (Möller et al., 2013a). LPS significantly increased frontal cortical DA levels, which was reversed by NAC (Fig. 5a), while it also normalized altered frontal cortical serotonin levels in both LPS and LPS+MA groups (Fig. 6a). The anti-oxidant properties of NAC in a neonatal LPS animal model are not new (Lanté et al., 2008), and here we extend these findings to a dual-hit paradigm as well. In line with our expectations, NAC significantly reduced striatal lipid peroxidation in the LPS alone and the

dual-hit models, and to a lesser degree in the frontal cortex (Fig. 8b and a respectively). NAC also normalized elevated ROS levels following LPS alone, although was less effective in the dual hit combination (Fig. 9). This apparent lack of efficacy following a dual hit paradigm is evident in both the lipid peroxidation (Fig 8a, c) and ROS (Fig. 9) assays, which may suggest the need for a higher NAC dose or extended treatment. Finally, NAC reversed decreased levels of IL-10 in the LPS alone and dual-hit models (Fig.10) and normalized the elevation in TNF- α levels in the LPS alone model (Fig. 11), thus confirming an immune-modulatory role for NAC (Chen et al., 2008; Khan et al., 2004; Möller et al., 2013a). In fact, NAC inhibits microglia and macrophage activation to prevent cytokine release (Tsai et al., 2009) and provides protection against prenatal inflammation-associated brain damage and its late onset sequelae (Beloosesky et al., 2012). These findings are consistent with a causal role for oxidative stress in MIA (LPS alone) and MIA+MA (LPS+MA) challenges and reinforce both the therapeutic role of NAC in schizophrenia and its potential in neuroprotection and prevention. Its mode of action in this regard might be linked primarily to mitochondrial effects (Möller et al., 2013a; Rajasekaran et al., 2015), and via its multifunctional antioxidant abilities, bolstering glutathione production and direct free radical scavenging activity (Zafarullah et al., 2003; Arakawa and Ito, 2007).

Concluding remarks:

Prenatal LPS and a combined LPS + postnatal MA elicited behavioural changes akin to schizophrenia, viz. altered SI and sensorimotor gating. Immune-redox changes, as seen in schizophrenia, were also noted in the LPS and the dual-hit model. The formation of positive feedback immune-redox loops can subsequently lead to progressive detrimental effects, causing structural brain changes that culminate in schizophrenia development and may additionally lead to neuroprogression in the disorder (Bitanirwe and Woo, 2011; Davis et al., 2014).

The generation of quinones via DA metabolism is widely recognised to contribute to ROS production (Hastings, 1995). Indeed, DA can decrease glutathione by 40% when added to cultured cortical neurons (Grima et al., 2003). In support of this, LPS induced significant increases in frontal-cortical DA and peripheral and central manifestations of oxidative stress. Concerning the observed depletion of frontal serotonin levels in LPS and dual-hit animals, it can be posited that indoleamine 2,3-dioxygenase (IDO) activation by pro-inflammatory cytokines will undermine serotonin synthesis via increased tryptophan-kynurenine metabolism (Dantzer et al., 2011).

Decreased fronto-cortical and striatal levels of NA was observed in LPS and dual-hit animals. Previously we described *increased* NA levels in these brain regions using a social isolation rearing paradigm (Möller et al., 2013b), which was correlated with increased oxidative stress (Möller et al., 2011). Since oxidative stress is evident under both conditions, these contrary findings are interesting. However, opposite to the findings of Möller et al. (2011), we found *increased* levels of social interactive behaviours in LPS and LPS+MA animals, although employed a different method of analysis. Noradrenergic hyperactivity is associated with certain anxiety conditions (Charney and Redmond Jr., 1983), while increased social behaviour is associated with decreased anxiety (File et al., 2005). Reduced limbic NA described here therefore provides a mechanistic explanation for the observed increase in social behaviour. Although social anxiety has a 17% prevalence rate in schizophrenia (Kingsep et al., 2003), thus contradictory to the above, reduced social anxiety is described in rodents after prenatal LPS administration (Harvey and Boksa, 2014). How MIA with LPS decreases noradrenergic activity is unclear and warrants further investigation, although it is clear that inflammatory-driven oxidative stress and altered monoamines are central to LPS and LPS+MA associated behavioural changes.

MA failed to evoke an added effect with LPS in any of the bio-behavioural parameters under study, which concurs with earlier work combining MA with post-weaning SIR (Strauss et al.,

2014). Although not demonstrated in this study, it is widely recognised that MA has pro-oxidant activity (Yamamoto and Zhu, 1998). It is possible that reactive adaptive mechanisms prevent excessive redox-related changes in DA release and neurotoxicity following two severe psychosocial stressors, with a consequential inhibitory effect on certain behavioural markers. Thus, although LPS and LPS+MA associated redox-immune changes may contribute to schizophrenia development later in life, their combined presence may be less severe than expected. Also of note is that apart from reducing sensorimotor gating, MA did not show a penchant to increase the risk of psychosis later in life, as has been suggested (Chen et al., 2005), which may impact current thinking on this matter.

Finally, NAC rescued deficits in almost all parameters following LPS or LPS+MA challenge, confirming its anti-oxidative effects on multiple markers of oxidative stress, as well as normalizing immune markers. By normalizing redox-inflammatory abnormalities, adjunctive NAC may provide protection against the later development of structural brain changes and ensuing monoamine alterations, thereby benefiting long term outcome. This work also provides impetus for its use as a therapeutic option in treating MA-associated psychosis.

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Conflicts of interest

The authors have no immediate conflicts of interest to declare. Except for income from the primary employer and research funding to BHH as described above, no financial support or compensation has been received from any individual or corporate entity over the past three years for research or professional services, and there are no personal financial holdings, that could be perceived as constituting a potential conflict of interest.

Figures

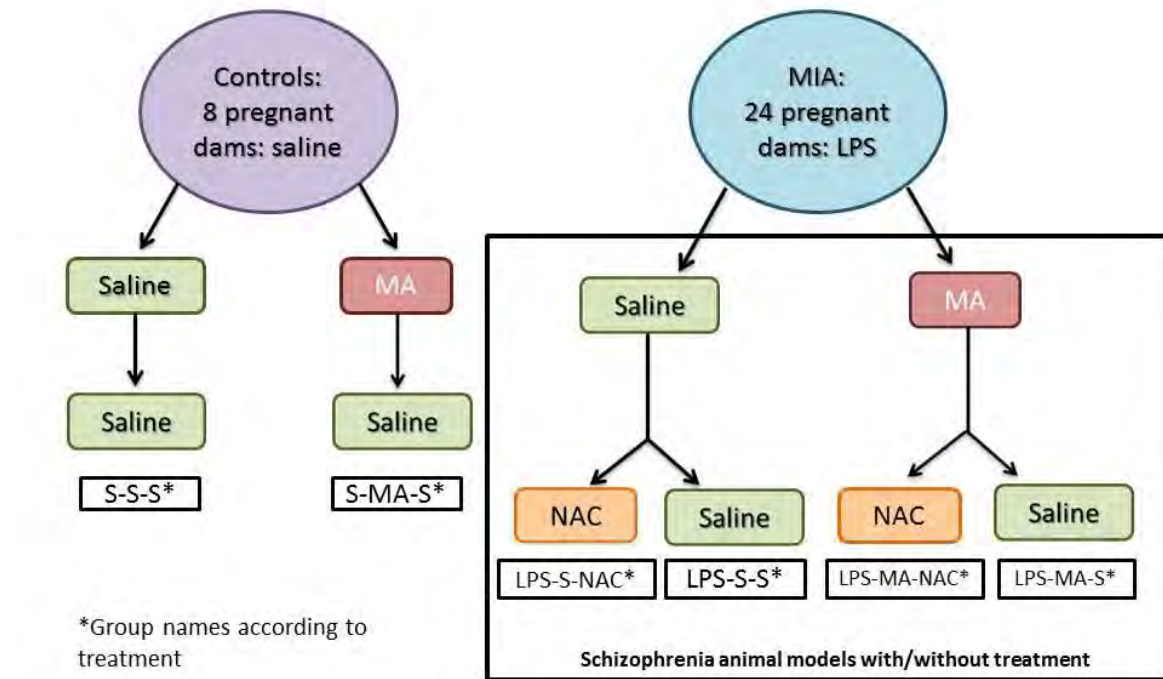


Figure 1

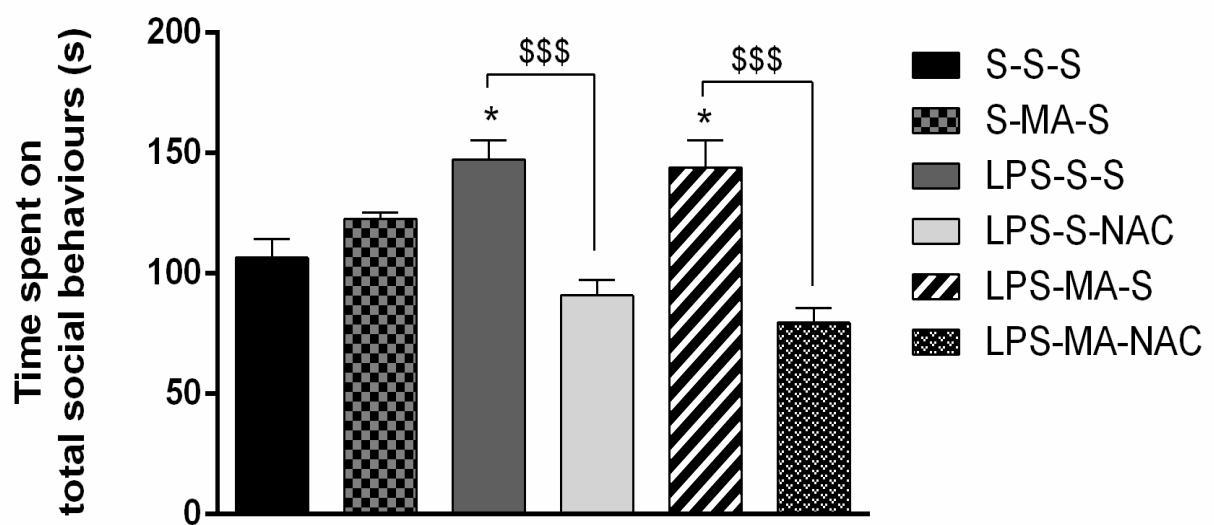


Figure 2

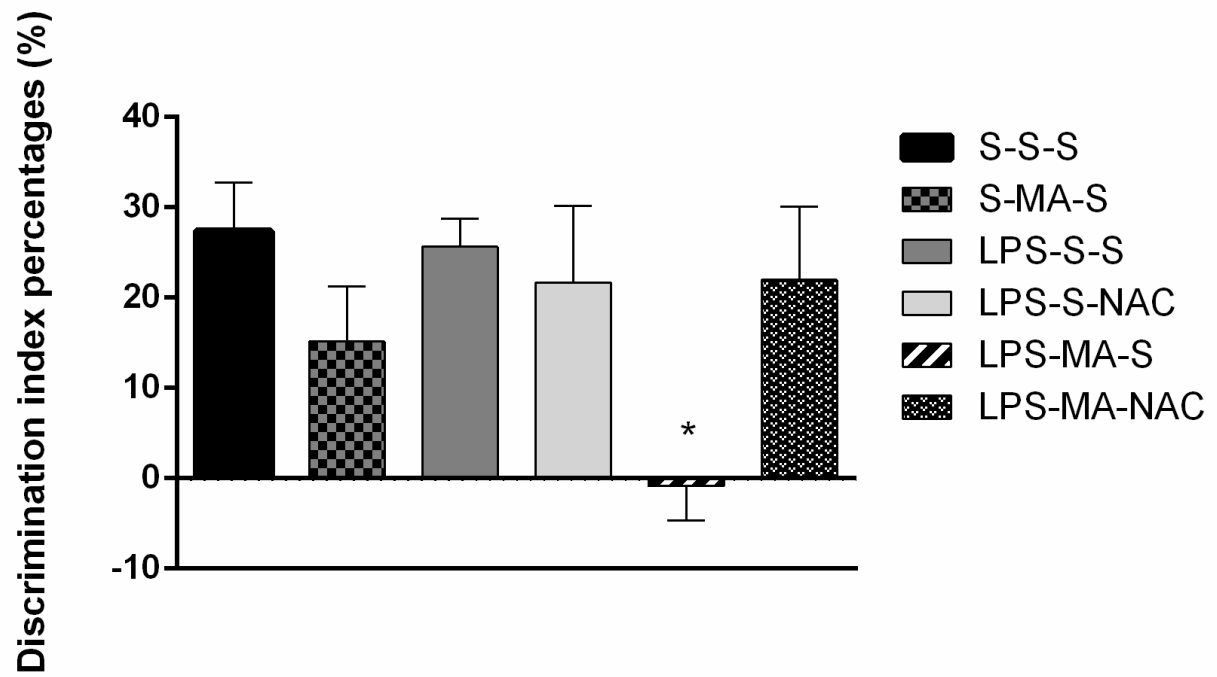


Figure 3

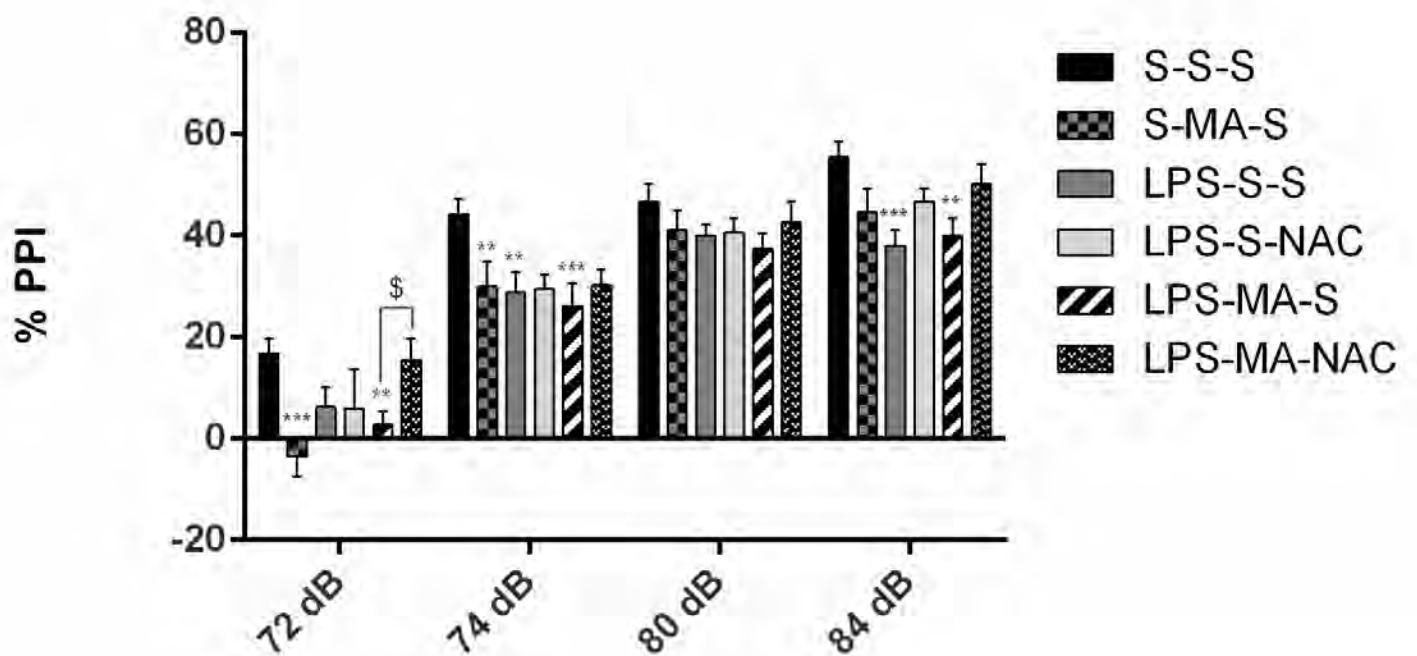


Figure 4

Fig. 5a

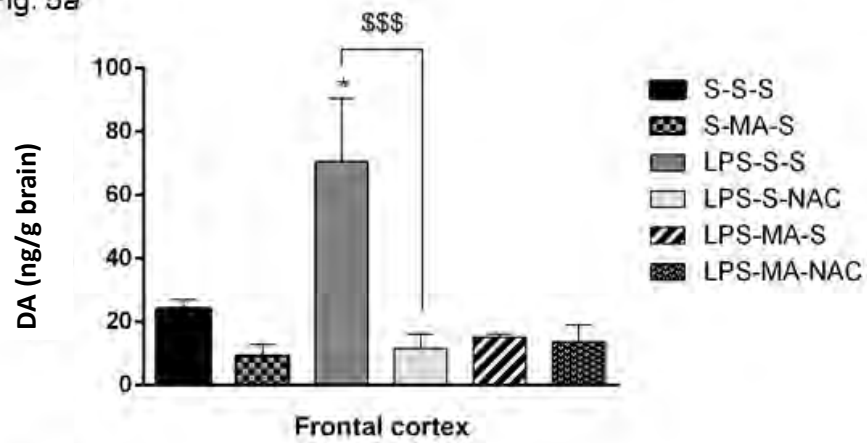


Fig. 5b

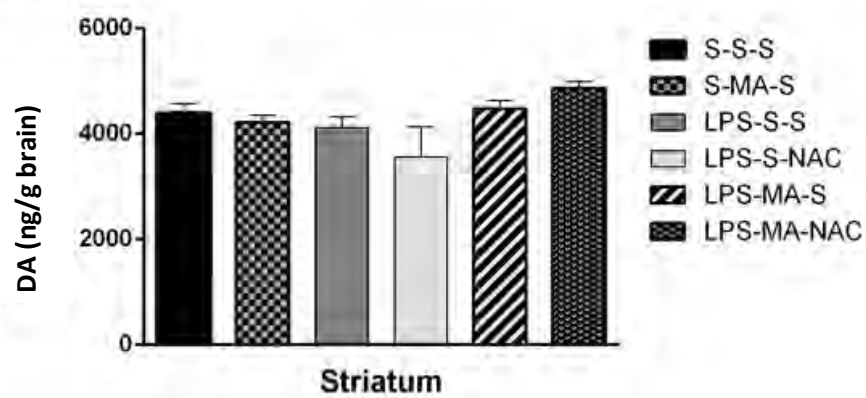


Figure 5

Fig. 6a

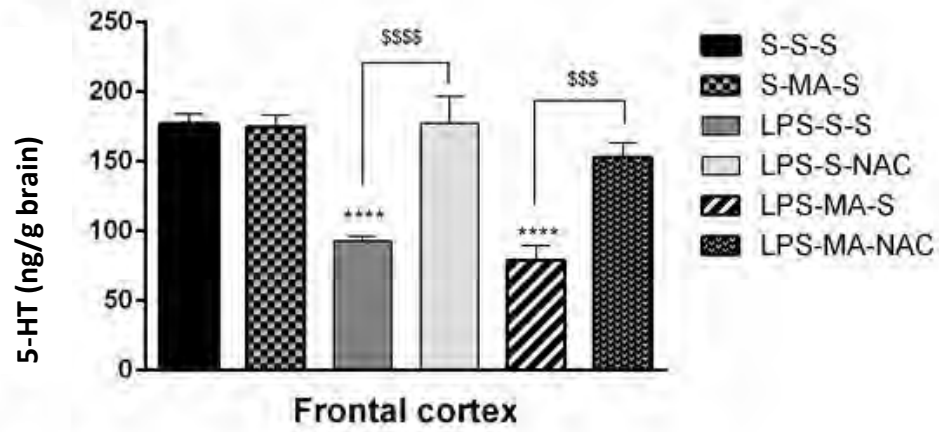


Fig. 6b

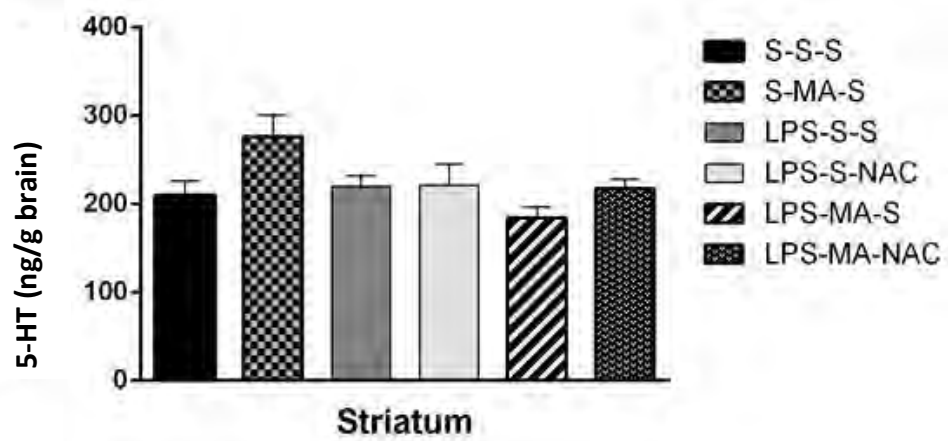


Figure 6

Fig. 7a

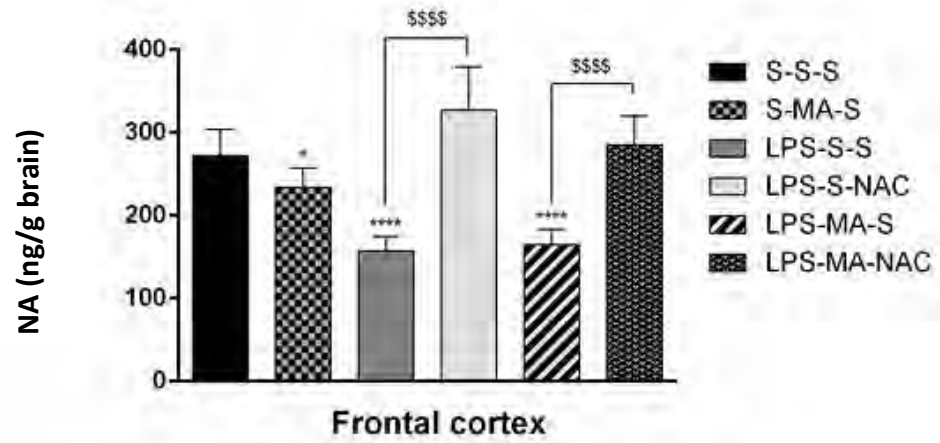


Fig. 7b

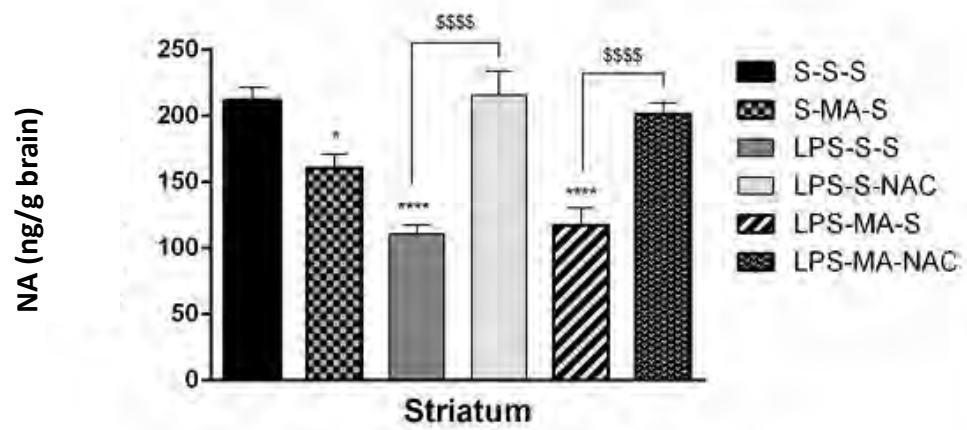


Figure 7

Fig. 8a

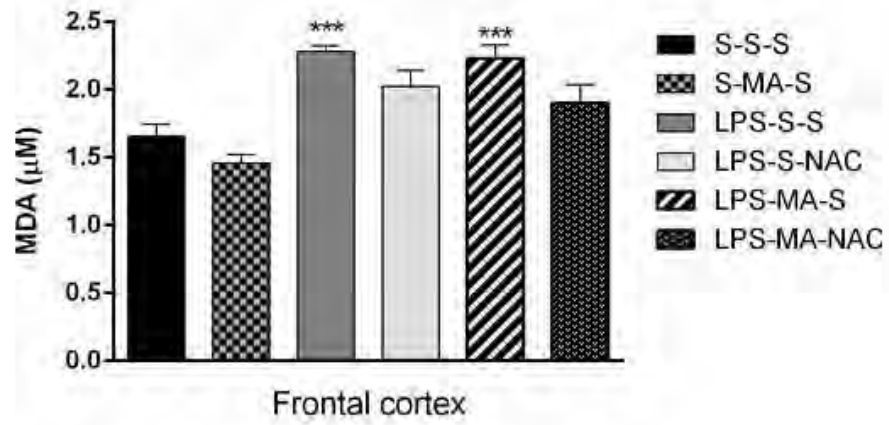


Fig. 8b

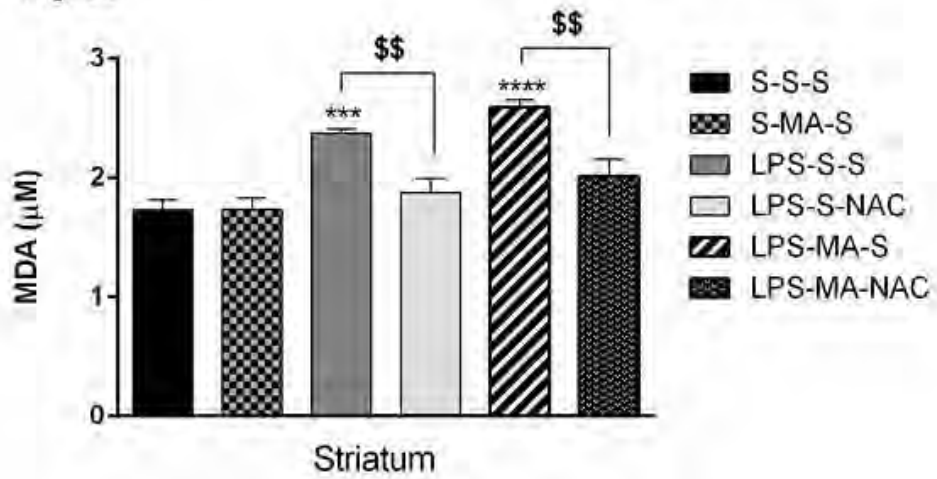


Figure 8

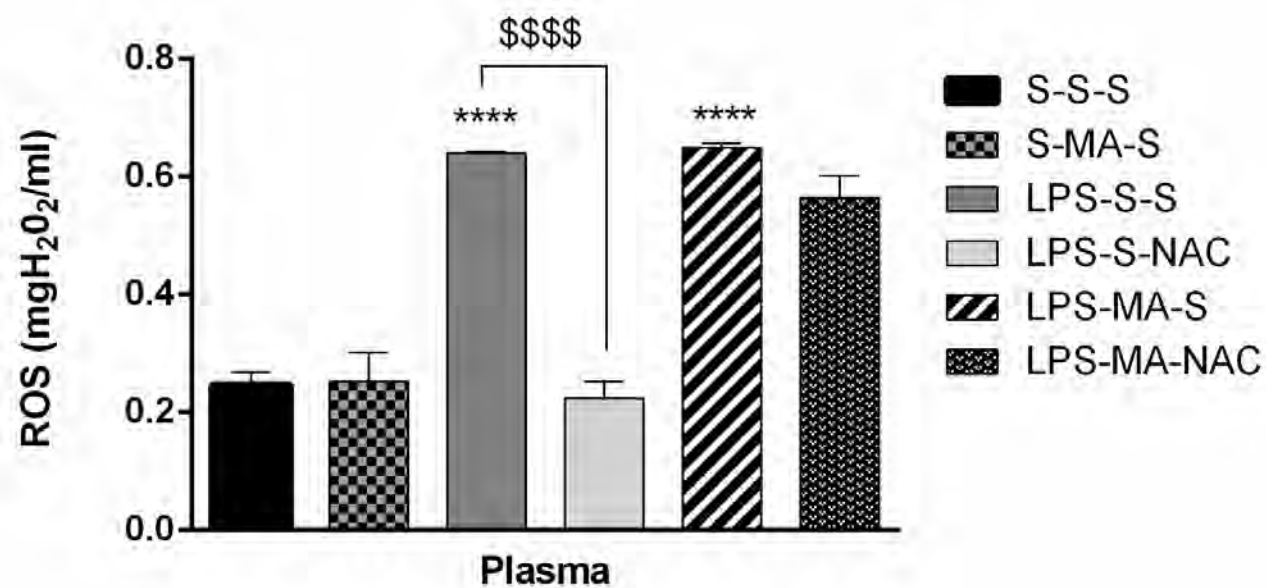


Figure 9

Fig. 10a

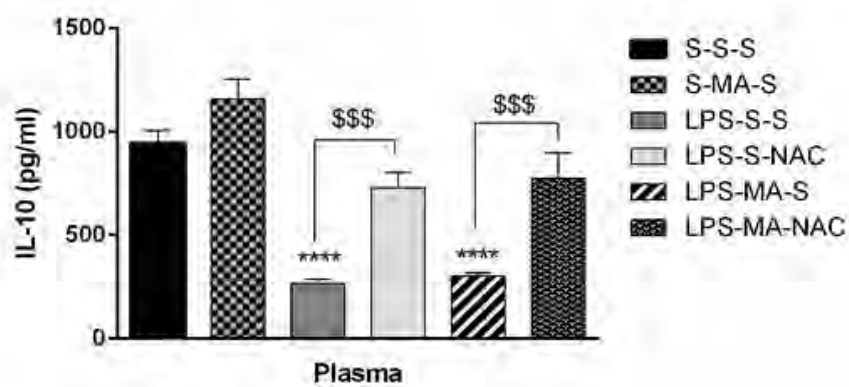


Fig. 10b

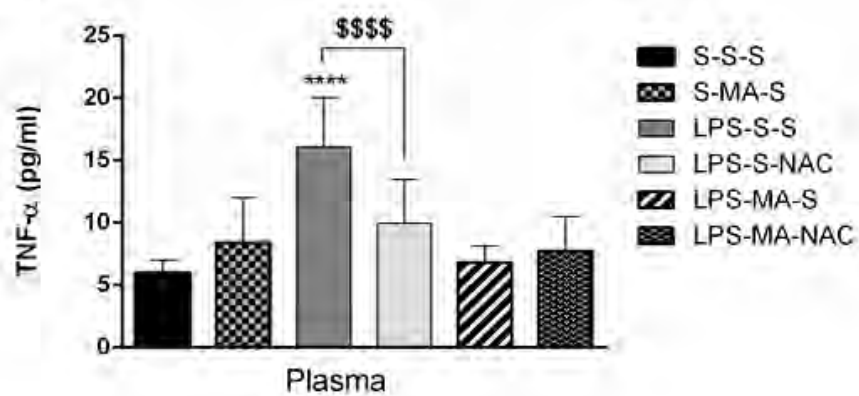


Figure 10

Figure legends:

Figure 1: Graphic presentation of study design.

Figure 2: Total social behaviours.

Figure 3: Novel object recognition test scores.

Figure 4: % prepulse inhibition of acoustic startle.

Figure 5: Dopamine levels in the (a) frontal cortex and (b) striatum.

Figure 6: Serotonin levels in the (a) frontal cortex and (b) striatum.

Figure 7: Noradrenaline levels in the (a) frontal cortex and (b) striatum.

Figure 8: Malondialdehyde (MDA) levels in the (a) frontal cortex and (b) striatum.

Figure 9: Plasma total reactive oxygen species (ROS).

Figure 10: Plasma (a) interleukin-10 (IL-10) and (b) tumor necrosis factor alpha (TNF- α) levels.

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Chapter 4: Published article

There is a growing body of evidence that inflammation and oxidative stress may be strongly involved in the underlying mechanisms of schizophrenia, and that these pathological processes may be mediated by neurodevelopmental insults. However, there remains a lack of knowledge regarding how these processes affect neurodevelopment to eventually lead to the full-blown illness later in life. In fact, this shortfall hinders progress in the discovery and development of improved pharmacological treatment and a deeper understanding is needed to attain an improved outcome. Subsequently, we set out to write an extensive review covering the most relevant neurodevelopmental animal models used to date (social isolation rearing and prenatal inflammation), as well as a more novel approach, viz. models based on the two-hit hypothesis, to act as a primer for more focused research on the subject. The focus of the paper is to report on the current state of the art with regard to inflammatory and oxidative biomarkers observed in these models, as well as associated effects on monoamine and behavioural data pertaining to schizophrenia. In addition, we reviewed the relevance of redox-inflammatory processes as novel drug targets. In essence, this novel review paper provides a summary of the current approaches and avenues in schizophrenia research, thereby enabling researchers to develop relevant future studies.

I contributed to the formulation of the concept and lay-out of the manuscript, as well as to the pre-submission version of the manuscript. Much of my contribution towards this work came from my effort in preparing the literature review for my MSc dissertation, except now much more focused and detailed for the article. In return, the review article contributed to my MSc dissertation by assisting me in writing a comprehensive literature review and, importantly, guided the planning of my project in the direction of relevant research avenues, i.e. selecting appropriate biomarkers of oxidative stress and inflammation and developing a two-hit study design. Guidance in its preparation was provided by the lead author, Dr. Marisa Möller-Wolmarans, and my study leader, Prof. Brian Harvey.

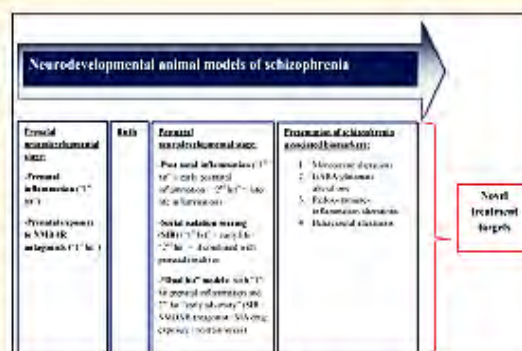
The review article, entitled “Neurodevelopmental animal models reveal the convergent role of neurotransmitter systems, inflammation, and oxidative stress as biomarkers of schizophrenia: Implications for novel drug development”, was published in *ACS Chemical Neuroscience*, and the PDF of this paper is enclosed in my dissertation for examination purposes.

Neurodevelopmental Animal Models Reveal the Convergent Role of Neurotransmitter Systems, Inflammation, and Oxidative Stress as Biomarkers of Schizophrenia: Implications for Novel Drug Development

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ABSTRACT: Schizophrenia is a life altering disease with a complex etiology and pathophysiology, and although antipsychotics are valuable in treating the disorder, certain symptoms and/or sufferers remain resistant to treatment. Our poor understanding of the underlying neuropathological mechanisms of schizophrenia hinders the discovery and development of improved pharmacological treatment, so that filling these gaps is of utmost importance for an improved outcome. A vast amount of clinical data has strongly implicated the role of inflammation and oxidative insults in the pathophysiology of schizophrenia. Preclinical studies using animal models are fundamental in our understanding of disease development and pathology as well as the discovery and development of novel treatment options. In particular, social isolation rearing (SIR) and pre- or postnatal inflammation (PPNI) have shown great promise in mimicking the biobehavioral manifestations of schizophrenia. Furthermore, the “dual-hit” hypothesis of schizophrenia states that a first adverse event such as genetic predisposition or a prenatal insult renders an individual susceptible to develop the disease, while a second insult (e.g., postnatal inflammation, environmental adversity, or drug abuse) may be necessary to precipitate the full-blown syndrome. Animal models that emphasize the “dual-hit” hypothesis therefore provide valuable insight into understanding disease progression. In this Review, we will discuss SIR, PPNI, as well as possible “dual-hit” animal models within the context of the redox-immune-inflammatory hypothesis of schizophrenia, correlating such changes with the recognized monoamine and behavioral alterations of schizophrenia. Finally, based on these models, we will review new therapeutic options, especially those targeting immune-inflammatory and redox pathways.

KEYWORDS: Redox-immune-inflammatory, social isolation rearing, inflammation models, “dual-hit” models, schizophrenia, monoamines



1. INTRODUCTION

Schizophrenia literally translates as “shattered mind”. It is among the world’s top 10 causes of long-term disability, affecting about 24 million people worldwide.¹ Furthermore, the majority of schizophrenia patients do not receive treatment, which contributes to the chronicity of the illness, while 20% of patients experience a relapse despite being on antipsychotic medication.¹ Moreover, the main hurdle in treating schizophrenia is nonadherence to antipsychotic medication, with 50% of patients being noncompliant, that escalates after the onset of the disorder.² All these factors emphasize an urgent need for new treatment strategies.

Various hypotheses have been suggested to explain the underlying biological mechanisms of schizophrenia, such as the first dopamine (DA) hypothesis,³ alterations in γ -aminobutyric acid (GABA) and glutamate function,⁴ as well as oxidative stress⁵ and inflammatory insults.⁶ In terms of the etiology of schizophrenia, a strong link to genetic susceptibility has been

suggested, and remains a contentious research issue.⁷ Adverse events experienced in early life, whether physiological (poor nutrition, obstetric complications, and compromised fetal blood supply), pharmacological (maternal drug abuse and early use of psychoactive drugs), or psychological (maternal loss and separation, social isolation, and sexual and physical abuse), play a critical role in the development of schizophrenia.⁸ Moreover, Weinberger⁹ first formulated the “neurodevelopmental hypothesis of schizophrenia” stating that abnormalities of early brain development increases the risk for subsequent emergence of clinical symptoms. A number of clinical studies have confirmed the presence of inflammation in patients

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suffering from schizophrenia^{10,11} with both prenatal and postnatal inflammation suggested to mediate a host of neurodevelopmental changes in the brain that have been regarded as prodromal events in the development of schizophrenia later in life.¹²

In line with the above-mentioned evidence, and with the aim of identifying potential new targets for drug treatment,¹³ researchers have identified translational developmental animal models specific to schizophrenia, focusing on epidemiological risk factors (e.g., prenatal viral insult and social isolation rearing (SIR)). Recently, these models have provided much vaunted information pertaining to the neurobiology and treatment of schizophrenia and in particular have emphasized the causal role of inflammation in this illness as well as revealing new opportunities for treatment.

Prenatal and postnatal infection (PPNI) has received a great deal of interest in the past decade as a causative factor in the development of schizophrenia, as noted above, which has turned the focus to an immune-inflammatory basis (reviewed in ref 14). Studies have employed several methods to induce infection in animal models, including the influenza virus,¹⁵ the synthetic viral polyriboinosinic-polyribocytidilic acid (Poly I:C),¹⁶ the bacterial endotoxin lipopolysaccharide (LPS),^{17,18} individual cytokine administration,¹⁹ or local inflammation using turpentine.²⁰ Of these methods, Poly I:C and LPS seem to be the most widely used methods in studies of PPNI. Poly I:C is a synthetic analogue of double-stranded RNA that stimulates the production and release of a variety of pro-inflammatory cytokines after injection through binding to the toll-like receptor (TLR)-3.²¹ LPS, on the other hand, is found in the cell membrane of Gram-negative bacteria (e.g., *Escherichia coli*) and thus mimics the immunological effects of Gram-negative infections.²⁰ LPS binds to TLR-4 on macrophages and other immune cells to trigger a signal transduction cascade leading to the activation of transcription factors such as nuclear factor kappa β NF- κ B, as well as genes coding for pro- and anti-inflammatory mediators such as cytokines, chemokines, and proteins of the complement system.²² These models have also demonstrated behavioral and neurochemical equivalence with schizophrenia as well as reversal of these manifestations with antipsychotic treatment (see ref 6 for review). Because of the popularity of these methods, they will be the most covered in this Review. However, certain sections also contain referrals to results obtained using other models as well.

Postweaning SIR is regarded as a translational model of schizophrenia, and has been reviewed extensively.^{23–25} Within a colony of rats, a strict social structure is at hand, allowing them to develop a hierarchy that plays a critical role in development and especially neurodevelopment.¹³ This postnatal developmental model explores the effects of environmental insults via social deprivation on the developing brain after birth.²⁶ The SIR model presents with numerous behavioral and neurochemical manifestations akin to schizophrenia and that can be reversed using a wide range of antipsychotics,^{13,23} thus confirming its validity for schizophrenia. Importantly, SIR has also revealed the presence of a pro-inflammatory state thus aligning itself with the earlier mentioned PPNI inflammatory models.^{27,28} However, the aforementioned PPNI model and SIR are not necessarily exclusive. Indeed, the “dual-hit” hypothesis has been developed to describe increasing evidence that an environmental insult in an already (prenatally or

postnatally) immune-compromised individual may translate into an even greater risk for developing schizophrenia.²⁹

More heuristic models have explored the use of pharmaceuticals to induce schizophrenia-like behaviors in rodents. These models are aimed at understanding the developmental neuropathology of the disease, such as neonatal *N*-methyl-D-aspartate (NMDA) receptor (NMDA-R) antagonist administration (reviewed in ref 25). The glutamate hypofunction model of schizophrenia was first proposed over two decades ago, based upon the observation that phencyclidine (PCP), ketamine, and similarly acting psychotomimetic compounds (dizocilpine (MK-801)) induced their unique behavioral effects by blocking neurotransmission at NMDA glutamate receptors.³⁰ The ability of PCP or ketamine to induce schizophrenia-like psychosis has become well established in schizophrenia research,³¹ although its value in the current Review will be its contribution toward the “dual-hit” hypothesis.

Earlier it was noted that schizophrenia may be subsequent to maternal drug abuse, or drug exposure. This propagated the idea that exposure to psychotogenic drugs such as methamphetamine (MA) may be useful to model schizophrenia in animals. This was first conceptualized after World War II when Japanese psychiatrists observed overlapping symptoms between the clinical manifestation of MA abuse and schizophrenia.³² Considering the earlier mentioned “dual-hit” hypothesis,²⁹ since MA abuse occurs mostly in adolescents and in adolescents with a troubled childhood, combined MA plus SIR or PPNI models may reveal much on the interplay between MA as an environmentally relevant adverse event that may worsen the outcome in individuals who have suffered an early life (psychosocial or inflammatory) insult. However, a MA model of schizophrenia has not received a great deal of attention in recent years, and as such valuable data regarding this model is limited. In this paper, we will review the behavioral and neurochemical impact of MA administration in rodents as part of the “dual-hit” hypothesis, while also highlighting how this method may provide valuable insights into the psychopathology of schizophrenia.

While most of the above-mentioned animal models of schizophrenia have been extensively reviewed,^{13,23,33} this Review will focus specifically on the role of inflammation and oxidative stress as biomarkers of schizophrenia, as presented in these models, and to discuss the relevance of these processes as novel drug targets. Moreover, despite the increasing evidence and support for a redox-immune-inflammatory basis for schizophrenia, it is imperative that altered neurotransmission be integrated into this theory, especially given the broad pharmacological and neuroimaging evidence that DA remains necessary and sufficient to explain the biobehavioral changes that characterize the illness.³⁴

2. NEUROTRANSMITTER THEORIES OF SCHIZOPHRENIA

2.1. Monoamine Alterations in Schizophrenia. The oldest and most established hypothesis of schizophrenia proposes that DA hyperactivity in the striatum is responsible for positive symptom expression, while DA hypoactivity in the frontal cortex mediates the cognitive and negative symptoms observed in schizophrenia patients.³⁵ Post-mortem studies have indeed confirmed this hypothesis with findings of frontal cortical hypo-DAergic and striatal hyper-DAergic described in schizophrenia.³⁶ However, based on recent clinical findings of DA involvement in schizophrenia patients, it is speculated that

multiple "hits" (i.e., adverse environment, infection, chronic substance abuse, etc.) interact in order to induce dysregulated DA transmission typical of the disorder.³⁷ Moreover, the effectiveness of especially conventional or typical antipsychotics, which block D₂ receptors, in ameliorating psychotic symptoms is also supportive of the hyper-DAergic hypothesis.³⁸ Furthermore, a clinical study has observed decreased plasma 3,4-dihydroxyphenylacetic acid (DOPAC) levels and decreased DA metabolic activity in relapsed patients with schizophrenia compared to first-episode patients, as well as decreased DA levels and increased DOPAC and DA transporters (DAT) in patients after risperidone treatment.³⁹ Interesting is that recent neuroimaging studies have indicated that DA transmission is altered even before psychotic symptoms are manifested.⁴⁰ Although the DA hypothesis has wide influence, a recent imaging study failed to find direct evidence for DAergic alterations in cortical and other extrastriatal regions in schizophrenia, thus warranting controlled studies in drug-naïve patients to verify these findings.⁴¹ However, the fact that D₂ receptor antagonist antipsychotics are ineffective in reversing cognitive and negative symptoms is an important shortcoming of the hyper-DAergic hypothesis of schizophrenia,⁴² emphasizing that further research is needed. Although dysregulated DA is insufficient to explain the full complexity of schizophrenia, it offers a direct relationship to symptom severity and to treatment effectiveness.

The serotonin (5-HT) hypothesis of schizophrenia found its place following the evidence that a partial or full agonist at 5-HT receptors, such as lysergic acid diethylamine (LSD), produce hallucinations in patients, prompting the idea that 5-HT transmission might be increased in schizophrenia patients.⁴³ Subsequent cerebrospinal fluid, genetic and neuroimaging studies have confirmed this hypothesis with evidence of increased central 5-HT-ergic transmission in schizophrenia patients,^{44,45} while increased striatal but diminished frontal cortical 5-HT uptake sites have also been described.⁴⁶ Furthermore, a post-mortem study observed decreased frontal cortical 5-HT_{2A} and increased 5-HT_{1A} receptor density in schizophrenia patients,⁴⁷ while other studies observed higher metabolic 5-HT activity in first-episode compared to relapsed schizophrenia patients.³⁹ Although positive symptoms of schizophrenia may be associated with an increase in 5-HT transmission in the striatum,⁴⁵ clinical studies have failed to describe the clinical efficacy of selective 5HT_{2A/C} antagonists in the treatment of schizophrenia-related psychosis.⁴⁸ On the other hand, novel antipsychotic agents that function as 5-HT_{2A/C} antagonists (such as clozapine) appear to be superior to first generation "typical" antipsychotics (often referred to as neuroleptics) for treating negative symptoms and treatment-resistant schizophrenia,⁴⁹ thus clearly implying a causal role for 5-HT in at least the negative and cognitive symptoms of the disorder. Moreover, some antipsychotics, such as ziprasidone, are partial 5-HT_{1A} agonists that would similarly abrogate excessive 5-HT activity.⁵⁰ Activation of 5-HT_{1A} receptors also inhibits NMDA receptor-mediated currents in the prefrontal cortex to subsequently modulate the effect of glutamate on subcortical/striatal release of DA (see review by Schwartz et al.⁵¹). This emphasizes the role of 5-HT in indirectly controlling emotional and cognitive processes mediated by DA.^{52,53} A recent review also observed that DAergic and 5-HTergic projections in the prefrontal cortex form a framework of complex neuronal interactions with glutamatergic projections that support cognitive function.⁵⁴ Importantly, these findings

link the 5-HT and DA hypotheses with the GABA-glutamate hypothesis (discussed in subsection 2.2). Serotonin also exerts an inhibitory effect on frontal cortical DA and NA release,⁵⁵ and it has been suggested that frontal cortical hypo-DAergia in schizophrenia, and associated negative and cognitive symptoms, is reversed following blockade of 5HT_{2A/C} receptors.⁵⁰ A clinical study also observed a relationship between 5-HT_{1A} agonist binding in the frontal cortex (with ziprasidone) and the degree of improvement in negative symptoms.⁵⁶ Interestingly, alterations of 5-HT transporters are generally not associated with schizophrenia.⁵⁷ Although there are some controversies, the studies mentioned above generally support the role of hyper-5-HTergic activity in the pathophysiology of schizophrenia, especially in the frontal cortical brain area.

A decade ago Yamamoto and Hornykiewicz posited evidence that hyperactivity and hypoactivity of noradrenaline (NA) transmission are associated with the positive and negative symptoms of schizophrenia, respectively.⁵⁸ Increased NA transmission has also been associated with the anxiety symptoms of schizophrenia,⁵⁹ and a recent study also observed higher NA turnover rate in undifferentiated patients in comparison to paranoid schizophrenia patients.³⁹ However, the role of NA in schizophrenia pathophysiology is not clearly understood and further studies are needed to conclude the presence of any brain-region-specific alterations. It could be argued, however, that the hyper-5-HTergic activity in the frontal cortex, noted above, inhibits NA release resulting in negative and cognitive symptoms.

2.2. GABA-Glutamate Alterations in Schizophrenia. As noted earlier, the hypo-glutamatergic hypothesis of schizophrenia was pivotal in its support that NMDA receptor antagonists induce behavioral alterations similar to symptoms observed in schizophrenia.³⁰ Despite its emphasis on glutamate, the dysfunctional glutamate transmission hypothesis of schizophrenia integrates GABA transmission and in particular the glutamate-GABA-glutamate feedback loops as the basis for psychopathology. In fact, GABA-glutamate interactions are directly responsible for modulating ascending 5-HTergic, NAergic, and DAergic pathways that ultimately drive the numerous symptoms of schizophrenia.⁶⁰

The glutamate-GABA-glutamate neurocircuitry of schizophrenia could be interpreted as explained below and illustrated in Figure 1 (reviewed in ref 51). The cortical brainstem glutamate projections are known as the corticocortical glutamatergic pathways and project from cortical pyramidal neurons in the prefrontal cortex to brainstem neurotransmitter centers to regulate neurotransmitter release, or descend from the prefrontal cortex to innervate the striatum and the nucleus accumbens. Thalamocortical glutamate pathways in turn project from the thalamus to innervate pyramidal neurons in the cortex. In schizophrenia, the cortical brainstem glutamate projection is compromised by suboptimal NMDA receptor activation. Normally a fully functioning primary prefrontal cortical glutamate neuron fires upon a GABA interneuron, but as a consequence of a poorly functioning NMDA receptor the GABA interneuron no longer fires adequately (hypo- or under-functioning GABA). This leads to a disinhibition of a secondary cortical glutamate neuron resulting in excessive glutamate-mediated activation of the mesolimbic DA pathway and the positive symptoms of schizophrenia. If the onset of negative symptoms is considered, again we have a dysfunctional NMDA receptor on cortical GABA interneurons culminating in the absence of sufficient GABA tone again resulting in an overactive

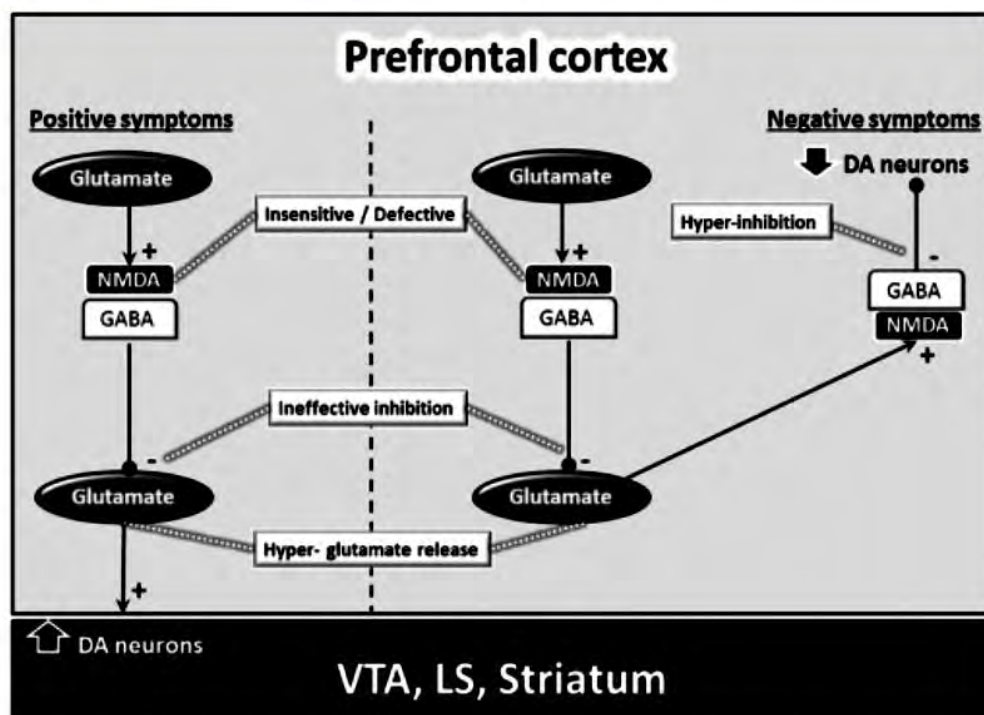


Figure 1. Simplified diagram depicting the neurocircuits involved in positive (left panel) and negative (right panel) symptoms observed in schizophrenia that arise due to neurodevelopmental abnormalities (Adapted from refs 51 and 40). DA activity in the midbrain is controlled by primary glutamate neurons in the PFC that activate NMDA receptors on inhibitory GABA interneurons. These GABA interneurons in turn synapse with secondary cortical glutamate neurons responsible for downstream neurotransmitter (DA, NA, 5HT) release in the VTA, LS, and striatum (left panel). In schizophrenia, PFC hypoglutamatergia will lead to excessive DA release in the VTA, LS, and striatum via the ineffective inhibition of the glutamate–GABA–glutamate loop, ultimately responsible for the positive symptoms (left panel). Regarding the negative symptoms of schizophrenia (right panel), as with the defect in the cortical glutamate–GABA–glutamate loop described for positive symptom development, the secondary glutamate neuron is again hyperactive (right panel). However, in this scenario, the secondary glutamate neuron impinges on another NMDA–GABA interneuron that connects with DA neurons in the PFC where the high glutamate tone will increase the release of GABA. The ensuing increase in GABA inhibits PFC DA release resulting in lowered DA mesocortical activity, eventually causing hypofrontality and negative symptoms (right panel). Abbreviations: dopamine (DA); prefrontal cortex (PFC); N-methyl-D-aspartate (NMDA); γ -amino-butyric acid (GABA); noradrenaline (NA); serotonin (5-HT); ventral tegmentum area (VTA); limbic system (LS).

secondary glutamate neuron. However, this secondary glutamatergic neuron impinges upon another GABA mediated interneuron responsible for regulating striatal DA release. This GABA interneuron is now overstimulated by glutamate to release more GABA that in turn inhibits the firing of the midbrain DA mesocortical pathway. The result is an underactive frontal cortical DA projection resulting in hypofrontality and negative symptoms (reviewed in refs 51 and 61); see Figure 1 for further explanation.

In line with the above hypothesis, numerous postmortem schizophrenia studies have observed deficits in glutamate systems in the temporal cortex, medial temporal lobe, and striatal regions (reviewed in ref 40), along with decreased glutamate uptake sites⁶² and increased NMDA receptors in the same brain regions.⁶³ However, a recent clinical study indicated elevated GABA and glutamate levels in the medial prefrontal cortex of unmedicated patients, with no alterations in medicated schizophrenia patients, suggesting possible normalization of GABA and glutamate levels after antipsychotic treatment.⁶⁴ Moreover, deficits in GABA-parvalbumin (PV) containing neurons in the frontal cortex and hippocampus⁶⁵ and GABAergic uptake sites in the hippocampus have been observed in schizophrenia patients.⁶⁶ Proton magnetic resonance spectroscopy (¹H-MRS) is a technique that allows the assessment of the glutamatergic index as a measure of

glutamate function in the brain (reviewed in refs 61 and 67). Interesting is that recent studies using ¹H-MRS in schizophrenia observed that unmedicated and medication-free patients present with elevated glutamatergic levels in the prefrontal cortex and basal ganglia (especially the striatum). A possible relationship was also observed between elevated glutamate and glutamine in the hippocampus and decreased hippocampal volume as compared to healthy controls (reviewed in refs 61 and 67). These studies are in contrast to the hypoglutamatergic hypothesis explained above. However, it could be debated that a hyperglutamatergic state brought about by the activation of neurotoxic oxidative stress processes (implicated in schizophrenia) underlie this observation (explained in subsection 3.2). It could also be theorized that both states (hyper- and hypoglutamate) exist, but at different glutamate neurons, where cortical hypoglutamatergia leads to hyperglutamatergia at a secondary interneuron (as explained above and illustrated in Figure 1). The presence of dysfunctional (hyper- or hypo-) glutamatergic neurocircuitry has also been linked to numerous genetic alterations observed in schizophrenia patients, such as ionotropic glutamate receptor genes (GRIN1, GRIN2A, GRIN2B, and GRIK3), metabotropic glutamate receptor genes (GRM3), the G72/G30 locus, d-amino acid oxidase (DAO) and G72 (DAOA) genes, as well

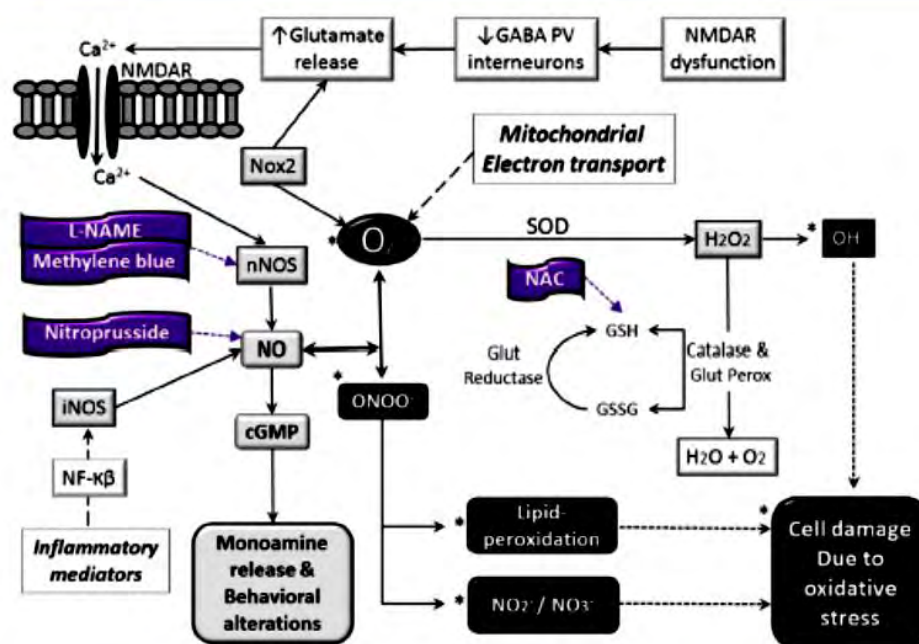


Figure 2. Activation of iNOS and nNOS and subsequent biological effects leading to changes in cellular redox status, monoamine release, and behavioral modulation (adapted from ref 149). Glutamate-directed activation of the NO-cGMP pathway via nNOS (Ca^{2+} dependent) will modulate monoamine release.¹⁴⁷ However, this pathway can also lead to oxidative stress and cell damage if excessive glutamate-mediated NO synthesis combines with O_2^- from aerobic metabolism via (Nox2)¹⁴⁹ to form ONOO^- . Also depicted is the effect of inflammatory mediators via $\text{NF-}\kappa\text{B}$, that promote Ca^{2+} -independent activation of iNOS leading to neurotoxic levels of NO being released thereby promoting the formation of cell-damaging reactive oxygen and nitrogen species. These pro-oxidative mechanisms can be abrogated by endogenous antioxidant systems such as SOD and GSH that act as a sink to quench excessive H_2O_2 and/or O_2^- . Furthermore, NMDAR dysfunction, observed in schizophrenia patients, leads to downregulation of GABA PV interneurons, which in turn causes an increase in glutamate release that over stimulates the NMDAR to increase oxidative stress mediators. Also depicted in this figure are L-NAME and methylene blue as nNOS inhibitors; nitroprusside as a NO donor; and NAC as a glutathione precursor, which will suppress activity or act as stimulants in the pathway. Abbreviations: nicotinamide adenosine dinucleotide phosphate (NADPH)-oxidase 2 (Nox2); superoxide (O_2^-); peroxynitrite (ONOO^-); cyclic guanosine monophosphate (cGMP); glutathione (GSH); reduced glutathione (GSSG); glutathione peroxidase (glut perox); inducible nitric oxide synthase (iNOS); nuclear factor kappa-beta ($\text{NF-}\kappa\text{B}$); neuronal nitric oxide synthase (nNOS); nitric oxide (NO); NMDA receptor (NMDAR); γ -aminobutyric acid parvalbumin (GABA PV); superoxide dismutase (SOD); L-NG-nitroarginine methyl ester (L-NAME); N-acetyl cysteine (NAC).

GABAergic genes (GAD1 and GABRB2) (reviewed in refs 68 and 69).

Another marker of glutamatergic neuronal function is *N*-acetyl aspartate (NAA), which is a precursor for the biosynthesis of *N*-acetylaspartylglutamate (NAAG), a neuropeptide with actions at NMDA and metabotropic glutamate receptors.⁷⁰ Decreased NAA has been observed in the temporal cortex, striatum, and hippocampus of schizophrenia patients,⁶⁵ which could be interpreted as decreased cortico-striatal glutamatergic function. Further support for glutamate dysfunction is provided by evidence for disturbances in the primary downstream messenger of the NMDA receptor, namely, the nitric oxide (NO)-cyclic guanosine monophosphate (cGMP) cascade.⁷¹ The enzyme nitric oxide synthase (NOS) generates NO via two isoforms, namely, neuronal NOS (nNOS) which is Ca^{2+} dependent and inducible NOS (iNOS) which is Ca^{2+} independent (see Figure 2) (reviewed in ref 72). Neuronal NOS is activated following stimulation of the NMDA receptor, while the expression of iNOS forms part of the immune-inflammatory cascade, illustrated in Figure 2 (reviewed in refs 71 and 72). Indeed, patients with schizophrenia present with numerous NO-related alterations that will be discussed in subsection 3.2.

3. IMMUNE-INFLAMMATORY-REDOX ALTERATIONS IN SCHIZOPHRENIA

3.1. Mitochondrial Alterations. The mitochondria are cell organelles responsible for many vital processes in the growth and functioning of an organism, such as energy production, intracellular calcium buffering, generation of reactive oxygen species (ROS) and apoptosis.⁷³ When the mitochondria of a specific cell does not function optimally, it can lead to changes in neuronal functions and plasticity, giving way to behavioral and cognitive abnormalities such as those observed in schizophrenia.⁷⁴ In fact, factors involved in cellular oxidative stress are known to evoke monoaminergic changes that may underlie various psychiatric manifestations.⁷⁵ In clinical studies, an array of observations has been made regarding the role of mitochondrial dysfunction in the development of schizophrenia, including deoxyribonucleic acid (DNA) mutations, functional abnormalities, and faulty adenosine triphosphate (ATP) production and storage.⁷⁶ Moreover, when abnormalities in mitochondrial function exist, ROS production, such as an increase in superoxide levels (indicated in Figure 2), increases, resulting in an amplified risk for oxidative damage to lipids, proteins, and DNA.⁷⁶ Thus, a close relation exists between mitochondrial dysfunction and oxidative stress that is observed in schizophrenia, and discussed below.

Having described the evidence of redox dysfunction in schizophrenia, it is worth noting that said oxidative status in schizophrenia may also in part be influenced by certain environmental factors, especially cigarette smoking. The prevalence of cigarette smoking in patients with schizophrenia is significantly higher than that compared to the general population (reviewed in ref 90). Cigarette smoking contributes greatly to oxidative stress, especially if one considers that every gram of tar found in cigarette smoke contains 10^{15} free radical molecules, while it also provokes the release of ROS by inflammatory cells in the lungs.⁹¹ Furthermore, cigarette smoking can also compromise endogenous antioxidant systems (reviewed in ref 92), rendering an organism susceptible to oxidative damage by free radicals. Schizophrenia also places tremendous stress on the psychosocial well-being of the patient, while stress in itself is a powerful pro-oxidant in both humans⁹³ and animals.⁹⁴

The above-mentioned findings provide a clear picture of oxidative stress in schizophrenia and have led to the clinical utility of antioxidants as adjunctive treatment in schizophrenia, as will be discussed in due course (subsection 5.2). Importantly, evidence for oxidative stress is also clearly evident in the neurodevelopmental models under discussion (subsections 4.1.3, 4.2.3, and 4.3.3).

3.3. Immune-Inflammatory Pathways. In line with the genetic predisposition of schizophrenia, it has recently been observed that patients and their first-degree relatives present with elevated proinflammatory cytokines and decreased anti-inflammatory cytokines,¹⁰ indicative of a proinflammatory state. First-episode schizophrenia patients also present with elevations in proinflammatory cytokines as well as a positive correlation between IL-6 (a proinflammatory cytokine) and the duration of illness.¹¹ In their 2008 meta-analysis, Potvin and colleagues provide a clear demonstration of the central role for cytokine profiles in schizophrenia.⁹⁵ Regarding the prominent place for the neurodevelopmental anomalies in schizophrenia, and indeed as applied in the SIR model, it is noteworthy that cytokines play a prominent role in the neurodevelopmental hypothesis due to their integral role in brain growth during fetal periods and thereafter (reviewed in ref 96). Indeed, preclinical studies have documented that circulating cytokines following a pathogen infection may enter the amniotic fluid and alter cytokine expression in the developing fetal brain.^{97,98} Furthermore, the anti-inflammatory cytokine IL-10 was found to be significantly decreased in the late stages of schizophrenia while IL-6 was significantly increased in the early stages of schizophrenia.⁹⁹ Elevated CSF levels of another proinflammatory cytokine, IL-1, have been suggested as a marker of acute psychotic relapse in schizophrenia patients.¹⁰⁰ Important to note here is that the inflammatory abnormalities observed in schizophrenia do not seem to relate to antipsychotic treatment.¹⁰⁰ Moreover, a positive correlation between the severity of cognitive deficits and IL-1 β , IL-6, and TNF- α levels has provided further evidence of the causal role of proinflammatory cytokines in schizophrenia pathology.¹⁰¹ Furthermore, microglia activation is central to the release of proinflammatory cytokines and is a key event in the activation of the innate immune system in the brain.¹⁰² Such a response has been implicated in the pathophysiology of schizophrenia¹⁰³ as well as during the acute phase of paranoid schizophrenia.¹⁰⁴ Previous postmortem studies have also observed activated microglia in the fronto-temporal regions of schizophrenia patients (reviewed in ref 105).

A very important link between the inflammatory cascades mentioned above and schizophrenia symptomatology is tryptophan metabolism via the kynurenine pathway¹⁰⁶ (Figure 3). Tryptophan is responsible for the synthesis of 5-HT via tryptophan-5-hydroxylase or kynurenine via tryptophan 2,3-dioxygenase (TDO) or indoleamine-2,3-dioxygenase (IDO) conversion, respectively.¹⁰⁶ The metabolism of kynurenine can then lead to the formation of 3-hydroxy-kynurenine (3HK), 3-hydroxy-anthranilic acid (3-OHAA), anthranilic acid, and quinolinic acid (QA) respectively, which have neurotoxic effects, or to kynurenic acid (KYNA) which has neuroprotective properties (Figure 3).¹⁰⁷ These actions on neuronal integrity are due to the opposing actions of QA and KYNA on NMDA receptor activity. QA is a NMDA receptor agonist while KYNA has NMDA receptor antagonistic properties.¹⁰⁸ These opposing effects of KYNA and QA on NMDA receptors also link to the glutamate hypotheses discussed in subsection 2.2, implying that a dysfunctional neurocircuitry of glutamate activity in schizophrenia can be engendered by an initial inflammatory insult with the kynurenine pathway being the comediator. Furthermore, increased KYNA (NMDA receptor antagonist) or QA (NMDA receptor agonist) levels could be implicated in a hypoglutamatergic or a hyperglutamatergic state respectively, as observed in schizophrenia.^{61,67} Postmortem schizophrenia studies have observed elevated levels of tryptophan, 3-OHAA, kynurenine and QA in various brain regions.¹⁰⁹ Previous studies have observed increased KYNA levels in the CSF of patients with schizophrenia¹¹⁰ and in post-mortem brain tissue of schizophrenia patients who had received antipsychotic treatment.¹¹¹ However, another study observed a significant reduction in plasma KYNA levels and a decrease in the neuroprotective ratio (a measure of the relationship between KYNA and kynurenine levels) in treatment-free schizophrenia patients.¹¹² These findings could indicate that antipsychotic treatment significantly improves the KYNA deficit observed in treatment-free schizophrenia patients. Moreover, schizophrenia patients also presented with increased levels of tryptophan in the CSF and plasma, which was independent of antipsychotic treatment.^{113,114} The above-mentioned evidence could point toward a decrease in 5-HT synthesis, but with concomitantly increased neurotoxic metabolites synthesized from tryptophan (Figure 3), ultimately leading to deregulated neuro-circuitries and neuro-degeneration, as observed in schizophrenia.¹¹⁵ These studies emphasize the importance of drug-free clinical studies to identify potential biomarkers, although data points toward a deficit in the neuroprotective KYNA metabolite, but which is amenable to treatment with an antipsychotic. Such a decrease in KYNA levels can also be linked to the hypo-glutamatergic state of schizophrenia (discussed in subsection 2.2).

Important enzymes in the kynurenine pathway that have been studied in psychiatric research are IDO and kynurenine 3-monooxygenase (KMO), IDO being responsible for the catabolism of tryptophan to kynurenine while KMO is responsible for the catabolism of kynurenine to 3HK (Figure 3).¹¹⁶ It has been observed that IDO is generally inhibited by anti-inflammatory cytokines (such as IL-10) or activated by proinflammatory cytokines (such as IL-1 and TNF- α), which in turn reduces peripheral tryptophan availability for the synthesis of 5-HT, and increases the synthesis of the neurotoxic metabolites of kynurenine, viz. 3-OHAA and QA (see Figure 3).¹⁰⁸ Recent evidence suggests that an alternate mechanism, namely a reduction in KMO activity in the periphery and/or in

the brain, may underlie the abnormalities observed in the kynurenine pathway in schizophrenia.^{117,118}

4. NEURODEVELOPMENTAL ANIMAL MODELS OF SCHIZOPHRENIA: BIOLOGICAL AND BEHAVIORAL EQUIVALENCE

The neurodevelopmental hypothesis of schizophrenia was first formulated by Weinberger nearly three decades ago.⁹ This hypothesis was based on (a) structural brain abnormalities observed at the onset of schizophrenia with no evidence for neurodegeneration in post-mortem studies; (b) the frequent occurrence at a young age of cognitive and motor abnormalities in patients who went on to develop schizophrenia later on; and (c) the idea that adult-onset disorders could have their origins in development, supported by studies in primates that showed that neonatal lesions can have delayed effects on behavior. Moreover, the abnormal developmental trajectory of the schizophrenia brain, which may be initiated and directed by environmental factors, appears to be established during gestation, long before clinical symptoms of the disease appear in early adult life.¹¹⁹

The neurodevelopmental hypothesis therefore suggests that brain development can be adversely affected at a critical time of life (pre- or postnatal), particularly through early life exposures to stress that may provoke the onset of psychosis in later adolescence or adulthood,^{120,121,24} along with associated neurochemical and redox-immune-inflammatory changes as discussed above. Thus, in order to further evaluate the key neurodevelopmental factors in the pathogenesis of schizophrenia, and to highlight potential new targets for novel drug treatment, analogous neurodevelopmental animal models of schizophrenia are needed,²⁴ such as the SIR model, inflammation models and “dual-hit” models. This review will now discuss each of these models with regards to their effect on monoamines, GABA-glutamate pathways, redox-immune-inflammatory activation and behavioral alterations, respectively.

An essential aspect of a translational animal model is the behavioral changes evident in the model and how these correlate with that of the human disorder. Some of the relevant behavioral paradigms used in translational animal models of schizophrenia replicate the psychological presentation of cognitive dysfunction, as well as positive and negative symptoms of schizophrenia.^{122,24,120} These behaviors can be assessed in animals using a specific array of behavioral tests, which include:

- social behavior (interactive, self-directed and aggression) relating to asociality symptoms,
- increased locomotor activity relating to hyper-activity or anxiety symptoms, as well as decreased locomotor activity possibly relating to the negative symptoms,¹²³
- sensorimotor gating (prepulse inhibition (PPI)) relating to the inability to gate intero- and exteroceptive stimuli,
- novel object recognition test (nORT) that assesses cognitive memory,
- spatial learning memory, as assessed in the Morris water maze test,
- spatial learning and working memory, as assessed in the food alternating dry maze matching-to-position paradigm, Y-maze, T-maze and win-shift task in a radial eight arm-maze,
- depressive symptoms, as assessed in the forced swim test (FST) or sucrose consumption test,

- cognitive deficits, as assessed in the latent inhibition test (refers to retarded conditioning to a stimulus that has repeatedly been presented without reinforcement),
- anxiety-like behaviors, as assessed in the elevated plus maze (EPM) test,
- altered water homeostasis, as assessed using the schedule-induced polydipsia test (relating to excessive nonphysiologic drinking observed in schizophrenia), and
- contextual and conditioned associative learning deficits, as assessed in the conditioned emotion response task (relating to deficits in fear-motivated associative learning as observed in schizophrenia).

4.1. Social Isolation Rearing (SIR). **4.1.1. Effect on Monoamines.** SIR decreases DA, DOPAC, and homovanillic acid (HVA) levels in the prefrontal cortex.^{28,124,125} However, this is contradictory to other SIR studies^{126,127} that have observed increased DA levels in this brain region. Previous studies have observed elevated DA, DOPAC and HVA levels in the striatum and nucleus accumbens (NAcc) following SIR,^{28,125,127} no change in striatal DA levels,¹²⁴ and increased DA turnover in the amygdala and NAcc.^{128,129} Moreover, a recent study observed that SIR in juvenile postnatal day (PND) 77 and adult (PND 181) rats increased DA release as well as increased DAT in the NAcc.¹³⁰ Increased DA release has also been observed in the prefrontal cortex of SIR rats after systemic administration of the atypical antipsychotics clozapine and olanzapine, but not the typical antipsychotic, haloperidol.¹³¹ A similar differential response following clozapine and haloperidol was also observed in SIR rats with respect to frontal cortical D₁ receptor binding.¹³² The latter two studies provide a possible explanation how atypical but not typical antipsychotics reverse the frontal cortical DA deficits observed in schizophrenia, thereby explaining the former presumed superior efficacy in treating negative symptoms.¹³³ Other results regarding DA receptor density in the SIR model include down-regulation of D₂ in the striatum,¹²⁹ and no change in D₂ receptor density in the mesolimbic,¹³⁴ hippocampal and prefrontal cortex or amygdala areas.¹³⁵ The general evidence discussed here suggests increased DA (and related metabolites) levels in the NAcc and striatum areas following SIR, whereas evidence in prefrontal cortical areas is varied and often contradictory. Inconsistencies also prevail with regard to DA receptor alterations in this model, also noted in clinical studies.³⁷ These conflicting findings in animal studies and the fact that imaging studies in clinical studies failed to show frontal cortical hypo-DAergia and striatal hyper-DAergia, emphasizes the need for more preclinical and drug-free clinical studies.

The inconsistencies observed using the SIR model could possibly be explained by the differences in SIR protocols used. A different SIR protocol, especially the commencement and duration of isolation, could significantly affect the release of several stress-related hormones, such as glucocorticoids, implicated in most of the stress-induced physiological changes in the brain (reviewed in 136), and in this manner differentially affect DA release. Another consideration is using the same animals for behavioral and neurochemical analysis as opposed to using different cohorts for both. For example, Han et al.¹²⁷ used the same animals, while Möller et al.²⁸ used separate animals for behavioral (social interaction test) and neurochemical analysis, which may underlie their differences in monoamine findings. Previous studies have indeed found that rodents subjected to social aggression (as observed in Han et

al.¹²⁷) developed increased anxiety, ultimately increasing glucocorticoid release and activation of the DA system,¹³⁷ whereas Möller et al.²⁸ observed significantly decreased social contact and interaction in the SIR rats (discussed in subsection 4.1.4). The housing condition of animals is also very important, as a previous study observed a significant positive correlation between plasma corticosterone levels and DA in the left NAcc of SIR rats housed in grid-floor cages (used by ref 127) compared to SIR rats housed in sawdust cages (used in ref 28).¹²⁸ Heidbreder et al. also observed a decreased basal turnover of DA in the medial prefrontal cortex of SIR rats in grid-floor cages compared to SIR rats in sawdust cages.¹²⁸ The nature and extent of corticosterone release following the isolation procedure may modulate physiological monoamine levels by altering 5-HT neurotransmission in the brain.¹³⁸ Corticosterone also increases NA transporters and DA hydroxylase (the enzyme that catalyzes DA to NA).¹³⁶

Another consideration is the role of oxidative stress and its effect on monoamine release.⁷⁵ Different SIR protocols have been found to differently affect oxidative stress parameters (as discussed in section 4.1.3) that could in turn affect DA release in a different manner. Indeed, DA dysfunction has been related to oxidative stress in SIR²⁷ and schizophrenia.¹³⁹ Thus, evaluating oxidative stress and glucocorticoid-induced modulation on neural systems may further clarify the differences observed in monoamine levels in the different SIR protocols of Han et al.¹²⁷ and Möller et al.²⁸ A final note of caution comes from a study by Trabace and colleagues¹²⁴ who highlighted differences in monoamine profiles in two rat strains in response to SIR, and who then conclude that strain-related monoamine differences in response to SIR may affect the ability of the model to replicate the biobehavioral changes typical of schizophrenia. Concluding, due consideration for applying the same SIR protocol with consistency across all studies is key to obtaining reproducible results.

With regard to 5-HT levels in the SIR model, the following has been observed: decreased 5-HT and 5-HIAA levels¹⁴⁰ as well as decreased 5-HT/5-HIAA¹²⁴ in the prefrontal cortex and increased 5-HT and 5-HIAA levels¹⁴⁰ as well as increased 5-HT turnover¹²⁷ in the striatum and NAcc. Conflicting results in other studies observed increased 5-HT levels;¹²⁷ and no effect¹²⁶ on 5-HT/5-HIAA in the prefrontal cortex and decreased¹²⁸ 5-HT turnover in the NAcc of SIR rats. The picture of 5-HT and 5-HIAA levels after SIR is therefore clear with regards to the findings in the striatum (increased 5-HT) and relates to increased 5-HT transmission observed in schizophrenia (section 2.1). However, data are varied when it comes to findings in the prefrontal cortex. Again, the conflicting results found in the prefrontal cortex could be due to different SIR protocols (same vs different animals used for behavioral and neurochemical analysis respectively; different cage conditions) that could possibly affect oxidative stress and glucocorticoid-induced monoamine regulation, as mentioned above. However, hyper-cortical 5-HT levels could be responsible for the inhibition of DA and NA release in the frontal cortex, also explaining the efficacy of 5-HT_{2A/C} antagonists in treating the negative symptoms observed in schizophrenia (as discussed in subsection 2.1).

Studies determining NA levels, metabolism, and function in the SIR model are generally rare. However, some very early studies on the SIR model observed increased NA turnover in the hippocampus, cerebellum, and cortex as well as enhanced hippocampal presynaptic α_2 -adrenoceptor function (reviewed

in ref 120). Additionally, a recent study in our laboratory observed elevated frontal cortical NA levels, elevated striatal NA and MHPG levels, and decreased frontal cortical MHPG levels following SIR.¹⁴⁰ The elevation in cortico-striatal NA could possibly be linked to the positive and anxiety symptomatology of schizophrenia (reviewed in ref 140). Furthermore, add-on therapy with α_2 adrenoceptor antagonists significantly improves not only the positive symptoms of schizophrenia but also the negative symptoms,¹⁴¹ emphasizing the role of NA modulation in schizophrenia.

4.1.2. Effect on GABA-Glutamate. Reduced glutamate function has been suggested to underlie the deficits observed in the nORT in SIR animals¹⁴² (explained in subsection 4.1.4). SIR in rats also decreases glutamate and glutamine levels in the hippocampus,¹⁴³ increases frontal cortical NMDA receptor binding¹³² and reduces glutamate receptors subunits GluR1 and NR1 in the prefrontal cortex.¹⁴⁴ Moreover, SIR decreases GABA PV- immunoreactivity (IR) in the hippocampus and prefrontal cortex.^{145,146} The latter observation linked to the downregulation of cortical GABA-PV neurotransmission described in schizophrenia²⁵ as well as to NMDA receptor dysfunction³¹ and excessive glutamate release¹⁴⁷ (see Figure 2 and discussion in subsection 2.2). Altered cortical NMDA receptor density seems to also show a differential response to typical vs atypical antipsychotics.¹³² Decreased NAA levels have been observed in the temporal cortex of SIR rats, in line with clinical findings in schizophrenia patients⁶⁵ and indicative of decreased glutamate function.

4.1.3. Effect on Redox-Immune-Inflammatory Processes. A study from our own laboratory observed mitochondrial dysfunction in SIR animals, evident by increased striatal ATP, together with decreased ATP in the frontal cortex.²⁸ These changes were also fully reversed by clozapine.²⁸ Importantly, Ben-Shachar⁷⁴ suggested a vital link between mitochondrial alterations and DA disruptions, and supported by decreased frontal cortical DA levels in our SIR study,²⁸ thus providing convincing evidence of mitochondrial dysfunction in schizophrenia pathology. Another important consequence of mitochondrial dysfunction and altered mitochondrial electron transport is oxidative stress via increased release of superoxide (see Figure 2).

Nicotinamide adenosine dinucleotide phosphate (NADPH) oxidase 2 (Nox2) generates superoxide and is a major source of ROS (see Figure 2) as well as plays a role in controlling glutamate release in the prefrontal cortex¹⁴⁸ (reviewed in ref 149; see Figure 2). Moreover, elevations in Nox2 are observed in the prefrontal cortex of rats exposed to SIR.¹⁴⁶ Another critical mechanism of oxidative stress is the down-regulation of cortical GABA-PV neurotransmission,²⁵ which in patients with schizophrenia appears to be attributable to the NMDA receptor dysfunction,³¹ leading to excessive glutamate release and subsequent cortical excitation and oxidative stress¹⁴⁷ (see Figure 2 and discussion in subsection 3.2). Interestingly, SIR decreased GABA PV- IR in the hippocampus¹⁴⁵ and in the prefrontal cortex, as noted earlier, while blockade of Nox2 abolishes these effects.¹⁴⁶ SIR also increases SOD activity, decreases oxidized:reduced ratio of GSH, and increases lipid peroxidation in both the striatum and frontal cortex of SIR rats.²⁷ Of particular significance is that the latter effects are reversed by clozapine treatment.²⁷ Another study described down-regulated GSH, decreased SOD activity, induced NF- κ B (a major transcription factor for the expression of genes involved in the inflammatory responses) and up-regulated

Table 1. Dopamine Alterations in Pre- and Postnatal Studies of Inflammation, in the Striatum, Frontal Cortex, NAcc, and Other Brain Tissue^a

prenatal immunogens	striatum	frontal cortex	NAcc	other
LPS ^{78,166,168,172,176–179}	↓DA ↓HVA ↑DOPAC	↓DA ↓DOPAC ↑HVA ↓D2-R function	↑DA	↓DA ↑HVA
Poly I:C ^{164,177,180,181}	↑D2-R function ↑D1-R function ↑TH	↑D2-R function ↑TH	↑D2-R function ↑D1-R function	↑DAT
cytokines ¹⁸²		↓D2-R function ↓D1-R function	↓DA ↑DOPAC ↑DA	
turpentine ^{183,184}			↑DOPAC ↑HVA ↑TH	

^aArrows: ↑ = increase, ↓ = decrease, ↔ = no change.

expression of iNOS in prefrontal cortex of rats exposed to SIR,¹⁵⁰ thus linking associated redox changes to the immune-inflammation cascade (see Figure 2). The activation of the (immunological) iNOS cascade is known to follow stressful conditions⁹⁴ and to involve the activation of NF- κ B signaling,¹⁵¹ therefore affirming how environmental adversity will have immunological consequences. The literature is however conflicting with regards to changes in SOD. Thus, Möller et al.²⁷ observed increased SOD while Zlatković and Filipović¹⁵⁰ observed decreased SOD. These variations could be due to different SIR protocols and rat strains used (e.g., 8 weeks SIR and Sprague–Dawley rats²⁷ 3 weeks SIR and Wistar rats¹⁵⁰). Importantly, 8 weeks of SIR can be considered a chronic stressor, known to deplete SOD and to engender a pro-oxidative state,¹⁵² while 3 weeks of SIR is more a subacute stressor that *increases* SOD release in an attempt to curb excessive production of ROS.¹⁵³ As noted earlier, oxidative stress may have profound effects on monoamine release, especially DA (reviewed in ref 154). Increased DA neurotransmission in turn can induce oxidative stress,¹⁵⁵ creating a positive feedback loop with escalating severity.

Elevated proinflammatory cytokines (TNF- α , IFN- γ) and decreased anti-inflammatory (IL-4) and dual-action (IL-6) cytokines has been described in the SIR model, and which are for the most part reversed by clozapine treatment.²⁸ Furthermore, the SIR model also presents with elevated plasma tryptophan, kynurenine, anthranilic acid, 3-OHAA and QA with reduced KYNA and neuroprotective ratio, with all alterations reversed with clozapine.¹⁵⁶ Considering the inter-relationship between tryptophan and kynurenine synthesis, these changes can be expected to have marked effects on behavior, as will be discussed shortly. The above redox-immune findings raise new hope for using mitochondrial regenerators, such as N-acetyl cysteine (NAC) and methylene blue, in the treatment of schizophrenia, while the central role for inflammation and kynurenine metabolism hints of the possible clinical utility of kynurenine modulators and antioxidants in the treatment of schizophrenia (see subsection 5.2).

4.1.4. Behavioral Manifestations of SIR. The SIR model produces a number of deficits in behavioral paradigms convergent with schizophrenia, including sensory-motor gating, object recognition memory in the nORT (reviewed in refs 27, 28, 120, and 130), anxiety-like behavior in the EPM¹³⁰ and depressive-like behavior in the FST.¹⁵⁷ In most instances, these

deficits are reversed by antipsychotic treatment.^{27,28} With regard to social interactive behaviors, previous studies observed a significant decrease in social behavior and an increase in self-directed behavior after SIR that could be reversed with antipsychotic treatment.^{28,157} However, Wongwitdecha and Marsden¹⁵⁸ observed a significant increase in aggressive behavior in SIR rats, as did Koike et al.¹⁵⁹ in SIR mice. Another study in female SIR rats also observed that SIR produced marked deficits in social interactive behaviors as well as increased anxiety-like behavior in the open-field test.¹⁴⁴ Important is that increased playful fighting (aggressive behavior) and social contact behaviors has been observed in SIR rats,¹²⁷ in contrast to the decreased social interaction observed by Möller and co-workers.^{27,28} However, the latter study simultaneously confirmed the presence of other behaviors congruent with schizophrenia, as well as presenting neurochemical data in line with frontal cortical hypo-DAergia and striatal hyper-DAergia.²⁸ These social interaction studies also emphasize the importance of the type of social interactive behaviors measured during the assessment of sociality. SIR also produces significant hyper-locomotor activity that is reversed by antipsychotic treatment^{27,28,120} as well as disrupted latent inhibition in associative learning.¹⁶⁰ Interestingly, SIR also increases the self-administration of ethanol in rats,¹⁶¹ which is similar to an increased risk for developing ethanol addiction in people exposed to early life stress,¹⁶² and increases polydipsia behaviors (under food restrictions).¹⁶³

4.2. Inflammation Models. 4.2.1. Effect on Monoamines. Many inflammation-based models have observed alterations in DA function and metabolism. These studies predominantly made use of prenatal immunogen treatment methods, considering that changes in fetal dopaminergic development were observed after maternal immune activation.¹⁶⁴ The studies reviewed here have incorporated varying immunogens with fluctuating results, and as such these outcomes are summarized in Table 1. The table includes effects on DA and DA-receptors (DA-R), as well as other markers, such as tyrosine hydroxylase (TH), the rate-limiting enzyme for DA synthesis,¹⁶⁵ DOPAC, HVA, and DAT. Only a few studies measured DA changes after postnatal immune challenge: one using LPS observed reduced DA in the striatum after treatment,¹⁶⁶ while the other found that direct cytokine administration decreased DA and increased DOPAC levels in the NAcc.¹⁶⁷ Table 1 reveals a great deal of controversy with regards to striatal and NAcc DA levels, as

determined in the PPNI models. Nevertheless, studies describe a clear deficit in frontal cortical DA and DA metabolite levels, thus congruent with the hypo-DAergic hypothesis of schizophrenia. However, decreased levels of DA in the striatum, as opposed to increased levels as proposed by the DA hypothesis, are paradoxical, although may be explained by a loss of DA neurons following an inflammatory insult.¹⁷ It has also been suggested that disruptions in DA function in different brain regions may fluctuate with the use of specific immunogens.¹⁶⁸ Clearly, further studies are needed to confirm these findings.

Prenatal immune exposure to Poly I:C and LPS significantly increased 5-HT and 5-HIAA in certain brain regions of offspring at PND 80 (adulthood), compared to controls.^{169,170} Elevated levels of 5-HIAA was also observed in the hippocampus and frontal cortex of prenatal Poly I:C challenged offspring,¹⁷¹ while increased 5-HT levels were observed in the frontal cortex,¹⁷² amygdala, hippocampus, and hypothalamus⁷⁸ of LPS-treated offspring. This hyper-5-HTergic state in the frontal cortex directly relates to the hypothesis that elevated levels of 5-HT decreases the release of specifically DA in the frontal cortex of patients with schizophrenia, leading to hypodopaminergia and hypo-frontality. Postnatal administration of LPS was also found to increase 5-HT and 5-HIAA in the hippocampus.¹⁷³ In the striatum no changes in either 5-HT or 5-HIAA levels were observed after prenatal LPS exposure.¹⁷² However, prenatal influenza administration led to a marked reduction in 5-HT in the cerebellum¹⁷⁴ and a significant increase in the density of 5-HT_{2A} receptors (but not 5-HT_{2C}) in the frontal cortex of the offspring.¹²³ Furthermore, direct administration of either IL-1 β or IL-6 enhanced 5-HT turnover in the prefrontal cortex and hippocampus.¹⁷⁵ However, another study that administered cytokines to adult rodents observed that IL-1 α administration increased, but IL-2 administration decreased 5-HIAA levels in the NAcc, suggesting diverse effects of different cytokines on 5-HT levels during adulthood.¹⁶⁷ As mentioned earlier, the evidence in PPNI models for 5-HT involvement in the frontal cortex seems to relate directly to the cortical hyper-5-HTergic theory of schizophrenia. However, 5-HTergic alterations induced by PPNI in the NAcc and striatal regions seem to be dependent on the type of inflammation insult and do not relate to the state of hyper-5-HTergic transmission observed in schizophrenia patients.

Postnatal administration of LPS to rats significantly increased NA and MHPG in the hippocampus,¹⁷³ as well as increased NA-levels in the nucleus of the tractus solitarius (NTS) and the locus coeruleus (LC).¹⁷⁰ Direct IL-1 β administration also enhanced NA turnover in the hypothalamus and hippocampus, while IL-2 only had a similar effect in the hypothalamus.¹⁷⁵ Unfortunately no evidence for PPNI induced frontal cortical changes in NA have been described in the literature, although the above-mentioned hippocampal, NTS and LC findings could relate to the hyper-NAergic hypothesis of schizophrenia responsible for the presentation of positive symptoms.

4.2.2. Effect on GABA-Glutamate. Because abnormalities in GABA function have been implicated in the pathology of schizophrenia,¹⁸⁵ it is considered an important biomarker in animal studies of inflammation. Prenatal Poly I:C challenge reduced GABA levels in the hippocampus of female offspring.¹⁶⁹ Furthermore, glutamic acid decarboxylase 67 (GAD67), the enzyme responsible for converting glutamate to GABA and that serves as a marker for GABAergic neurons,¹⁸⁶ was significantly decreased in the hippocampus of

offspring exposed to prenatal LPS administration.¹⁸⁷ A reduction in GAD67 was also observed in the prefrontal cortex of prenatal Poly I:C-challenged rodents.¹⁸⁸ In contrast, prenatal LPS and Poly I:C administration significantly increased GAD67 in the hippocampus of the offspring.¹⁸⁶ These contradictory findings could be due to the different gender and age of rats used as well as the different brain regions analyzed in these studies (viz., Boksa¹⁸⁹ and Richetto et al.¹⁸⁸) while Harvey and Boksa¹⁸⁶ showed that changes in GAD67 levels can also vary between bacterial and viral immune challenges. Prenatal Poly I:C administration in mice also reduced PV interneurons in the medial prefrontal cortex, and up-regulated GABA_A receptor subunit α_2 expression in the ventral hippocampus of offspring.¹⁹⁰ The latter increase in the GABA_A receptor subunit expression was also observed in the ventral dentate gyrus and amygdala of prenatal Poly I:C treated offspring.¹⁹¹

It has frequently been reported that prenatal exposure to an immune challenge induces abnormalities in the glutamate system, especially data supportive of the NMDA glutamate hypo-function hypothesis of schizophrenia. Consequently, prenatal Poly I:C exposure decreased levels of glutamate in the hippocampus and the prefrontal cortex of rodents.¹⁶⁹ In contrast, neonatal Poly I:C treatment in mice results in elevated levels of glutamate in the hippocampus, while K⁺-induced glutamate release in this brain region was significantly diminished compared to that of saline-treated animals.¹⁹² A possible explanation for this result is that activity-dependent changes in extracellular glutamate levels are inhibited in the hippocampus of immune-challenged rodents, leading to a poorer signal/noise ratio in glutamatergic neurotransmission in adulthood.¹⁹² Importantly, this dysfunction of glutamatergic neurotransmission in the hippocampus may be related to the cognitive deficits seen in schizophrenia.¹⁹³ Furthermore, the NMDA glutamate hypo-function hypothesis of schizophrenia predicts that reduced NMDA-subtype glutamate receptor activity in the hippocampus will give way to decreased stimulation of GABAergic inhibitory neurons, leading to elevated glutamate release in the prefrontal cortex.¹⁹⁴ Accordingly, researchers have observed that extracellular basal glutamate are increased in the prefrontal cortex of Poly I:C offspring, and that this effect was successfully reversed after both risperidone and paliperidone treatment.¹⁹⁵ Different studies of prenatal inflammation showed decreased levels of the NMDA- and glutamate-receptor subunits, GluN1,^{196,21} NR1,¹⁹⁰ and mGlu2.¹²³ Two studies incorporating LPS as the prenatal immunogen observed that the NMDA/AMPA ratios^{196,197} were significantly decreased in the hippocampus of offspring. These ratios are important measures of glutamate synapses and a decrease in these ratios as mentioned above directly relates to a hypo-glutamatergic hypothesis of schizophrenia. Prenatal LPS administration has also been found to impair NMDA synaptic currents and long-term potentiation in the hippocampus of offspring.¹⁹⁸ This supports the findings that decreased NMDA receptor expression leads to behavioral alterations akin to that observed in schizophrenia.¹⁹⁹

4.2.3. Effect on Redox-Immune-Inflammatory Processes. A study using Poly I:C to induce inflammation during gestation observed that ATP production in spleen mitochondria of offspring was significantly reduced.²⁰⁰ Numerous studies on the offspring of LPS-challenged rats (pre- or postnatal) demonstrate a significant and delayed decrease in hippocampal GSH,^{166,198,201–203} as well as an increase in oxidized glutathione (GSSG).^{166,198,203} Interestingly, administration of

Table 2. Cytokines Found to Be Elevated in Pre- and Post Natal Studies of Inflammation, in Different Plasma and Tissue Samples

pre/postnatal insult	maternal plasma	amniotic fluid and/or placenta	fetal/offspring plasma	fetal brain	adult brain (postnatal treatment)	adult plasma (postnatal treatment)
LPS ^{97,98,166,178,204,205,208,209,214,216–232}	IL-6, IL-1 β , TNF- α	IL-6, IL-1 β , TNF- α	IL-6, TNF- α , IL-2	IL-6, IL-1 β	IL-6, IL-1 β , IL-1, TNF- α , IFN- γ , IL-2	IL-6, TNF- α , IFN- γ , IL-10
Poly I:C ^{97,171,192,207,210,211,233–235}	IL 1 β , TNF- α , IL-6	IL-6, IL-1 β , TNF- α	IL-6, IL-2, IL-5, TNF- α , IL-3, IL-12, IL-1 β , IFN- γ	IL-1 β , IL-10, IL-12, IFN- γ , IL-6	IL-6, IL-1 β , TNF- α	IL-6
influenza no data turpentine ¹⁸³	IL-6	IL-6				

clozapine was unable to reverse these alterations in GSH-levels after postnatal treatment.²⁰² However, considering clozapine has been effective in reducing redox related changes in other postnatal models, such as SIR,²⁷ these findings are of interest and might point toward the specific period of development when clozapine may offer therapeutic benefit. Numerous postnatal inflammation models also present with increased lipid peroxidation when compared to controls,^{202–204} while elevated levels of malondialdehyde (MDA) (a marker of lipid peroxidation) in a postnatal, acute inflammation model has been found to be successfully reduced by earlier administration (30 min before the LPS administration) of risperidone.²⁰⁴ Still other authors could not reverse these changes with clozapine,²⁰² illustrating a controversy in the predictive validity of these models. In a prenatal model of inflammation, LPS administration led to increased lipid peroxidation especially in the midbrain of aging offspring.²⁰³

Postnatal inflammation studies also showed increased levels of iNOS in the hippocampus, striatum and prefrontal cortex following Poly I:C,²⁰² with similar results noted in the hippocampus²⁰⁵ and cerebral cortex following LPS challenge.²⁰⁴ Ribeiro et al.²⁰² were able to reverse these changes with clozapine. Using this same model, MacDowell et al.²⁰⁴ successfully used risperidone to reduce levels of oxidative stress. Furthermore, mice exposed to prenatal PolyI:C had decreased GABA-PV-IR in the prefrontal cortex,¹⁹⁰ with neonatal LPS exposure also able to decrease PV-IR interneurons in the hippocampus of adult rats.²⁰⁶ Following LPS injection during gestation, male fetuses exhibited increased protein carbonyl levels and reduced levels of α -tocopherol.¹⁹⁸

Cytokine analyses after immunogen treatment have revealed an array of outcomes. Table 2 summarizes the various cytokines that have been found to be increased in different tissues and plasma following a specific immunogen (LPS being the most studied). From this data, IL-6 seems to play a significant role in the PPNI model, while elevated levels of cytokines such as IL-1 β , IFN- γ and TNF- α have also been observed frequently. Of these studies, a few have demonstrated successful reversal with antipsychotic treatment, such as clozapine, olanzapine²⁰⁷ and risperidone.^{204,207} Although the data for the different cytokines remain inconsistent between these studies, possibly due to the different immunogens used, various treatment regimens (pre- or postnatal) and varying times of analyses, it is clear that each of these cytokines play an important role in an inflammatory animal model of schizophrenia. Also worth noting is that studies using a prenatal immune challenge with either a systemic endotoxin or viral mimic vs an inducer of local inflammation suggests that maternal infection may affect fetal neurodevelopment through circulating cytokines and/or fever, rather than via direct effects of the infectious agent on the

fetus.²⁰ Moreover, the nature of the infectious agent may also illicit different cytokine responses.²⁰⁸

Interestingly, Bison et al.²⁰⁹ observed increased levels of IL-10 in the plasma of adult rats after a LPS challenge. IL-10, an anti-inflammatory cytokine, may have been released as a physiological protective measure to counteract inflammatory damage. This was also an acute study with LPS administered to adult rats, which could be responsible for the acute activation of the inflammatory response and release of IL-10.²⁰⁹ Other studies, however, have shown contrasting results, namely, PPNI studies have revealed a decrease in IL-10 levels in the fetal central nervous system,²¹⁰ placenta,²¹¹ maternal serum,^{98,212} and the plasma of adult-rats exposed to an immunogen,²⁰⁷ pointing to a decreased anti-inflammatory capacity after immunogen treatment. The latter are similar findings to that described in late stage schizophrenia (discussed in subsection 3.3). Furthermore, Sugino et al.²⁰⁷ showed that olanzapine, clozapine, and risperidone reversed decreased levels of IL-10 after an immune challenge, suggesting therapeutic anti-inflammatory activity. The apparent paradoxical findings with IL-10 indicate how important time of sampling is post PPNI, where earlier sampling will display an immune response that is challenged yet coping, while later analysis may reveal an immune system under severe pressure, with evidence of maladaptation and a developing pro-oxidative state.

Numerous studies of prenatal inflammation have demonstrated increased microglia or activated microglia.^{105,213} However, this body of evidence too is controversial, with opposing findings noted in the literature.^{210,214} In response to an unexpected finding of decreased microglia activation after prenatal LPS challenge, Cai and colleagues suggested that prenatal LPS may result in altered immunoreactivity of microglia.²¹⁴ In terms of postnatal immune challenges, several studies observed increased microglia activation,^{171,182,202,215} which could be successfully reversed with clozapine.²⁰² LPS was also able to increase cyclooxygenase (COX)-2 (see subsection 5.4) protein and mRNA in male adult rats, which was also successfully prevented by preadministration of risperidone.²⁰⁴

In evaluating the kynurenine pathway, it was observed that prenatal Poly I:C resulted in decreased serum levels of the neuroprotective metabolites, kynurenine and KYNA and elevated levels of the neurotoxic metabolite, QA.²³⁶ An interesting finding of this study was that the KYNA/kynurenine ratio (neuroprotective ratio) was unaltered,²³⁶ which is in contrast to findings following SIR (subsection 4.1.3), as well as in patients with schizophrenia (subsection 3.3). This phenomenon could be clarified by a possible physiological attempt to compensate for the increased levels of neurodegenerative QA at this developmental stage. The authors also argue that an age-dependent shift in the balance of the

kynurenine pathway could be due to developmentally specific changes in NMDAR function in the Poly I:C model.²³⁶ Another study observed that postnatal Poly I:C led to a marked increase in IL-6, TNF- α and IL-1 β , three cytokines known for their ability to induce IDO.¹⁷¹ Elevated IDO was confirmed in the hippocampus and frontal cortex of postnatal Poly I:C immune-challenged rodents.¹⁷¹ A number of other studies have also shown increased IDO after immune activation using either Poly I:C or LPS in adult rodents.^{219,228} Connor et al. furthermore confirmed increased KMO mRNA, the enzyme responsible for the degradation of kynurenine into 3HK (see Figure 3), in the cortex and hippocampus of rats 24 h after LPS administration.²¹⁹

4.2.4. Behavioral Manifestations of Inflammation Models.

Increased locomotor activity was observed in rats that received influenza A at neonatal age,²³⁵ while a number of studies^{190,237} have demonstrated reversal of said hyperlocomotor activity with clozapine and risperidone. Similarly, prenatal administration of LPS to rodents led to increased locomotor activity in offspring at different postnatal ages.²³⁸ However, a recent study observed that prenatal infection with Poly I:C reduced locomotor activity in the offspring, without any preceding drug administration,²³⁹ while decreased locomotor activity was also evident after postnatal administration of either IL-2 or the influenza virus to rodents.^{19,123} The latter observations are in contrast not only to a number of preclinical studies noted above and elsewhere,^{172,240,241} but also with clinical studies where agitation and hyper-activity is observed in certain patients with schizophrenia.¹²² However, it should be considered that such a decrease in locomotor activity is a general feature of sickness behavior induced by a pathogen,²⁴² while hypo-locomotion may also relate to the *negative symptoms* observed in schizophrenia.

Both prenatal and postnatal LPS^{103,229} as well as a prenatal influenza¹⁵ or Poly I:C¹⁶⁹ reduce spontaneous social activities in early and late adulthood in rodents. In contrast, the administration of IL-1 α to rat pups resulted in increased social interaction in adulthood, which might be due to a cognitive deficit in recognizing an unfamiliar intruder caused by this specific cytokine.¹⁹ Various studies have revealed deficits in PPI after the prenatal administration of either Poly I:C or LPS,^{168,172,177,178,190,202,234,243,244} or postnatally following either subcutaneous¹⁹² or direct intrahippocampal administration.^{103,223} Additionally, some of these deficits were reversed with clozapine^{202,218,243} and haloperidol.²¹⁸ In the study by Meyer et al., PPI deficits were only observed in animals that were subjected to an immune insult (Poly I:C) at GD 9 in comparison to GD17.¹⁹⁰ This suggests that infection early in pregnancy renders offspring more vulnerable to develop sensorimotor gating deficits, and is supported by previous results using LPS.^{168,178} However, in contrast to this explanation, both Ozawa et al., and Wolff and Bilkey demonstrated PPI deficits when immune activation (Poly I:C) took place at later gestational periods (GD 12–17 and GD 15, respectively).^{177,244} These differences in PPI deficits could possibly be explained by the activation of endogenous cytokines as opposed to the immunogen itself. Such a suggestion is supported by a study where turpentine was administered to pregnant rodents, activating an endogenous inflammatory response and resulting in a reduction in PPI in the offspring.²⁴⁵ Indeed, it has been suggested that cytokines differ with respect to their detrimental effects²⁴⁶ and that the behavioral alterations following different immunogens could be attributed to their

ability to induce different biological processes.¹⁶ In a study using direct cytokine administration (IL-1 α , -2, -6, or IFN- γ) to newborn rat pups, interleukin IL-1 α was the only cytokine that caused PPI deficits.¹⁹ However, two recent studies show PPI deficits in offspring after administering IL-6 to pregnant mice on GD 12.5.^{211,246} Furthermore, different behavioral outcomes, as observed in the IL-6 study by Tohmi et al.¹⁹ might be dependent on the time point of administration, in this case suggesting that IL-6 only has detrimental effects on sensorimotor gating during gestational periods. Finally, other forms of prenatal inflammation (viz., Borna disease virus, cytomegalovirus, and influenza) also induce PPI deficits in rodent offspring, thereby providing a common link with an important behavioral manifestation of schizophrenia.^{15,247,248}

Prenatal inflammation studies with Poly I:C have revealed deficits in latent inhibition that could be reversed with a variety of antipsychotic treatments (haloperidol, clozapine, and risperidone).^{237,243,249} Interestingly, abnormally enhanced latent inhibition has also been observed in the Poly I:C model which the authors correlated with severe negative symptoms of schizophrenia.¹⁶⁹

Impaired memory, as measured in the nORT, was observed in both pre- and postnatal Poly I:C and LPS models^{177,193,213,250} and after a prenatal influenza challenge.¹⁵ Previous studies have also observed that the Poly I:C model¹⁹⁰ and the LPS model^{201,226} impair memory in the Morris water maze. The dry maze matching-to-position paradigm also showed impaired working memory in Poly I:C offspring compared to controls,¹⁸⁸ as did the Y-maze task in rats receiving Poly I:C at PND 5–7.²⁰² Moreover, excessive cognitive switching has been observed in the T-maze after prenatal Poly I:C treatment,^{237,249,250} but that could be normalized with risperidone treatment.²³⁷

A few studies also observed increased time spent in the outer sector of the open field test (an indication of anxiety-like behavior) in prenatal and postnatal Poly I:C administration,^{192,211} postnatal LPS administration,¹²¹ prenatal influenza,¹⁵ and IL-6 administration.²¹¹ Interestingly, administration of Poly I:C on GD 17 and 15 also led to a significant decrease in sucrose consumption in offspring, indicative of depressive-like symptoms in these rodents.^{169,210}

4.3. "Dual-Hit" Animal Models of Schizophrenia. A multifactorial or "dual-hit" conceptualization of schizophrenia suggests that a combination of genetic susceptibility, an environmental stressor or inflammatory processes in early life coupled with a distinct developmental insult can prime an individual for a later event that ultimately leads to the development of schizophrenia wherein the full clinical syndrome may be presented. This association has indeed been observed in animal models and in human studies (reviewed in ref 251). Moreover, a variety of specific adverse environmental conditions, such as biological and psychosocial risk factors, during the antenatal and perinatal periods, early and late childhood, adolescence and early adulthood, along with genetic susceptibility, have been implicated in the etiology of schizophrenia. Numerous studies have also linked later in life infections (such as *Toxoplasma gondii* and retroviruses),^{252,253} hormone imbalances (such as cortisol and estrogen),²⁵⁴ and structural brain changes due to synaptic pruning¹⁸⁹ with a subsequent increase in the incidence of schizophrenia. The above-mentioned later in life insults could be seen as the "2nd hit" while genetic susceptibility or early life environmental insults could be seen as the "1st hit" of the "dual-hit"

Table 3. Application of “Dual-Hit” Protocols as Neurodevelopmental Models for Schizophrenia and/or Psychosis

application	1st hit	2nd hit	behavior augmented
neonatal NMDAR antagonist exposure + neurodevelopmental insult	neonatal PCP	SIR	PPI deficits and conditioned emotional response freezing ²⁶⁴
neurodevelopmental insult + later life drug exposure	neonatal MK-801	SIR	no studies done
	SIR	MK-801	increased or no added effect on locomotor activity ^{265,253}
	SIR	MA	no added effect on PPI or social interaction ²⁵⁶
	SIR	ketamine	no studies done
prenatal inflammation insult + later life stress exposure	maternal separation	MA	no studies done
	prenatal LPS	restrain stress	increased anxiety behaviors ¹²¹
	prenatal Poly I:C	restrain stress	PPI deficits ¹⁸¹
	prenatal Poly I:C	unpredictable stress	no studies done
prenatal inflammation insult + later life drug exposure	prenatal LPS/ Poly I:C	amphetamine/ MA/MK-801	increased locomotor activity ^{20,177,188,190,237,236,243}

hypothesis. A recent review also observed that structural, molecular, and functional changes in glial cells (oligodendrocytes, astrocytes, and microglia cells) that follow the above challenges contribute significantly to the neurodevelopment–neurodegeneration hypotheses of schizophrenia.²⁵⁵

Based on the above-mentioned “dual-hit” hypothesis, numerous researchers have proposed that by exposing neurodevelopmental animal models to an experimental manipulation that mimics the effects of environmental risk factors associated with schizophrenia, it may be possible to better model some forms of this mental illness. In this “dual-hit” animal model, early life insults (such as maternal immune activation or SIR) or genetic factors are seen as the “1st hit” that disrupt neurodevelopment and, in so doing, produce long-term vulnerability to a “2nd hit” that is responsible for precipitating the onset of schizophrenia-like pathophysiology and its associated behavioral alterations. Such a “2nd hit” is represented by later life adversity such as drug abuse, with for example, NMDAR antagonists (ketamine, PCP etc.) or MA, or exposure to an environmental stressor (e.g., restraint stress or maternal separation in rodents, as well as abuse, infections later in life as noted above, structural brain changes due to synaptic pruning and hormone imbalances in humans etc.).

Thus, combining neurodevelopmental models and environmental insults with biobehavioral testing will allow new ways to model schizophrenia in rodents and assist in developing the next generation of antipsychotic drugs, as will be discussed below. However, it should be mentioned that the structure of a “dual-hit” model will depend on the specific hypothesis of schizophrenia that the researcher aims to mimic (see Table 3). In this regard, maternal separation or SIR could be a “1st hit” if coupled with a later in life environmental insult (such as drug abuse), or be a “2nd hit” if coupled with a prenatal insult (such as prenatal inflammation) as indicated in Table 3. For the purpose of this Review, and in line with the neurodevelopmental hypothesis of schizophrenia, we will consider “dual-hit” animal models to represent the following: combined neurodevelopmental factors (either SIR or PPNI), representing the “1st hit” plus postnatal conditions representative of a severe psychosocial stressor, representing the “2nd hit”, such as late life infection or drug abuse (e.g., either exposure to NMDAR antagonists or MA). In some cases neonatal or maternal drug exposure (PCP or MK-801) as the “1st hit” combined with SIR as a “2nd hit” will be discussed as well.

4.3.1. Effect on Monoamines. To the best of our knowledge, published studies evaluating the effect of “dual-hit” models on monoamine alterations are limited, clearly

pinpointing an urgent need for future studies. One recent study using postweaning SIR and postnatal (PND 35–50) MA exposure observed a trend toward further elevations in frontal cortical (but not striatal) DA levels in SIR-MA animals compared to SIR alone animals, although this increase was not significant and no effect on other brain monoamines was evident.²⁵⁶ The authors argue that combining potent neurophysiological challenges, such as SIR-MA, may reach a ceiling beyond which further effects are not attainable, possibly explaining the lack of an additive effect, although this warrants further study. Nevertheless, their findings do emphasize a central role for DA in SIR-MA regulation of frontal cortex-driven behaviors (discussed in subsection 4.3.4). Another study used maternal separation in combination with MA administration later in life (PND 60–70) and observed that this combination significantly decreased striatal DAT and TH levels in comparison to MA administration alone.²⁵⁷ These findings are in contrast to the increased striatal DAT and TH observed after SIR and PPNI (indicated in Table 4). A possible mechanism whereby maternal separation affects DA regulation differently to SIR could be due to the high levels of glucocorticoids present in the immediate postnatal period that assures DAergic transmission in the mesolimbic, mesocortical and nigrostriatal systems, as determined by the ontogenetic state of development at this early lifetime point.²⁵⁸ In support of this, studies have observed a significant difference between the effect of early postnatal stressors vs postweaning stressors on the hypothalamic-pituitary-adrenocortical and related glucocorticoid synthesis in rats.²⁵⁹ In this regard, it should be noted that maternal separation is generally associated with late-onset depression and anxiety studies,²⁵⁷ not schizophrenia per se.

4.3.2. Effect on GABA-Glutamate. A recent study observed that SIR followed by a ketamine challenge significantly reduced prefrontal cortical GABA levels, in comparison to group reared animals also exposed to a ketamine challenge.²⁶⁰ This observation is in line with the decreased prefrontal cortex GABA levels observed in unmedicated schizophrenia patients, and draws a parallel with the dysfunctional glutamate hypothesis of schizophrenia. In a study where mice were exposed to a single injection of MK-801 on PND 7, prior to SIR (a second hit design), a significant decrease in prefrontal cortical GABA-PV interneuron expression was observed, together with a down-regulation of the GAD67 gene, compared to group reared rats also receiving MK-801.²⁶¹ These studies suggest that SIR combined with NMDAR blockade in early life may modulate glutamatergic function and predict glutamatergic

Table 4. Overview of the SIR, PPNI, and “Dual-Hit” Animal Models of Schizophrenia with Regard to Monoamine, GABA-Glutamate, Redox-Immune-Inflammatory, and Behavioral Alterations, Including Response to Antipsychotics and the Identification of Novel Treatment Strategies^a

animal models	SIR	PPNI	“Dual-hit” models	targeted treatment strategies ^b
markers				
monoamines	↓DA, DOPAC, HVA (frontal cortex) ^{26,124,125} ↓DA (frontal cortex) ^{126,127} ↓DA, DOPAC, HVA, DAT (striatum, NAcc) ^{28,125,127,130} ↔DA (striatum) ¹²⁴ ↓DA turnover (amygdala, NAcc) ^{128,129} ↑↑↔S-HT and S-HIAA (frontal cortex) ^{126,127,140} ↓S-HT and S-HIAA (striatum, NAcc) ^{140,127} ↓S-HT/SHIAA (NAcc) ¹²⁸ ↑NA and turnover (frontal cortex, cerebellum, hippocampus) ¹²⁰ ↑NA, ↓MHPG (frontal cortex) ¹⁴⁰ ↑NA and MHPG (striatum) ¹⁴⁰	↓DA, DOPAC (frontal cortex) ¹⁷ ↓DA, ↓DOPAC (frontal cortex) ¹⁷⁵ ↑↓DA; ↓DOPAC (striatum) ^{166,172,176} ↑TH (striatum) ¹⁸⁰ ↑TH, DA, DOPAC, HVA, DAT (NAcc) ^{168,183,184} ↑S-HT, S-HIAA (hippocampus, frontal cortex, hypothalamus) ^{171,F3} ↓↑S-HIAA (NAcc) ¹⁶⁷ ↓S-HT (cerebellum) ¹⁷⁴ ↔S-HT, S-HIAA (striatum) ¹⁷² ↑NA, MHPG (hippocampus) ¹⁷³ ↑NA (NTS, LC) ¹⁷⁰ ↑NA turnover (hypothalamus, hippocampus) ¹⁷⁵	↑DA trend (frontal cortex) ¹²⁷ ↓DAT ; TH (striatum) ²⁵⁷	atypical antipsychotics NAC group II metabotropic glutamate receptor agonist (LY354740)
GABA-glutamate	↓glutamate function ¹⁴² ↓NAA (temporal cortex) ⁶⁵	↓GABA, (hippocampus) ¹⁶⁹ ↓↑GAD67 (hippocampus, cortex) ^{187,188} ↑glutamate (hippocampus, frontal cortex) ^{169,192} ↓ATP (spleen) ²⁰⁰	↓GABA (prefrontal cortex) ²⁶⁰ ↓GAD67 (prefrontal cortex, striatum) ^{181,261}	atypical antipsychotics LY354740
redox-immune-inflammatory pathways	mitochondrial ↓ATP (striatum) ²⁸ ↓ATP (frontal cortex) ²⁸		↑↓energy metabolism, redox processes and neurotrophic proteins (NAcc) ^{263,128,263}	atypical antipsychotics minocycline NAC
oxidative stress	↑Nox2 (frontal cortex) ¹⁴⁸ ↑iNOS (frontal cortex) ¹⁵⁰ ↓GABA PV-IR interneurons (hippocampus, frontal cortex) ¹⁴⁶ ↓SOD (frontal cortex) ¹⁵⁰ ↑SOD (striatum, frontal cortex) ²⁷ lipid peroxidation (striatum, frontal cortex) ²⁷	↑iNOS (hippocampus, striatum, frontal and cerebral cortex) ^{202,205} ↓GABA PV-IR interneurons (hippocampus, frontal cortex) ^{206,164} lipid peroxidation (midbrain) ^{202–204} ↓GSH, ↑GSSG (hippocampus) ^{201,203,166} ↑protein carbonyl and ↓α-tocopherol ¹⁹⁸	↓GABA PV-IR interneurons (frontal cortex) ²⁶¹	sodium nitroprusside methylene blue L-NAME ketamine memantine minocycline Ro 61-8048 NAC NAC-amine oleanolic acid selenium mangiferin
inflammation	↑NF-κB ¹⁵⁰ ↑proinflammatory cytokines (TNF-α, IFN-γ) (plasma) ¹⁴⁰	↑ pro-inflammatory cytokines (IL-1β, IFN-γ, TNF-α) (see Table 2)	no studies done	ketamine minocycline

Table 4. continued

animal models	SIR	PPNI	"Dual-hit" models	targeted treatment strategies ^b
redox-immune-inflammatory pathways				
kynurenine pathway	↓anti-inflammatory cytokines (IL-4, IL-6) (plasma) ¹⁴⁰	↓anti-inflammatory cytokines (IL-10, IL-6) (CNS, serum, plasma) ^{96,207,210,211}		NAC
	↓tryptophan, kynurenine, anthranilic acid, 3-OHAA and QA (plasma) ¹⁵⁶			mangiferin
	↓KYN and neuroprotective ratio (plasma) ¹⁵⁶	↑IL-10 (plasma) ²⁰⁹ ↑COX2 protein (plasma) ²⁰⁴		γ-mangostin celastrol aspirin estradiol
		↑QA (serum) ²¹⁶	no studies done	ketamine
behavior		↓KYN and kynurenine (serum) ²¹⁶		Ro 61-8048
		↔neuroprotective ratio ²³⁶		allopurinol
		↑IDO and KMO (hippocampus, frontal cortex) ^{17,121,228}		NAC
	↓sensory motor-gating (PPI) ^{28,120}	↓sensory motor-gating (PPI) ^{172,177,192,234}	↓↔sensory motor-gating (PPI) ^{256,264}	antipsychotics
	↓recognition memory (nORT) ^{28,130}	↓recognition and learning memory (nORT, morris water maze) ^{15,177,192}	↓ conditioned emotional response freezing behavior ²⁶⁴	ketamine
	↓mobility (FST) ¹⁵⁷	↓working memory (Y-maze, T-maze, dry maze matching-position) ^{202,237,249}		mementine minocycline
	↓social interaction ^{27,28}	↓social interaction ^{15,169,215,229}		allopurinol
	↓self-directed behaviors ²⁷	↓social interaction ¹⁹		NAC
	↓aggressive and social contact behaviors ^{127,158}			oleanolic acid sodium nitroprusside
	↓latent inhibition ¹⁶⁰	↓latent inhibition ^{237,243,249} ↓latent inhibition ¹⁶⁹		methylene blue L-NAME
	↓anxiety (EPM, OFT) ¹³⁰	↓anxiety (OFT, EPM) ^{121,192,211}	↓anxiety (OFT, EPM) ¹²¹	celecoxib
	↓locomotor activity ^{27,28,120}	↓locomotor activity ^{123,235,239}	↔locomotor activity ^{256,265}	estradiol LY354740
	↓ethanol self-administration ¹⁶¹ and polydipsia ¹⁶³	↓sucrose consumption ^{169,210}		

^aArrows: ↑ = increase, ↓ = decrease, ↔ = no change. ^bSee text for specific references.

and GABAergic alterations later in life. These findings relate directly to observations in clinical studies. Moreover, a reduction in GAD67 levels was also observed in the cortex and striatum of Poly I:C offspring subsequently challenged with restraint stress.¹⁸¹ Another study in mice that combined prenatal Poly I:C exposure with unpredictable stress from PND 30–40 observed a significant decrease in GABA-PV interneurons in the ventral dentate gyrus of the hippocampal formation, whereas none of the stressors alone had an effect on this marker.²⁶² Furthermore, prenatal Poly I:C exposure also induced a blunted response (absence of elevated extracellular glutamate) to MK-801 administration in the offspring, supporting the hypothesis that NMDA receptor function is decreased in immune challenged animals.¹⁹⁵

4.3.3. Effect on Redox-Immune-Inflammatory Processes. A recent study observed that maternal separation in rats caused a reduction of cytoskeletal proteins in the shell of the NAcc and altered cytoskeletal, signaling, energy metabolism and redox proteins in the core of the NAcc.²⁶³ Furthermore, when the authors combined maternal separation with MA exposure, they observed more altered proteins involved in energy metabolism, redox regulatory processes, and neurotrophic proteins in the NAcc, compared to maternal separation or MA exposure alone.²⁶³ These observations support the theoretical rationale for added impact following early life adversity plus drug abuse and their subsequent impact on oxidative stress. In terms of postnatal immune challenges, another study also observed that LPS exposure was only able to increase microglia in offspring obtained from dams that were exposed to stress during gestation.²²¹

4.3.4. Behavioral Manifestation of “Dual-Hit” Animal Models. In line with the “dual-hit” hypothesis, a recent study observed that acute neonatal PCP administration combined with SIR produced significant PPI deficits, compared to PCP administration alone.²⁶⁴ Furthermore, conditioned emotional response freezing behavior (which examines contextual and conditioned associative learning) was significantly reduced by SIR but an even greater effect occurred when combined with neonatal PCP treatment.²⁶⁴ With regards to locomotor activity, it was observed that SIR combined with subchronic MK-801 exposure (for the last week of SIR) significantly enhanced locomotor activity compared to SIR alone.²⁶⁵ However, other studies observed that repeated postweaning MK-801 injections during the 8 weeks of SIR produced no additive or synergistic effects on locomotor activity, suggesting that these two perturbations may work through different mechanisms.^{266,267} Similarly, SIR-induced drinking behavior was *not* bolstered following the addition of MK-801 to the SIR paradigm, suggesting that SIR induces a predisposition to polydipsia without the need for another stressor, or second “hit”.¹⁶³ Moreover, postweaning SIR and MA exposure from PND35–50 also failed to show an exacerbation of SIR-associated deficits in social behaviors and sensorimotor gating.²⁵⁶ It could however be argued that some of these environmental stressors/“hits” are equally potent in inducing behavioral alterations and that an additive effect may have been abrogated by a ceiling effect or by specific autoregulatory processes that are brought “on line” following severe adverse challenges. For example, where chronic MA reduces central monoamine levels,²⁶⁸ the combination of MA+SIR may actually reverse the effect of the MA or have no effect on specific monoamines (see subsection 4.1.1). Strauss and colleagues advocate that

redox related processes mediate a reactive protective mechanism that prevents an additive effect.²⁵⁶

Prenatal LPS administration followed by restraint stress in adulthood led to heightened anxiety-like behavior in the open field test and the EPM when compared to prenatal LPS treatment alone.¹²¹ Prenatal challenge with LPS or Poly I:C also increases hyper-locomotor activity after postnatal amphetamine,^{20,188,190,237,243} MA¹⁷⁷ or MK-801 administration^{190,236} in adulthood, compared to LPS or Poly I:C alone. Furthermore, a recent study in juvenile animals receiving prenatal Poly I:C administration only experienced PPI deficits after subsequent restraint stress.¹⁸¹

The above evidence indicates that the “dual-hit” models present with more robust behavioral alterations akin to schizophrenia (such as PPI deficits, hyperlocomotor activity, deficits in contextual and associated learning, and anxiety-like behaviors) than a “single-stressor” neurodevelopmental model (see Table 3). However, some conflicting results have been observed in the SIR + MA and SIR + MK-801 models that warrants further study. Moreover, the validity of an apparent ceiling effect when utilizing combined stressors needs further clarification and the mechanisms delineated.

5. TARGETING IMMUNE-INFLAMMATORY-REDOX PATHWAYS IN SCHIZOPHRENIA: NOVEL TREATMENTS

Two previous antipsychotic clinical trials (e.g., CATIE, CUtLASS) highlighted the restrictions of current pharmacological therapies for schizophrenia, and the critical need for novel targets in drug research and development (reviewed in ref 269). Moreover, animal models (SIR, PPNI, and “dual-hit”), as reviewed thus far, may be extremely useful in identifying novel treatment targets. A number of putative targets relating to immune-inflammatory responses, oxidative stress, NO, COX, estrogen regulation, and glutamate receptor activation, have been revealed using these models and have formed the basis for novel drug design. Some of these ideas will now be discussed.

5.1. Compounds Targeting Immune-Inflammatory Responses. The NMDAR antagonist, ketamine, induces biobehavioral alterations^{25,270} and inflammation²⁷¹ in rodents akin to that of schizophrenia. However, in contrast to the pro-inflammatory effects observed with ketamine, a recent review discussed its possible anti-inflammatory properties in preclinical studies, noting especially that ketamine administration decreases TNF- α and IL-6 levels and improves the IL-6/IL-10 ratio in rats after an acute insult with LPS or *E. coli*^{272,273} as well as suppression of NO.²⁷³ Moreover, ketamine is able to improve synaptogenesis by acting on NMDA and AMPA receptors, and probably through activation of mammalian target of rapamycin (mTOR), which in turn triggers the translation of synaptic proteins required for neuronal plasticity (reviewed in ref 273). Additionally, ketamine reduces inflammation, either via an inhibitory effect on NF- κ B (see Figure 2) or by preventing IDO activation in the kynurenine pathway (see Figure 3) and decreasing the synthesis of neurodegenerative QA (reviewed in ref 273). With evidence indicating significant deficits in synaptic plasticity²⁷⁴ and oxidative stress in schizophrenia, and that ketamine exerts rapid antidepressant effects within hours,²⁷⁵ ketamine may be of value in treating the negative, depressive-like symptoms and inflammation associated with schizophrenia, although further studies are needed. However, a recent case report indicated that another NMDAR antagonist, memantine, significantly improved the negative

symptoms of schizophrenia when used as add-on therapy.²⁷⁶ Adjunctive memantine together with clozapine also significantly improved not only the negative symptoms but also positive symptoms in refractory schizophrenia patients.²⁷⁷ The authors argue that this improvement could be related to memantine's NMDAR antagonistic mechanisms, preventing a toxic influx of Ca^{2+} into neuronal cells and as such abrogating oxidative stress (see Figure 2).^{276,277}

Another possible anti-inflammatory adjunctive treatment that has been evaluated in schizophrenia is minocycline. Minocycline is an antibiotic that has anti-inflammatory and antioxidant properties by inhibiting $\text{TNF-}\alpha$ release and iNOS activation, respectively.²⁷⁸ An extensive review of minocycline noted that it decreases the release of cytokines (IL-1 β , IL-6, and $\text{TNF-}\alpha$), cytochrome *c* and apoptotic factors; it also decreases mitochondrial membrane permeability and inhibits COX-2-induced NO release, among other properties, possibly explaining its neuroprotective properties (see Figure 3).²⁷⁹ Randomized double-blind placebo-controlled clinical trials have indeed observed that the addition of minocycline to antipsychotic treatment early in the course of schizophrenia predominantly improves negative symptoms.^{280,281} This supports evidence that the negative symptoms of schizophrenia is positively correlated with oxidative stress and a proinflammatory state (reviewed in ref 282). Furthermore, other interesting preclinical studies have observed that minocycline treatment reverses MK-801-induced cognitive abnormalities (PPI and memory deficits), an effect it shares with haloperidol, therefore demonstrating possible antipsychotic properties.²⁸⁰ Minocycline treatment also reverses LPS induced deficits in PPI²³⁴ as well as MA induced deficits in recognition memory.²⁸³

Altered kynurenine metabolism plays a prominent role in schizophrenia pathology. It is therefore of interest that IL-1 β induced deficits in neurogenesis in rats is reversed following cotreatment with the KMO inhibitor, Ro 61-8048.¹¹⁶ Ro 61-8048 is known to increase KYNA levels and holds promise as a treatment to abrogate neurotoxicity and oxidative stress in schizophrenia. Ro 61-8048 is also associated with an increase in the neuroprotective balance (Figure 3).^{156,284} Another recent study observed that allopurinol, a TDO inhibitor (Figure 3), significantly reversed immobility in the FST and increased hepatic kynurenine levels in a chronic stress animal model of depression.²⁸⁵ These studies indicate that targeted inhibition and possible activation of the kynurenine pathway to increase KYNA production may provide new therapeutic approaches to reverse inflammatory-induced reduction in neurogenesis in schizophrenia. However, some studies have demonstrated elevated levels of KYNA in schizophrenia.¹¹⁰ An important consideration here is that the NMDA receptor antagonist activity of KYNA could assist in driving a hypoglutamatergic state that is proposed to underlie schizophrenia, as discussed in subsection 2.2. Thus, correction of KYNA levels could also significantly improve glutamate dysfunction in schizophrenia. Further studies evaluating such therapy are warranted.

5.2. Compounds Targeting Oxidative Stress. The impact of oxidative stress in the pathophysiology of schizophrenia has been discussed throughout this Review, which brings us to the possible clinical utility of antioxidant treatment. Indeed, previous studies have observed that NAC, a well-known antioxidant and glutamatergic modulator (reviewed in ref 286) improves negative symptomatology in schizophrenia patients when used as an adjunctive treatment.^{287,288} Preclinical studies have also observed that NAC treatment successfully

reverses LPS-induced decline in the GSH/GSSG ratio in male fetuses and prevents these oxidative insults when given as pretreatment.¹⁹⁸ More interesting is that NAC was found to augment selected behavioral, neurochemical, mitochondrial, redox, and anti-inflammatory responses to clozapine²⁸ and to reverse cortico-striatal monoamine alterations induced by SIR.¹⁴⁰ NAC also reversed memory impairment in the Morris water maze in offspring following maternal exposure to LPS.²⁰¹ In rodents, MA induced hyper-locomotor activity was also reversed with NAC treatment.²⁸⁹ Furthermore, MA induced elevations in lipid peroxidation and protein carbonyls as well as decreased GSH levels and glutathione peroxidase activity, were reversed by the administration of NAC-amide, a more lipid soluble form of NAC that aids brain penetration.²⁹⁰

Clinical studies have observed that the combination of the antioxidants, omega-3 fatty acids with vitamins E and C can significantly reduce negative symptoms in schizophrenia.²⁹¹ However, combined omega-3 fatty acids and α -lipoic acid was ineffective in preventing relapse in patients who had responded well to antipsychotic treatment after a single episode of psychosis.²⁹² Other recent reviews of clinical trials concur that dietary supplementation of patients with schizophrenia with omega-3 fatty acids does not improve the symptomatology of these patients.^{293,294} However, it is worth mentioning that a recent study did show that omega-3 fatty acids reduces the progression of at-risk individuals to the full-blown manifestation of psychosis.²⁹⁵ Consequently, the value of omega-3 fatty acids as a preventive measure in schizophrenia needs further study. Some earlier clinical work also hinted at the possible benefit of natural antioxidants.²⁹⁶ However, this goal has not always been reachable. In fact, attempting to predict therapeutic effects of antioxidants is complex, especially comparing animal to human studies. Antioxidants such as α -lipoic acid, NAC, and others may perform differently depending on the cellular milieu, acting either as an antioxidant or as a pro-oxidant depending on the redox status of the organism.^{297,298} Moreover, such redox status very likely relates to illness severity and its progression over time that in turn will predetermine how an antioxidant will perform and at what dose. Thus, conditions such as the applied dose of the antioxidant as well as illness state/severity will have a role in determining the clinical outcome. Preclinical studies, however, have observed that oleanolic acid (an antioxidant and anti-inflammatory agent) normalizes MK-801-induced behavioral (PPI and memory deficits) alterations,²⁹⁹ thus suggestive of some benefits of antioxidant treatment in schizophrenia. Another antioxidant, selenium, has also been proven to reverse MA induced elevation of 3-nitrotyrosine (3-NT) in the rat striatum,³⁰⁰ while pretreatment of rats with the plant extract, mangiferin (MGF), decreased ketamine-induced elevations in brain IL-6 levels and lipid peroxidation.³⁰¹ MGF is a natural xanthone and by virtue of its antioxidant and anti-inflammatory properties has been speculated to be neuroprotective.³⁰¹ Another xanthone, γ -mangostin, also increased expression of 5-HT_{2A/C} receptors and has anti-inflammatory properties *in vitro*.³⁰² However, no other schizophrenia related studies have been undertaken and further research into this promising area of therapeutics is warranted.

5.3. Compounds Targeting Nitric Oxide. NO contributes significantly to oxidative stress (Figure 2) as well as modulates DA release, and has gained growing interest in the pathophysiology of schizophrenia.⁷¹ Clinical studies have begun to reveal the potential of NO-modulators, such as methylene blue and sodium nitroprusside (SNP), in the treatment of

schizophrenia. SNP is a NO donor (Figure 2), and is used clinically to treat hypertension. However, recent preclinical^{303,304} and a clinical study³⁰⁵ have observed its novelty in treating schizophrenia where psychosis-like behavior and c-fos expression in the brain of PCP treated rats³⁰³ as well as amphetamine-induced PPI deficits in rats,³⁰⁴ were reversed with SNP infusion. Moreover, SNP infusion rapidly improved positive and negative symptoms in patients with schizophrenia within hours.³⁰⁵ The authors speculate that the antipsychotic mechanism of SNP might be mediated via NMDA-NO-cGMP signaling pathways (see Figure 2), exerting an inhibitory effect on DAT, which in turn would correct frontal cortical hypodopaminergia. Through feedback loops, SNP may also correct the hyperdopaminergic levels in striatal regions (see Figure 1).³⁰⁵ On the other hand, the NOS-guanylyl cyclase inhibitor, methylene blue, has demonstrated therapeutic efficacy in schizophrenia.³⁰⁶ Via its inhibition of the NO-cGMP pathway, methylene blue modulates the glutamatergic system and could, as an extension of its action, modulate DA and 5-HT release (reviewed in ref 307). Preclinical studies have indeed observed that methylene blue increases 5-HT and DA levels via nNOS inhibition in the rat ventral hippocampus, which may underlie its anxiolytic and antidepressant mechanisms, indicating its clinical utility in possibly treating the negative symptoms of schizophrenia (reviewed in ref 307). Deutsch and colleagues observed a statistically significant decrease in the severity of psychopathology in schizophrenia patients following methylene blue treatment that worsened after discontinuation of the drug.³⁰⁶ In addition, preclinical studies have showed that methylene blue reverses PCP-induced behavioral (PPI and hyperlocomotor activity) alterations in mice,³⁰⁸ while another animal study observed that L-nitroarginine methyl ester (L-NAME), also a nNOS inhibitor, reversed PCP induced PPI deficits³⁰⁹ (see Figure 2). Work in our own laboratory has demonstrated the structural characteristics of various methylene blue analogues, confirming that their antidepressant properties reside in the positively charged dimethylamino group.^{310,311} This property enables these compounds to modulate the mitochondrial electron transport chain, affording them with potent antioxidant and neuroprotective properties.

5.4. Compounds Targeting Cyclooxygenase (COX). As early as the late 1970s, evidence has accumulated in support of the role of arachidonic acid and its metabolites in the etiology of schizophrenia.³¹² When evaluating the redox-immune-inflammatory pathways in schizophrenia, the prostanoid class of fatty acid derivatives (including prostaglandins, thromboxanes, and prostacyclins) produced from arachidonic acid in the COX pathway needs serious consideration. The immunological imbalance observed in schizophrenia, as discussed throughout this Review, has previously been linked to disturbances in the COX pathway, including increased prostaglandin E₂ (PGE₂) production as well as a possible increase in COX expression.³¹³ Interestingly, adjunctive treatment with celecoxib, an anti-inflammatory agent and selective COX-2 inhibitor (Figure 3), has proved to be valuable in improving symptomatology in schizophrenia patients.³¹⁴ However, COX-2 expression is not upregulated in the hippocampus of schizophrenia patients, suggesting that celecoxib may affect the pathophysiology of schizophrenia through COX-2-independent actions rather than by inhibiting the activity of up-regulated COX-2 protein.³¹⁵ Moreover, preclinical evidence indicates that celecoxib treatment prevents hyperlocomotor activity in rats exposed to prenatal Poly I:C combined with later

life MK-801 administration.²³⁶ The nonselective COX inhibitor, aspirin, has also gained some interest recently, demonstrating in clinical studies that it too can significantly improve schizophrenia symptomatology.³¹⁶ However, no preclinical evidence in support of this finding is currently available.

5.5. Other. The early life neurodevelopmental aspect of schizophrenia and its presentation during puberty and predominantly in male subjects raises a question as to the role of estrogen. The estrogen theory posits a protective effect for estrogen in schizophrenia, with periods of protection occurring when estrogen levels are high, for example, during puberty and pregnancy.³¹⁷ Treatment studies using estrogen or related hormones have shown some benefit in treating schizophrenia and related cognitive impairments.^{318,319} Further, there is evidence in multiple systems that estrogens exhibit neuroprotective effects and can interact with immune processes to reduce CNS inflammation and to activate t-helper cell-2 response (reviewed in ref 320). Although there is some evidence that estrogens may have proinflammatory effects as well (reviewed in ref 321), further exploration of its benefits and clinical application have been limited by worrying side effects such as breast and uterine cancer.³¹⁹ Preclinical studies have observed that estradiol treatment has a protective effect against MK-801 induced PPI disruptions,³²² as well as against PCP induced cognitive deficits in the nORT.³²³ Interestingly, a recent study observed some sex specific neurochemical and behavioral alteration in SIR rats,³²⁴ the findings of which could, in part, indicate that sex hormones represent a novel drug target in schizophrenia.

Another interesting treatment option evaluated in schizophrenia research is LY354740, a group II metabotropic glutamate receptor agonist, which has been shown to reverse ketamine-induced increases in NA levels in the hippocampus of rats.³²⁵ Furthermore, LY354740 also blocked the behavioral effects induced by PCP and MK-801 as well as the increased glutamate release elicited by PCP and ketamine in rodents (reviewed in ref 326). These studies indicate that LY354740 could correct the glutamate dysfunction in schizophrenia, and possibly also decrease glutamate-NMDA associated oxidative stress and immune-inflammatory pathways (see Figure 2). However, no clinical studies have yet been undertaken.

6. FINAL SUMMARY AND CONCLUSIONS

To date, new antipsychotics with adjunctive mechanisms other than an action on monoamine regulation remains disappointing, while many patients suffering from schizophrenia are nonresponsive to current treatments. Furthermore, the diagnosis of schizophrenia mainly relies on behavioral symptomatology observed in patients, with no laboratory or biological tests available to confirm and/or extend the diagnosis. In this Review, we have evaluated the neurodevelopmental theories of schizophrenia, focusing specifically on SIR, PPNI, and "dual-hit" animal models of schizophrenia in an attempt to reveal new biomarkers for diagnosis and/or to shed new light on novel treatment targets as well as targets for possible adjunctive treatment strategies, summarized in Table 4.

Monoamine alterations observed in these models are outlined in the first row of Table 4. The SIR and PPNI models present with general hypocortical and hyperstriatal DA activity as well as increased NAcc activity, with some opposing studies noted. Opposing (increased or decreased) 5-HTergic activity is also evident in the same brain regions in both models,

while a general hyper-NAergic activity is evident in the hippocampus, hypothalamus, striatum, cortex, and cerebellum, also in both SIR and PPNI models. Overall, atypical antipsychotics are the most effective in reversing the above-mentioned monoamine alterations in these models. However, new treatment options such as NAC and LY354740 were also effective in reversing monoamine alterations in the SIR and ketamine models, respectively (Table 4).

Evaluating GABA–glutamate alterations (Table 4), a decrease in GABA and GAD67 (one study observed an increase in GAD67) was observed in the PPNI models, but appears to be more pronounced in the “dual-hit” models. Glutamate function was decreased in the SIR models but decreased or increased in the PPNI models, emphasizing some opposing findings in the PPNI models. Decreased NAA was observed in the SIR model (not accessed elsewhere), which is in line with clinical findings. Again, atypical antipsychotics appear to be the mostly effective in reversing some of these alterations. LY354740 also reversed PCP induced increases in glutamate release (Table 4).

With regards to the redox-immune-inflammatory pathways (Table 4), alterations in ATP levels have been observed in the SIR model (increased in the striatum and decreased in the frontal cortex), in the PPNI model (decreased in the spleen), as well as in the “dual-hit” model (altered energy metabolism, redox processes, and neurotrophic proteins). SIR and the PPNI models are associated with a general increase in Nox2 and iNOS, lipid peroxidation, pro-inflammatory cytokines and the neurotoxic kynurenine metabolites, along with a general decrease in PV-IR interneurons, GSH, anti-inflammatory cytokines (although one PPNI study observed an increase in IL-10), and the neuroprotective kynurenine metabolites. In addition, SIR results in an increase and decrease in SOD while PPNI is associated with an increase in protein carbonyl and a decrease in α -tocopherol. Some differences that have been observed between these models include a decrease in the neuroprotective ratio following SIR while no change in respect of this parameter was observed in the PPNI models. Some new treatment strategies that may be identified from these redox-immune-inflammatory studies, apart from atypical antipsychotics, include: minocycline, NAC, SNP, methylene blue, L-NAME, ketamine, memantine, the KMO inhibitor (Ro 618048), allopurinol, NAC-amide, oleanolic acid, selenium, mangiferin, γ -mangostin, celecoxib, aspirin, estradiol (Table 4).

In the behavioral alterations section (Table 4), SIR, PPNI and “dual-hit” models (except for one study), generally engender a deficit in PPI and an increase in anxiety-like behavior and locomotor activity (except for one PPNI study that observed decreased locomotor activity), making this the most valid and reliable behavioral changes across all three models. Furthermore, deficits in recognition memory, social interaction and latent inhibition (except for one PPNI study that observed increased latent inhibition), were observed in the SIR and PPNI models. Behavioral alterations that have to-date only been observed in SIR include a decrease in mobility in the FST, increased self-directed, aggressive-like and social contact behavior, increased ethanol self-administration and polydipsia. Behavioral alterations noted in PPNI models include decreased working memory and sucrose consumption, while decreased conditioned emotional response freezing behavior has been observed in the “dual-hit” model. These behavioral differences are generally only due to the lack of consistent behavioral tests performed across all the models, emphasizing that further

studies are needed in this regard. Overall, it is well-known that antipsychotics are effective in reversing most of these behavioral alterations in at least the SIR and PPNI models, confirming their predictive validity. New treatment strategies that also reversed some of these behavioral alterations include: ketamine, memantine, minocycline, allopurinol, NAC, oleanolic acid, SNP, methylene blue, L-NAME, celecoxib, estradiol, and LY354740 (Table 4). However, further work in this regard is recommended.

It should, however, be noted as a general shortcoming that numerous conflicting results are evident in the SIR, PPNI, and “dual-hit” models throughout this Review, which emphasizes the need for further research in order to standardize these models as translational resources in the evaluation of schizophrenia. Thus, the translation of findings taken from these animal models to clinical practice needs careful consideration. A recent review that evaluated various adjunctive treatments found that aspirin, estrogens and NAC were the only treatment strategies that showed significant efficacy on symptom severity in schizophrenia patients.³¹⁶ Indeed, these findings are very much in line with most of the findings discussed in this review, indicating that, while translational animal models are essential in drug discovery, investigators need to be cognisant of their limitations. In fact, many such models can effectively reproduce only certain symptoms of the human illness, may respond to nonspecific treatments, or show variable biomarkers responses. Given these restrictions, this Review has shown that future use of different established schizophrenia animal models *in combination* might provide a more robust “dual-hit” approach in translating pathological findings in humans and for improving the search for new drug treatments. Earlier we discussed the dilemma of attempting to predict the therapeutic effects of antioxidants by inferring animal data are immediately transferrable to human studies. Indeed, illness severity, comorbidities and dose of the drug in question have a definitive role in determining outcome within a given clinical scenario.

The neurodevelopmental animal models (SIR and PPNI models) discussed in this Review have proven themselves to be of significant value in schizophrenia research. Furthermore, a “dual-hit” hypothesis built on the latter paradigms, followed by a specific later life insult or drug abuse, has provided a growing body of evidence in support of this theory as well as neurobiological data in respect of its construct and predictive validity. Future studies should therefore incorporate SIR or PPNI with the “2nd hit” more representative of a specific clinical condition, for example, SIR combined with MA to study the link between early life adversity and drug abuse (as illustrated in Table 3). Schizophrenia research is needful of a “new champion”, and given the data discussed here redox and inflammatory markers, especially mitochondrial and kynurenine pathways, may provide the melting point for the next generation of antipsychotic drugs. The goal of this Review is to mobilize ideas in order to discover and develop more beneficial and effective treatments for this life altering disease. Although it is too soon to predict the outcome, the novel treatments discussed here show great promise and more studies focusing on their underlying biological mechanisms and effects on behavior are warranted. To this end, “dual-hit” paradigms designed around neurodevelopmental models and the immune-inflammatory redox hypothesis of schizophrenia are likely to provide a wide array of new therapeutic options. Furthermore, these models in turn will provide the thrust for the search for

much needed biomarkers of the disease that will benefit diagnosis and treatment of schizophrenia.

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Chapter 5: Conclusion and future recommendations

Schizophrenia is a debilitating disease with widespread adverse effects; strongly influencing the quality of life of those it touches (Di Forti *et al.*, 2007:S101). What makes matters worse is that our understanding of its origin and pathological pathways remain unclear (Sawa & Snyder, 2002:692), while current treatment regimens are unsatisfactory (Buckley & Stahl, 2007:93; Erhart *et al.*, 2006:234). Schizophrenia presents with positive symptoms, such as auditory hallucinations and delusions, and is usually also the most easily treated component of the illness (Kapur & Remington, 2001:873). However, the negative symptoms and cognitive impairments that constitute the full syndrome remain the most difficult to treat, hindering successful management of the illness (Buckley & Stahl, 2007:93; Erhart *et al.*, 2006:234).

Regarding the pathological mechanisms of schizophrenia, evidence for a dysfunctional state between important neurochemical pathways, such as dopamine (DA), glutamate, gamma-aminobutyric acid (GABA) and serotonin (5-HT) pathways, remain compelling (Carlsson *et al.*, 2001:237). In addition, a strong link exists between an altered redox-inflammatory state and schizophrenia pathology (Bitanirwe & Woo, 2011:878; Kneeland & Fatemi, 2012:35). It should also be taken into account that because the brain is extremely sensitive to oxidative insults (Bitanirwe & Woo, 2011:878), the influence of a negative redox balance in the pathology of schizophrenia cannot be ignored. When considering aetiological factors, apart from a possible genetic predisposition, environmental factors during sensitive pre- or postnatal periods have been strongly implicated in its development (Lewis & Levitt, 2002:409). Of these, prenatal inflammation has delivered robust evidence as an aetiological role player in the manifestation of the illness, both in epidemiological (Brown & Derkits, 2010:261) and preclinical studies (Möller *et al.*, 2015:987). This makes sense when one takes into consideration the important part cytokines play during prenatal brain development (Feigenson *et al.*, 2014:72). Abuse of potent psychotropic drugs, specifically methamphetamine (MA), has also been shown to cause enduring psychological manifestations, such as psychosis, depression and paranoia (Chen *et al.*, 2003:1407). These drug-induced occurrences, which mimic significant symptoms of schizophrenia, hint at a possible existence of pre-existing risk factors in certain individuals (Chen *et al.*, 2005:87). In line with this, the two-hit hypothesis of schizophrenia suggests that a genetic susceptibility

combined with a particular developmental insult can prime a person for a later event, eventually giving way to schizophrenia presentation (Bayer *et al.*, 1999:543). Although a genetic susceptibility is proposed to be the “first hit”, researchers have suggested that prenatal inflammation can also initiate the process of disease progression (Meyer & Feldon, 2010:285).

Preclinical studies using animal models to reveal the full nature of schizophrenia play an immense role in our understanding and treatment of the disorder. Several studies of prenatal inflammation have provided us with an enhanced perception of its aetiology and pathology (reviewed in Möller *et al.*, 2015:987). Similarly, pharmacological animal models of schizophrenia, usually by means of MA exposure, have contributed greatly to our current knowledge (Machiyama, 1992:107). However, to date studies incorporating a two-hit animal model of schizophrenia is scarce, despite a strong indication towards the hypothesis’ validity. Consequently, we designed a study using both a prenatal inflammation model and a two-hit model (prenatal inflammation combined with postnatal MA abuse) of schizophrenia to address these shortfalls in existing knowledge. We investigated the effects of these models on several redox-inflammatory markers, as well as essential regional brain monoamines. We correlated these biochemical changes to alterations in schizophrenia-associated behavioural paradigms, namely sensorimotor gating (by assessing prepulse inhibition or PPI), social interaction and memory function (by assessing novel object recognition). Since the current treatment options for schizophrenia, which functions mainly on dopaminergic and serotonergic pathways (Sumiyoshi *et al.*, 2013), remain inadequate, we aimed to introduce a pharmacological agent that targets the proposed developmental basis for the disease, particularly that centred on the immune-inflammatory hypothesis. Additionally, with the above in mind, the thought that an anti-oxidant, N-acetyl cysteine (NAC), may serve as a promising novel treatment option was also introduced. Subsequently, we applied NAC treatment to the prenatal immune-inflammatory model with and without postnatal MA challenge in an attempt to reverse the behavioural and biochemical changes induced by these models.

A brief overview of the findings in chapter 3, 4 and the addendum:

Our review article confirmed the value of including neurodevelopmental animal and dual-hit animal models in preclinical studies. Both the social isolation rearing and prenatal inflammation models studied to date have contributed greatly to our current knowledge by eliciting changes in important monoamines, oxidative pathways, immune markers and behavioural paradigms. Although studies incorporating dual-hit models are limited, those

reviewed in this article provide valuable bio-behavioural data that support the construct and predictive validity of this hypothesis. In addition, this review highlighted a vital role for diverse pathological pathways in schizophrenia pathology and by implication, its treatment. The novel treatments discussed here demonstrates great promise, motivating future studies to focus on their effects and mechanisms with regards to schizophrenia research. In conclusion, this review served to provide a summary of our current knowledge with regards to neurodevelopmental and dual-hit aetiology and novel treatments, and will be provocative for new and exciting future research.

Outcomes achieved in the current study:

- *Objective:* Establish whether prenatal LPS administration on GD 15-16 can induce oxidative stress in offspring in later life, akin to redox changes observed in schizophrenia.
Outcome: We observed that prenatal inflammation with LPS alone caused significant elevations in frontal cortical, striatal and hippocampal lipid peroxidation, as well as a significant increase in plasma total reactive oxygen species (ROS) levels, compared to the control group.
- *Objective:* Establish whether postnatal administration of MA (relating to MA abuse) can induce oxidative stress, akin to redox changes observed in schizophrenia.
Outcome: We discovered that postnatal chronic MA administration did not significantly alter the oxidative status of experimental animals, compared to the control group.
- *Objective:* Determine whether a dual-hit model can exacerbate oxidative changes in rodents.
Outcome: Compared to controls, the dual hit model exhibited significant elevations in plasma ROS levels, as well as in frontal cortical and striatal lipid peroxidation levels. However, when compared to either MA or LPS alone, we were unable to demonstrate a significant worsening of redox changes in combined LPS+MA treated animals (i.e. the two-hit model). Indeed, only the striatal lipid peroxidation results revealed an exacerbated effect after two hits, in comparison with LPS or MA alone.

- *Objective:* By only focusing on models that are able to induce redox imbalances, we aimed to establish whether NAC can reduce or reverse oxidative stress in these models.

Outcome: A MA+NAC group was excluded as MA alone failed to evoke a noteworthy pro-oxidative state (see above). NAC engendered a significant anti-oxidant effect by reversing the elevation of striatal lipid peroxidation levels in both the prenatal inflammation (i.e. LPS+NAC) and the dual-hit (i.e. LPS+MA+NAC) models, as well as reversing the plasma ROS levels in the prenatal inflammation (LPS+NAC) group.

- *Objective:* Establish whether a prenatal LPS alone model and a postnatal MA alone model can cause abnormalities in SI, %PPI and visual memory, akin to schizophrenia symptomology, and whether the dual-hit model (i.e. LPS+MA) will worsen these changes.

Outcome: Prenatal LPS administration revealed marked deficits in sensorimotor gating and caused significant elevations in total social behaviours, compared to the control group, although it did not markedly alter memory function. Chronic MA administration caused significant deficits in sensorimotor gating, but showed no effect on social behaviours or memory function. The dual-hit revealed a significant increase in total social behaviours, although this was not exacerbated over that induced in the single-hit models. However, the dual-hit model did exacerbate sensorimotor gating deficits (%PPI) and was also the only model to cause significant memory impairments in experimental animals.

- *Objective:* Establish whether a prenatal LPS alone model and a postnatal MA alone model can alter monoamine levels in the brain tissue of animals, and whether the dual-hit model (i.e. LPS+MA) will worsen these changes.
- *Outcome:* Prenatal inflammation (LPS alone) induced significant changes in various monoamines, compared to the control group: increased frontal cortical DA, reduced frontal cortical 5-HT and decreased noradrenaline (NA) in frontal cortical and striatal tissue. MA administration reduced NA levels in frontal cortical and striatal tissue, in comparison to the control group. Although the dual-hit model did not exacerbate monoamine irregularities, it significantly reduced frontal cortical 5-HT levels, as well as frontal cortical and striatal NA levels. NA levels were also significantly elevated in the hippocampus of dual-hit animals compared to controls. Analysis of monoamine metabolites across all three groups delivered little of note, but nonetheless served to support the monoamine changes observed in the different brain regions.

- *Objective:* Establish whether a prenatal LPS alone model and a postnatal MA alone model can cause changes in the pro- and anti-inflammatory cytokine profile of rodents, and whether the dual-hit model (i.e. LPS+MA) will worsen these changes.
Outcome: The prenatal inflammation model significantly reduced plasma levels of the anti-inflammatory cytokine, interleukin 10 (IL-10), and increased plasma levels of the pro-inflammatory cytokine, tumor necrosis factor-alpha (TNF- α), all in comparison to the control group. MA alone did not alter the cytokine levels of experimental animals. The dual-hit model failed to exacerbate the effects caused by LPS alone, but did significantly reduce IL-10 levels compared to controls.

- *Objective:* Determine whether NAC can reduce or reverse behavioural, inflammatory and neurochemical alterations in the prenatal inflammation and the dual-hit animal (i.e. LPS+MA) models of schizophrenia.
Outcome: NAC was successful in reversing dual-hit induced %PPI deficits observed at a prepulse of 72 dB, as well as the increase in total social behaviours induced by LPS alone and the dual-hit. NAC treatment failed to normalize memory impairments in the dual-hit group or sensorimotor gating deficits in the LPS alone and dual-hit models at prepulses of 74 and 84 dB. We observed that NAC successfully reversed all of the monoamine changes observed in the LPS alone and the dual-hit animals. Interestingly, NAC induced significantly elevated levels of hippocampal 5-HT and NA in the LPS alone group, which we correlated with alterations in redox pathways. NAC was successful in normalizing all immune changes in both the LPS and dual-hit models.

In conclusion, we have found that both prenatal inflammation (LPS alone) and a dual-hit model (LPS+MA) elicited behavioural changes akin to schizophrenia symptomology. Furthermore, the dual-hit model revealed a superior ability in altering memory function, a well-known feature of schizophrenia (Meyer, 2013:20). Immune-redox changes have been strongly linked to the development of schizophrenia (Bitanirwe & Woo, 2011:878). Here we confirmed that both prenatal immune activation and a two-hit model caused significant alterations in immune markers and oxidative status. Furthermore, irregularities in important monoamine and metabolite levels, driven by the aforementioned oxidative changes, confirmed that both these animal models may alter neuro-function. This study confirms that prenatal inflammation may alter brain development leading to the initiation of the pathological processes involved in schizophrenia development. Coupled with a second hit later in life, cognitive disabilities become more apparent, suggesting that dual-hit aetiology

may be involved in the full manifestation of schizophrenia. However, in most cases, we observed that the dual-hit failed to induce additive effects on the bio-behavioural markers studied, as was expected (see Aims and Objectives, Chapter 1). In addition, we have found that MA may be less prone to causing bio-behavioural changes related to psychosis than originally thought. That a significant effect was not observed in these two models warrants further investigation. Finally, NAC had beneficial effects on both behavioural and redox-inflammatory markers, proposing that this specific anti-oxidant treatment may be of value as adjunctive treatment in schizophrenia.

Future recommendations:

Whereas the current dissertation successfully addressed all of the study objectives outlined in Chapter 1, there were a number of limitations to the study, for which we supply recommendations that should be incorporated in future studies:

- 1) By including a group that receives NAC alone (S-S-NAC), as well as a group receiving MA plus NAC (S-MA-NAC), future studies may better elucidate the mechanisms by which NAC functions, as well as its effects on chronic MA abuse. However, it needs to be borne in mind that this study demonstrated that MA alone is for the most part ineffective in inducing bio-behavioural changes related to schizophrenia, thereby casting doubt on the value of performing such a study. This observation, although in hindsight, also places doubt on its incorporation in a two-hit design (see below).
- 2) By including a well validated schizophrenia treatment agent (such as clozapine), future studies can compare its effects with that of NAC in both the prenatal inflammation and the dual-hit model. Better yet, combining the two drugs in both the prenatal inflammation and the dual-hit model should deliver impactful results of translational relevance for schizophrenia and its treatment.
- 3) Revision of the MA alone and dual-hit protocols (adjustments to dosages, dosing intervals or the inclusion of other relevant “dual hits” such as LPS plus post-weaning social isolation) may provide a better 2-hit design, as well as shed light on why we failed to see significant effects in these models.
- 4) Since the redox and immune-inflammatory results obtained in this study could be linked to a role for both glutathione and the kynurenine pathway, it is recommended that future studies analyse these markers in the prenatal inflammation and dual-hit models as well.

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Addendum A: Additional results

In this addendum additional data not included in Chapter 3 (Article) is presented. The main reason for presenting this data as an addendum is to streamline the concept article according to the submission requirements of the journal, and to better package the dissertation in a more coherent manner. Moreover, some of the less impactful data were excluded from the chapter 3 for reasons that will become clear.

One of the main study objectives of the current study (Chapter 1, Section 2) was to determine the effects of prenatal inflammation alone, and then prenatal inflammation combined with postnatal methamphetamine (MA) abuse (two-hit model) on lipid peroxidation, as well as regional brain monoamines, viz. dopamine, serotonin and noradrenaline and their metabolites. Brain regions include the frontal cortex, striatum and hippocampus. We then attempted to reverse these effects by means of chronic N-acetyl cysteine (NAC) administration. In addition, we also measured the effects of MA administration alone on the above markers. In this addendum, we've included the lipid peroxidation and monoamine results obtained from hippocampal tissue. Furthermore, all regional brain monoamine metabolite data (3,4-dihydroxyphenylacetic acid, homovanillic acid and 5-hydroxyindoleacetic acid) that were excluded from the article will be presented here.

Lipid peroxidation in all brain regions were measured with the Parameter™ TBARS assay and all monoamine and metabolite quantification was done with the same HPLC method, and is fully described in Addendum B.

The additional data not included in the article and presented in this addendum include:

- Malondialdehyde (MDA) measurements, a product of lipid peroxidation, in the hippocampus of all treatment groups.
- Monoamine measurements in the hippocampal tissue of all treatment groups.
- Dopamine metabolites, namely 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), and the serotonin metabolite, 5-hydroxyindoleacetic acid (5-HIAA), in all brain regions for all of the treatment groups

A.1.1 Results: Hippocampal lipid peroxidation

One-way ANOVA revealed a significant treatment*lipid peroxidation interaction in the hippocampus ($F(5, 66) = 20.66, p < 0.0001$) (Fig. A-1). Bonferroni post-hoc analysis showed that only the LPS alone (LPS-S-S) group revealed significantly increased levels of MDA ($p < 0.001$), which was successfully reversed by NAC (LPS-S-NAC) ($p < 0.0001$) (Fig. A-1).

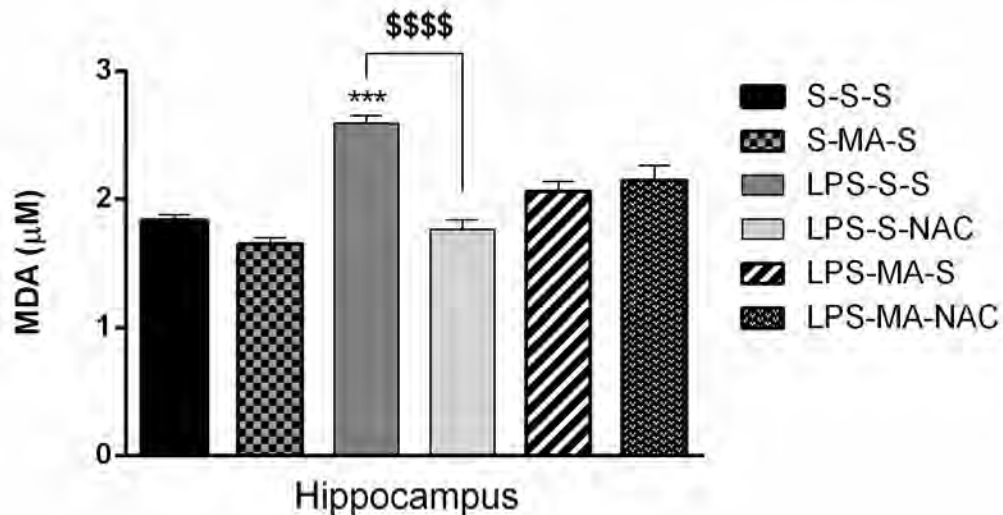


Figure A-1: Malondialdehyde (MDA) levels in the hippocampus.

*** $p < 0.001$; **** $p < 0.0001$ (One-way ANOVA, Bonferroni post-hoc test).

A.1.2 Discussion: Hippocampal lipid peroxidation

We observed that prenatal infection alone (LPS-S-S) significantly increased lipid peroxidation in the hippocampus (Fig. A-1), in line with a previous study (Zhu *et al.*, 2007:671). Interestingly, neither MA alone nor the dual-hit model altered lipid peroxidation in the hippocampus (Fig. A-1). As discussed in Chapter 3, we suggest that our MA protocol may be sub-optimal to induce lasting oxidative damage. This, however, does not explain why LPS failed to increase MDA levels in the dual-hit model. We propose that after two serious psychosocial insults, reactive adaptive mechanisms come in play, preventing the expected excessive redox-related changes.

A2.1 Results: Hippocampal monoamines:

Dopamine:

One-way ANOVA produced no treatment*dopamine interaction in hippocampal tissue ($F(5, 30) = 0.87$, $p=0.51$). Bonferroni post-hoc test revealed no significant differences between any of the treatment groups, although a trend towards a reduction can be seen in the MA alone, LPS alone, LPS+MA, as well as the NAC treated groups, compared to controls (S-S-S) (Figure A-1.1).

Serotonin:

One-way ANOVA produced a significant treatment*serotonin interaction in the hippocampus ($F(5, 66) = 2.56$, $p=0.04$). Bonferroni post-hoc analysis revealed a significant increase in serotonin in the prenatal inflammation group treated with NAC (LPS-S-NAC), compared to the maternal inflammation (MIA) group (LPS-S-S) ($p<0.05$) (Fig. A-1.2). None of the other groups revealed any significant changes in serotonin levels.

Noradrenaline:

One-way ANOVA revealed a significant treatment*noradrenaline (NA) interaction in the hippocampus ($F(5, 66) = 14.91$, $p<0.0001$). Bonferroni post-hoc test revealed that LPS+MA induced a significant increase in NA, compared to controls ($p<0.001$) (Fig. A-1.3). We also observed that NAC treatment significantly increased NA levels in the LPS alone model (LPS-S-NAC) compared to the MIA animals (LPS-S-S) ($p<0.0001$). None of the other treatment groups altered NA levels (Fig. A-1.3).

Fig. A-2.1

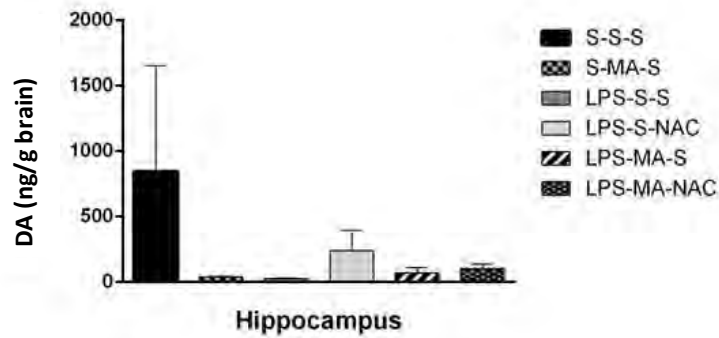


Fig. A-2.2

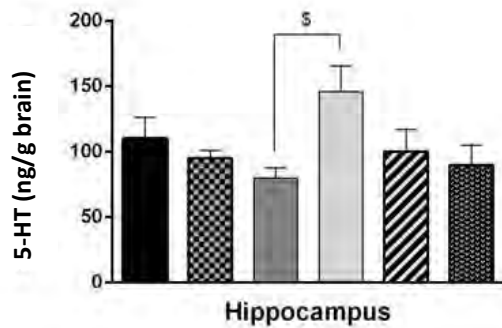


Fig. A-2.3

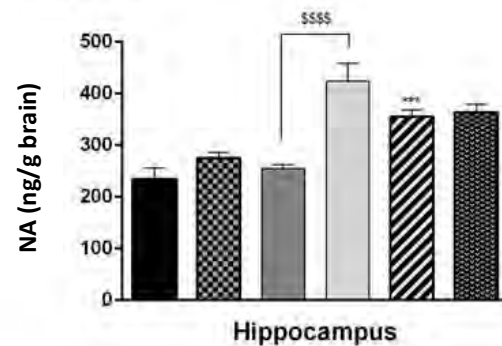


Figure A-2: Hippocampal levels of (A-1.1) dopamine, (A-1.2) serotonin and (A-1.3) noradrenaline.

*** $p < 0.001$ vs. controls (S-S-S); \$: $p < 0.05$; \$\$\$\$: $p < 0.0001$ (One-way ANOVA, Bonferroni post-hoc test).

A.2.2 Discussion: Hippocampal monoamines

Although no significant results were observed in hippocampal DA levels, all of the treatment groups, including the NAC treated animals, showed a trend towards reduced levels of DA, compared to controls (Fig. A-1.1). Abnormal neurotransmission of hippocampal DA is thought to be involved in certain aspects of cognitive dysfunction (Borgkvist *et al.*, 2012:531). Our data suggests that prenatal inflammation and the dual-hit model altered DA function in the hippocampus, which may relate to cognitive disabilities as seen in schizophrenia (reviewed in Tandon *et al.*, 2009:1). This is in line with our sensorimotor gating data (presented in Chapter 3) in which both MIA and MIA+MA reduced % prepulse inhibition (PPI). Disappointingly, only the dual-hit model induced significant deficits in visual memory,

as evinced by the novel object recognition test (nORT) (Chapter 3). Still, it is clear that both these models inhibited DA activity in the hippocampus. NAC failed to reverse these changes, suggesting that it possibly lacks effect in hippocampal regions with regards to DA modulation (Fig. A-1.1), although further work is needed. MA alone tended to deplete DA (not significant), also in line with the literature (Cass *et al.*, 2006:261).

It is peculiar that NAC increased hippocampal serotonin in the prenatal inflammation group (LPS-S-NAC) (Fig. A-1.2), even more so when we consider that the same results were not obtained in the dual-hit model (Fig. A-1.2). The hippocampus plays a distinct role in anxiety, independent of its roles in learning and memory (reviewed in Engin & Treit 2007:365). A possible interaction between N-methyl-D-aspartate (NMDA) and serotonin receptors has been described in brain regions associated with anxiety (eg. the dorsal periaqueductal gray) (Moraes *et al.*, 2008:181). Indeed, Moraes' review noted that intra-hippocampal infusions of glutamatergic or serotonergic compounds produce significant anxiolytic effects (Engin & Treit, 2007:365). Since NAC is able to modulate glutamatergic function (Dean *et al.*, 2011:78), it is possible that glutamate modulation by NAC affects serotonergic activity in the hippocampus. This is in line with our social interaction data (presented in Chapter 3) where we described increased social interaction in MIA (LPS-S-S) animals, which indicate towards reduced anxiety-like behaviour. The elevation in hippocampal serotonergic activity may possibly drive anxiolytic behaviour. On the other hand, the dual-hit model (LPS-MA-S) did not deliver the same results (Fig. A-1.2), and one wonders whether MA administration suppressed the LPS-induced rise in serotonin. This corroborates with the trend towards lowered serotonin in the hippocampus following MA administration (S-MA-S) (Fig. A-1.2). Indeed, MA has been shown to deplete serotonin levels in rodent brain (Yu & Liao, 2000:419). However, this does not relate to our social interaction data of dual-hit animals, seeing as they also exhibited anxiolytic behaviour (Chapter 3). For this we suggest that perhaps social behaviour in the dual-hit group was related to other factors, such as hyperactivity or aggression, both known features of MA abuse (Sekine *et al.*, 2006:90; Yukio *et al.*, 2009:7).

The dual-hit model significantly increased hippocampal NA levels (Fig. A-1.3). Previous studies reported that oxidative stress may stimulate NA release (Milusheva *et al.*, 2003:771), thus it is plausible that oxidative pathways in this model could be responsible for this rise in NA. Surprisingly, NAC caused an even greater increase in hippocampal NA in the MIA model (LPS-S-S) (Fig. A-1.3). For now, the mechanisms involved in this phenomenon are still unclear and warrants further investigation.

A.3.1 Results: Monoamine metabolites

A.3.1.1 DOPAC

Frontal cortex:

One-way ANOVA revealed a significant treatment*DOPAC interaction in the frontal cortex ($F(5, 66) = 4.55, p = 0.001$). Bonferonni post-hoc test revealed no significant changes in the LPS alone and LPS+MA groups when compared to controls (S-S-S), but a visible trend towards increased DOPAC can be seen in both these models (Fig. A-2.1). NAC significantly reversed these changes in the LPS alone model (LPS-S-NAC) ($p < 0.05$) and tended to do the same in the dual-hit model, although narrowly missing significance (LPS-MA-NAC) ($p = 0.08$) (Fig. A-2.1). MA alone (S-MA-S) caused no significant changes in DOPAC levels.

Striatum:

One-way ANOVA showed a significant treatment*striatal DOPAC interaction ($F(5, 66) = 4.44, p = 0.002$). However, Bonferonni post-hoc analysis revealed no significant changes in striatal DOPAC levels between any of the treatment groups (Fig. A-2.2).

Hippocampus:

By means of one-way ANOVA, no significant treatment*DOPAC interaction was evident in the hippocampus ($F(5, 66) = 0.70, p = 0.63$) (Fig. A-2.3).

Fig. A-3.1

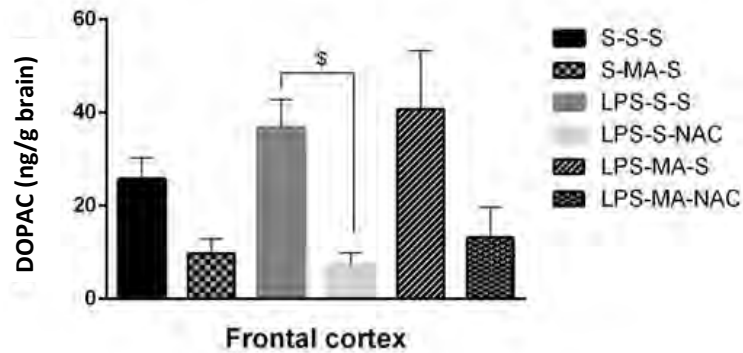


Fig. A-3.2

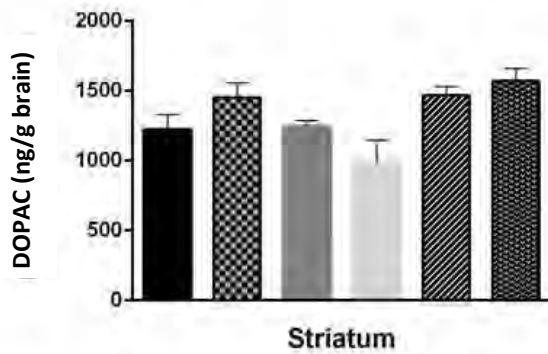


Fig. A-3.3

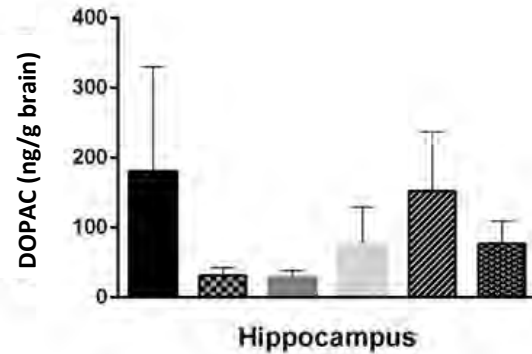


Figure A-3: Levels of 3,4-dihydroxyphenylacetic acid (DOPAC) in the (A-2.1) frontal cortex, (A-2.1) striatum and (A-2.3) hippocampus.

\$: $p < 0.05$ (One-way ANOVA, Bonferroni post-hoc test).

A.3.1.2 HVA

Frontal cortex:

One-way ANOVA revealed a significant treatment*HVA interaction in the frontal cortex ($F(5, 66) = 4.70$, $p = 0.001$). Bonferroni post-hoc analysis revealed no significant changes in frontal cortical HVA levels in the MIA (LPS-S-S) and dual-hit models (LPS-MA-S) compared to the control (S-S-S), but a trend towards elevation can be seen in both groups (Fig. A-3.1). NAC significantly reversed this change in the MIA model (LPS-S-NAC) ($p < 0.05$), however

NAC showed a trend towards reversing the increased HVA levels in the dual-hit model (LPS-MA-S), although significance was not attained (LPS-MA-NAC) (Fig. A-3.1).

Striatum:

One-way ANOVA revealed a significant treatment*HVA interaction in the striatum ($F(5, 66) = 8.78, p < 0.0001$). Bonferroni post-hoc test showed that the dual hit model (LPS-MA-S) induced significant elevations in striatal HVA ($p < 0.01$) compared to the control group (S-S-S) (Fig. A-3.2). None of the other groups revealed any significant changes with regards to striatal HVA levels.

Hippocampus:

One-way ANOVA revealed no significant treatment*HVA interactions in the hippocampus ($F(5, 66) = 0.84, p = 0.52$) (Fig. A-3.3).

Fig. A-4.1

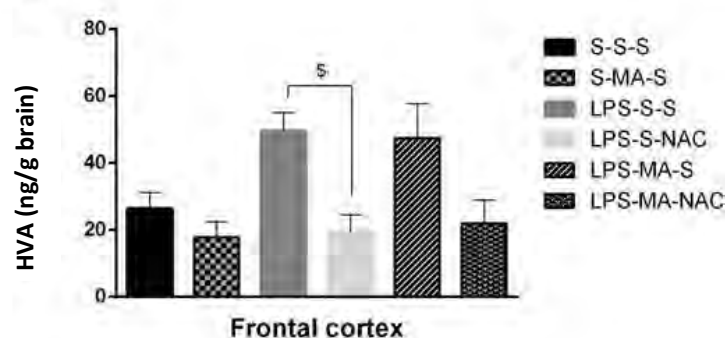


Fig. A-4.2

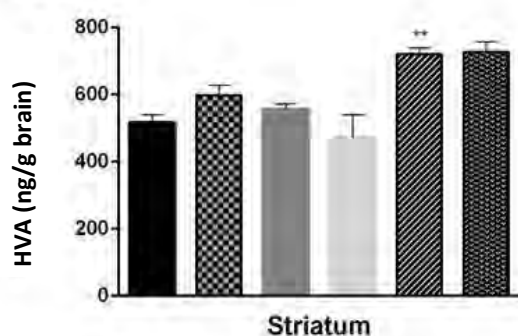


Fig. A-4.3

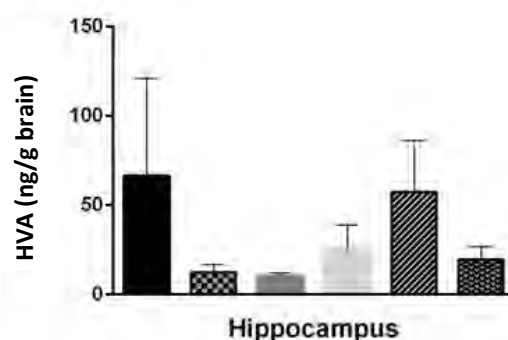


Figure A-4: Homovanillic acid (HVA) levels in the (A-3.1) frontal cortex, (A-3.2) striatum and (A-3.3) hippocampus.

** $p < 0.01$; \$: $p < 0.05$ (One-way ANOVA, Bonferroni post-hoc test).

A.3.1.3 5-HIAA

Frontal cortex:

One-way ANOVA revealed no significant treatment*5-HIAA interactions in the frontal cortex ($F(5, 66) = 1.13, p = 0.35$) (Fig. A-4.1).

Striatum:

One-way ANOVA revealed no significant treatment*5-HIAA interactions in the striatum ($F(5, 66) = 2.22, p = 0.06$) (Fig. A-4.2).

Hippocampus:

One-way ANOVA revealed a significant treatment*5-HIAA interaction in the hippocampus ($F(5, 66) = 12.33, p < 0.0001$). Bonferonni post-hoc test revealed that NAC induced a significant elevation in hippocampal 5-HIAA levels in the dual-hit model (LPS-MA-NAC), compared to the LPS-MA-S group ($p < 0.0001$) (Fig. A-4.3). None of the other groups revealed any significant changes in hippocampal 5-HIAA levels

Fig. A-5.1

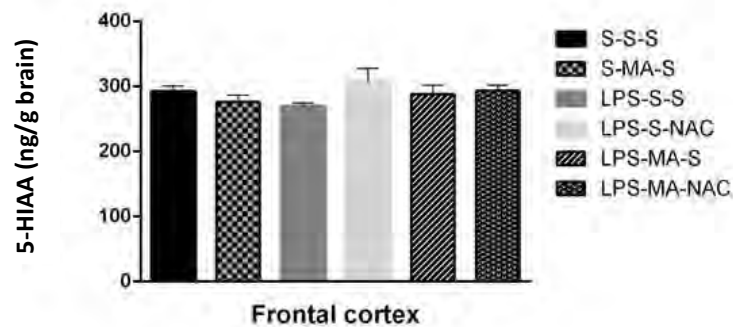


Fig. A-5.2

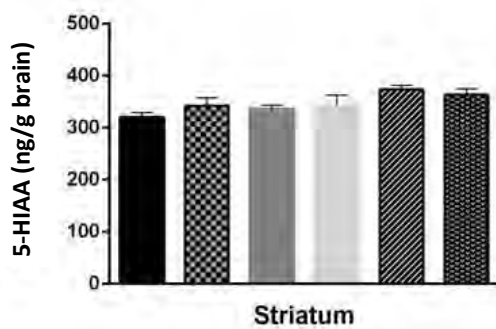


Fig. A-5.3

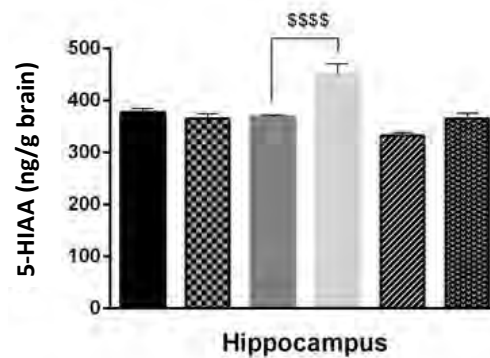


Figure A-5: Levels of 5-hydroxyindoleacetic acid (5-HIAA) in the (A-4.1) frontal cortex, (A-4.2) striatum and (A-4.3) hippocampus.

****: $p < 0.0001$ (One-way ANOVA, Bonferroni post-hoc test).

A.3.2 Discussion: Monoamine metabolites

In the frontal cortex, we observed increased levels of DOPAC in the MIA (LPS-S-S) and dual-hit models (LPS-MA-S), although this did not reach statistical significance (Fig. A-2.1). Our frontal cortical DA data (Chapter 3) revealed that prenatal LPS treatment induced a significant increase in DA. Taken together, these data suggests that prenatal LPS administration caused increased turnover of DA in the frontal cortex of experimental animals, which is confirmed by our HVA data, where LPS alone triggered an increase in HVA levels (however not significant) (Fig. A-3.1). This is in line with a previous study, which detected an increased production and turnover rate of DA in the prefrontal cortex of schizophrenia patients (Lindstrom *et al.*, 1999:681). The same results, however, did not occur in the dual-

hit model (LPS-MA-S) (Fig. A-2.1 and A-3.1). Although both DOPAC and HVA levels in the dual-hit model (LPS-MA-S) were increased (insignificantly) in the frontal cortex, DA levels were reduced in this group in the frontal cortex (Chapter 3). This suggests that augmented metabolism of DA resulted in decreased levels of DA in this brain region. Seeing that MA is known to cause long-term depletion of DA (Cass *et al.*, 2006:261), it can be put forward that perhaps this aspect of the dual-hit model is responsible for elevated DA metabolism in the frontal cortex. Unfortunately, NAC failed to reverse any of these alterations in either schizophrenia model (Fig. A-2.1 and A-3.1).

In the striatum, no significant changes could be detected for DOPAC levels (Fig. A-2.2). HVA levels, on the other hand, were significantly elevated in the dual-hit model only (Fig. A-3.2), with no changes in striatal DA levels noted (Chapter 3). We suspect that again increased production *and* metabolism took place in this model. Unfortunately, NAC failed to normalize this altered state in the dual hit model.

In the hippocampus, DOPAC and HVA were increased in the dual-hit animals but did not reach statistical significance (Fig. A-2.3 and A-3.3). The hippocampal DA data (Fig. A-1.3) showed a trend towards decreased DA levels in the dual hit model although it too was not significant. This is in line with our observation in the frontal cortex of dual-hit animals, suggesting that the combination of LPS and MA induced an increase in DA metabolism, resulting in reduced levels of this monoamine. No changes were observed for any of the other treatment groups.

When we consider data on frontal cortical serotonin levels (Chapter 3), both prenatal inflammation alone (LPS-S-S) and in combination with MA (LPS-MA-S) resulted in significantly decreased levels of serotonin. However, no changes in 5-HIAA levels occurred between any of these experimental groups in the frontal cortex (Fig. A-4.1), suggesting that depletion of serotonin levels occurred via a different route than increased metabolism rates. A possible explanation for these results could be the involvement of the kynurenine pathway in serotonin synthesis, where pro-inflammatory cytokines activate indoleamine 2,3-dioxygenase (IDO) leading to greater tryptophan metabolism via this pathway and depletion of serotonin as a result (Dantzer *et al.*, 2011:426). In the striatum, no changes in 5-HIAA (Fig. A-4.2) or serotonin levels (Chapter 3) were seen between any of the groups, suggesting that striatal serotonin was unaffected by either model. In the hippocampus, neither LPS alone (LPS-S-S) nor the dual-hit model (LPS-MA-S) caused any significant changes in 5-HIAA levels, compared to the control group (S-S-S) (Fig. A-4.3). Interestingly, NAC increased 5-HIAA levels in the LPS-alone model (LPS-S-NAC) (Fig. A-4.3), confirms our earlier reasoning that glutamate may modulate serotonin levels in the hippocampus.

To conclude, the hippocampal monoamine data revealed a possible significant interaction between glutamate modulating agents (i.e. NAC) and serotonin function. DA was insignificantly suppressed by MA alone, LPS alone and in combination (LPS+MA), although NAC failed to reverse these alterations. Finally, data on three different monoamine metabolites (DOPAC, HVA and 5-HIAA) served to support our monoamine (DA and serotonin) findings in the different brain regions described in chapter 3.

To conclude, it is clear that the data presented here contributed to a lesser degree to the overall study, motivating our decision to exclude it from the article. However, these results still contribute to our current knowledge and could inspire future investigation.

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Addendum B: Methods of neurochemical and peripheral analyses

This addendum serves to provide the reader with supplementary information regarding the materials and methods used for the neurochemical analyses, as described in chapter 3.

B.1 Regional brain monoamine analysis

B.1.1 Introduction:

The quantification of regional brain dopamine (DA), serotonin (5-HT) and noradrenaline (NA), as well as their metabolites, were carried out using a high performance liquid chromatography (HPLC) system with electrochemical detection (HPLC-EC), according to an established method (Harvey *et al.*, 2006:881, Harvey *et al.*, 2010:1580). The monoamine concentrations were calculated by comparing the area under the peak of each monoamine to that of the internal standard, isoprenaline (range 5–50 ng/ml; Agilent 1200 series HPLC, equipped with an isocratic pump, autosampler, coupled to an ESA Coulochem Electrochemical detector and Chromeleon® Chromatography Management System version 6.8). Linear standard curves (regression coefficient greater than 0.99) were found in this specific range. Monoamine concentrations were expressed as ng/g wet weight of brain tissue.

B.1.2 Chromatographic conditions:

Analytical instrument:

An agilent 1200 series HPLC, equipped with an isocratic pump, autosampler, coupled to an ESA Coulochem III Electrochemical detector (with Coulometric flow cell) and Chromeleon® Chromatography Management System version 6.8 was used.

Column / stationary phase:

A unisol C18, 2.1 x 150mm, 5µm, 100Å (Agela Technologies, Wilmington, USA) column was used.

Guard column:

A 4.0 x 3.0 mm C18 SecurityGuard™, HPLC Guard Cartridge System, Phenomenex was used.

Mobile phase:

- 0.1 M (6.801g/L) sodium formate buffer
- 5 mM (1.01125g/L) sodium heptane sulphonic acid
- 0.17 mM (20 mg/L) ethylenediaminetetraacetic acid (EDTA disodium salt; Na₂EDTA)
- 40ml (4%) acetonitrile.

The pH of the mobile phase was set at ± pH 3.5-4.1 with orthophosphoric acid (85%). Important is to note that this method is highly pH sensitive.

Flow rate:

The flow rate was set at 0.20 ml/min.

Injection volume:

The total injection volume per sample was 10 µl.

EC Detector settings:

- ESA 5011A Analytical Cell Potential settings:
Volts: E1: +150 mV; E2: +750 mV
- Gain range: 500nA
- Polarity: Positive
- Reaction: Oxidation
- Guard cell potential setting: +350mV

B.1.3 Materials

Solution A preparation:

This solution was used in the preparation of all the standards.

Reagents:

- 0.5 mM sodium metabisulphite
- 0.3 mM Na₂EDTA
- 0.1 M perchloric acid (60% strong solution).

Preparation:

- 1) Dissolve 0.09505 g sodium metabisulphite and 0.111672 g Na₂EDTA in 800 ml distilled water.
- 2) Measure and add 10.87 ml of perchloric acid to the solution and make up to 1000 ml with distilled water.

Standard preparation:

- i. 3-Methoxy-4-hydroxyphenylglycol (MHPG):

MHPG hemipiperazinium salt = 454.5 MW

MHPG = 184.09 MW (40.50%)

Dissolve 2.47 mg MHPG hemipiperazinium salt in solution A, 40.50% of the 2.47 mg will present 1 mg of MHPG.

- ii. Noradrenaline (NA):

L-Noradrenaline hydrochloride = 205.6407 MW

Noradrenaline = 169.1798 MW (82.27%)

Dissolve 1.22 mg in solution A, 82.27% of the 1.22 mg will present 1 mg NA.

- iii. 3,4-Dihydroxyphenylacetic acid (DOPAC):

3,4-Dihydroxyphenylacetic acid = 168.15 MW

Dissolve 1 mg of DOPAC in solution A.

iv. 5-Hydroxyindole-3-acetic acid (5-HIAA):

5-Hydroxyindole-3-acetic acid = 191.19 MW

Dissolve 1 mg 5-HIAA in solution A.

v. Dopamine (DA):

3-Hydroxytyramine hydrochloride = 189.64 MW

Dopamine = 153.18 MW (80.77%)

Dissolve 1.24 mg in solution A, 80.77% of the 1.24 mg will present 1 mg DA.

vi. Homovanillic acid (HVA):

HVA = 182.18 MW

Dissolve 1 mg HVA in solution A.

vii. Serotonin (5-HT):

Serotonin creatinine sulphate or 5-hydroxytryptaminecreatinine sulphate = 405.43 MW

5-HT = 176.2 MW (43.46%)

Dissolve 2.30 mg in solution A, 43.46% of the 2.30 mg will present 1 mg 5-HT.

Internal standard preparation:

- 1) Dissolve 1 mg of 3,4-Dihydroxy-benzylamine in 10 ml of solution A. This is the stock solution for the internal standard.
- 2) Take 10 µl of the stock solution and make up to 2 ml with 0.25 M perchloric acid solution. This is the working internal standard solution with a final concentration of 500 ng/ml.

B.1.4 Sample preparation:

- 1) The pre-frozen brain samples were thawed and weighed and 1 ml of solution A was added to each tube.
- 2) Brain tissue was ruptured by sonication (2 x 12 seconds, at an amplitude of 14 µ).
- 3) Samples were left to rest on ice for 20 minutes to complete perchlorate precipitation of proteins and the extraction of monoamines.
- 4) Samples were then centrifuged at 4°C in an ultra-centrifuge for 20 minutes at 16 000 rpm (24 000 g).
- 5) The supernatant was subsequently removed and the sample pH adjusted to 5 by adding 1 ml of 10 M potassium acetate.
- 6) Using a pipette, 200 µl of the tissue extract was removed and placed in a new tube.
- 7) 20 µl of the internal standard was then added to each sample and vortexed.
- 8) The samples were then transferred to HPLC vials.
- 9) Finally, 10 µl of each final sample was injected into the HPLC column.

Results are expressed as ng/g wet weight of tissue.

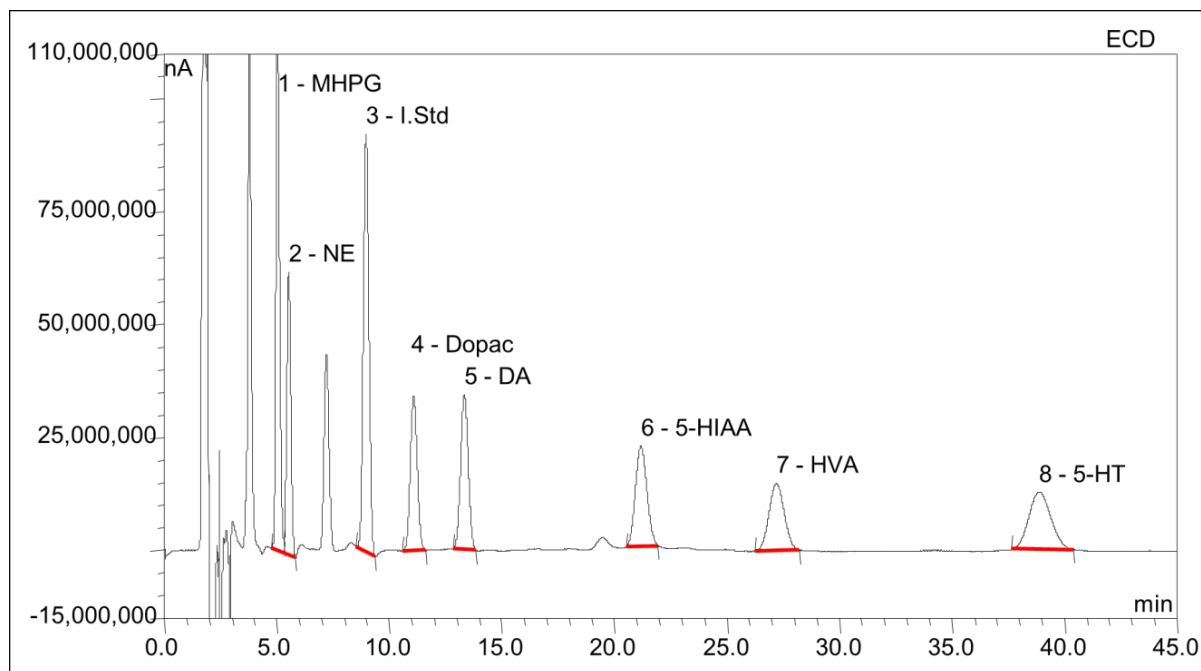


Figure B-1: Sample of chromatogram obtained from HPLC.

B. 2 Regional brain lipid peroxidation analysis

B.2.1 Introduction:

The Parameter™ TBARS assay from R&D Systems (Minneapolis, USA; catalogue number KGE013) was used to analyse lipid peroxidation in brain tissue. Oxidizing agents can alter lipid structures, which creates lipid peroxides that result in the formation of malondialdehyde (MDA). MDA can be measured as Thiobarbituric Acid Reactive Substances (TBARS), a common and convenient method for determining the relative lipid peroxide content of sample sets (Benzie, 1996:233; Ohkawa *et al.*, 1978:1053). Lipids that are multi-unsaturated (three or more double bonds) are both most likely to form peroxides and the most reactive in the TBARS assay (Benzie, 1996:233; Lykkesfeldt, 2007:50; Rael *et al.*, 2004:749). Free MDA is typically fairly low and requires the release of MDA by acid treatment of proteins and the breakdown of peroxides by both heat and acid to facilitate colour development in the TBARS reaction (Benzie, 1996:233; Lykkesfeldt, 2007:50; Meagher & Fitzgerald, 2000:1745). In addition, removal of proteins by precipitation eliminates potentially interfering amino acids that may react with thiobarbituric acid (Benzie, 1996:233; Meagher & Fitzgerald, 2000:1745).

The Parameter TBARS assay is a 2-3 hour chemical analysis designed to measure TBARS in cell culture supernates, cell lysates, serum, plasma, tissue and urine.

Principle of method:

When acid and heat is present, MDA reacts with TBA to produce a coloured end-product that absorbs light at 530-540 nm (indicated in Figure B-2). The intensity of the colour at 532 nm correlates to the level of lipid peroxidation in the sample. Unknown samples are compared to the standard curve.

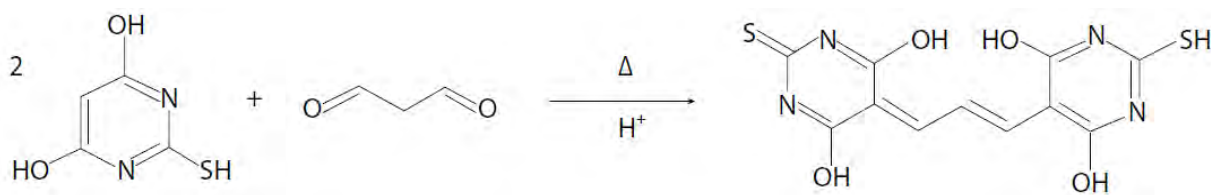


Figure B-2: *In the presence of acid and heat, two molecules of 2-thiobarbituric acid react with MDA to produce a coloured end-product that can be easily quantified.*

B.2.2 Materials:

Materials provided:

- Two 96 well, uncoated microplates (12 strips of 8 wells)
- TBA reagent: 15 ml of thiobarbituric acid in an aqueous solution.
- TBARS standard: 1 ml of 500 μ M 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
- Four plate sealers

Materials required:

- Microplate reader (absorbance at 530-532 nm)
- 45-50 $^{\circ}$ C incubator
- Microcentrifuge capable of $\geq 12,000 \times g$
- Deionized or distilled water
- Pipettes and pipette tips
- Microcentrifuge tubes for acid treatment
- Test tubes for dilution of standards

- TBARS Controls (optional; available from R&D Systems)

B.2.3 Sample preparation:

Preparation of phosphate buffered solution:

A phosphate buffered solution (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 Na₂HPO₄ 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 harvested 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 subsequently ultrasonically homogenized, after which the resulting homogenates were centrifuged at 800 x g for 10 min, at 4°C (Pereira *et al.*, 2009:188). The supernatants were then used for the TBARS assay (Župan *et al.*, 2008:771).

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 were added to a microcentrifuge tube and mixed.
- 2) The mixture was then incubated at room temperature for 15 minutes.
- 3) Sample mixtures were then centrifuged for 4 minutes at $\geq 12,000 \times g$.
- 4) The supernate of each sample mixture was carefully removed and retained.
- 5) Samples were assayed immediately.
- 6) The concentration read off the standard curve was multiplied by the dilution factor, 2.

B.2.4 Reagent preparation:

All reagents must be at room temperature before use

TBARS Standard:

The standard was converted to MDA by adding 100 μL of TBARS Standard to 200 μL of TBARS Acid Reagent. The standards were then left to rest for a minimum of 30 minutes with agitation. This produced a stock solution of 167 μM . Using a pipette, 900 μL of deionized water was placed into the 16.7 μM tube. 500 μL of deionized water was then placed in the remaining tubes. The stock solution was then used to produce a dilution series (Figure B-3). Pipette tips were changed after each transfer and every tube was thoroughly mixed. The 16.7 μM standard served as the high standard and deionized water served as the 0 μM standard.

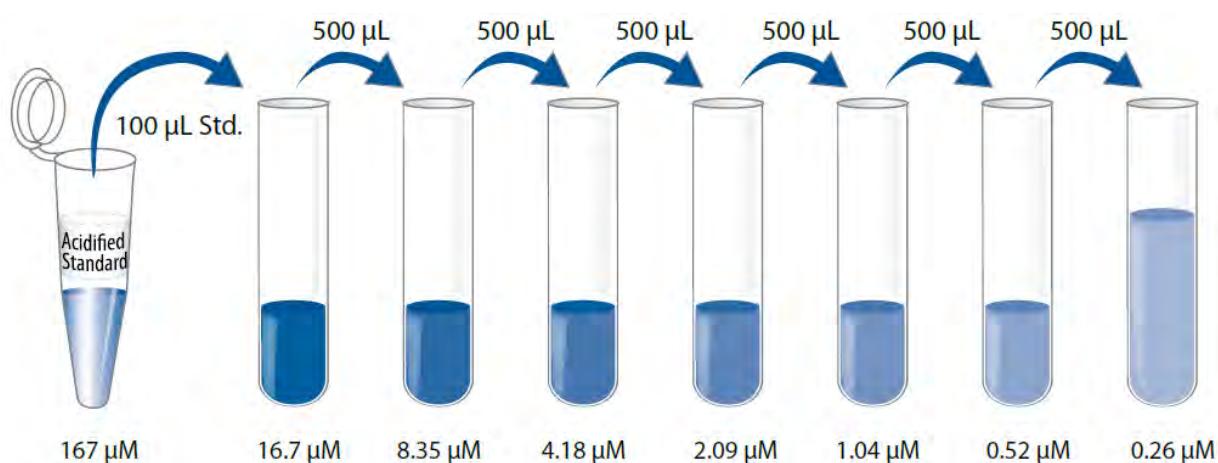


Figure B-3: Dilution series

B.2.5 Assay procedure:

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

- 1) Initially, all reagents, TBARS standards, and samples were prepared as discussed above.

- 2) Any excess microplate strips were removed from the plate frame and resealed in the original foil pouch for later use.
- 3) 150 µl of standards and samples were added to each well.
- 4) 75 µl of TBA Reagent was then added to each well.
- 5) The optical density of each well was pre-read using a microplate reader set to 532 nm.
- 6) The provided adhesive strip was then used to cover the microplate, which was then incubated for 2-3 hours at 45-50 °C.
- 7) The optical density of each well was determined using a microplate reader set to 532 nm.
- 8) Finally, the pre-reading was subtracted from the final reading to correct for the sample's contribution to the final absorption at 532 nm.

B.2.6 Calculation of results:

- 1) The optical densities obtained for each standard, sample and control, prior to the incubation with the TBA reagent, were subtracted from the optical densities for the same wells after incubation.
- 2) The corrected duplicate readings for each standard, control, and sample were then averaged.
- 3) Using computer software capable of generating a linear curve fit, a standard curve was created by reducing the data (Figure B-4).
- 4) Seeing that samples have been diluted, the concentration read from the standard curve have been multiplied by the dilution factor.

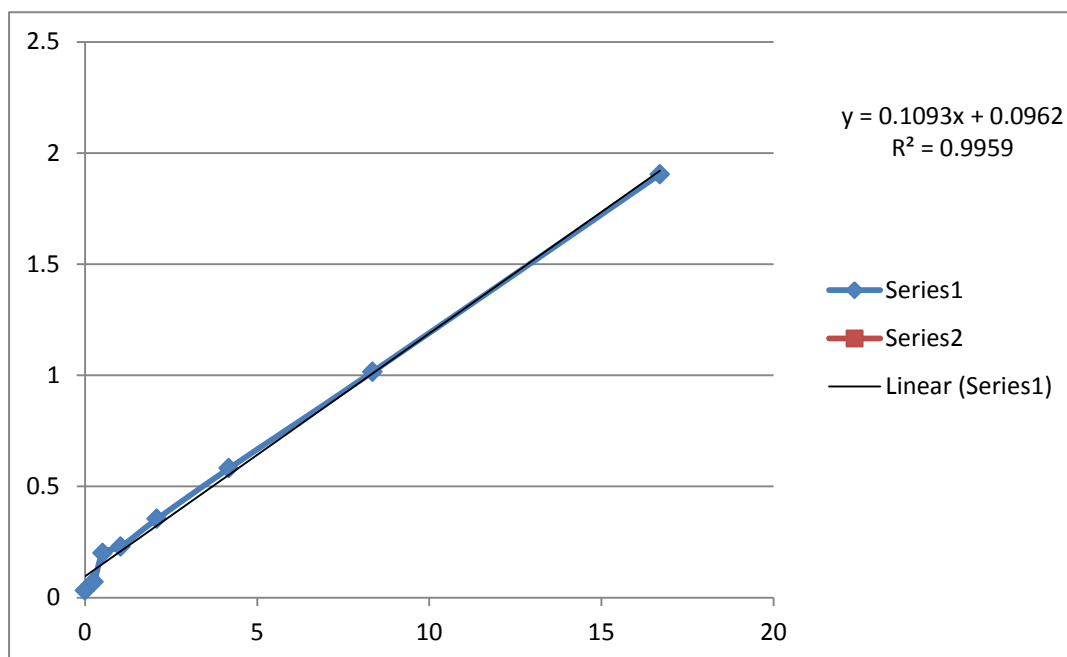


Figure B-4: Standard linear curve for lipid peroxidation.

B.3 Plasma reactive oxygen species analysis

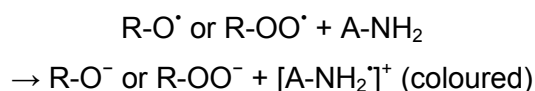
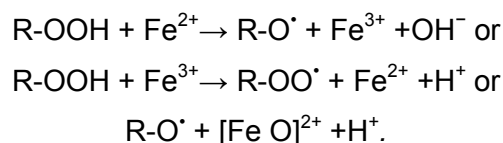
B.3.1 Introduction:

Reactive oxygen species (ROS) is a collective noun referring to a diversity of molecules and free radicals resulting from molecular oxygen (Turrens, 2003:335). When the balance between these oxidative species and antioxidant systems is tipped towards the aforementioned, oxidative stress develops and subsequent damage to DNA, lipids and proteins occur (Betteridge, 2000:3; Kohen & Nyska, 2002:620). The ROS relevant to biological systems include superoxide radical anion, hydroxyl radicals, hydrogen peroxide, alkoxy radicals, and peroxy radicals and singlet oxygen (Hayashi *et al.*, 2007:55). A well-established method for measuring oxidative stress in biological samples is the derivatives of reactive oxygen metabolites (dROM) test (Hayashi *et al.*, 2007:55). The improved version of the dROM test as suggested by Hayashi *et al.* (2007:55) was later adjusted by Botha in 2007 (unpublished results) and renamed the “ROS assay”. This assay facilitates automated analyses of numerous samples, increasing throughput, while still delivering accurate and reproducible results. In addition, the ROS assay requires reduced quantities of both reagents and samples.

Principle of the method:

In an acidic medium, ROS will react with transition metals (e.g. iron) to form alkoxy and peroxy radicals. These radicals are able to oxidize a chromogenic substrate, such as N,N-diethyl-para-phenylenediamine (DEPPD) to its corresponding radical cation. The concentration of the cation can then be measured at 546 nm using a microplate reader.

Reactions of the system (Hayashi *et al.*, 2007:55):



R-OOH is generic peroxide, R-O[•] is the alkoxy radical of a generic peroxide, R-OO[•] is the peroxy radical of generic peroxide, A-NH₂ is the chromogenic substrate (DEPPD) and [A-NH₂]^{•+} is the coloured radical cation of the chromogenic substrate (Hayashi *et al.*, 2007:55).

B.3.2 Materials:

Reagents:

- DEPPD
- Sodium acetate (anhydrous) (C₂H₃O₂Na)
- Hydrogen peroxide (H₂O₂)
- Ferrous sulphate (FeSO₄·7H₂O)

Solutions and buffer:

- Sodium acetate buffer (0.1 M, pH 4.8): Dissolve sodium acetate in ultrapure water (Milli-Q®) and adjust the pH to 4.8 using acetic acid.
- Hydrogen peroxide standard series (0; 60; 120; 180; 240 and 300mg/L): Dilute 30% hydrogen peroxide in ultrapure water to make up the standard series at different concentrations.

- Reagent 1 (R1): Dissolve DEPPD in the constituted sodium acetate buffer for a final concentration of 100 Mm and cover with aluminium foil.
- Reagent 2 (R2): Dissolve ferrous sulphate in the sodium acetate buffer for a final concentration of 4.37 μ M and cover with aluminium foil.

B.3.3 Methods and data calculation:

Firstly, 140 μ l sodium acetate buffer (0.1 M; pH 4.8) was added to each well of a 96-well microplate. Next, 2.5 μ l of either the different standard solutions or samples (rat plasma, thawed on ice) were added to each well (in duplicate). The plate was incubated at 30 $^{\circ}$ C for 10 minutes. A mixture of R1 and R2 was constituted at a ratio of 1:25 and incubated at 30 $^{\circ}$ C in a water bath for 10 minutes. 100 μ l of the mixed R1:R2 solution was added to each well of the microplate. Using the The Bio-Tek FL600 Microplate Fluorescence Reader (Bio-Tek, Instruments, Inc., Highland Park, Winooski, VT, USA), the absorbance was measured at 546 nm for a fixed time from 1 to 10 minutes at 1 minute intervals. ROS plasma levels were then extrapolated from the H_2O_2 standard curve as indicated in Figure B-6, and the following formula used to calculate ROS units:

1 Unit = 1.0 mg H_2O_2 /L

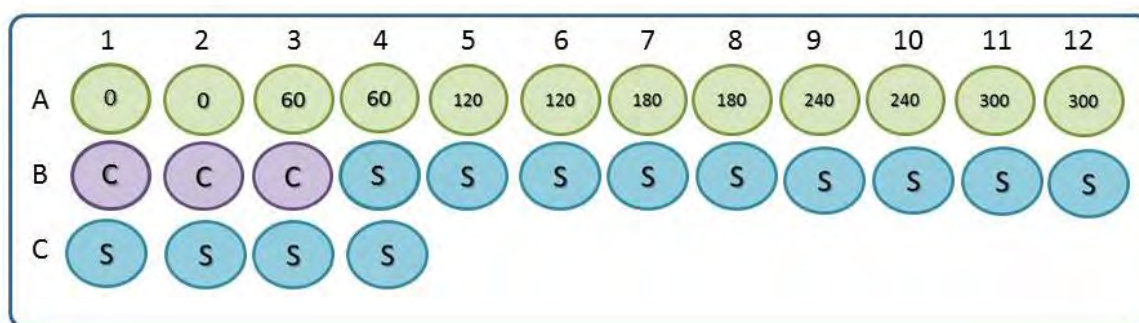


Figure B-5: Layout of 96-well plates for the ROS assay. A1-12 (in green): H_2O_2 standards; B1-3 (in purple): control samples; rest of the plate (in blue): samples in triplicate

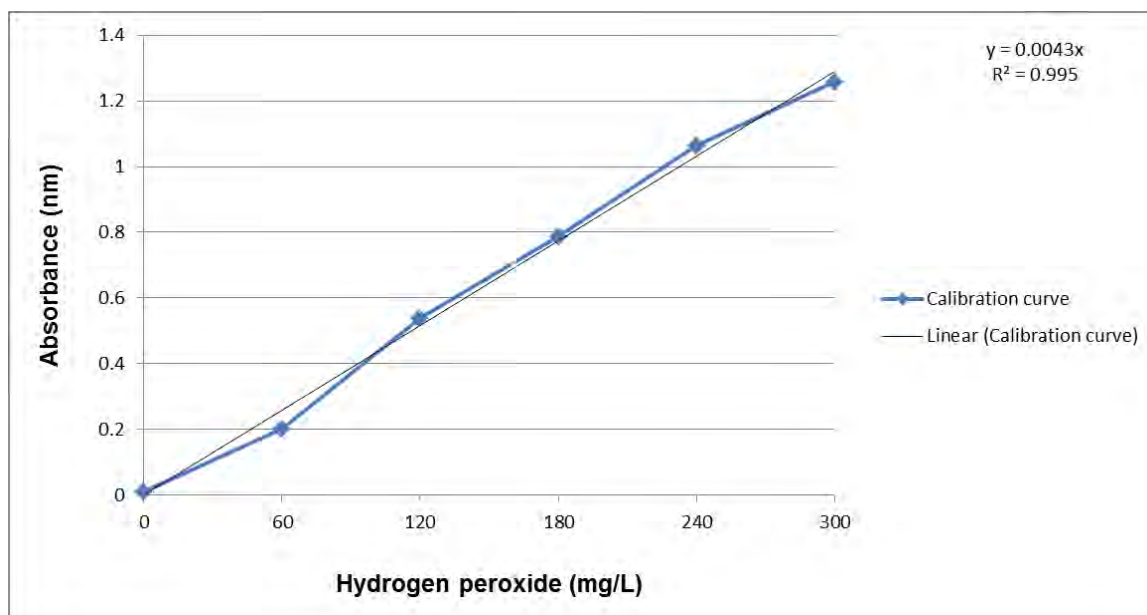


Figure B-6: ROS calibration curve

B. 4 Plasma tumor necrosis factor alpha analysis

B.4.1 Introduction:

Plasma tumour necrosis factor- α (TNF- α) was measured by means of the Rat TNF- α ELISA MAX™ Deluxe Set (catalogue number 438204) from Bio Legend (San Diego, USA). TNF- α is a powerful and multifunctional cytokine that is able to exert regulatory, inflammatory and cytotoxic effects on a wide range of cells.

Principle of method:

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 TMB substrate solution, producing a blue colour in proportion to the concentration of TNF- α present in the sample. Finally, 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.4.2 Materials

Materials provided:

- 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 MaxisorpTM 96 MicroWell Plates

Materials required:

- PBS (Phosphate-Buffered Saline): 8.0 g NaCl, 1.16 g Na₂HPO₄, 0.2 g KH₂PO₄, 0.2 g KCl, add deionized water to 1 L; pH to 7.4, 0.2 μ m filtered.
- Wash Buffer: BioLegend Cat. No. 421601 is recommended, or PBS + 0.05% Tween-20.
- Stop Solution: BioLegend Cat. No. 423001 is recommended, or acid solution, e.g. 2N H₂SO₄
- 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 μ l 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.4.3 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.4.4 Assay procedure:

- 1) One day prior to running the ELISA, the Capture Antibody in 1X Coating Buffer was diluted as described in Reagent Preparation. Then, 100 µl of this Capture Antibody solution was added to all wells of the 96-well plate provided in this kit. The plate was 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 were run in duplicate. A standard curve is required for each assay.
- 3) The plate was 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 were performed similarly.
- 4) To block non-specific binding and reduce background, 200 µl 1X Assay Diluent A were added to each well.
- 5) The plate was 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 were performed similarly.
- 6) While the plate is being blocked, the standards and samples were prepared (sample dilutions were not necessary).
- 7) The plate was then washed 4 times with wash buffer.
- 8) For measuring serum and plasma samples: 50 µl Matrix Diluent A was added to the standard wells and 50 µl Assay Diluent A to the sample wells. Then, 50 µl standard were added to the standard wells or 50 µl sample to the sample wells.
- 9) The plate was sealed and incubated at room temperature for 2 hours with shaking.
- 10) The plate was washed 4 times with wash buffer.

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

B.4.5 Calculation of results:

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

B. 5 Plasma interleukin-10 analysis

B.5.1 Introduction:

Levels of interleukin-10 (IL-10) were measured using the Rat IL-10 ELISA Kit (catalogue number RAB0246-1KT) from Sigma-Aldrich (Johannesburg, South Africa). IL-10, expressed by various immune cells, inhibits macrophage-mediated cytokine synthesis, activates the response of the T-helper cell, TH2 and suppresses of the delayed-type hypersensitivity response.

B.5.2 Materials:

Materials provided:

- Rat IL-10 Antibody-coated ELISA plate (Item A)
- 20X Wash buffer (Item B)
- Lyophilized rat IL-10 protein standard (Item C)
- ELISA 1X Assay/Sample Diluent Buffer A (Item D1)
- ELISA 5X Assay/Sample Diluent Buffer B (Item E1)
- Biotinylated Rat IL-10 Detection Antibody (Item F)
- HRP-Streptavidin (Item G)
- ELISA Colorimetric TMB reagent (HRP substrate) (Item H)
- ELISA Stop solution (Item I)

Materials required:

- Microplate reader capable of measuring absorbance at 450 nm
- Precision pipettes (2 μ l – 1 ml)
- Adjustable pipettes (1-25 ml)
- 100 ml and 1 L graduated cylinders
- Absorbent paper
- Distilled / deionized water
- Software capable of performing four-parameter logistic regression models
- Tubes for standard and sample preparation

B.5.3 Methods

Reagent preparation

- 1) Dilute plasma samples 2 fold with Assay Diluent A (Item D).
- 2) Dilute Assay Diluent B 5-fold with deionized water.
- 3) Briefly spin the vial containing Item C and then add 400 μ l Assay Diluent A into the vial of Item C to prepare a 100ng/ml standard. Gently mix to dissolve the powder.
- 4) Add 60 μ l IL-10 standard from the vial of Item C into a tube with 940 μ l Assay Diluent A to prepare a 6,000 pg/ml stock standard solution.
- 5) Pipette 400 μ l Assay Diluent A into each tube.
- 6) Use the stock standard solution to produce a dilution series (Figure B-7). Mix thoroughly. Assay Diluent A serves as the zero standard (0 pg/ml)

- 7) Dilute 20 ml of Item B concentrate into deionized / distilled water to yield 400 ml of 1x Wash Buffer.
- 8) Briefly spin Item F and then add 100 μ l of 1x Assay Diluent B to prepare a detection antibody concentrate. Pipette up and down to gently mix.
- 9) Briefly spin Item G and pipette up and down to mix. Then, dilute Item G 140-fold with 1x Assay Diluent B.

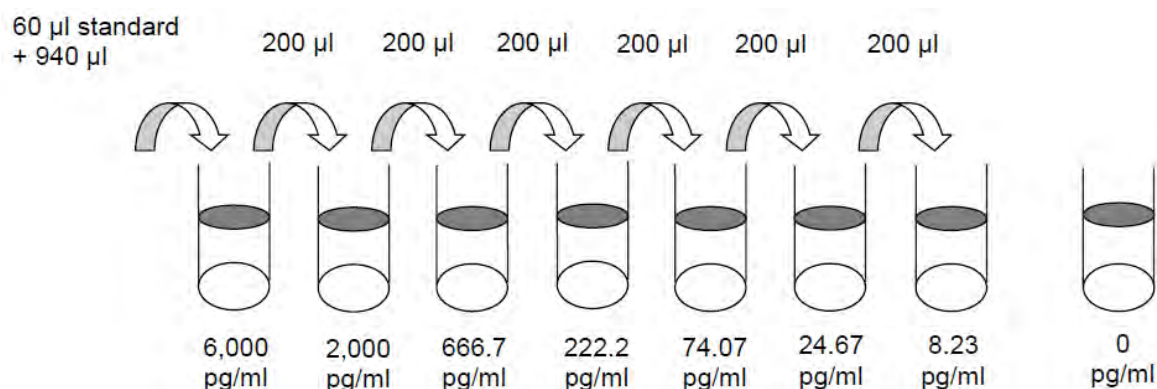


Figure B-7: Dilution series for standards

B.5.4 Assay procedure:

- 1) All reagents and samples were at room temperature before use. Standards and samples were run in duplicate.
- 2) 100 μ l of each standard and sample (thawed on ice and diluted 2-fold) were added into appropriate wells. Plate was then covered and incubated at room temperature for 2.5 hours.
- 3) The solution was discarded and washed 4 times with 1 x Wash Solution. After the last wash, any remaining Wash Buffer was removed by aspirating or decanting. The plate was inverted and blotted against clean paper towels.
- 4) 100 μ l of 1x prepared Biotinylated Detection Antibody was added to each well and incubated at room temperature for 1 hour with gentle shaking.
- 5) The solution was then discarded and washed as in step 3.
- 6) 100 μ l of ELISA Colorimetric TMB Reagent was added to each well and incubated at room temperature in the dark for 30 minutes with gentle shaking.
- 7) 50 μ l of Stop Solution was added to each well and read immediately at 450 nm.

B.5.5 Calculation of results:

The mean absorbance for each set of duplicate standards, controls and samples were calculated and the average zero standard optical density subtracted. The standard curve was plotted using appropriate software, with standard concentration on the x-axis and absorbance on the y-axis. The best-fit curve was drawn through the standard points.

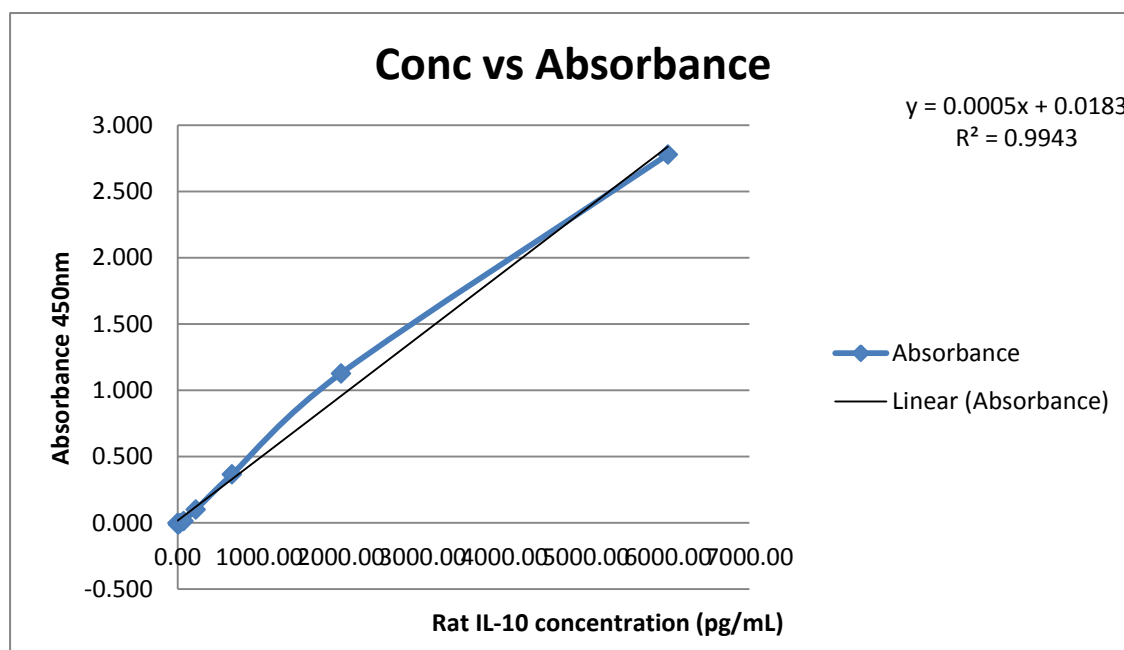


Figure B-8: Standard linear curve for IL-10

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