

Oxidative status in rats exposed to social isolation
rearing: Behavioral pharmacology studies and relevance
for schizophrenia

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“Ek is tot alles instaat deur Hom wat my krag gee.”

Phil 4:13

“I can do everything through Him who gives me strength.”

Abstract

Purpose:

Psychotic (positive) symptoms are the most distinctive feature of schizophrenia, although negative symptoms such as emotional flattening, social withdrawal and cognitive disturbances are the most treatment resistant manifestation of the illness. Schizophrenia is a progressive degenerative illness that has been causally linked to environmental and neurodevelopmental factors, as well as dysfunctional redox balance. Validated animal models are useful in identifying and studying novel neurobiological targets for neuropsychiatric illnesses. Post weaning social isolation rearing (SIR) in rats has been proposed to model the neurodevelopmental aspects of schizophrenia. We validated the SIR model with respect to effects on sensorimotor gating and social interaction, deficits of which are core symptoms of schizophrenia. Following this, effects on the levels of oxidative stress were determined in the frontal cortex and striatum of rats exposed to SIR, two brain regions strongly implicated in the pathology of schizophrenia. Finally, in order to more closely relate these bio-behavioral changes to the human condition, we studied the overall effect of sub-chronic treatment with the atypical antipsychotic, clozapine, on the above described behavioural and neurochemical parameters.

Methods:

Male Sprague-Dawley (SD) rats (10 rats/group) were used. In a non-treatment arm, four groups of rats were randomly separated at weaning and exposed to either 8 weeks SIR or 8 weeks social rearing. At the respective time point of 8 weeks, two groups were subjected to behavioral testing of mean startle amplitude (at 120dB) and percentage prepulse inhibition (%PPI) of the acoustic startle (AS) reflex (at 72, 76, 80 and 86dB prepulse), and various social interactive and self-directed behaviors were accessed using the open field test (OFT). The remaining two groups were sacrificed at 8 weeks and brain tissue was harvested for analysis of superoxide dismutase activity, oxidized (GSSG) versus reduced

GSH) glutathione ratio, and levels of lipid peroxidation, in the frontal cortex and striatum. In the treatment arm, consisting out of eight groups of animals, four groups of SIR rats received either saline or clozapine (5mg/kg i.p.) for the last 11 days of SIR. The remaining four groups were socially reared and also received either saline or clozapine treatment as above. At 8 weeks, four groups were subjected to behavioral testing as described above and a parallel neurochemical study was performed using the same layout as above, except that after the 8 weeks, neurochemical redox analysis were done as described above. Mixed statistical modeling with repeated measures and appropriate post hoc tests were used to access the effects of SIR with and without treatment on PPI and mean startle. Social interaction in SIR and socially reared animals, with and without treatment, was analyzed using 1-way ANOVA with suitable post hoc testing. Mixed linear models with repeated measures and appropriate post hoc tests were used for analysis of the redox data in SIR and socially reared animals, with and without treatment.

Results:

In the non-treatment arm, %PPI was significantly reduced in SIR versus socially reared rats. Deficits in various social interactive behaviors were observed in SIR versus group-housed rats, as well as increased locomotor activity and self-grooming. Superoxide dismutase activity and oxidized versus reduced glutathione ratio were significantly decreased, together with a significant increase in products of lipid peroxidation, in isolation reared versus socially reared rats.

Following clozapine treatment, %PPI in isolates was significantly elevated by clozapine versus saline treatment (i.e. reversed the effect of SIR). %PPI was unaltered in socially reared animals receiving either treatment. As with the non-treatment group, social interactive behaviors were significantly impaired in isolates receiving saline, while locomotor activity and self-grooming were increased. SIR rats receiving only saline showed similar altered redox state as the non-treatment groups, while clozapine treatment effectively reversed deficits in %PPI, aberrant social behaviors and redox alterations in the SIR rats, with limited to no effects in the socially reared controls.

Conclusion:

SIR thus significantly disrupts sensorimotor gating and social behaviors in male Sprague-Dawley rats, while at the same time evokes a significant disruption of redox state in both the frontal cortex and striatum of these animals, with distinct evidence for increased oxidative stress in these brain regions. Importantly, both altered behavior and redox state are reversed by sub-chronic clozapine treatment. SIR is therefore a useful, non-lesion and non-pharmacological neurodevelopmental animal model of schizophrenia that presents with robust face, predictive and possibly construct validity for schizophrenia.

Keywords: social isolation, prepulse inhibition, social interaction, clozapine, schizophrenia animal model.

Opsomming

Doel:

Positiewe simptome soos psigosos oorheers dikwels die beeld van skisofrenie, alhoewel die negatiewe simptome soos kognitiewe en sosiale versteurings dikwels die moeilikste is om te behandel. Skisofrenie is 'n progressiewe degeneratiewe siekte, wat moontlik veroorsaak word deur omgewings- en senuwee-ontwikkelingsfaktore, asook 'n versteurde redoksbalans. Dieremodelle wat gevalideer is, is dus nodig om nuwe en nuttige neurobiologiese merkers vir psigiatriese siektes te identifiseer en te ondersoek. 'n Model wat die senuwee-ontwikkelingsaspekte van skisofrenie moontlik kan voorstel, is sosiale isolasie-geïnduseerde stres (SSI) by pas- gespeende rotte. Ons het die SSI -model gevalideer met betrekking tot sensoriese motoriese seleksie en sosiale interaksie, twee kernsimptome van skisofrenie. Die effek van oksidatiewe stres is hierna geëvalueer in twee breinareas wat sterk geïmpliceer word in die patologie van skisofrenie, nl. die frontale korteks en striatum van die SSI rotte. Om 'n moontlike verwantskap te vind tussen die bio-gedragsveranderinge en die menslike siekte, het ons die algemene effek van sub-kroniese behandeling met die atipiese antipsigotiese middel, klosapien bestudeer op bogenoemde parameters.

Metodes:

Manlike Sprague-Dawley (SD) rotte is gebruik (10 rotte / groep). Die diere wat geen behandeling ontvang het nie, is met spening onwillekeurig in vier groepe verdeel en blootgestel aan 8 weke se SSI of groeps-(sosiale) behuising. Na die 8 weke is twee van hierdie groepe onderwerp aan die volgende gedragstoets: persentasie prepulsinhibisie (% PPI) van die skrikreaksie (teen 72, 76, 80 en 86dB prepuls) en verskeie sosiale interaksie, asook selfgerigte gedragspatrone in die oop- veld toets (OVT). Die oorblywende twee groepe is onthoof na 8 weke, die breine is verwyder en die aktiwiteit van superoksied dismutase (SOD), geoksideerde teenoor gereduseerde glutatioon verhouding (GSSG/GSH), en die vlakke van

lipiedperoksiedasie is bepaal in die frontale korteks en striatum. By die behandelde groep diere is agt groepe rotte gebruik, vier van die groepe is geïsoleer (SSI) vir 8 weke, en met 'n soutoplossing of klosapien (5 mg/kg) behandel vir die laaste 11 dae van SSI. Die ander vier groepe is in groeps- (sosiale) behuising geplaas vir 8 weke en het ook 'n soutoplossing of klosapienbehandeling ontvang soos hierbo beskryf. Na 8 weke was vier van die behandelingsgroepe onderwerp aan gedragstudies, soos hierbo beskryf, terwyl daar terselfertyd 'n parallelle neurochemiese studie (redoks-analise) gedoen is op die oorblywende vier groepe soos hierbo beskryf.

Die effek van SSI met of sonder behandeling op PPI en gemiddelde skrikreaksie is geanaliseer met behulp van gemengde statistiese modelle, gevolg deur toepaslike post hoc- toetse. Een- rigting ANOVA met geskikte post hoc toetse is gebruik om die effek van SSI met of sonder behandeling te analiseer met betrekking tot die sosiale interaksie gedragspatrone. Om die effek op die redoksbalans te analiseer in die SSI rotte met of sonder behandeling, is gemengde lineêre modelle met herhaalde metings en geskikte post hoc toetse gebruik.

Resultate:

In die diere wat geen behandeling ontvang het nie, is 'n betekenisvolle verlaging in % PPI in die SSI -rotte opgemerk in vergelyking met die rotte wat in groepe (sosiaal) gehuisves was. Betekenisvolle verskille is ook tussen hierdie twee groepe rotte gevind in verskeie sosiale interaksie gedragspatrone wat 'n toename in lokomotoriese aktiwiteit en selfversorgings-bewegings in die SSI- rotte in vergelyking met die groeps- (sosiaal) gehuisveste rotte. Daar was ook 'n betekenisvolle verlaging in die mate van SOD- aktiwiteit en GSSG/GSH verhouding met 'n betekenisvolle verhoging in lipiedperoksidase in die SSI- rotte in vergelyking met die sosiaal- gehuisveste rotte. In die diere wat behandeling ontvang het, is 'n betekenisvolle verhoging in % PPI opgemerk in die SSI- rotte wat klosapien ontvang het in vergelyking met die wat net soutoplossing ontvang het. % PPI was onveranderd in die sosiaal- gehuisveste rotte ongeag of 'n soutoplossing of klosapien toegedien is. Soortgelyk aan die onbehandelde diere, is 'n betekenisvolle verlaging in sosiale interaksie en verhoging in lokomotoriese aktiwiteit en

selfversorgingsbewegings verkry in die SSI -rotte wat slegs die soutoplossing ontvang het. Die SSI- rotte wat slegs die soutoplossing ontvang het, het ook dieselfde veranderinge in redoksbalans getoon as die SSI -rotte wat geen behandeling ontvang het nie, terwyl klosapien die veranderinge in % PPI, sosiale gedragsspatrone en redokswanbalans in die SSI- rotte suksesvol omgekeer het, met min tot geen effekte in die sosiaal- gehuisveste kontrole rotte.

Gevolgtrekking:

Sensoriese motoriese seleksie en sosiale gedragsspatrone is dus betekenisvol ontwig in manlike Sprague Dawley rotte wat blootgestel is aan SSI. SSI het ook betekenisvolle veranderinge in redoksbalans veroorsaak in beide die frontale korteks en striatum van die rotte, wat op 'n verhoogte oksidatiewe stresreaksie in die spesifieke breinareas dui. Belangrik is dat sub-kroniese klosapien hierdie gedrag- en redoksveranderinge suksesvol omgekeer het. SSI kan derhalwe beskou word as 'n nuttige, senuwee-ontwikkelingsmodel van skisofrenie, wat nie deur farmakologiese of senuwee-ontwikkelingstrauma veroorsaak is nie. SSI voldoen ook aan validasie vereistes t.o.v gesig-, voorspelbare- en moontlike konstruktiewe waarde vir skisofrenie.

Sleutelwoorde: sosiale isolasie, pre-puls inhibisie, sosiale interaksie, klosapien, skisofrenie, dieremodel.

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REARING INDUCED CHANGES IN FRONTAL
CORTICAL N-METHYL-D-ASPARTATE AND D1
RECEPTOR BINDING IN RATS.**

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List of Abbreviations

5HT₋₂: Serotonin 2

Ach: Acetylcholine

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

CAT: Catalase

CNS: Central nervous system

D₁₋₄: Dopamine 1-4 receptor

DA: Dopamine

DOPAC: 3,4-dihydroxyphenylacetic acid

DSM-IV: Diagnostic and statistical Manual of Mental Disorders 4th edition.

DUP: Duration of untreated psychosis

EPS: Extrapyramidal side effects

GABA: γ -amino butyric acid

GSH: Oxidized glutathione

GSH-Px: Glutathione peroxidase

GSSG: Reduced glutathione

H₂O₂: Hydrogen peroxide

LCMS: Liquid chromatography, mass spectrometry

LI: Latent inhibition

LS: Limbic system

LSD: Lysergic acid diethylamide

MAO: Monoamine oxidase

MDA: Malondialdehyde

MRI: Magnetic resonance imaging

NAA: N-acetylaspartate

NAcc: Nucleus accumbens

NAC: N-acetyl L-cystein

NBT: Nitro blue tetrazolium

NICE: National Institute for Health and Clinical Excellence

NMDA: N-methyl-D-aspartate

NO: Nitric oxide

O₂⁻: Superoxide

OFT: Open field test

•OH: Hydroxyl radical

ONOO⁻: Peroxynitrite

PBS: Phosphate buffered saline

PCP: Phencyclidine

PET: Positron emission tomography

PFC: Prefrontal cortex

PND: Post natal day

PPI: Prepulse inhibition

PUFAs: Poly-unsaturated fatty acids

RNS: Reactive Nitrogen species

ROO⁻: Peroxyl radical

ROS: Reactive oxygen species

SD: Sprague-Dawley

SOD: Superoxide dismutase

TBA: Thiobarbituric acid

TBARS: Thiobarbituric reactive substances

TD: Tardive dyskinesia

VTA: Ventral tegmentum area

XO: Xanthine oxidase

Chapter 1

Introduction

1. Problem statement

On a global scale, schizophrenia is among the top ten causes of disease related, long-term disability (World Health Organization, 2001), with about 1% of the population affected by schizophrenia, with similar rates across different countries, cultural groups and sexes (Weiss & Feldon, 2001). The illness becomes evident between the ages of 16 and 30 years, and for the most part will persist throughout the patient's lifetime (Seisdedos et al., 1999). The impact of the illness on the patient, his/her family and on health care providers is enormous. Approximately 50% of discharged patients will be rehospitalised within a year (Rabinowitz et al., 2001). Less than 20% of schizophrenia patients are employed at one time and 12% of patients with paranoid subtype schizophrenia will commit suicide (Fenton et al., 1997). More staggering is that 20% of patients experience a relapse despite antipsychotic medication, while approximately two-thirds of patients on typical antipsychotic medication for schizophrenia experience persistent Parkinsonism (Harvey et al., 1999). Schizophrenia patients are also stigmatized, probably more than with any other mental disease (Carpenter & Koenig, 2008).

The symptoms of schizophrenia mainly consist of positive symptoms that include hallucinations, delusions and thought disorder, and negative symptoms comprised of affective flattening and social withdrawal, and profound cognitive deficits in attention, learning, memory and behavioural flexibility (Ross et al., 2006, Lewis & Gonzales-Burgos, 2008). Recent research has indicated that while antipsychotics (typical and atypical) are effective for controlling positive symptoms, negative symptoms remain a major problem. This has prompted a greater demand for research into improved treatments that not only address the positive symptoms of schizophrenia, but especially the negative and cognitive symptoms (Keefe et al., 2006).

An important symptom in patients with schizophrenia is the inability to filter sensory information and is ascribed to deficits in sensory motor-gating, presenting with hyperalertness and poor discrimination (Martinez et al., 2002). One operational measurement of altered sensory motor-gating is the prepulse inhibition (PPI) paradigm (Martinez et al., 2002).

Numerous neuroanatomical and neurochemical hypotheses involving the pathophysiology of schizophrenia have been developed, with hyper-dopaminergia being the most well-supported hypothesis. Recently, however, the emergence of glutamate as an important neurotransmitter in the neurobiology of schizophrenia has received significant attention (Goff & Coyle, 2001). Current evidence supports the view that the dysfunctional state of glutamate in schizophrenia is region specific, with the cortical brain regions predominantly hypoglutamatergic (Hirsch et al., 1997), but with hyperglutamatergia in the sub-cortical regions (Konradi & Heckers, 2003 for review). Altered glutamate activity not only will impact on the illness by its ability to modify the release of dopamine in the cortex and striatum (Konradi & Heckers, 2003), but is also essential for regulating the expression of anti-oxidant enzymes and to strongly influence regional redox balance in the brain (Smythies, 1999). Recent studies indicate that increased oxidative stress may be an important cornerstone in the development of schizophrenia, with studies describing an increase in lipid peroxidation products (thiobarbituric reactive substances, or TBARS; Akyol, 2002) in the blood of schizophrenia patients, as well as an increase in catalase (CAT) activity and decreased superoxide dismutase (SOD) activity (Rachkauskas, 1998).

Early life adversity has been found to affect neuronal growth and differentiation (Bloom, 1993; Murray, 1994; Weinberger, 1987; Weiss and Feldon, 2001) and is deemed an important risk factor for the later development of schizophrenia (Lipska & Weinberger, 2000). Early adverse experiences may “shape” a pre-existing genetic vulnerability to stress and disease (Heim & Nemeroff, 2001). Schizophrenia has been causally linked to genetic, environmental and neurodevelopmental factors (Weiss & Feldon, 2001), with epidemiological studies showing increased incidence of schizophrenia in patients subjected to different forms of pre- and perinatal stress (Van den Buuse et al., 2003). However, it is not yet fully understood how exposure of the brain to adverse events during early development contributes to the expression

and/or exacerbation of the physical and psychological aspects of schizophrenia (Lipska & Weinberger, 2000).

In order to closely model the neurodevelopmental hypothesis of schizophrenia and to glean more knowledge on the role and identity of neurodevelopmental factors in the pathogenesis of schizophrenia, the development of well-validated analogous animal models of schizophrenia are needed. Not only will these animal models aid in our understanding of the illness, but will be invaluable in identifying new targets suitable for rational drug development. One animal model that closely resembles the neurodevelopment aspects of schizophrenia is the social isolation rearing (SIR) model. Like schizophrenia, SIR in rodents presents with altered sensory-motor gating deficits that is amenable to treatment with antipsychotic drugs, which provides the model with important face and predictive validity for the illness (Weiss and Feldon, 2001). However, it is incumbent upon behavioural neuroscientists to now extend the model to new levels of validation, particularly with respect to more robust behavioral assessment that will more closely portray the diverse behaviors typical of schizophrenia, as described earlier. Furthermore, it is critical to now consider aspects of construct validity, which ultimately will provide much needed detail concerning the underlying causal factors in schizophrenia.

2. Project hypothesis, aim and objectives

Hypothesis:

Earlier papers have extensively studied the face validity of SIR in rodents by determining sensory motor gating changes (prepulse inhibition of startle, or PPI) (Geyer et al., 1993; Varty & Geyer, 1998; Weiss et al., 2000), a measure of cognitive performance, as well as locomotor activity (Weiss et al., 2000) and explorative behavior (Ferdman et al., 2007). However, another important aspect of schizophrenia is the pronounced deficits in social behavior (Heidbreder et al., 2000; Pinkham et al., 2003), so that studying social interactive behaviors in rats following SIR would be a valuable criterion for face validity. Moreover, to simultaneously study SIR-induced changes on more than one behavior akin to schizophrenia would raise

the level of face validity significantly. Consequently, we propose that SIR in rats will induce significant deficits in inwardly- and outwardly directed social behaviors, as well as simultaneously suppress PPI. Moreover, we propose that sub-chronic treatment with clozapine will rectify these behavioral changes similar to that observed in socially housed animals. Since recent evidence has highlighted the importance of altered redox state in schizophrenia (Do et al., 2009; Akyol, 2002), we also propose that the above-mentioned behavioral changes induced by SIR are accompanied by an increase in regional brain oxidative stress. Finally, we suggest that sub-chronic treatment with clozapine, that is effective in addressing the altered behaviors seen in SIR rats, will simultaneously reverse altered brain redox state in SIR animals.

Study Aims:

The first aim of this study is to investigate whether SIR in rats is associated with deficits in cognitive and social behaviors, and whether these behaviors are causally related to altered redox state in the frontal cortex and striatum of rats reared in isolation. This latter study will address novel aspects of face and construct validity for the model. The second aim will be to study the predictive validity of the model by determining whether sub-chronic treatment with clozapine can reverse the above-mentioned behavioral changes observed in rats reared in isolation, as well as reverse any changes in cortico-striatal redox balance.

Objectives of this study:

- To establish whether 8 weeks of SIR in rats induces deficits in inwardly- and outwardly-directed social interactive behaviors, as well as deficits in cognitive performance, compared to socially reared animals using the open field test (OFT) and PPI of startle, respectively.
- To establish whether any changes in sensory-motor gating (i.e. PPI) and social interactive behaviors induced by SIR are accompanied by regional brain changes in redox state, as determined by superoxide dismutase activity, oxidized vs. reduced glutathione levels, and accumulation of products of lipid peroxidation, in the frontal cortex and striatum of rats reared in isolation versus socially reared controls.

- To determine whether any changes in sensory-motor gating (i.e. PPI) and social interactive behaviors induced by SIR can be reversed by sub-chronic treatment with the atypical antipsychotic, clozapine (5 mg/kg/day x 11 days).
- To establish whether sub-chronic treatment with clozapine (5 mg/kg/day x 11 days) can reverse any observed changes in regional brain redox state in isolation reared animals.

3. Project layout

The study will consist of two arms, a non-treatment cohort and a treatment cohort. Male Sprague-Dawley rats (10/group) will be used throughout the study.

3.1 The non-treatment cohort.

As indicated in Figure 1, for the behavioral study animals will be randomly separated at weaning (post natal day (PND) 21) into two groups. One group of animals (n=10) will be placed into isolation. A parallel socially reared control group (n=10) will be run concurrently where animals will be group housed and only exposed to normal daily handling for 8 weeks. At 8 weeks after PND 21, both groups of animals will be subjected to behavioral testing of PPI and social interaction, with one day of rest between the PPI and the OFT test. Animals will be sacrificed immediately thereafter.

For the neurochemical study, an additional two groups of animals will be randomly assigned to the same groupings as described above, following the above described protocol except that at 8 weeks the animals will be sacrificed and the brains rapidly dissected for regional brain redox analysis.

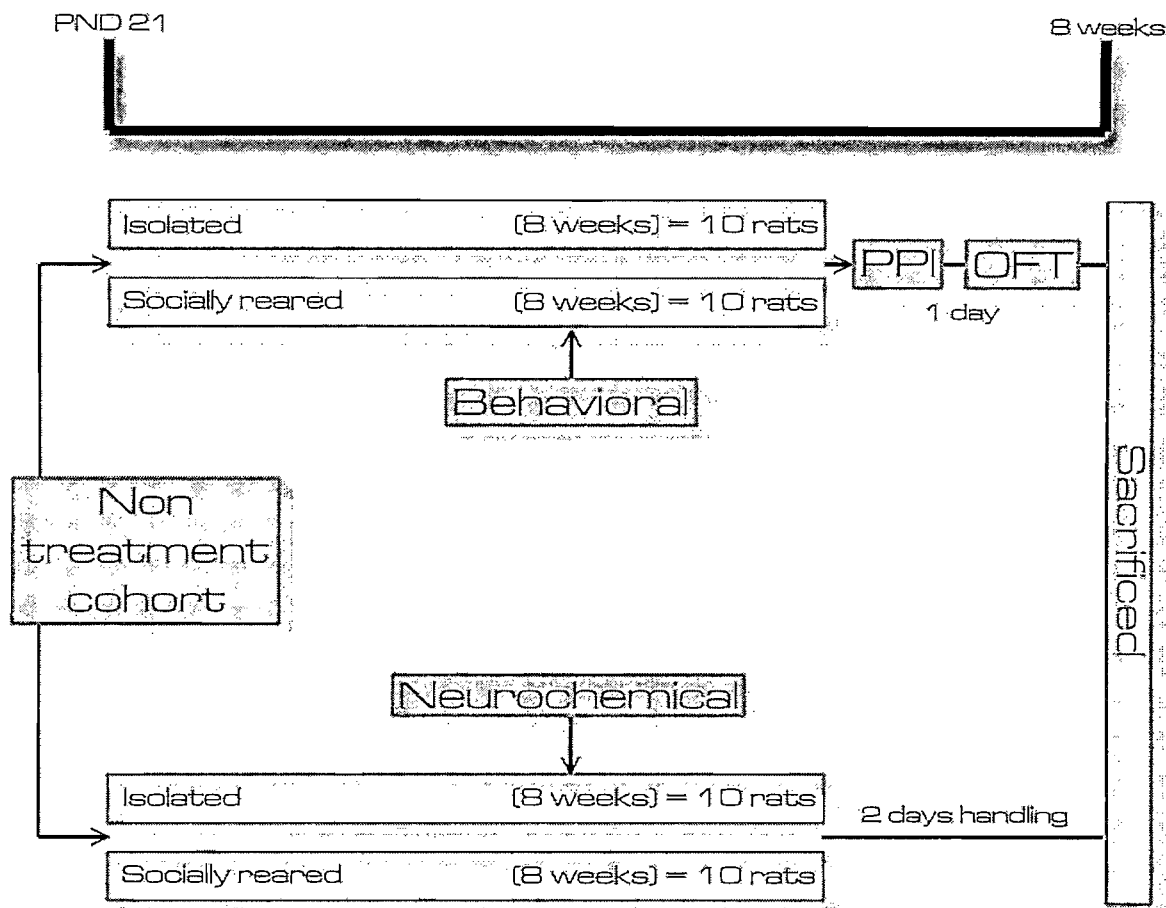


Figure 1: Study design for the behavioral and neurochemical studies in SIR and socially reared rats in the non-treatment cohort.

3.2 The treatment cohort:

As indicated in Figure 2, animals will be separated at weaning into four groups for behavioral testing. Two of these groups will be set aside for 8 weeks social isolation rearing and will receive either saline or clozapine treatment (5 mg/kg/day ip x 11 days), both using a maximum volume of administration of 0.5 ml. The remaining two groups will be socially reared for 8 weeks, and will receive either saline or clozapine treatment, as described above. Animals will be treated with drug or saline during the last 11 days of SIR, as per our previous protocol (Toua et al., 2009). At 8 weeks after

PND 21, the four groups of animals will be subjected to behavioral testing in the OFT and for PPI, with one day of rest between the OFT and PPI tests. Animals will be sacrificed immediately thereafter.

An additional four groups of animals will be set aside for the neurochemical study. These animals will be randomly assigned to the same groupings as described above following same drug treatment protocols (saline vs. clozapine), except that at 8 weeks after PND 21, the animals will be sacrificed and the brains rapidly dissected for regional brain redox analysis.

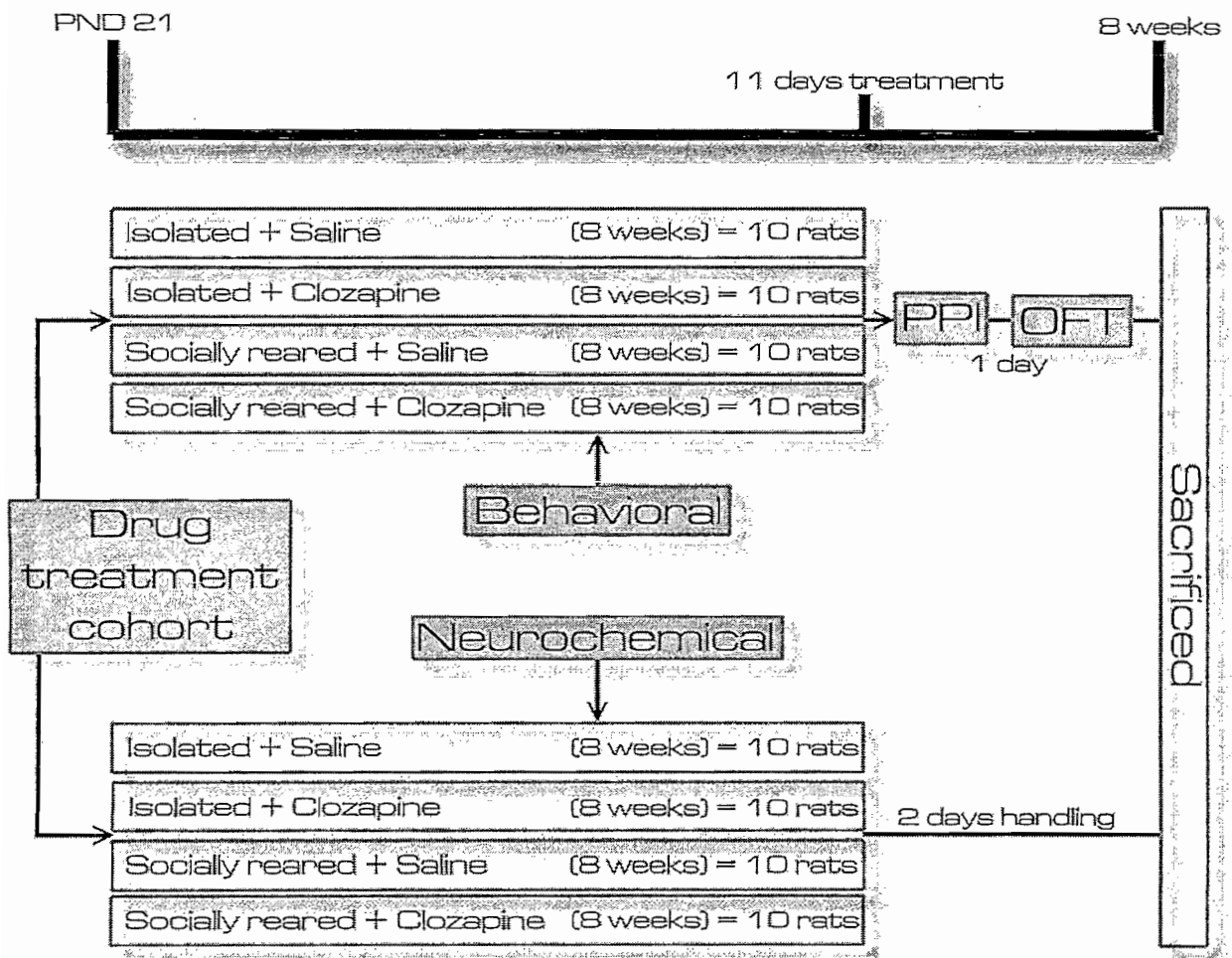


Figure 2: Study design for the behavioral and neurochemical studies in SIR and socially reared rats in the drug treatment cohort, with SIR and socially reared rats treated with either saline or clozapine (5 mg/kg/day x 11 days).

4. General points

This dissertation has been written and submitted in the article format for thesis/dissertation submission, as approved by North-West University. The format includes an introductory chapter, a chapter covering the relevant literature overview, chapter/s containing one or more full length articles for submission to a peer-review neuroscience journal, and a chapter describing the conclusion of the study, as well as providing recommendations for future study. The article chapter has been carefully prepared to present the most novel and impactful data from the study. To this end, the article will be prepared according to the house style and author instructions of that particular journal. This house style and the instructions to authors are provided in Addendum C. All other work performed during this study, including additional validations as well as work performed during the course of the study but not included in the journal article, will be provided in the addenda.

Data from the behavioral studies (the PPI and OFT assessments), regional brain redox data (viz. %SOD activity, oxidized (GSSG) versus reduced (GSH) glutathione ratio, and lipid peroxidation), as well as response of all the behavioral and redox parameters to antipsychotic treatment, will form the focus of a full length research paper intended for submission to *European Neuropsychopharmacology* (Springer). Additional work performed during the course of this study, including the validation of the PPI and OFT paradigms using the dizocilpine (MK-801) challenge model, as well as the setting up of the superoxide dismutase assay, will be presented in Addendums A and B respectively.

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Chapter 2

Literature Review

1. Introduction

The term 'schizophrenia' comes from the Greek and it translates roughly as 'shattered mind'. Schizophrenia is a mental illness that is among the world's top ten causes of long-term disability (World Health Organization, 2001). About 1% of the population is affected by schizophrenia, with similar rates across different countries, cultural groups and sex (Weiss & Feldon, 2001). The illness tends to develop between the ages of 16 and 30 years, and mostly persists throughout the patient's lifetime. Approximately 50% of discharged patients will be re-hospitalised within a year (Weiden et al., 1996). Less than 20% of schizophrenia patients are employed at one time, 10% of patients with schizophrenia will commit suicide (Weiden et al., 1996) and 20% of patients experience a relapse despite antipsychotic medication (Fleischhacker & Hummer, 1997). In addition to severely disrupting the life of the patient and their family, schizophrenia incurs a great cost to society in terms of lost productivity and treatment-related expenses. Among psychiatric disorders, schizophrenia occupy about 25% of all psychiatric hospital beds (Terkelsen & Menikoff, 1995) and represent 50% of admissions to hospital (Geller et al., 1991).

The primary manifestations of schizophrenia are an inability to filter incoming sensory information, disturbances in thinking, mood and overall behavior (Eisendrath & Lichtmacher, 2005). Converging results also suggests that schizophrenia present with at least three distinct dimensions or symptoms, namely *negative*, *psychotic* and *disorganized* (Andreasen, 1995, Guillem et al., 2002). Negative symptoms severely disrupt the cognitive, intellectual and psychomotor functioning of the patient, and significantly impact on the patient's every day life (Weiss & Feldon, 2001). Different combinations of symptoms with varying degrees of severity, as well as varying

responses to antipsychotic treatment, are observed in schizophrenia patients, while the illness generally presents with poor long-term prognosis (Harvey et al., 1999).

The heterogeneity of schizophrenia is often considered a major obstacle, involving environmental, neurodevelopmental and genetic factors, and has led to schizophrenia being described as a multifaceted disease (Weiss & Feldon, 2001). While there is strong evidence for genetic transmission of vulnerability to schizophrenia (Harrison and Weinberger, 2005; Tsuang et al., 2001), the heterogeneity and complexity of clinical phenotypes pose great obstacles for research into understanding the molecular and genetic basis of susceptibility for developing schizophrenia, indicating that other factors also contribute to the development of this devastating illness (Karayiorgou & Gogos, 1997, Horan et al., 2008).

The current treatment regime for schizophrenia mainly comprises the typical and atypical antipsychotics, but despite their apparent effectiveness, 20% of patients experience a relapse within a treatment year, regardless of treatment (Fleischhacker & Hummer, 1997). In the last few decades, significant attempts have been made to improve the treatment of schizophrenia. Approximately two-thirds of patients on typical antipsychotic medication for schizophrenia experience persistent Parkinsonism (Harvey et al., 1999), while up to 70% of patients using typical antipsychotics develop acute extrapyramidal side effects (EPS) (Chakos et al., 1994). Recent studies have also indicated that almost all patients experience undesirable side effects during the treatment with antipsychotics (Fakhoury et al., 2001) such as weight gain and its metabolic consequences, which unfortunately results in discontinuation or switching of medication (Lieberman et al., CATIE-study 2005; Kahn et al., 2008). Treating schizophrenia should therefore focus on providing the best quality of life for the patient. The means to achieve this would be to effectively and lastingly decrease the severity of psychotic symptoms (desired effect) with little to no undesired effects, to adequately address negative and cognitive symptoms, and to allow the patient to reintegrate into society (Kasper, 2006). Thus, there is a greater urgency for research to better understand the illness in order to develop new and improved treatment regimes.

2. Symptoms and clinical description of schizophrenia

People diagnosed with schizophrenia usually experience a combination of positive, negative and cognitive symptoms. Four basic dimensions of schizophrenic illness or unique domains of psychopathology (presumably with a distinctive pathophysiology and treatment) can be discerned and are depicted in Figure. 1 (Tandon & Maj, 2008).

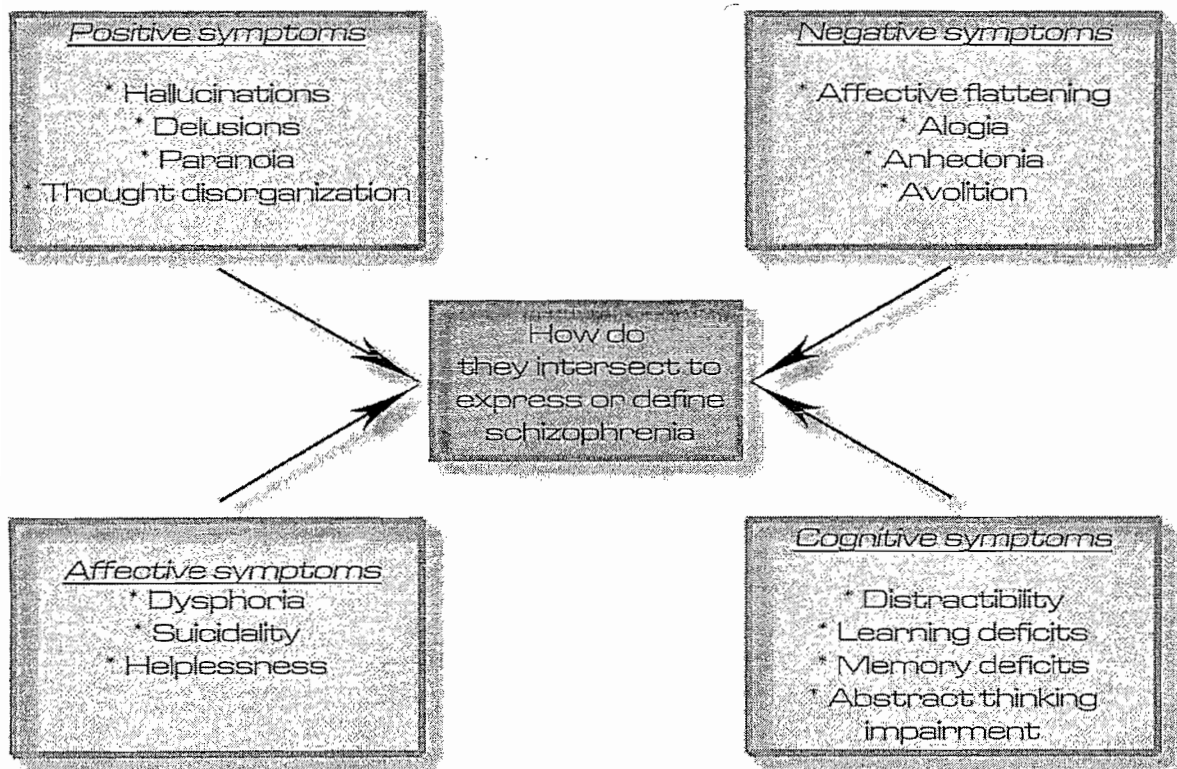


Figure 1: The four domains of schizophrenia symptoms: Positive symptoms, negative symptoms, cognitive symptoms and affective symptoms (Adapted from Tandon & Maj, 2008).

In the 1970s, numerous concepts aimed at differentiating positive and negative forms of schizophrenia, have developed based on anatomical and clinical correlations (Crow, 1985; Andreasen and Olsen, 1982). Positive symptoms can be described as reflecting an excess of normal function, while the negative symptoms are a loss of normal function (Fuller et al., 2003). Investigating the relationship

between schizophrenia symptoms and personality, both in the acute phase of the illness and longitudinally may provide potentially important clues in understanding the pathophysiology of symptom expression (Guillem et al., 2002). But let us first discuss the various symptoms domains of schizophrenia.

2.1 Positive symptoms

Positive symptoms involve impaired reality testing, and include delusions, hallucinations, racing thoughts and other reality distortions.

2.1.1 Delusions and hallucinations

Delusions can be defined as “firmly held erroneous beliefs”, due to distortions or exaggerations of reasoning and/or misinterpretations of perceptions or experiences (Geyer & Vollenweider, 2008). Numerous varieties of delusions can occur with varying degrees of persistence and systematization, influencing the schizophrenia patient’s functioning to different extents (Tandon et al., 2009). Although delusions of control, thought insertion, withdrawal and broadcasting (all so-called Schneiderian first-rank symptoms; Mellor, 1981) are traditionally linked to schizophrenia, persecutory delusions and delusions of reference are most frequent (Bentall et al., 2001). A variety of other delusions can also occur, with the content of delusions being influenced by the person's life and socio-cultural setting. Hallucinations are distortions or exaggerations of sensory perception, although auditory hallucinations (hearing voices, distinct from ones own thoughts) are the most common, followed by visual hallucinations (Mueser et al., 2007). Delusions of control, for e.g. belief that others can interfere with your thoughts, and grandiose delusions, e.g. the person believes that he is Jesus Christ, and somatic delusions, e.g. the person believes that his brain is rotting away (Mueser & Mc Gurk, 2004), are the most common.

2.1.2 Disorganized and catatonic behaviors

Grossly disorganized behavior includes unpredictable agitation, difficulty in goal-directed behavior, social dysfunction, or behaviors that are odd or inappropriate to

society (DSM –IV, American Psychiatric association, 1994). Catatonic behaviors are characterized by a marked decrease in reaction to the immediate surrounding environment, for e.g. motionless and apparent unawareness, rigid or bizarre postures, or aimless excessive motor activity.

2.1.3 Disorganized speech and thought

Disorganized speech or thinking, also described as thought disorder or loose and indirect associations, is a very important presenting symptom of schizophrenia (reviewed in Subotnik et al., 2006). Disorganized thinking is usually assessed primarily based on the person's speech. Therefore, loosely associated, or incoherent speech that is severe enough to substantially impair effective communication is used as an indicator of thought disorder (DSM –IV, American Psychiatric association, 1994).

2.2 Negative symptoms

The negative symptoms of schizophrenia typically include anhedonia, flat or blunted affect, poverty of speech (alogia), avolition (lack of initiative), and asociality (Andreasen & Olsen, 1982; Kay et al., 1986). Negative symptoms are relatively common (Fenton and McGlashan, 1994) and are independent from positive, disorganized, and affective symptoms (Andreasen et al., 1995; Emsley et al., 2003; Smith et al., 1998). In addition, negative symptoms demonstrate unique associations with social functioning, neurocognition, and neurobiology (for a detailed review see Earnst and Kring, 1997). Negative symptoms involve a blunting or loss of a range of affective and cognitive functions. These include impairments in affective experience and expression, abulia (loss of motivation), alogia (poverty of speech), anhedonia (inability to experience pleasure), avolition, apathy (lack of interest), and reduced social drive (Crow, 1980; Andreasen & Olsen, 1982; Carpenter et al., 1988). Since a range of causes can contribute to the expression of negative symptoms, it is important to distinguish between primary and secondary negative symptoms (Carpenter et al., 1988; Kirkpatrick et al., 2006). Primary negative symptoms are fundamental or intrinsic to schizophrenic illness, while secondary negative symptoms are caused by 'extrinsic' factors linked to schizophrenia, such as environmental

deprivation, neuroleptic treatment and depression. The pathophysiology of negative symptoms is poorly understood (Keshavan et al., 2008) and they remain relatively treatment-refractory as well as the most debilitating component of schizophrenia (Erhart et al., 2006; Stahl and Buckley, 2007).

2.2.1 Affective flattening, alogia and avolition

Affective flattening is the reduction in the range and intensity of emotional expression, including facial expression, voice tone, eye contact, and body language (Kane et al., 2009). Alogia (poverty of speech) is a deficit in speech fluency and productivity, thought to resemble slow or inadequate thoughts, and often manifested as short, empty replies to questions (Iversen et al., 2008). Avolition is the deficit or inability to persist or initiate in goal-directed behavior (Iversen et al., 2008). Examples of avolition include no longer being interested in going out or meeting with friends, or no longer being interested in activities that normally would prompt enthusiasm (Moller, 2007).

2.2.2 Social withdrawal

Patients with schizophrenia are unable to integrate into society, while showing a marked lack of social interaction skills and social cognition (Couture et al., 2006 for review). Consequently, impairments in social functioning represent a core behavioral feature of schizophrenia (Pinkham et al., 2003), and are among the most debilitating and treatment refractory aspects of schizophrenia (Bellack et al., 2007). A wide range of deficits in social and interpersonal functioning have been documented in schizophrenia, such as verbal ability, verbal memory (Addington et al., 2000), verbal fluency, appropriate communication, eye contact and the extent to which the individual appears involved in a conversation (Pinkham & Penn, 2006). Early studies had proposed that premorbid social functioning is a strong predictor of long-term functioning (Foerster et al., 1991) and that social and occupational functioning tended to vary independent of each other and with respect to psychotic symptoms (Strauss & Carpenter, 1977). More recent studies have concurred that deficits in social functioning may be premorbid markers and short- and long-term predictors of functional capacity, course and outcome in schizophrenia (Green et al., 2004 for detailed review).

3. Diagnosis of schizophrenia

The diagnosis of schizophrenia requires at least 1-month duration of two or more positive symptoms, unless hallucinations or delusions are especially bizarre, in which case one positive symptom would suffice for diagnosis, with at least 6 months of occupational or interpersonal social dysfunction (DSM –IV, American Psychiatric association, 1994). Exclusion criteria includes: psychosis secondary to general medical conditions, psychosis secondary to substance abuse as well as schizoaffective disorder and mood disorder with psychotic features, where either (1) no major depressive, manic, or mixed episodes have occurred concurrently with the active-phase symptoms; or (2) if mood episodes have occurred during active-phase symptoms, their total duration has been brief relative to the duration of the active and residual periods (DSM –IV, American Psychiatric association, 1994).

4. Epidemiology and etiology of schizophrenia

Epidemiology is the study of distribution and determinants of disease (MacMahon and Pugh, 1970). By distinguishing characteristics and experiences of individuals who develop a disease from those who do not, allows one to identify factors related to causation of the disease (MacMahon and Pugh, 1970). With schizophrenia, both genetic and environmental risk factors need to be considered since both are important in the etiology of schizophrenia and neither appears to operate in isolation (Tsuang et al., 2004). The distribution of a disease is generally expressed in terms of incidence (new cases) and prevalence, which refers to the total number of cases, existing and new (Tandon et al., 2008). The estimated risk of developing schizophrenia over one's lifetime ranges from 0.3–2.0% (Saha et al., 2005). A meta-analysis of 24 studies found a median lifetime prevalence estimate for schizophrenia to be in the order of 4.0 per 1000 persons (Tandon et al., 2008).

Although the basis of the above findings is not well understood, they do aid in developing and testing hypotheses concerning the cause of schizophrenia (McGrath, 2007), especially interpreting what these distribution patterns say about the specific genetic and environmental risk factors of schizophrenia, as well as about the neurobiological mechanisms involved (Khashan et al., 2008). Schizophrenia aggregates in families, although over two-thirds of the cases occur sporadically. Nevertheless, having an affected family member substantially increases the risk of developing schizophrenia (Tandon et al., 2008 for review). This risk increases as the degree of genetic affinity with the affected family member increases (Kendler et al., 1993). Thus, if one monozygotic twin is afflicted with schizophrenia the other twin has a 50-70% risk of developing the illness as well (Goldberg et al., 1995). A variety of specific environmental exposures have been implicated in the etiology of schizophrenia. These include both biological and psychosocial risk factors during the antenatal and perinatal periods, early and late childhood, adolescence and early adulthood (Maki et al., 2005). In the antenatal period, maternal infections and nutritional deficiency during the first and early second trimesters of pregnancy are associated with an increased liability for developing schizophrenia (Penner and Brown, 2007; Meyer et al., 2007). Severe nutritional deficiency (St Clair et al., 2005) and severe adverse life events (Khashan et al., 2008) experienced by the mother during the first trimester of pregnancy have been linked to an increased risk for developing schizophrenia. These effects are hypothesized to be mediated by “stress sensitization” (Koenig et al., 2005) and a predisposition to subsequent hyperdopaminergia (Lipska et al., 1993). Considering the child, it has been suggested that a range of obstetric and perinatal complications may in fact increase the risk of developing schizophrenia in the offspring (Tandon et al., 2008), while birth during late winter or early spring has a 5–10% greater likelihood of developing schizophrenia (Torrey et al., 1997; Davies et al., 2003). Another significant risk factor for developing schizophrenia is cannabis abuse (Henquet et al., 2005).

Apart from the above-mentioned causally related factors, less well defined environmental risk factors, such as immigrant status (reviewed in McDonald & Murray, 2000) have been linked to an increased liability to develop schizophrenia, although their exact relevance remains unclear (McDonald & Murray, 2000).

Pathophysiology

5.1 Neuroanatomy

Since the symptoms of schizophrenia are so divergent, it is difficult to relate a single brain structure or network to the behavioural and psychic aberrations of the illness (Fallon et al., 2003). In an attempt to explain the brain circuitry involved in schizophrenia, an integrated neuroanatomical model has been put forward based on what is currently known about its neuroanatomy and chemistry (Lipska, 2004; Leonard 2003; Figure 2 B), compared to the brain circuitry in healthy subjects (Figure 2 A). This model places the primary deficit in the subcortical neurons projecting from the ventral tegmental area (VTA) to the cerebral cortex, postulating that a primary lesion, evoked by a hitherto unknown event before or after birth, later mediates a decreased activity of prefrontal cortex (PFC) (Figure 2 B). The latter is either due to neuronal atrophy or degeneration of neurogenesis, resulting in reduced neuronal connectivity in the PFC (Duman & Newton, 2007; Figure 2 B). Prevailing evidence would now suggest that decreased PFC activity is expressed as hypofunction of critical dopaminergic and glutamatergic pathways. Since the PFC is involved in the top-down control over activity of sub-cortical brain regions, the result of this is a weaker cortical feedback control on the VTA neurons and, simultaneously, in less effective cortical regulation of the limbic systems (LS), particularly the nucleus accumbens (NAcc). As a result, increased dopaminergic drive (from the partially disinhibited VTA neurons) acting on the NAcc, which at the same time is now less inhibited by the PFC (due to decreased glutamatergic activity), will allow greater VTA-directed stimulation of the NAcc (Figure 2 B). Increased (disinhibited) dopaminergic activity projecting from the VTA is now less effective in driving the activity of PFC under such conditions, especially in lieu of the existing primary glutamatergic (excitatory) deficiency (Figure 2 B).

Although useful conceptually, this model may require further modification and refinement to account for additional characteristics of schizophrenia, such as the time course of the illness or the role of stressful events in triggering the disease (Holcomb et al., 2004; Moghaddam, 2002). However, the salient feature of the model, viz. dopaminergic and glutamatergic deficits in the PFC upstream from

hyperdopaminergic activity in the LS (Holcomb et al., 2004), has important construct and heuristic value in explaining both the positive (hyperactive LS; Figure 2 B) and negative symptoms, as well as the cognitive deficits, of schizophrenia. These deficits are known to be accompanied by a reduced activity in the PFC in patients with schizophrenia, as well as in associated brain structures such as the mediodorsal nucleus of the thalamus (Yang et al., 2003; Lehrer et al., 2005), and that drive the fragmentation of cognitive processing.

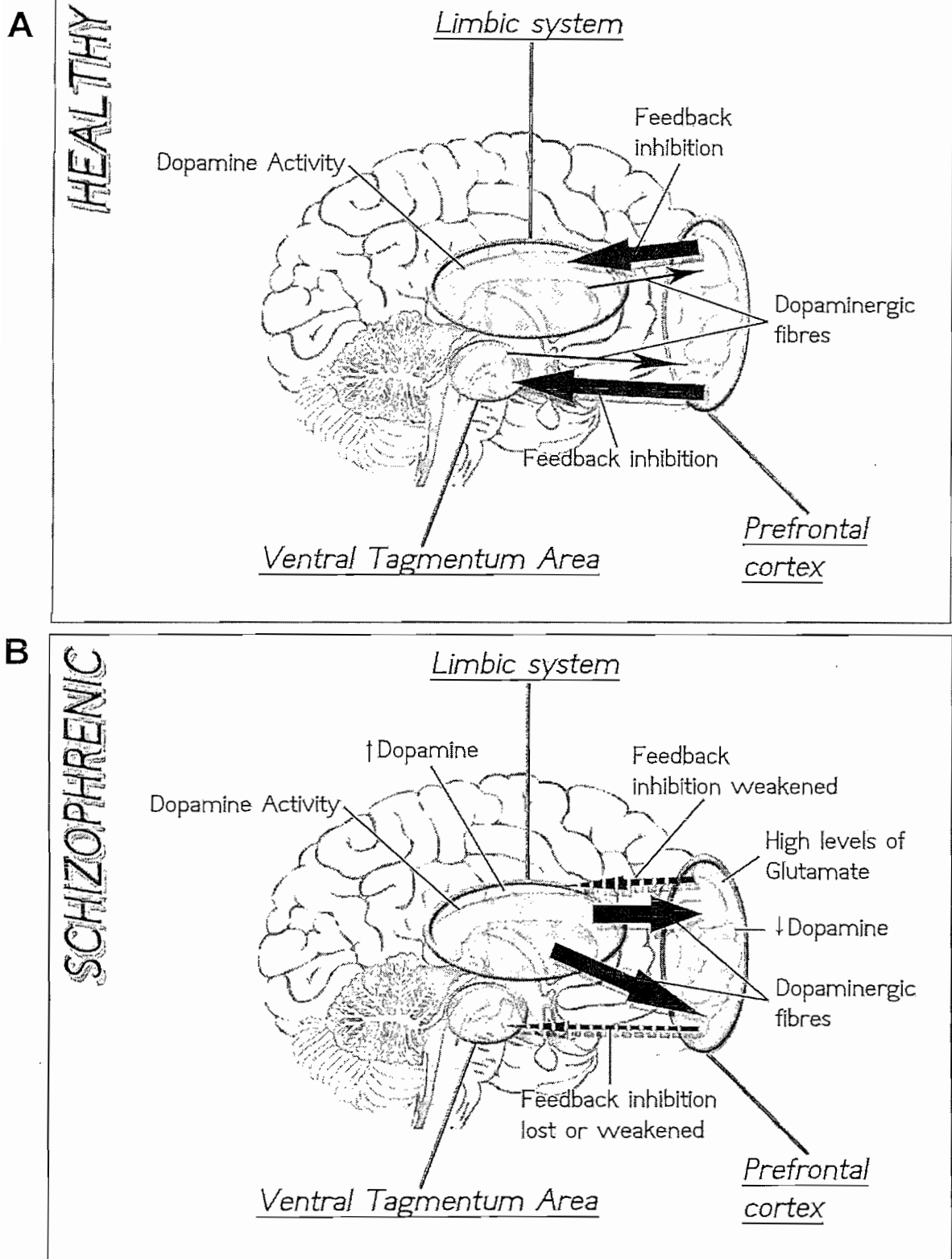


Figure 2: The brain circuits involved in schizophrenia in (A), healthy subjects compared to (B), schizophrenia patients (Adapted Leonard, 2003; Nanitsos et al., 2005).

The most frequent neurobiological finding in schizophrenia is enlargement of the ventricular system (Wright et al., 2000). Ventricular enlargement is accompanied by overall reductions in brain volume and cortical grey matter (Andreas et al., 1994). Magnetic resonance imaging (MRI) studies have found reduced grey matter in schizophrenia patients compared to healthy controls, in specifically the prefrontal cortex (Sigmundsson et al., 2001; Thompson et al., 2001) as indicated in Figure 3. These three-dimensional maps of brain changes over time have been derived from high-resolution magnetic resonance images (MRI scans) acquired from the same group of subjects. This illustration reveals significant, progressive gray matter loss in schizophrenia patients over time (as indicated on the right side of Figure 3). The average rate of gray matter loss from 13 to 18 years of age is displayed in both the schizophrenia patients and healthy controls. In Figure 3, this severe loss is observed (red and pink; up to 5% annually) in the parietal, motor, and temporal cortices, whereas inferior frontal cortices remain stable (blue; 0–1% loss). Dynamic loss is also observed in the parietal cortices of normal adolescents, but at a much slower rate (Thompson et al., 2001).

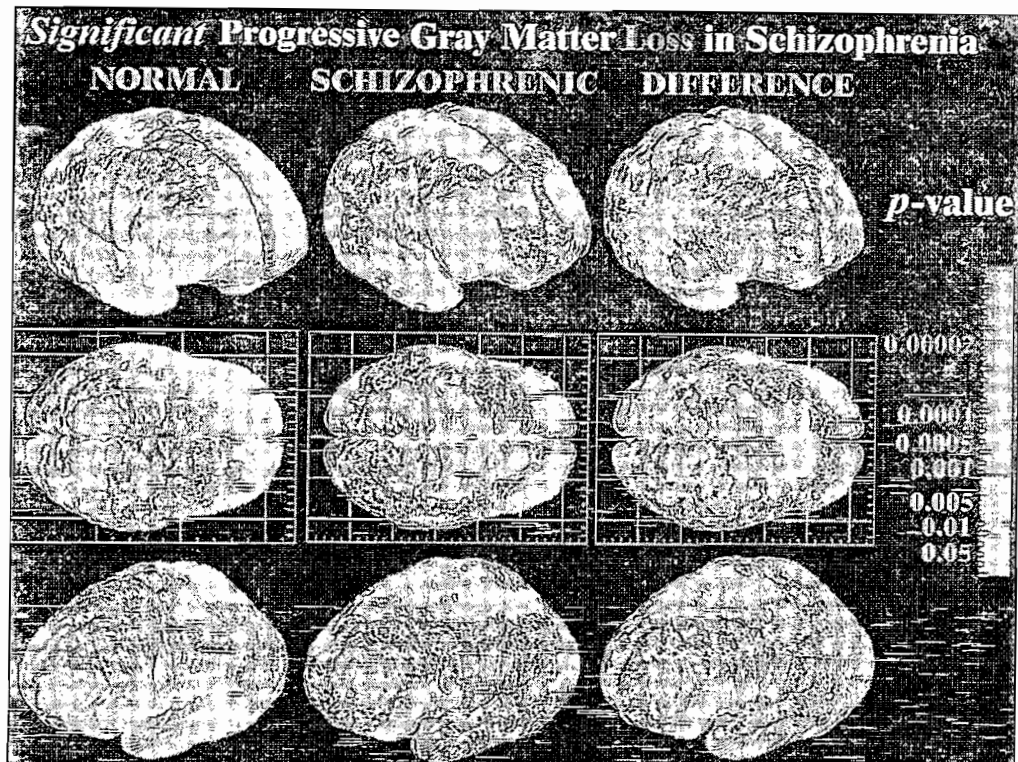


Figure 3: Significance of dynamic gray matter loss in normal adolescents and in patients with schizophrenia. Highly significant progressive loss occurs in schizophrenia in parietal, motor, supplementary motor, and superior frontal cortices. Broad regions of the temporal cortex, including the superior temporal gyrus, experience severe gray matter attrition (Thompson et al., 2001).

Abnormalities in blood flow have also been shown in the frontal regions, thalamus and cerebellum of schizophrenia patients in positron emission tomography (PET) studies (Andreasen et al., 1996). This hypofrontality with respect to blood flow can be linked to diminished dopamine activity and therefore decreased cognitive functioning, as is observed in the pathophysiology of schizophrenia (Mueser & McGurk, 2004).

Recent studies have also found a decrease in the dendritic spine density on the hippocampus and the medial part of the prefrontal cortical pyramidal neurons in schizophrenia patients compared to healthy controls (Glantz & Lewis, 2000).

The neuroanatomical development of schizophrenia therefore appears to have a direct relationship with the deficits shown in imaging data of specifically the subcortical regions (nucleus accumbens and hippocampus) and the frontal cortex in schizophrenia patients (Weiss & Feldon, 2001; Shad et al., 2006). But what is the basis for the initial lesion in early development, as well as the mechanisms underlying the progressive degeneration of these brain regions post diagnosis? This is discussed in the following section, and indeed is the focus of this study.

5.2 Neurodevelopmental anatomy in schizophrenia

Adverse events experienced in early life may contribute to the expression or exacerbation of a variety of physical and psychological disorders, and is particularly valuable for our understanding of schizophrenia (Lipska & Weinberger, 2000). The “neurodevelopmental hypothesis of schizophrenia” states that abnormalities of early brain development increase the risk for subsequent emergence of clinical symptoms (Marenco et al., 2002). Since schizophrenia does not develop acutely, but through a gradual prodromal phase that takes place over a prolonged period, it may be important to intervene early on in the developmental phase of the illness and to identify pivotal neurobiological markers that drive the pathophysiology of schizophrenia. The initial prodrome of schizophrenia describes a period of time which begins with the first changes in the person and extends up to the development of the first psychotic episode (Yung and McGorry, 1996).

Prodromal symptoms include attenuated positive symptoms (illusions, ideas of reference, magical thinking, superstitiousness), brief limited intermitted psychotic symptoms (hallucination and delusion less than 7 days), so called cognitive basic symptoms (thought interference, tendency of self reference, changed language expression), and negative symptoms identified by gradual social withdrawal (Yung and McGorry, 1996; McGlashan, 1996; Klosterkotter et al., 2001). Early and late neurodevelopmental disturbances in schizophrenia and their functional consequences involve structural brain abnormalities that may already be apparent in the premorbid stage (Pantelis et al., 2003). Indeed, MRI imaging and novel neuranatomical marker studies all agree that schizophrenia may be a neurodevelopmental disorder (Sawa et al., 2002).

Underlying the macroscopic changes in schizophrenia, two very important histological alterations are noted (Arnold et al., 1996). Firstly, the cortical cytoarchitecture is altered, with neurons being misplaced, abnormally sized, and disorganized (Harrison et al., 1997). These abnormalities are highly indicative of an early developmental origin with an onset no later than infancy. Secondly, the neurodegenerative outline of schizophrenia in the absence of glial reactions (Weinberger & Marengo, 2003) confirms that the neuropathological changes in schizophrenia are prenatal rather than post natal. Glial reactions are associated with most adult-onset brain injuries, as well as with neurodegenerative disorders such as Alzheimer's and Parkinson's disease, and are not found in neurodegenerative disorders that arise during early brain development (Weinberger & Marengo, 2003). In addition, neuropsychological studies in children also support an early onset brain abnormality that later leads to the development of schizophrenia. These children present with distinct neuromotor, neuropsychological and intellectual abnormalities in early childhood, even before any psychiatric symptoms appear (Done et al., 1994; Cannon et al., 1994).

Another important finding is that brain asymmetry (specifically lack of normal hemispheric volume asymmetries) is reduced in schizophrenia (Bilder et al., 1994). A developmental origin is the most plausible explanation for this, given the normally asymmetrical growth of the cerebral hemispheres (Bakalar et al., 2009). Abnormal low levels of neuropil, abnormalities in synaptic, dendritic, axonal and white matter tract organization and abnormal glutamatergic neurotransmission (Coyle, 1996; Zaidel et al., 1997), may also indicate the neurodevelopmental time line in the brain of the schizophrenia patient, which are consistent with defective connectivity between brain regions such as the midbrain, nucleus accumbens thalamus, temporo-limbic and prefrontal cortices (Arnold et al., 1999; Selemon & Goldman-Rakic, 1999).

The neurodevelopmental etiology of schizophrenia links genetic risk to environmental risk factors such as perinatal insults (Coyle et al., 2004), as described in section 4. These insults ultimately leads to gamma-aminobutyric acid (GABAergic) neuronal damage (Figure 4), evidence of which being being reduced expression of presynaptic markers in subpopulations of GABAergic interneurons in the frontal

cortex and the hippocampal formation of schizophrenia patients (Lewis & Frangou, 2003 for review). These GABAergic neurons play an important role in regulating the activity of the projecting glutamatergic pyramidal cells (Benes & Berretta, 2001). GABAergic neuronal damage therefore will lead to disinhibition of glutamatergic neurons, as indicated in Figure 4. Alternatively, glutamate levels are reduced in schizophrenia patients (Kim et al., 1980), which is argued to then cause a reduced stimulation of the above GABAergic neurons, again resulting in an increase in subcortical glutamate activity. Glutamate in turn, controls the excitation of neurons and glia through the activation of various glutamate receptors (Konradi & Heckers, 2003 for review), and is therefore critically involved in neuronal development, neuroplasticity, neurotoxicity and formation of a sufficient number of synapses (Goff & Coyle, 2001; see Konradi & Heckers, 2003 for review). Reduced levels of glutamate, as indicated in Figure 4, or hypoactivity at NMDA receptors, will ultimately impact on the number of synapses established, resulting in abnormalities in brain development, brain circuitry and deficient synaptic connectivity, all linked to the neurodevelopmental theory of schizophrenia (Lewis & Lieberman, 2000). Goff & Coyle, 2001 also proposed that a primary hypoactive glutamate system in schizophrenia could influence the formation of neuronal connections in the cortical and subcortical brain areas early in life, which fits well with the anatomical abnormalities found in the adult schizophrenia brain.

However, as indicated in Figure 4, the disinhibition of glutamatergic neurons will also increase subcortical dopamine function. In fact, dopamine neurons in the substantia nigra and the VTA receive glutamatergic inputs (Carr et al., 1999), while glutamate can excite dopaminergic activity (Meltzer et al., 1997). Conversely, the glutamate system can also be inhibited by dopamine, or be facilitated by the inhibition of D₂ receptors (Konradi & Heckers, 2003 for review). This hyperdopaminergic activity is in turn strongly implicated in the neurochemistry of schizophrenia (see section 4.3.).

Another important finding is that hydrogen peroxide (H₂O₂), a reactive oxygen species (ROS), is formed during the metabolism of dopamine and can inhibit glutamate release, ultimately leading to oxidative stress, as discussed in section 7 and 8. (Tretter et al., 2003 for review). Altered glutamate will not only have an impact on schizophrenia by its ability to modify the release of dopamine in the cortex and

striatum (Konradi & Heckers, 2003), but is also essential for regulating the expression of anti-oxidant enzymes and to strongly influence regional redox balance in the brain (Smythies, 1999), further influencing oxidative stress.

Concluding, the neurodevelopmental hypothesis therefore suggests that brain development can be adversely affected at a critical time of life (see Figure 4), particularly through early life exposures to stress, which may provoke the onset of psychosis in later adolescence or adulthood (Weiss & Feldon, 2001), along with changes in neurochemistry (summarized in Figure 4). Increased (sub-cortical) and decreased (cortical) glutamate, reduced cortical GABA and increased (sub-cortical) and decreased (cortical) dopaminergic pathways are all central to this hypothesis.

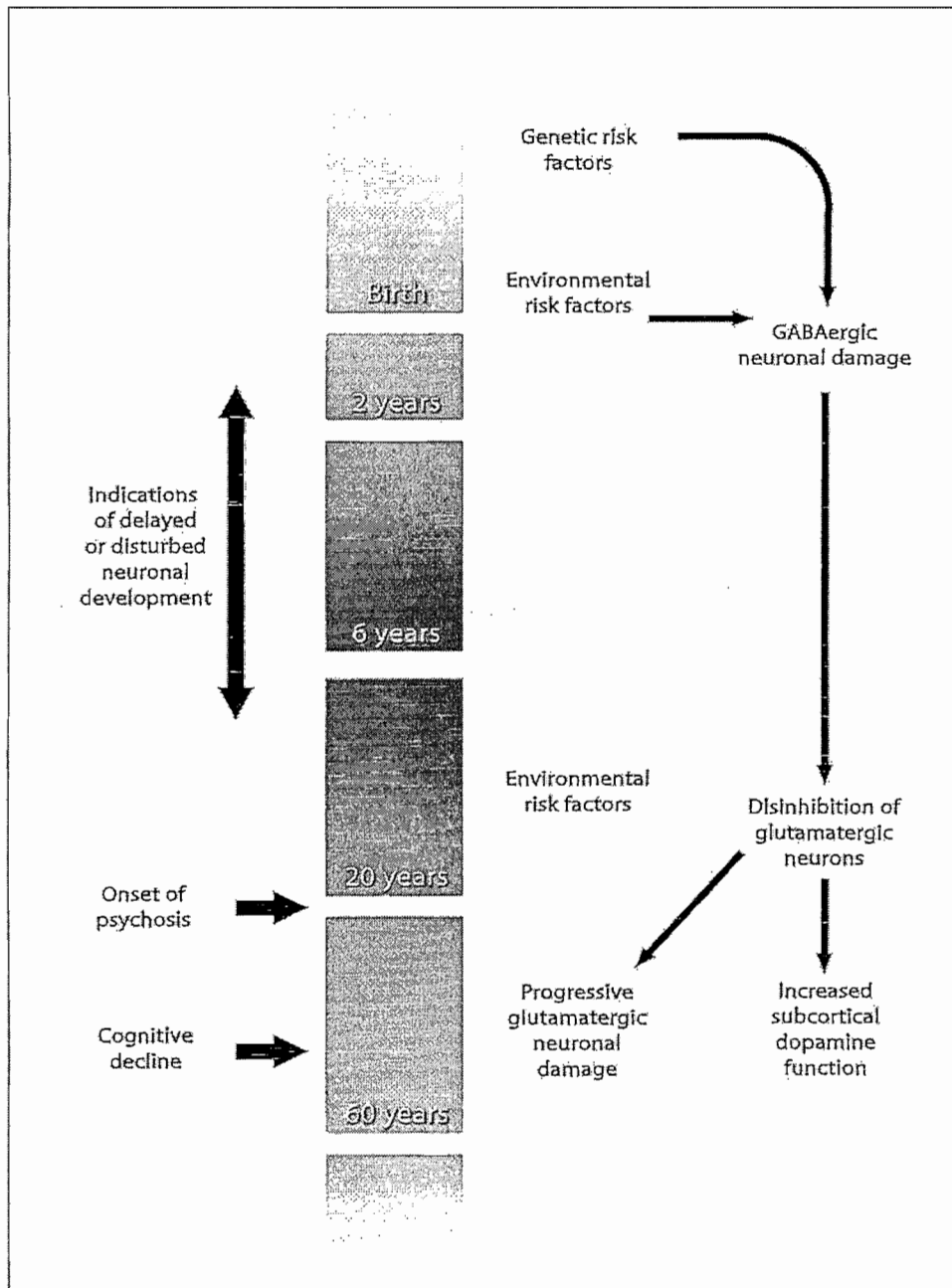


Figure 4: A depiction of the neurodevelopment of schizophrenia, with onset of psychosis and how various neurotransmitter systems are involved (Adapted from: Reynolds, 2005).

5.3 Neurochemistry

In concurrence with the neurodevelopmental hypothesis of schizophrenia (Figure 4), is the dysfunction of a number of neurotransmitter systems that has formed the principle construct dominating neuropharmacological research into new drug

development. The following hypotheses have been developed to evaluate the extent to which neurochemical findings reflect primary or secondary mechanisms involved in the illness. Today these theories form the basis for explaining the mode of action of all currently used drugs for the treatment of schizophrenia, and in many ways still determine the way forward for new drug development.

5.3.1 The Dopamine hypothesis

The classical “dopamine hypothesis of schizophrenia” postulates a hyperactivity of dopaminergic transmission at the D₂ receptor, in the mesencephalic projections to the limbic system (Carlsson, 1988). This hypothesis was first based on the ability of dopamine agonists, for e.g. amphetamines which stimulate dopamine release, to induce psychosis with schizophrenic features in healthy subjects, and at very low doses provoke psychotic features in schizophrenia patients (Miyamoto et al., 2003). In animals, amphetamine is used in the dopamine sensitization model of schizophrenia, described in section 8.4 (Tenn et al., 2003 for review). This notion was also supported by the correlation between the therapeutic doses of conventional antipsychotics and their affinities of the D₂ subtype(s) of dopamine receptors (Miyamoto et al., 2001). Subsequently, the dopamine hypothesis has received strong support from PET studies, indicating a higher density of D₂ receptors in post-mortem brain from schizophrenic patients (Wong et al., 1986), as well as imaging studies indicating the close correlation between D₂ receptor binding and efficacy of these drugs to decrease psychosis (Corripio et al., 2005; Carlsson et al., 1997 for review). This work led to the formulation of a modified dopamine hypothesis in which elevated D₂ receptors were proposed to underlie the positive symptoms of schizophrenia (Reynolds et al., 2005).

Numerous studies have revised the dopamine hypothesis to include the cortical and subcortical components of the brain (Grace et al., 1991; Davis et al., 1991). Evidence that patients with schizophrenia have higher levels of dopamine in the brain has also been found in the striatum post mortem (Guillin et al., 2007). The current dopamine hypothesis, however now postulates that there is an imbalance between subcortical and cortical dopamine levels (Duncan et al., 1999; Tzschentke,

2001). A hyperactivity of dopamine prevails in the mesolimbic dopamine projections and in the dopamine cell bodies located in the VTA, resulting in hyperstimulation of D₂ receptors and ultimately causing psychotic, positive symptoms. On the other hand, a hypodopaminergic state, caused by mesocortical hypoactive dopamine projections, is observed in the frontal cortical terminal fields, resulting in the negative symptoms of the illness (Guillin et al., 2005). Despite the importance and relevance of the dopamine hypothesis in explaining the neurobiology and pharmacology of schizophrenia, there are still noteworthy limitations that need to be considered.

Firstly, there is no direct evidence of pathological dopamine neuronal activity, for e.g. increased levels of dopamine, increased dopamine metabolites or up or down-regulated receptors, in schizophrenia. This is mainly due to the fact that presynaptic dopamine function in the frontal cortex is not at present accessible to noninvasive imaging studies (Carlsson et al., 1988; Guillin et al., 2005). Secondly, no differences in the percentage of D₂ receptor occupancy has been found in responders compared to non-responders to antipsychotic treatment (Coppens et al., 1991). Furthermore, only 30% of schizophrenia patients respond to typical D₂ receptor antagonists (Chavez-Noriega et al., 2002). Thirdly, the development of low potency atypical antipsychotics such as clozapine and quetiapine, demonstrate exceptionally low affinity for D₂ receptors in relation to their therapeutic dose (Kerwin & Dumon, 1994; Harvey et al, 1999). When clozapine reaches its therapeutic dose in plasma, only approximately 30-60% of D₂ receptors are occupied, whereas 80-90% 5-HT₂ receptors are occupied (Farde et al., 1992; Nyberg et al., 1996). Later on, clozapine was found to be a more potent blocker of the D₄ receptor (Harvey et al., 1999). Furthermore, the introduction of new atypical antipsychotics similar to clozapine emphasized the important role of serotonin, but also a host of other signaling pathways, such as the cholinergic and adrenergic systems (see Harvey et al., 1999 for review), which together brought question on the immediate importance of D₂ receptor binding for adequate antipsychotic action. However, later evidence that D₄ blockers are ineffective antipsychotics (Kramer et al., 1997), and that D₂ blockade is necessary and sufficient for antipsychotic efficacy (Kapur & Ramington, 2001), re-affirmed the important role of dopamine in the neurobiology of schizophrenia, and indeed of the role of the D₂ receptor in antipsychotic drug action.

If one could summarize the latest thinking on how dopamine is involved in schizophrenia, it would be as follows (Kapur, 2003; Kapur & Ramington, 2001).

Limbic dopamine mediates the detection and response to unpredicted reward, such as food, sex etc, as well as aversive stimuli such as stressors, and by doing this, dopamine controls the level of significance (salience) of the environment and how it is represented internally. Hyperdopaminergia essentially increases the sense of novelty. At the same time, if there is inadequate control of salience by the frontal cortex, there is an incorrect assignment of significance to environmental stimuli resulting in delusions and hallucinations. Delusions are an attempt by the person to make sense of inappropriately significant experiences and associations ("a new awareness"), while hallucinations are a direct result of experiencing the inappropriate environmental stimulus. Dopamine dysregulation thus provides the driving force of the psychosis, but the subject's own cognitive, psychodynamic and cultural context gives form to the experience. Important to note is that antipsychotic drugs do not remove the symptoms, but modify the degree to which these ideas/perceptions occupy the mind, so that symptomatic improvement requires further psychological and cognitive resolution over time. Recovery is thus treatment dependent, and is put in place by a process of extinction and unlearning that is dependent on continuous dampening of inappropriate salient stimuli.

While the role of dopamine cannot be disregarded, it is clear that pharmacological properties other than D₂ receptor antagonism may contribute to effective treatment of schizophrenia, making the dopamine hypothesis in some ways incomplete in explaining the pathology of the illness.

5.2.2 Serotonin hypothesis

Considering the remarkable efficacy in treating schizophrenia, shown by atypical antipsychotics (Meltzer and Fatemi, 1996), which often act via the serotonergic system, and the possibility that other neurotransmitters such as serotonin might also contribute to the etiology of schizophrenia, research on the serotonergic system has been gaining in attention and importance. The first hypotheses concerning the involvement of 5-HT in schizophrenia was advanced by Wooley and Shaw (1957)

and Gaddum (1951), and was based on the psychotomimetic effects of lysergic acid diethylamide (LSD). LSD is structurally related to 5-HT, and was proposed to be an antagonist at brain 5-HT receptors. Since the drug is a powerful hallucinogen, causing psychotic symptoms in healthy subjects, investigators proposed that serotonergic activity might be decreased in schizophrenia. Indeed, this has credence since 5-HT_{2A} and 5-HT_{1A} receptors are altered in cortical brain areas of schizophrenia patients (Harrison et al., 1999; Lieberman et al., 1998). However, an inherent drawback to this hypothesis is that the primary effect of LSD is to produce visual hallucinations, which are relatively rare in schizophrenia, and not auditory hallucinations which are the most common perceptual disturbance in schizophrenia (Aghajanian & Marek, 2000). Another concern with the LSD hypothesis is that LSD is a full or partial agonist rather than an antagonist at many 5-HT receptors, as originally predicted (Shaw & Woolley, 1956 as reviewed by Aghajanian & Marek, 2000). However, the psychotic symptoms induced by glutamate NMDA receptor antagonists are blocked by atypical antipsychotics (e.g., clozapine) and selective 5-HT_{2A} antagonists. Indeed, both hallucinogens and NMDA antagonists enhance glutamatergic transmission via stimulation of 5-HT_{2A} receptors (Aghajanian and Marek, 2000), so that the primary mediator of psychosis is the glutamatergic system. Nevertheless, postmortem-studies, as well as the examination of 5-HT in the cerebrospinal fluid, genetic studies and neuroimaging findings, have demonstrated an increase in central serotonergic neurotransmission in schizophrenia (Harrison, 1999; Ngan & Liddle, 2000; Eastwood et al., 2001; Dean, 2003). A more general statement of the role of 5-HT in schizophrenia is that functional alterations in the serotonergic system (including both pre-and postsynaptic function) affect multiple neurotransmitter systems (e.g., glutamate, GABA, norepinephrine, acetylcholine, and dopamine) and therefore does contribute to the various behavioral disturbances in schizophrenia (Fallon et al., 2003).

5.2.3 Glutamate and gamma-aminobutyric acid (GABA) hypothesis:

GABA and glutamate are respectively the most common inhibitory and excitatory neurotransmitters in the brain, so that a disturbance in glutamate and/or GABA in the brain of schizophrenia patients are an obvious consideration. Indeed, as alluded to earlier, psychosis is evoked by the administration of antagonists of the NMDA

receptor such as phencyclidine (PCP) and ketamine, both non-competitive NMDA antagonists (Wang et al., 2007 for review). Moreover, the psychotogenic effects of drugs mimicks that of schizophrenia, including negative symptoms, positive symptoms and cognitive deficits (Abi-Saab et al., 1998), thus providing a closer representation of the overall symptoms of schizophrenia (Reynolds, 2005; Adler et al., 1998; Krystal et al., 1994).

The conclusion to the above is that a predisposing factor in schizophrenia may therefore be decreased cortical NMDA receptor function (Javitt et al., 1991), specifically NMDA receptor hypofunction, that precipitates the cognitive and negative symptoms observed in schizophrenia (Carlsson et al., 2000). Glutamatergic hypofunction in frontal-cortical areas is therefore seen as the initiating factor evoking a reactive increase in dopaminergic function in limbic brain regions (see Figure 4) (Holcomb et al., 2004). Confirming this, partial deletion of the NMDA receptor 1 subunit in mice is associated with behavioral alterations akin to that observed in PCP treated mice (Mohn et al., 1999). In post-mortem studies of schizophrenia, deficits of glutamate systems have been described in the temporal cortex, medial temporal lobe and striatal regions (Bauer et al., 2008; Goff & Coyle, 2001), with losses of glutamate uptake sites (Aparicio-Legarza et al., 1997) and increases in NMDA receptors (Nudmamud-Thanoi & Reynolds, 2004). Similar changes have been observed in animal models of schizophrenia, with decreased glutamate release in the frontal cortex of the Homer1 mutant mice (Szumlinski et al., 2005), and decreased expression of glutamate receptors in the prefrontal cortex, with chronic PCP administration in rats (Barbon et al., 2007). In a recent study we demonstrated an increase in NMDA receptor binding in animals subjected to isolation rearing, a putative model of schizophrenia (see Section 8.7) (Toua et al., 2009). Interestingly, changes in glutamate is thought to be mediated by a hypofunction of GABAergic neurons, on which NMDA receptors are located, resulting in disinhibition and hence overactivity of downstream glutamatergic neurons, with evidence of significant losses in cortical and hippocampal GABA-containing neurons (Fallon et al., 2003; Lewis, 2000).

The above discussion emphasizes that schizophrenia is a severe, disabling disorder with multiple neurotransmitter dysfunctions, as summarized in Table 1. However, the role of other signaling systems, such as cannabinoids, opioids etc, have also been

found to be involved in schizophrenia, and needs to be considered (De Fonseca, et al., 2001; Schmauss & Emrich, 1985). Moreover, the role of oxidative stress and altered redox balance in schizophrenia is becoming increasingly relevant (Berk et al., 2008).

Table 1: Summary of the neurochemical/neurotransmitter findings in schizophrenia (Adapted from Miyamoto et al., 2003).

Neurotransmitter	Strength of evidence
Dopamine	
Striatal D ₂ receptors ↑	++++α
Dopamine content or metabolism ↑	+++α
Amphetamine-stimulated dopamine transmission↑	+++
Cortical D ₁ receptors ↓	+
Cortical D ₃ receptors ↑	+
D ₄ receptors ↑	+/-
Abnormal configuration of D ₂ receptors	+/-
Altered dopamine receptor–G protein coupling	+/-
Serotonin	
Cortical 5-HT _{2A} receptors ↓	+++
Cortical 5-HT _{1A} receptors ↑	++
CSF 5-HIAA concentrations related to negative symptoms	+
Glutamate	
Expression of non-NMDA receptors in the temporal cortex and hippocampus ↓	++
Cortical expression of some NMDA receptor subunits↑	++
Glutamate reuptake in frontal cortex ↑	+
Cortical glutamate release ↓	+
Altered concentrations of glutamate and metabolites	+/-

+/- = weak; + = moderate; ++ = good; +++ = strong; ++++ = very strong shown by meta-analysis.

↑ = increase; ↓ = decrease; α = Much of the increase is due to antipsychotic medication.

5. Treatment

The management of schizophrenia is extremely difficult. Although schizophrenia can not be cured by drug therapy, symptoms such as thought disorder, emotional withdrawal and hallucinations or delusions, can be attenuated by antipsychotic treatment (Kane et al., 1993). In many patients, these symptoms can be completely eliminated. Once these symptoms have been controlled, maintenance treatment can minimize the likelihood of relapse. It should be emphasized that schizophrenia is a life-long disorder that is progressive over time (Peuskens et al., 2004), and therefore needs protracted treatment. Moreover, treatment is often complicated by side effects that vary in severity from one patient to the next, and between different drugs (Prior et al., 1999). Recent research has therefore begun to focus on improving the overall benefit-to-risk ratio of long-term antipsychotic treatment by establishing optimal strategies for maintenance therapy (Wood, 1996). The value of maintenance treatment is now well established, based on numerous double-blind, placebo-controlled trials (Davis et al., 1975; Kane et al., 1987). Treatment strategies in schizophrenia vary according to the phase and severity of the illness (Miyamoto et al., 2005), and because the etiology of schizophrenia is still unknown, current treatments mainly focus on eliminating the symptoms of the disease by means of pharmacological treatment (mainly antipsychotics) and various psychosocial interventions. Psychosocial treatment, such as psychoeducation, supported employment and cognitive behavior therapy are very useful (Kane et al., 1996; Miyamoto et al., 2005 for review), but still does require pharmacological intervention to be maximally effective (Miyamoto et al., 2005). However, early and effective intervention is critical, as this may improve long-term outcomes. Indeed, first-episode schizophrenia patients respond better to antipsychotic treatment compared to chronic schizophrenia patients, possibly because first episode patients are more sensitive to treatment (Emsley et al., 2008).

The pharmacological treatment of schizophrenia mainly consists of the typical (first-generation) and atypical (second-generation) antipsychotics. This will be briefly discussed below. However, in the interest of brevity, in-depth discussion of the

different agents will be limited to clozapine, the drug that will be the focus in the current study.

6.1 Typical antipsychotics

Chlorpromazine, the first typical antipsychotic, was synthesized in 1950 (Healy, 2003). Typical, or first generation, antipsychotics comprises the following chemical subgroups: phenothiazines (e.g. chlorpromazine), butyrophenones (e.g. haloperidol), thioxanthenes (e.g. thiothixene), dibenzoxazepines (e.g. lozapine), and dihydroindoles (e.g. molidone) (Kane et al., 2009). The common pharmacological property of all typical antipsychotics is their high affinity for the dopamine D₂ receptor (Marder, 1995). In fact, there is a strong correlation between the therapeutic dose of these drugs and their binding affinity for the D₂ receptor (Kapur et al., 2000). Recent imaging studies have also found that therapeutic doses of the typical antipsychotics produce high occupancy of the D₂-like receptors in both the limbic areas and the striatum (Xiberas et al., 2001), which explains the penchant of these agents to induce severe motor side effects at therapeutic doses.

Blockade of 60–70% of D₂ receptors is required to reach a threshold of antipsychotic activity, beyond which there is little evidence of enhanced antipsychotic efficacy, except an increase in adverse effects (Kapur et al., 2006). These typical antipsychotics produce a number of problematic side-effects, including extra pyramidal side effects (akathisia, tremors) and tardive dyskinesia due to blockade of the striatal D₂ receptors, as well as cardiovascular, autonomic and other central effects due to non-specific binding to cholinergic, adrenergic and histaminergic receptors (Harvey et al., 1999) (see section 5.3).

6.2 Atypical antipsychotics

Cognitive dysfunction in schizophrenia remains an unresolved problem in the successful management of the illness, emphasising the need for improved treatments targeted at cognition (Marder and Fenton, 2004). Although second-generation antipsychotics (“atypical”), have an apparent improved negative symptom efficacy over earlier first-generation agents (Lidow, 2000), this has been challenged by the

Clinical Antipsychotic Trials of Intervention Effectiveness (CATIE) study. Essentially, what this study is saying is that first- and second-generation antipsychotic drugs are similar in mechanism of action and efficacy for psychotic symptoms, but also with regards lack of efficacy for avolition and impaired cognition (Keefe et al., 2007). It is only when considering adverse effect profiles that the second generation agents show superiority. However, clozapine was confirmed the most effective drug for refractory individuals (Swartz et al., 2008).

The introduction of clozapine into clinical psychiatry represented a major step forward in schizophrenia treatment. Because of its unique ability to address both positive and negative symptoms, and also to treat refractory patients, it became widely recognised as the definitive atypical, second generation antipsychotic (Harvey et al, 1999), “atypical” because it was regarded as being “different from those before”. Its success led to the development of other atypical antipsychotics, including risperidone, olanzapine, quetiapine, ziprasidone, sertindole and zotepine (Miyamoto et al., 2005). There is growing evidence that the atypical antipsychotics offer distinct advantages over the typical antipsychotics, such as greater improvement in negative symptoms and cognitive impairment, improved relapse prevention, improved social and functional capacity, as well as fewer EPS and less tardive dyskinesia, that together will improve patient compliance and quality of life (Lieberman et al., 2003; Miyamoto et al., 2003). But, the atypical antipsychotics present with a general increased risk of weight gain and metabolic consequences (Lieberman et al., 2003; Miyamoto et al., 2003). Atypical antipsychotics are therefore prescribed as first-line therapy in countries where they are affordable, for acute and maintenance therapy for schizophrenia (Buckley et al., 2001), as recommended by the National Institute for Health and Clinical Excellence (NICE) (Edwards et al., 2009).

The pharmacological mechanisms underlying the improved response to atypical antipsychotics remain to be identified, although extensive research in recent years has led to a greater understanding of how these antipsychotics work (Miyamoto et al., 2005). The mechanism of action of the atypical antipsychotics is mainly explained by the serotonin-dopamine (5-HT₂-D₂) antagonism theory (Meltzer, 1989), which suggests that a higher 5HT₂:D₂ binding ratio explains the enhanced efficacy

and reduced EPS liability of atypical antipsychotics (Duncan et al., 1999; Harvey et al., 1999; see section 2.5.3), although other mechanisms are also evident (see Harvey et al., 1999 for review).

Clozapine can be classified as a dibenzodiazepine, and is a known multi-receptor antagonist, with affinity for D_1 , D_2 , D_4 , $5HT_{1A}$, $5-HT_2$, α -1, α -2, muscarinic, and histamine- H_1 receptors (Nordstrom et al., 1995; Wetterling, 2001; Harvey et al., 1999). Clozapine has been credited for improved efficacy over typical agents for the negative symptoms of schizophrenia, which can be explained by its significant antimuscarinic actions, α -2 antagonism, high $5-HT_2/D_2$ ratio allowing D_1 receptor disinhibition and /or its mesolimbic-specific action via D_4 antagonism (Sanyal et al., 1997; Harvey et al., 1999). Clozapine has a well established reputation for causing little to no EPS due to a high $5-HT_2/D_2$ ratio and its low affinity for striatal D_2 receptors (Nyberg & Farde, 1996; Kapur et al., 2000, reviewed by Steward, 2002). Important to its therapeutic profile is that clozapine occupies more than 80% of cortical $5-HT_{2A}$ receptors at therapeutic doses in humans (Nordstrom et al., 1995). This is supported to have a number of benefits, including a low EPS profile as well as benefits in negative symptoms and cognition. This can be explained through clozapine's ability to disinhibit cortical-striatal dopamine neurons via actions on $5HT_{2A}$ and $5HT_{1a}$ receptors.

Serotonergic projections from the dorsal raphe nuclei project to the substantia nigra where they act on somatodendritic $5HT_2$ heteroreceptors on dopaminergic neurons to inhibit their firing (see Figure 5) (Kapur & Remington, 1996). Accordingly, serotonergic projections from the dorsal raphe project via the median forebrain bundle to the striatum and cortex, outlined in Figure 5, to inhibit neuronal firing by decreasing synthesis and / or release of dopamine. The actions of serotonin are thus to impair frontal cortical dopamine function, resulting in cognitive disturbances and depression, and/or motor disturbances as a result of modulation of striatal dopamine function (Harvey et al., 1999). Clozapine counters these effects by increasing dopamine release through stimulation of presynaptic $5HT_{1a}$ autoreceptors and thereby attenuating serotonin release, or by blocking post-synaptic $5HT_{2A/C}$ receptors and this disinhibiting dopamine function. Furthermore, these effects on $5HT_{1a}$ and $5HT_2$ receptors may also constitute an antidepressant-like action, contributing to their unique efficacy against negative symptoms (Harvey et al., 1999 for review). Clozapine also has a high affinity for $D_{3/4}$ receptors which are preferentially

distributed within the limbic system, so that by blocking these receptors clozapine is able to effectively treat psychosis without severe motor or cognitive side effects (Liégeois et al., 1995)

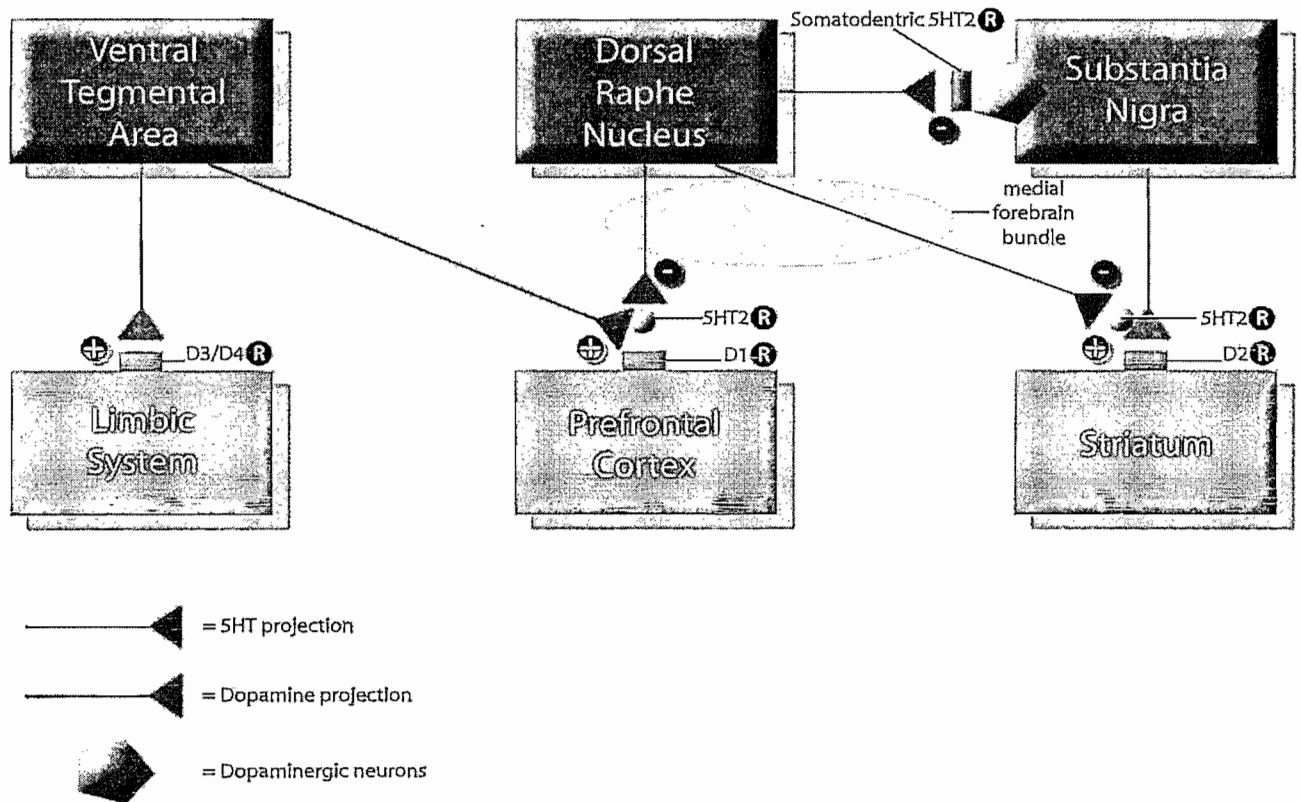


Figure 5: Mechanism of clozapine on serotonergic (5HT) and dopamine (D) receptors (R) in the substantia nigra, limbic system, prefrontal cortex and striatum (Adapted from Harvey et al., 1999).

However, a serious side effect of clozapine is agranulocytosis, which is a significant lowering of the white blood cell count, that could be fatal if not detected early on (Miller, 2000). Other side effects associated with clozapine are cardiomyopathy, constipation, dysphagia, myocarditis, seizures, urinary incontinence, urinary retention and weight gain (Henderson et al., 2000). More recently, emphasis on the metabolic side effect profile of atypical antipsychotics has made many clinicians less enthusiastic about these agents, evidence being that hypertriglyceridemia (Ahmed et al., 2009), obesity (Bustillo et al., 1996), and diabetes (Wirshing et al., 1998), all risk factors for cardiovascular diseases, are associated with atypical antipsychotics

(Henderson et al., 2000 for review). Clozapine is therefore mainly prescribed for the treatment of persistent psychotic and negative symptoms, treatment-refractory psychosis and patients with suicidal ideation (Lieberman, 2003; Meltzer et al., 2003).

6.3 Neurochemical mechanisms in antipsychotic treatment

It has been proposed that activity in the meso-limbo-cortical dopamine system explains the mechanism of antipsychotic treatment (Casey, 1996; Kapur et al., 2006). Dopamine neurons with cell bodies in the VTA and substantia nigra in the brain stem project via the meso-limbo-cortical and nigro-striatal dopamine systems to the nucleus accumbens, amygdala, the neocortex and basal ganglia, as seen in Figure 6.

The meso-cortical pathway (1 in Figure 6) innervates the frontal cortical regions, and regulates higher cognitive functions such as working memory, learning and reward (Janhunen & Ahtee, 2007). Hypodopaminergic activity of the mesocortical dopamine pathway is associated with the negative symptoms and cognitive deficits observed in schizophrenia (Knable et al., 1997). Stimulating or sustaining D₁ receptor activity in the frontal cortex will improve deficits in learning, memory and cognition in schizophrenia patients, while blocking this receptor will have the opposite effect (McLean et al., 2009). Thus, for e.g., haloperidol, which blocks both D₁ and D₂ receptors, will cause neuroleptic induced deficit syndrome and possibly worsen negative symptoms (Harvey et al., 1999; Toua et al., 2009).

The mesolimbic pathway (2 in Figure 6) innervates the limbic system and is involved in regulating emotion, reward and motivation (Leonard, 2003). Hyperdopaminergic activity at D₂ receptors in the NAcc has thus been associated with the positive symptoms of schizophrenia (Carlsson et al., 2004), as well as in mediating the euphoric effects of drugs of abuse, so that by blocking the D₂ receptors, an antipsychotic will suppress these psychotic manifestations (Keshavan et al., 2008).

Dopamine cells in the substantia nigra also project to the caudate putamen via the nigrostriatal system (3 in Figure 6), which controls posture and motor behavior (Janhunen & Ahtee, 2007). D₁ and D₂ receptors are predominant in this brain region, so that disturbances in dopamine function here will be associated with disorders of motor function, such as Parkinson's disease, dyskinesia, dystonia, stereotypies etc.

Blocking striatal dopamine D₁ and D₂ receptors will result in extrapyramidal side effects (EPS) and tardive dyskinesia (Janhunen & Ahtee, 2007).

The fourth dopamine projection is the tuberoinfundibular pathway (4 in Figure 6), projecting from the dopamine cell bodies in the hypothalamus to innervate the pituitary gland, regulating the secretion of growth hormone and prolactin (Dubuc et al., 2002). Thus, blockade of D₂ receptors in this pathway will result in hyperprolactinaemia, galactorrhoea and sexual dysfunction (Meany et al., 2002; Halbreich & Kahn, 2003).

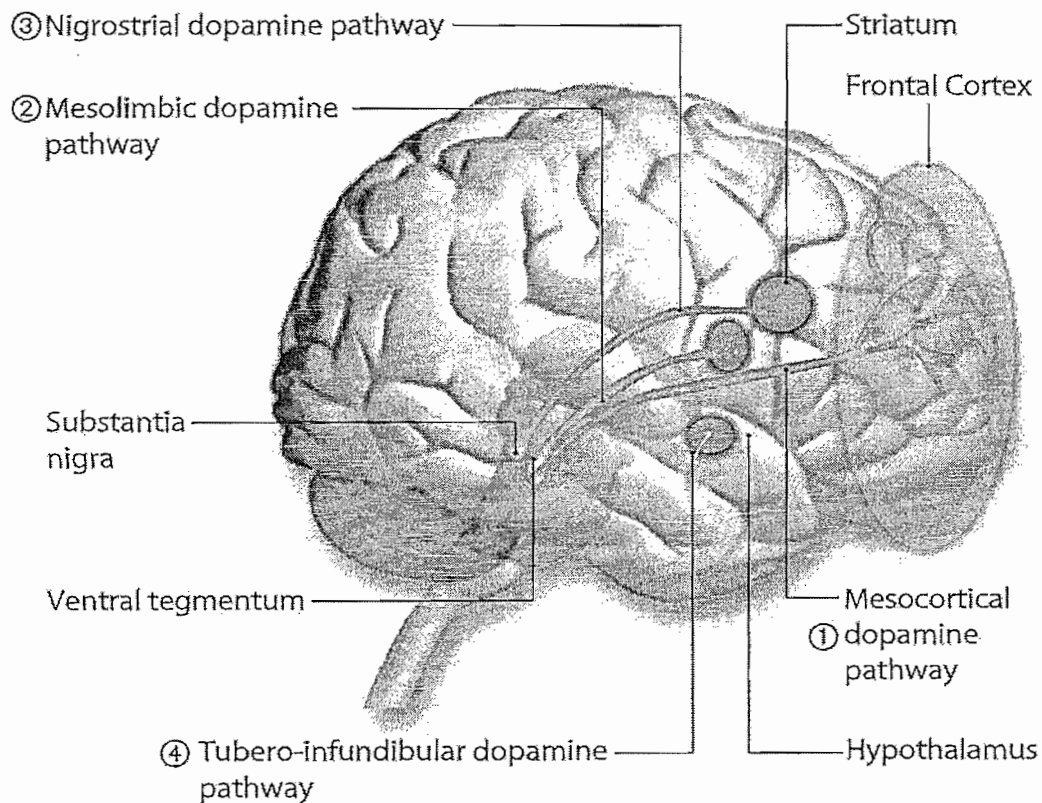


Figure 6: The four principle dopaminergic projections in the brain (Adapted from Leonard, 2003). Pathways: 1, Mesocortical projection. 2, Mesolimbic pathway. 3, Nigrostriatal pathway. 4, Tubero-infundibular pathway.

Dopamine receptors can be classified into D₁-like (D₁ and D₅) and D₂-like receptors (D₂, D₃, D₄). Moreover, each of these receptor subtypes may have variants that

have the potential for explaining heterogeneity in drug responses (Emilien et al., 1999).

Thus, antipsychotic treatment with preferential activity at meso-limbo-cortical dopamine sites would result in clinical effectiveness with fewer neurological side effects. However, all antipsychotics have affinity for striatal dopamine receptors, yet the reason why atypical antipsychotics have fewer EPS is related to their additional effects on 5-HT_{2A} receptors, as discussed earlier (Figure 5), which increases dopamine turnover in the striatum. This same mechanism explains their beneficial effects on dopamine functioning in the cortex. In addition, agonism of 5-HT_{1A} receptors also contributes to enhancing prefrontal dopamine release (Figure 5; Ichikawa et al., 2001). Consequently, by acting on cortical serotonergic receptors, clozapine will augment dopamine and norepinephrine release in the prefrontal cortex relative to the subcortical regions thereby preventing or treating hypofrontality, and improving efficacy for addressing negative symptoms and cognitive dysfunction (Li et al., 1998).

The prefrontal cortex also contains high densities of 5-HT_{1A} and 5-HT_{2A} receptors located on afferents to and on pyramidal neurons (Martin-Ruiz et al., 2001). Activation of these 5-HT_{2A} receptors promote the release of glutamate (Aghajanian & Marek, 2000) and thus address diminished cortical glutamate function. Serotonin also inhibits the release of glutamate via the activation of 5-HT_{1A} receptors (Tanaka & North, 1993), so that 5-HT_{2A} antagonism and/or 5-HT_{1A} agonism by clozapine effectively regulates the neurochemical and physiological balance between excitatory and inhibitory inputs onto the prefrontal cortical pyramidal neurons (Millan et al., 2000; Martin-Ruiz et al., 2001), and thus benefit frontal lobe function.

6.4 Other considerations in treating schizophrenia

A recent study indicated that only 47% of schizophrenia patients are treated with antipsychotics, while 43% of patients receive one additional class of medication, and 10% of patients receive 2 additional classes of medication in addition to their antipsychotic medication (Cascade et al., 2008). These additional medications mainly consists out of antidepressants (28%), mood stabilizers (18%), agents to treat

EPS (7%) and lastly sleeping aids (5%) (Cascade et al., 2008), indicating that other pharmacological treatments may assist with the successful overall management of schizophrenia.

Another important clinical aspect of schizophrenia that has been recently identified is that the duration of untreated psychosis (DUP), which is generally long in schizophrenia, appears to be causally linked to an increased variability in response to drug treatment (Gunduz-Bruce, et al., 2005). Thus, patients with a shorter duration of untreated psychosis have a better adherence to treatment, while they also respond more quickly to treatment than patients with a longer duration of untreated psychosis (Chow et al., 2005). A study by Amminger and colleagues, 2002, also found that a long DUP may be associated with greater cognitive deterioration in patients with first episode psychosis. The emphasis today should be on early intervention, since it is now evident that treating first episode schizophrenia patients, even with a low dose of typical antipsychotic, can achieve sustained remission after a first psychotic episode, compared to patients suffering from chronic schizophrenia (Emsley et al., 2008).

The main hurdle in treating schizophrenia is non-adherence to antipsychotic medications, with 50% of patients being non-compliant, which escalates after the onset of the disorder (Fenton et al., 1997). A fundamental symptom of schizophrenia is impaired insight which in turns leads to poor adherence, which is the greatest risk factor for relapse or persistence of symptoms (Cooke et al., 2007; Rocca et al., 2009). Mechanisms aimed at improving compliance, decreasing the risk of recurrence of psychotic symptoms and ultimately improving the quality of life in the schizophrenia patient, are critical outcomes and are described below (section 2.6).

6. Quality of life in schizophrenia

Broadly defined, quality of life can be described as satisfaction in different areas of life, and in objective criteria such as social functioning, activities of daily living and physical health (Lehman, 1988). The quality of life for schizophrenia patients is associated with overall levels of general psychopathology, increasingly seen as an important indication of daily functioning (Huppert et al., 2001).

People suffering from schizophrenia have a substantially lower quality of life than healthy people (Carlsson et al., 2002a), indicating that numerous factors such as compliance with antipsychotic treatment (Coldham et al., 2002), antipsychotic side-effects, increased psychosis, and anxiety may be involved (Yen et al., 2008). It is therefore, crucial for the schizophrenia patient to obtain insight into this debilitating disorder, to realize that self-management of their illness and its treatment through better compliance, is a crucial step to reducing debilitating symptoms and to improve psychosocial functioning, ultimately resulting in a better quality of life (Kozuki et al., 2005; Rocca et al., 2009). Depression in schizophrenia patients significantly contributes to a number of indices of subjective quality of life (Galletly et al., 1997; Huppert et al., 2001). Insight is also related to depression, hopelessness, lower self-esteem (Cooke et al., 2007; Rocca et al., 2009) and lower quality of life (Hasson-Ohayon et al., 2006). Studies have found that compliance to antipsychotics has no direct effect on quality of life, but rather an indirect effect through a reduction of psychotic symptoms (Puschner et al., 2009). Thus, the balance between adverse effects and reduction in symptoms are extensively associated with the benefit a patient perceives in terms of improved quality of life.

7. Reactive oxygen species (ROS) and natural defence mechanisms

A major source of radicals in biological systems is superoxide ($O_2^{\cdot-}$) (Figure 6), and the radicals originating from $O_2^{\cdot-}$, namely hydrogen peroxide (H_2O_2), singlet oxygen (O_2), hydroxyl radical ($\cdot OH$) and peroxy radical ($ROO\cdot$), while reactive nitrogen species (RNS) include nitric oxide ($NO\cdot$) and the highly toxic peroxynitrite ($ONOO^-$) anion (Figure 6) (Akyol, 2002). The production of oxygen radical in the body follows the reduction of oxygen, which is a normal process in the body, being ultimately necessary for generating energy (Lohr & Caligiuri, 2003) In the mitochondria, arachidonic acid metabolism and enzymes such as xanthine oxidase, nitric oxide synthase (NOS), monoamine oxidases all have the ability to produce ROS in the brain (Schulz et al., 2000).

The antioxidant defence systems in the body consist of enzymes such as superoxide dismutase (SOD) (in the mitochondria), glutathione peroxidase (GSH-Px), (an

endogenous antioxidant-enzyme), catalase (CAT), and nonenzymatic antioxidants which such as glutathione (GSH) (Figure 6), ascorbic acid (vitamin C), α -tocopherol (vitamin E), carotenoids and flavonoids (Akyol, 2002). SOD is a group of potent protective enzymes that can selectively scavenge the superoxide radical by catalyzing its dismutation to hydrogen peroxide and molecular oxygen (Figure 6) (Herken et al., 2001). CAT and GSH-Px aid to decompose H_2O_2 to water, as indicated in Figure 6 (Fridovich, 1983).

GSH is one of the most vital antioxidants in the human body. It maintains the redox state of critical protein sulfhydryls that are necessary for redox-sensitive processes (Jones, 2008), such as receptor activation (e.g. NMDA receptor (Lipton et al., 2002)), and cell cycle regulation and differentiation (Shi et al., 2000). The main protective roles of GSH against oxidative stress can be described as follows:

- GSH is a cofactor for a number of detoxifying enzymes that protect against oxidative stress, including glutathione peroxidase and glutathione transferase.
- GSH detoxifies hydrogen peroxide and lipid peroxides by converting $\bullet OH$ and singlet oxygen (O_2) directly by the catalytic action of glutathione peroxidase (Figure 6).
- GSH is involved in neurodevelopment (Dringen, 2000; Shi et al., 2000).
- GSH is able to convert most important antioxidants, vitamin C and E, back to their active forms.
- In particular, a deficiency in GSH or a increase in ROS, lead to NMDA receptor hypofunction (Do et al., 2009 for review).

A GSH deficiency will therefore results in oxidative stress. When the production of ROS overwhelms the capacity of endogenous free radical scavengers, ROS attack the polyunsaturated fatty acids (PUFAs) (Figure 6) in the membrane lipid of cells resulting in lipid peroxidation and the release of malondialdehyde (MDA), a product of cell damage (Akyol et al., 2002). Under these conditions, the defence system cannot prevent the escape of ROS, particularly from mitochondria, and engendering deleterious effects on other intracellular compartments. Oxidative and nitrosative stress therefore result from an imbalance between the overproduction of ROS and RNS on the one hand, and deficiency of enzymatic and non-enzymatic antioxidants on the other, leading to extensive peroxidation of lipids, proteins and DNA strands

(Valko et al., 2007), ultimately compromising mitochondrial function and lipid metabolism (Do et al., 2009; Keller & Glaze, 2001).

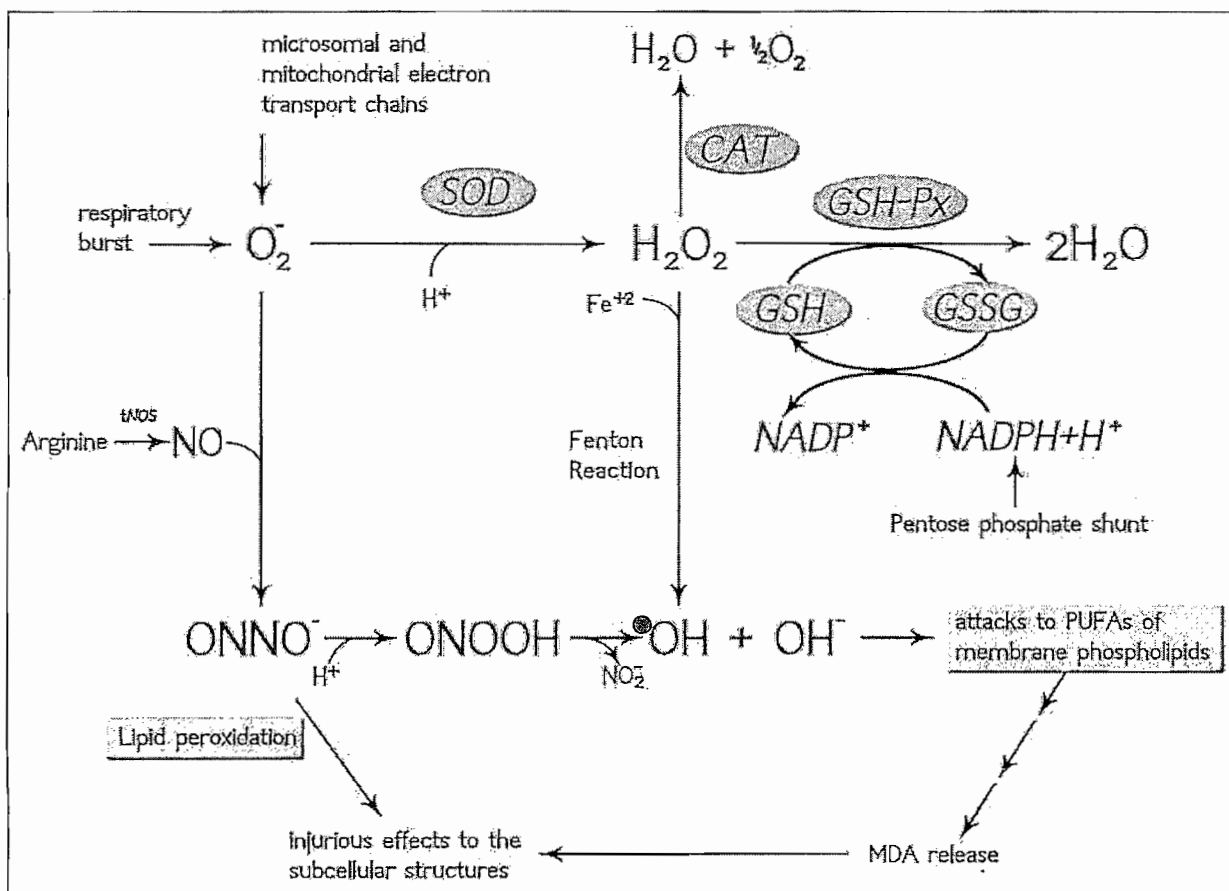


Figure 6: Schematic representation of cellular reactive oxygen and enzymatic antioxidant systems with lipid peroxidation processes (Adapted from Akyol, 2002).

The brain is particularly vulnerable to oxidative damage because of its high oxygen utilization (e.g. catecholamine degradation, etc.), low levels of antioxidant defence enzymes, high content of oxidizable membrane polyunsaturated fatty acids (PUFAs), a high ratio of membrane surface area to cytoplasmic volume and the presence of redox-active metals (Cu and Fe) (Do et al., 2009; Akyol, 2002). Dopamine (DA), one of the major sources for ROS in the central nervous system, can be oxidized to DA semiquinone/quinone, which have potent oxidizing properties (Janaky et al., 1999)

by reacting with cellular thiols including DA or glutamate transporters (Berman et al., 1996) or with mitochondrial proteins (Berman & Hastings, 1999). In addition, via the action of monoamine oxidase (MAO) (Sinet et al., 1980), or via oxidized DA through redox cycling (Brunmark & Cadenas, 1988), DA can also induce the generation of H_2O_2 and O_2^- , leading to oxidative stress and impairment of cellular processes (Hastings & Zigmond, 1996). Considering the significant disadvantage of the brain under conditions of oxidative stress, it is not surprising that numerous central nervous system disorders, including Alzheimer's disease, Huntington's disease, Parkinson's disease, tardive dyskinesia, schizophrenia, depression and autism, have all been associated with oxidative stress/ redox dysregulation (Bilici et al., 2001; Kuloglu et al., 2002; Berk et al., 2008).

8.1 Oxidative stress in schizophrenia

Converging evidence indicates that schizophrenia is a neurodevelopmental disorder (section 4.2), while various anatomical findings point to a vastly distributed neuropathology, possibly involving oxidative stress (Do et al., 2009).

The neurodevelopment of schizophrenia is believed to be affected by early environmental insults, which include malnutrition, exposure to toxins, maternal infection, obstetrical complications (preeclampsia and hypoxia), and maternal or early-life stress (Brown & Susser, 2008). Importantly, these insults result in an increase in ROS, lipid and protein peroxidation and DNA damage, and a decrease in GSH and antioxidant defence systems (Akyol, 2002). Early life insults also lead to increased inflammation (Garcia-Bueno et al., 2005) emphasizing the close link between inflammation, oxidative stress and the neurodevelopmental hypothesis of schizophrenia.

The developmental dysregulation of GSH synthesis is proposed by Do et al., 2009 to be of genetic origin in schizophrenia, and when combined with environmental risk factors that can boost levels of oxidative stress, it can play a critical role in inducing deficits in neural connectivity and synchronization evident in the disease (Do et al., 2009). According to the glutamate hypofunction hypothesis, developmental stages that are affected by exposure to oxidative stress, and that are dysfunctional in

schizophrenia, may be mediated by hypoactive NMDA receptors, developmental impairment of GABA interneurons and anomalies in myelination (Do et al., 2009).

Evidence has accumulated in recent years that antioxidant systems are impaired in schizophrenia (Mahadik & Mukherjee, 1996). Thus, an increase in products of lipid peroxidation in the blood (Akyol, 2002), and decreased CAT activity associated with a decrease SOD activity in different forms of schizophrenia (Rachkauskas, 1998), have been described. Another study found significantly lower SOD activity in erythrocytes of schizophrenia patients, although with no differences in CAT and GSH-Px activities (Mukherjee et al., 1996). Herken and colleagues (2001) found increased CAT and GSHPx activities, but not SOD activity, as well as significantly increased peroxidation in the erythrocytes of schizophrenia patients. Taking into account the important antioxidant properties of GSH, a study by Do and colleagues (2000) found a 52% decrease in GSH levels in the prefrontal cortex of schizophrenia patients. The mitochondria therefore represent an important target during oxidative stress under conditions of decreased GSH.

DA, via monoamine oxidase (MAO) activity (Maker et al., 1981), or oxidized DA, through redox cycling (Brunmark & Cadenas, 1988), can also induce the generation of H_2O_2 and $O_2^{\cdot -}$, which are known to evoke lipid peroxidation, DNA modifications and protein oxidation (Grima et al., 2003 for review). Thus, the efficacy of antipsychotics and their ability to target DA metabolism via blockade of D_1/D_2 receptors, may play an important role in suppressing ROS formation, resembling indirect antioxidant properties (Grima et al., 2003).

The aforementioned studies are therefore adamant that both oxidant and antioxidant systems, and oxidant/antioxidant balance, play a pathophysiological role in schizophrenia. It would thus be interesting to study the efficacy of antioxidant drugs in the treatment of this disease, alone and in combination with the more classical treatments, as suggested in previous studies (Adler et al., 1998; Zhang et al., 2001). A recent double-blind placebo controlled study using the antioxidant and glutathione replenisher, N-acetylcysteine (NAC), in combination with antipsychotics found that treatment increased GSH plasma levels, improved negative symptoms, and reduced side effects (akathisia) in schizophrenia patients (Berk et al., 2008). Thus, adding an antioxidant to existing antipsychotic treatment demonstrates a definite improvement

in treatment response. However, it is unclear if the oxidative stress in schizophrenia is due to excess production of ROS or deficient antioxidant mechanisms, or what the source of raised ROS may be.

8. Animal models

Epidemiological studies have shown increased incidence of schizophrenia in patients subjected to different forms of pre- and perinatal stress (Van den Buuse et al., 2003). It is not yet fully understood how disruption of early brain development may ultimately lead to malfunction years later. In order to identify the key neurodevelopmental factors in the pathogenesis of schizophrenia, and to highlight potential new targets for drug treatment, analogous animal models of schizophrenia are needed (Van den Buuse et al., 2003).

Numerous animal models of schizophrenia have been developed, all seeking to replicate one or more of the symptoms of the illness (Dawe et al., 2009). However, no particular model has yet been developed that is able to completely replicate all the symptoms observed in schizophrenia. Numerous behaviors, such as hyperactivity, hyper-locomotion and stereotypic behavior in animal models are considered to be related to the positive symptoms observed in schizophrenia (Bikcel & Javitt, 2009). Although negative symptoms of schizophrenia are extremely difficult to model in animals, this form of behavior in man is considered to be related to decreased social behavior in animals (Meyer & Feldon, 2009). Prepulse inhibition (PPI) and latent inhibition (LI) on the other hand are measures of deficits in information-gating at a preattentive sensorimotor reflex level and of attentional habituation and processing respectively (Marcotte et al., 2001; Kapur, 2003), while cognitive functions in animals are often tested by various maze tasks, e.g. Y maze, Morris water maze (Marcotte et al., 2001). Current animal models of schizophrenia are not intended to serve as an absolute animal equivalent of the human disorder, but rather to assist in evaluating specific causative or mechanistic hypotheses regarding schizophrenia (Marcotte et al., 2001). Before they have utility in pre-clinical research, these models need to be validated according to certain criteria, as described below.

9.1 Validation of animal models

- Face validity

Face validity describes how accurately the model reproduces the clinical symptoms of the human illness, such as behavior, as well as the degree of similarity (Marcotte et al., 2001; Meyer & Feldon, 2009).

Face validity is also considered to be the most difficult to establish (Marcotte et al., 2001), due to the fact that animals have their own species-specific behaviors, with little resemblance to the behaviors in humans (van der Staay et al., 2006).

- Construct validity

The construct validity of an animal model assesses whether the model is in agreement with current theoretical rationale regarding the human illness it is attempting to model (Marcotte et al., 2001). This form of validity often involves studying the similarity of neurobiological mechanisms in the animal model and the mechanisms known to be involved in the human disorder (Van den Buuse et al., 2003). However, this form of validity has its limitations in what is known about the human disorder, since in many psychiatric disorders the exact neurobiological basis is unknown.

- Predictive validity

Predictive validity reflects how well the model can predict a given response in the human disorder (Lipska & Weinberger, 2000). In this regard, the ability of the model to identify drugs that have therapeutic significance in humans is a particularly valuable aspect of validity (Geyer & Markou, 1995).

According to the schizophrenia research forum (Taylor et al., 2009), there are approximately 87 animal models that have bearing on schizophrenia, each with a

substantial body of published research to back it. However, I will only focus on some of the most widely used animal models, but in particular the model that is of relevance to my study.

9.2 NMDA receptor antagonist models:

Neurodevelopmental models of schizophrenia emphasize the construct of glutamate hypofunction in schizophrenia, and especially NMDA receptor inhibition. Administration of an NMDA receptor antagonist, such as dizocilpine (MK 801), PCP or ketamine, in late fetal and early postnatal period of life in the rat increases neuronal death by apoptosis (Takadera, et al., 2006). On the contrary, administration of these substances to rats at an adult age will increase neuronal damage by necrosis with subsequent gliosis (Manahan-Vaughan et al., 2008). PCP, ketamine and MK-801 administration to (3 month old) animals produces impairments in cognitive function (Pitsikas et al., 2008), while similar treatment perinatally significantly impairs spatial memory performance, as well as causes alteration in social behaviour, hyperactivity and sensorimotor gating deficits (Moghaddam et al., 1997; Wiley et al., 2003).

9.3 Amphetamine sensitization model:

Based on the endogenous dopamine sensitization hypothesis of schizophrenia, as described in section 4.3.1, rodents treated with the psycho-stimulant, amphetamine, develop a state of behavioral sensitization, a phenomenon that is characterized by a progressive augmentation on behaviors and increased dopamine levels in the ventral striatum as a response to subsequent drug challenges (i.e. sensitisation), chronic or continuous treatment with moderate to high doses of amphetamine in nonhuman primates elicits a subset of behaviors that approximate the visual or auditory hallucinations in both amphetamine psychosis and schizophrenia (Castner et al., 2005 for review).

The positive symptoms observed in schizophrenia are therefore, closely replicated in rats and mice following the administration of amphetamine, which evokes dopamine release. In this case, the animal responds with hyperactivity and behavioral

disinhibition, as well as decreased PPI and LI (Kilts, 2001). These deficits in PPI and LI compare well to the same deficits observed in schizophrenia patients (Powell et al., 2003; Geyer & Ellenbroek, 2003; Powell & Geyer, 2007). However, a limitation of the amphetamine treated models is that they are not able to induce social withdrawal, thus lacking the ability to relate to the negative symptoms of the disorder (Featherstone et al., 2007), making the amphetamine model unsuitable as an analogous model of schizophrenia.

9.4 Hippocampal lesions:

Neonatal excitotoxic lesioning of the rat ventral hippocampus may serve as a heuristic model of schizophrenia (Lipska, 2003; Tseng et al., 2009; Chambers & Self, 2002). The model mimics a spectrum of neurobiologic and behavioral features of schizophrenia, such as disrupted sensory motor gating, and reduced PFC spine density (Marquis et al., 2008; Pen et al., 2003). This form of functional pathology is created in a critical brain region associated with the hippocampal formation, namely, the striatum, nucleus accumbens and the prefrontal cortex implicated in schizophrenia (Tseng et al., 2009).

Compared to healthy controls, neonatal ventral hippocampal lesion (NVH) rats display deficits in social interactive behavior at postnatal day (PND) 35, and at postnatal day 56 they show motor hyper responsiveness to stressful stimuli as well as elevated stereotype behavior (Daenen et al., 2002). The NVH model also demonstrates an increased sensitivity to the NMDA antagonists, PCP and dizocilpine, including deficits in PPI and latent inhibition, impaired social behavior and deficits in working memory, with all deficits observed in schizophrenia and normalized by antipsychotic treatment (Lipska, 2000; Lipska, 2004; Berg & Chambers, 2008). The limitation of this model, however, is that the human schizophrenia brain does not show any signs of a comparable lesion and thus, while NVH rats may be useful for testing the efficacy of antipsychotic drugs, it cannot be regarded an analogous model of schizophrenia (Lipska et al., 2000; Marcotte et al., 2001).

9.5 Social isolation reared (SIR) model:

The social isolation rearing (SIR) model is the subject of this investigation. This postnatal developmental model explores the effects of environmental injuries via social deprivation to the developing brain after birth (Ferdman et al., 2007). SIR gained significant interest as a developmental model after it was found to induce pronounced deficits in prepulse inhibition (PPI) in rats (Varty & Geyer, 1998; Heidbreder et al., 2000; Weiss & Feldon, 2001), which is a test of pre-attentional sensorimotor gating that also shows impairments in schizophrenia (Braff & Geyer, 1990; Swerdlow et al., 1994). Rearing rats in isolation has since been shown to be a useful paradigm for studying the impact of early life stress on subsequent behavioral changes in adulthood, and also to understand the etiology of depression and related affective disorders (Heidbreder et al., 2000). Importantly, antipsychotics can reverse PPI disruption in SIR rats (Geyer et al., 1993 for review), providing SIR with important predictive validity for schizophrenia and hence of value for pharmacological studies and for testing antipsychotic drugs (Toua et al., 2009).

Over and above impairments in PPI, SIR also presents with a number of post-pubertal behavioural and neurochemical changes, some of which are similar to those observed in schizophrenia, including locomotor hyperactivity in the open field (Weiss et al., 2000), altered responsiveness to psychostimulants like amphetamine (Hall et al., 1998) and impaired cognitive functions, such as attentional shifting (Schrijver & Wurbel, 2001) that are akin to the cognitive deficits observed in schizophrenia patients (Geyer et al., 1993; Cilia et al., 2005). Moreover, we have recently shown in our laboratory that rats reared in isolation demonstrate opposing changes in NMDA and D₁ receptor binding in the frontal cortex of rats, with remarkable similarity to that observed in schizophrenia patients (Toua et al., 2009). Earlier studies have noted a reduction in the expression of NMDA NR₁ receptor subunit mRNA in the hippocampus of schizophrenia patients (Gao et al., 2000 reviewed in Toua et al., 2009). A study by Okubo and colleagues (1997) also found reduced D₁ binding in schizophrenia patients, while a recent study by Carlsson (2002b) and Abi-Dargham and colleagues (2002) reported no change or increased D₁ binding in the frontal cortex. Moreover, these receptor changes are oppositely affected by typical and

atypical antipsychotics, thus providing novel evidence of predictive validity (Toua et al., 2009).

Some of the neurochemical alterations observed following SIR includes reduced cortical DOPAC (3,4-dihydroxyphenylacetic acid)/DA turnover in the frontal cortex (Blanc et al., 1980; Heidbreder et al., 2000), reflecting lower DA metabolism.

This is also in agreement with altered D₁ receptor binding described in the frontal cortex of SIR rats (Toua et al., 2009). When considering neuroanatomical changes, SIR rats show a significant decrease in the density of dendritic spines in layer 3 pyramidal neurons of the PFC (Silva-Gomez et al., 2003). The behavioral and neurochemical changes induced by isolation rearing in rats, as well as their reversal by antipsychotic drugs provide a non-lesion and non-pharmacological model with face, predictive and construct validity for schizophrenia. The model therefore has great potential for enhancing our understanding of the developmentally linked emergence of neural and behavioral abnormalities that is characteristic of schizophrenia (Weiss & Feldon, 2001).

9. Conclusion

Schizophrenia is a severe, disabling multifactorial disease involving genetic, environmental and neurodevelopmental factors (Weiss & Feldon, 2001).

Despite the marked improvement of positive, and to some extent, negative symptoms in schizophrenia patients with antipsychotic treatment, lack of efficacy and side-effects hampers compliance and thus favors relapse (Lipkovich et al., 2007). Numerous hypotheses have been developed to define and include the various brain circuits and neurochemical aberrations present in schizophrenia. However, further research is needed in order to improve our understanding of the illness, to stimulate new and improved treatment, ultimately to improve the quality of life in the schizophrenia patient. To this end, validated animal models are an urgent need.

One of the most difficult aspects of schizophrenia to model in animals has been the lack of a clear and explicit pathophysiological framework for this illness. Despite the importance and relevance of the neurodevelopmental theory, it has remained difficult to develop specific hypotheses that can be tested in animals (Meyer & Feldon,

2009). Future research should therefore unite the various neurodegenerative–neurodevelopmental and genetic–environmental aspects of the disorder. Multiple factors may contribute to abnormal brain maturation up to puberty, with the ensuing emergence of symptoms and their progression over time. A greater understanding of the various genetic factors involved and the environmental forces that modulate their expression over time may help us to develop more sophisticated animal models. Ultimately, such models may help to expand our knowledge of this poorly understood disorder (Marcotte et al., 2001).

10. Summary of aims and objectives

As has been outlined in Chapter 1 section 2 (Aims and Objectives), the SIR model has not yet been studied with regards to effects on social interaction. In addition, SIR has not been studied for face validity using two behavioral measures that are simultaneously related to schizophrenia, in this case social interaction and sensorimotor gating using PPI. Predictive validity with respect to antipsychotic drug treatment on both these behaviours has also not been determined, which will significantly strengthen the validity of the model. The aim of this study was therefore to investigate a possible relationship between SIR, as a neurodevelopmental model of schizophrenia, and its effects on the above behaviours, as well as their response to sub-chronic treatment with the atypical antipsychotic, clozapine (5 mg/kg/day). Finally, in order to extend the construct validity of the model for new theoretical rationale relating to schizophrenia, behavior and treatment response in SIR and socially reared animals were correlated with frontal cortex and striatal markers of oxidative stress, including the activity of the antioxidant defence enzyme SOD, the oxidized (GSSG)/ reduced (GSH) ratio and levels of lipid peroxidation.

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Chapter 3

Article

Article for submission for publication in *European Neuropsychopharmacology*.

Introduction:

This chapter presents the full-length manuscript for submission to *European Neuropsychopharmacology*, publisher by Elsevier. The manuscript is presented in the required format prescribed by *Instructions to the Authors*. As per the journal website:

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The complete Instructions to the Authors have been reproduced and included in the dissertation under Appendix C. Thus, the manuscript will begin with the title, contributing authors and affiliations on a separate page, followed by an Abstract, also on a single page. Thereafter will follow the main body of the manuscript, including Introduction, Experimental procedures, Results, Discussion, Acknowledgements, References, Legends to Figures and Figures. As per the journal submission format, all figures are separate, and placed at the end of the manuscript.

Isolation rearing induced deficits in sensory motor gating and social interaction in rats is causally related to cortico-striatal oxidative stress, and are reversed by sub-chronic clozapine administration

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Abstract:

Social isolation rearing (SIR) in rats models the neurodevelopmental aspects of schizophrenia. Schizophrenia is associated with increased oxidative stress. In this study we demonstrate that SIR increases superoxide dismutase (SOD) activity, decreases oxidized versus reduced glutathione ratio, and increases lipid peroxidation, in both the frontal cortex and striatum of isolation reared rats. Moreover, these redox changes are associated with deficits in prepulse inhibition (PPI) as well as social and self-directed interactive behaviours, symptoms akin to that in schizophrenia. Clozapine treatment (5 mg/kg/day x 11 days) reversed PPI and social interaction deficits, as well as corrected cortico-striatal redox disturbances. SIR thus presents with robust face and predictive validity for schizophrenia, with evidence for increased oxidative stress providing novel construct validity.

Keywords: social isolation, prepulse inhibition, social interaction, clozapine, schizophrenia animal model.

1. Introduction

Early adverse experiences may “shape” a pre-existing genetic vulnerability to stress and disease (Heim & Nemeroff, 2001). Schizophrenia has been causally linked to genetic, environmental and neurodevelopmental factors (Weiss & Feldon, 2001), with epidemiological studies showing increased incidence of schizophrenia in patients subjected to different forms of pre- and perinatal stress (Van den Buuse et al., 2003). Schizophrenia is characterized by positive symptoms that include hallucinations, delusions and thought disorder, by negative symptoms comprised of affective flattening and social isolation, and by cognitive deficits in attention, learning, memory and behavioural flexibility (Ross et al., 2006, Lewis & Gonzales-Burgos, 2008). Another important presenting symptom is anxiety, which occurs in conjunction with positive symptoms (Tandon et al., 2009). While antipsychotics (typical and atypical) control positive symptoms; little therapeutic advances have been made in alleviating negative and cognitive symptoms (Keefe et al., 2007; Marder & Fenton, 2004).

Glutamate N-methyl-D-aspartate (NMDA) receptor dysfunction and excitotoxicity is involved in a variety of acute and chronic neurological disorders (Waxman and Lynch, 2005), and is also central to explaining the neurobiology of schizophrenia (Krystal et al., 2005; Olney et al., 1999), including positive, negative and cognitive symptoms (Krivoy et al., 2008). Glutamatergic neurons are present in both the striatum and cortex (Amadio et al., 2004; Haroutunian et al., 2003), where excessive release of glutamate, activation of NMDA receptors, and the resulting surge in calcium influx, will alter dopamine release (Moore et al., 1999) as well as have implications for cellular redox balance (Smythies, 1999). The end result is an increase in reactive oxygen species (ROS) (Love, 1999), depletion of cellular energy and a further release of glutamate and eventually apoptotic cell death (Betzen et al., 2009). Indeed, altered redox state is evident in schizophrenia, including reduced superoxide dismutase (SOD), catalase and glutathione peroxidase (Mukherjee et al., 1996), as well as increased lipid peroxidation (Kuloglu et al., 2002).

Early life adversity has been found to affect neuronal growth and differentiation (Bloom, 1993; Murray, 1994; Weinberger, 1987; Weiss and Feldon, 2001) and is deemed an important risk factor for the later development of schizophrenia (Lipska & Weinberger, 2000). In rodents, social isolation rearing (SIR) leads to long-lasting behavioral alterations (Andreas et al., 2004), and has been used to study aberrant behaviors akin to that observed in schizophrenia (Heidbreder et al., 2000), such as neophobia (Fone, 2008), locomotor hyperactivity (Levine et al., 2007; Weiss & Feldon, 2001), learning and attentional shifting (Andreas et al., 2004), and sensorimotor gating deficits, such as in prepulse inhibition (PPI; Schubert et al., 2009; Geyer & Ellenbroek, 2003). PPI reflects the basic inhibitory process of sensorimotor gating that regulates the input of sensory stimuli to the brain, thus preventing cognitive fragmentation and sensory overload typical of schizophrenia (Young et al., 2009). SIR induced behavioral changes has been correlated to a hyperactive mesolimbic dopaminergic system (Shao et al., 2009), as well as with reduced input to the prefrontal cortex. The latter is perceived to lead to weakened functional connections resulting in dendritic atrophy, decreased spine density of pyramidal neurons in the prefrontal cortex and hippocampus (Silva-Gomez et al., 2003), and an associated reduction in volume (Schubert et al., 2009). SIR induced changes in frontal cortical NMDA and dopamine D₁ receptor binding (Toua et al., 2009) has been used to explain the altered state of cognitive functioning in these animals, and possibly in schizophrenia. These bio-behavioral and neuro-anatomical consequences of SIR, and that antipsychotics can reverse SIR-induced attenuation of PPI (Pen & Moreau, 2002) as well as selectively altering D₁-NMDA receptor binding (Toua et al., 2009), emphasizes the important face, construct and predictive validity of SIR for schizophrenia, as well as for studying antipsychotic drug action (Andreas et al., 2004; Geyer & Ellenbroek, 2003; Silva-Gomez et al., 2003). Moreover, with SIR increasing frontal cortical NMDA receptor density (Toua et al., 2009) as well as prefrontal cortex NR_{2A} NMDA expression (Turnock-Jones et al., 2009) suggests that isolation rearing profoundly changes NMDA receptor function, with distinct implications for altered redox state.

In the current study we studied the association between social interaction and PPI in rats subjected to 8 weeks SIR, with changes in frontal-cortical and striatal levels of

SOD activity, oxidized versus reduced glutathione ratio (GSSG/GSH) and malondialdehyde (MDA) accumulation (a marker of lipid peroxidation). In order to more specifically relate these bio-behavioral changes to schizophrenia and with antipsychotic response, we also investigated the above with respect to response to sub-chronic treatment with the atypical antipsychotic agent, clozapine.

2. Experimental procedures

2.1 Animals

Male Sprague-Dawley rats, initially weighing 160 – 190g were obtained from the animal research centre of the North-West University (Potchefstroom Campus). All groups consisted of 10 rats / group. At weaning (postnatal day 21), animals were randomly separated into social isolation-reared (SIR) animals (one animal/cage) and socially reared control animals (groups of 3-4 rats/cage). The rats were reared in identical cages containing sawdust (Weiss & Feldon, 2001) and housed under controlled ambient conditions (temperature: $21 \pm 5^{\circ}\text{C}$, humidity: $50 \pm 10\%$). Free access to food and water were allowed with full spectrum cold white light (350 – 400 lux) provided over a 12 hour light/dark cycle. Positive air pressure was maintained in the facility with air filtration 99.7 % effective for a particle size of 2 micron and 99.9 % for a particle size of 5 micron. During the period of isolation, animals were maintained with minimal handling, and no environmental enrichment. Cages were changed once a week with fresh sawdust. The behavioural testing was carried out during the light phase of the cycle (between 08:00 and 17:00 h). Ethics approval was obtained from the Animal Ethics Committee of the North-West University (Ethics approval number NWU-0035-08-S5), and all animals were handled according to the code of ethics in research, training and testing of drugs as laid down by this committee.

2.2 Drugs and drug treatment protocol

Clozapine (Sigma-Aldrich, Johannesburg, South Africa) was administered intraperitoneally at a dose of (5 mg/kg ip), according to a previous protocol (Toua et al., 2009), while control groups received saline injection (pH 5). Clozapine was dissolved in saline containing a small amount of glacial acetic acid, made up to volume with 0.9 % sodium chloride solution, warmed and buffered to a final pH of 6 with 1M sodium hydroxide (Toua et al., 2009). Clozapine and saline were administered in the last 11 days of the rearing period. The treatment period of 11 days was elected as increasing evidence points to an early response at 2 weeks being a specific (89%) and accurate (72%) predictor of subsequent response (Kapur et al., 2005; Ascher-Svanum et al., 2008). This study therefore applied an 11 day treatment period as opposed to 28 days or longer. Nevertheless, it has been shown that on a relative scale, 7 days in the life span of a rat is approximately equivalent to 6 months in the human life span (Adamec et al., 1997). An injection volume of 0.5 ml was used and drugs were freshly prepared each day before experimental testing and stored in glass bottles covered with aluminum foil.

2.3 Experimental design

2.3.1 Non-treatment cohort

This arm consisted of a behavioral and a neurochemistry study, each comprising 2 groups of animals.

Behavioral study: Animals were randomly separated at weaning (21 days post natal) into two groups. One group of animals (n=10) were placed into isolation, while a parallel socially-reared control group (n=10) was run concurrently, but only exposed to normal daily handling for 8 weeks. After 8 weeks, both groups of animals were subjected to behavioral testing in the OFT and PPI test, as described below, with one day allocated between the two tests. Animals were sacrificed immediately thereafter by decapitation without the prior use of an anesthetic agent.

Neurochemical study: An additional two groups of animals, randomly assigned to the same groupings as described above, followed the above protocol except that at 8 weeks, the animals were sacrificed and the brains rapidly dissected for regional brain neurochemical analysis.

2.3.2 Drug-treatment cohort

As with the above, the study comprised a behavioral and a neurochemical study, each consisting of 4 groups of animals.

Behavioral study: Animals were randomly separated at weaning (21 days post natal) into four groups. Two of these groups (n=10 each) were destined for 8 weeks isolation rearing or social rearing and received saline treatment. The remaining two groups (n=10) were also destined for 8 weeks isolation rearing or social rearing but received clozapine treatment. At 8 weeks, the four groups of animals were subjected to behavioral testing in the OFT (2 groups) and PPI testing (2 groups), as described below, with one day of rest between the OFT (on day 9 of treatment) and PPI (on day 11 of treatment) tests. Animals were sacrificed immediately thereafter.

Neurochemical study: An additional four groups of animals, randomly assigned to the same groupings as described above, followed the above drug treatment protocols (saline vs. clozapine) except that at 8 weeks, the animals were sacrificed and the brains rapidly dissected for regional brain neurochemical analysis, as outlined below.

2.4 Body weight

The body weights of all rats in the isolation-reared and socially reared animals were determined at PND 21, and again on day 1 and day 11 of drug treatment, the latter being the last day of the housing conditions (i.e. week 8).

2.5 Behavioral analyses

2.5.1 Prepulse inhibition testing

PPI was assessed in two illuminated and ventilated sound-attenuated startle chambers (SR-LAB, San Diego Instruments, San Diego, USA), as described previously (Van den Buuse et al., 2003; Powell et al., 2009). Each startle chamber consists of a stabilimeter system composed of a transparent Plexiglas cylinder (diameter 8.2 cm, length 20 cm) mounted on a Plexiglas frame. A speaker mounted 24 cm above the cylinder provides the acoustic noise bursts. The startle responses of the rat within the cylinder is detected and transduced by a piezoelectric accelerometer mounted below the frame. During each session stimuli are delivered and startle responses measured by the SRLab software (San Diego Instruments) running on a personal computer. Startle amplitudes were defined as the average of one hundred 1-ms stabilimeter readings collected from the stimulus onset. The stabilimeter was calibrated before each test session.

An identical protocol was used for all the experiments, adapted from previously described methods (Geyer et al., 1998; Wang et al., 2003). The startle session started with a 5-min acclimatization period, with a 68-dB background noise level that continued throughout the test session. To evaluate the basal startle response, 10 trials consisting of a single 40ms 120 dB white-noise startle stimuli were presented to the rat. Next, the test session continued with 80 trials which consisted of a random delivery of twenty 120 dB pulse-alone trials, 10 trials during which no stimuli was delivered, and 50 prepulse trials. Prepulse trials included a single 120 dB pulse preceded by a 20 ms non-startling prepulse-stimulus with intensities of 72, 76, 80 or 84 dB. The time interval between the prepulse offset and the pulse onset was 80 ms. Percent PPI (% PPI) for the four prepulse intensities were calculated according to the formula: $\% \text{ PPI} = [100 - (\text{startle response for PREPULSE} + \text{PULSE trial}) / (\text{startle response for PULSE ALONE trial}) \times 100]$ (Van den Buuse et al., 2001). The last 10 trials were single 40 ms 120 dB pulse-alone startle stimuli. The total of 100 trials was delivered with an average interval of 25 s. 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 (Wang et al., 2003; Van den Buuse et al., 2001).

2.5.2 Social interaction test

The open field test (OFT) is performed as a measurement of spontaneous activity in rodents (Gonzalez et al., 1996; Shimazaki & Chaki, 2005; Ferdman et al., 2007). Briefly, the apparatus consisted of a black square (70cm x 70cm x 40cm) arena that is divided into 35cm x 35cm equal squares drawn onto the floor of the arena (Sherif & Orelund, 1995). The arena was illuminated with red light (40 W), with a digital video camera mounted above the arena. For the treatment cohort, rats were injected with their daily dose of clozapine or saline 30 min prior to each session. Two rats were placed in the centre of the floor and were allowed 30s for adaptation. Thereafter, the time spent in active social interactive behaviors, namely rearing, anogenital sniffing, approaching and staying with the partner (Gonzalez et al., 1996), as well as self directed behavior, including squares crossed and self grooming (Gonzalez et al., 1996), were scored for a period of 10 min. Of the two rats tested, one of these animals was tested for social interaction with an unknown test partner that did not differ by more than 10 g in weight. Both members of a test-pair had received the same treatment and the same prior experience. Subsequent video analyses were performed by two independent observers blind to the study, with the average of the two observers' scores used for analysis. Testing was performed between 08.00 and 12.00 h. The researcher remained outside the testing room during the 10 min testing period. The arena was cleaned with 10% ethanol solution after each test (Ferdman et al., 2007).

2.6 Neurochemical analyses

2.6.1 Preparation of brain tissue

Animals were sacrificed by decapitation. The brains were then immediately dissected on an ice-cooled slab. The frontal cortical tissue was dissected following the removal

of the olfactory bulb from the cortex and cutting around the anterior tip of the corpus callosum (Toua et al., 2009). For the dissection of the striatum, the dorsal side of the brain was placed side up, the two cerebral hemispheres split and the striatum dissected with the external walls of the lateral ventricles as internal limits and the corpus callosum as external limits. The frontal cortex and striatum were then immediately, fixed in liquid nitrogen and stored in the -72°C freezer, until the day of assay. On the day of assay, the frontal cortex and striatum were removed from the freezer and allowed to thaw on ice. The frontal cortex and striatum were weighed and homogenized to prepare a 10% tissue homogenate with phosphate buffered saline (PBS), using a Teflon homogenizer. A protein content of the brain homogenate was determined on the day of superoxide dismutase and lipid-peroxidation assays as described by the Bradford method (Bradford, 1976).

2.6.2 Assessment of redox state:

Superoxide dismutase (SOD) activity

The SOD activity (as percentage) was measured in the brain homogenates using a commercially available kit (SOD Assay Kit-WST(Sigma Aldrich)) which assays SOD by utilizing Dojindo's highly water-soluble NBT salt, WST-1 (2-(4-Iodophenyl)- 3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt). The latter produces a water-soluble formazan dye upon reaction with superoxide anion, the rate of the reaction being linearly related to xanthine oxidase (XO) activity, and is inhibited by SOD. The % SOD activity is then determined by a colometric method according to the manufacturer's protocol. One unit (U) of enzyme activity was defined as the quantity of SOD required to cause a 50% inhibition of the absorbance change per min of the blank reaction (diluent rate). SOD activity in the frontal cortex and striatum were given as U/mg protein.

Oxidized glutathione (GSSG) versus reduced (GSH)

GSSG and GSH in rat frontal cortex and striatum were analyzed utilizing a modified version of the liquid chromatography/mass spectrometry (LC/MS) method of Harvey et al 2008. Briefly, for sample preparation the frontal cortex and striatum were accurately weighed and placed into a 1.5 ml eppendorf tube. Derivatization solution

(300 µl) were added together with 25 µl internal standard (200 ng/ml of gabapentin), vortexed and sonicated for 10 seconds and then left on ice for 15 minutes. Sulfosalicylic acid solution (150 µl) were added (10% w/v) to precipitate proteins. The supernatant was transferred to 200 µl inserts, in autosampler vials, and analysed using an Applied Biosystems API 2000 triple quadrupole LC/MS with atmospheric pressure electron ionisation (Turbo ion spray source), positive ion mode. The column used was a Gemini C18 column, 150 x 2mm, 5 µm (Phenomenex, Torrance CA), with the mobile phase as follows: A= 0.1% formic acid in water, B= 0.1% formic acid in acetonitrile. The flow rate was 250 µl/min, with an injection volume of 10 µl. Multiple reaction monitoring (MRM) scan were used with the following ion pairs as quantifier and qualifier, respectively: GSH: (366.1/134.2, 366.1/83.9), GSSG: (613.21/231.0, 613.21/177.1) and Gabapentin (internal standard): (172.04/137.2). Data are expressed as GSSG:GSH.

Lipid peroxidation

Frontal cortex and striatum lipid peroxidation was determined using the thiobarbituric acid (TBA) assay with butanol extraction, as previously described (Ottino and Duncan 1997; Harvey et al., 2008). The TBA assay is widely used to determine the index of lipid peroxidation in cells and is based on the reactivity of malondialdehyde (MDA), a colourless end product of lipid peroxidation, with TBA. At low pH and high temperatures, MDA reacts with TBA in a nucleophilic addition reaction, generating a red, fluorescent 1:2 MDA:TBA product that is extracted with butanol and the absorbance read at 532 nm. Tetramethoxypropane is used as a standard and expressed as pmol/mg protein using a 10% protein concentration for each assay (Harvey et al., 2008).

2.7 Statistical analysis

Graphpad Prism version 4 for windows (Graphpad software, San Diego, USA) and SAS/STAT® Software were used for the statistical analysis and graphical presentations.

Behavioural analysis:

To model % PPI and mean startle amplitude with repeated measures in the SIR and socially reared conditions with both non-treatment and treatment cohort, a mixed statistical model approach with Newman-Keuls or unpaired student t-test was followed. However, for the social interaction (OFT) tests, the unpaired student t-test was used in the non-treatment cohort with SIR and socially reared conditions. One way factorial analysis of variance (ANOVA) with repeated measures was used, followed by Newman-Keuls in the treatment cohort, with SIR and socially reared conditions.

Neurochemistry:

Unpaired student t-tests were used for the analysis of the non-treatment cohort in the SIR and socially reared conditions. A mixed linear statistical model was used with repeated measures for the different brain regions, with Newman-Keuls in the treatment cohort, for the SIR and socially reared conditions.

Body weights

A one-way (ANOVA) with repeated factors of treatment (saline and clozapine) and housing conditions were used to compare body weights, followed by Newman-Keuls post-hoc analyses.

3. Results

3.1 Behavioral studies

3.1.1 Sensory motor-gating

3.1.1.1 Prepulse inhibition (PPI) in the non-treatment cohort

The mean startle amplitudes of the socially-housed controls and isolated rats during four consecutive blocks of ten 120 dB stimuli are shown in Figure 1A. There were no significant overall differences in mean startle amplitude between the group-housed controls and SIR rats ($F(3,54)=1.12$, $p=0.35$). Furthermore, no significant habituation was observed in the group housed ($F(3,54)=6.198$, $p=0.24$) or SIR rats

($F(3,54)=6.82$, $p=1.0$) with repeated stimulation between BLOCK 1 and BLOCK 4 of startle (Figure 1A).

Mixed model analysis revealed a significant PPI*housing condition interaction ($F(3,72)=2.81$, $p=0.045$) (Fig 1B). Figure 1B shows that the %PPI increased significantly as the prepulse intensities, from 72 dB to 84 dB increased, in both the socially reared ($p=0.0004$) and SIR ($p<0.0001$) animals. However, %PPI was significantly reduced in the SIR rats compared to the socially reared controls, with a significant overall effect for each prepulse intensity: 72 dB ($p<0.0001$), 76 dB ($p<0.0001$) 80 dB ($p<0.0003$) and 84 dB ($p=0.001$) (Figure 1B).

3.1.1.2 PPI in the treatment cohort

A overall significant BLOCK*treatment interaction, was observed in socially reared rats ($F(3,54)=2.91$, $p=0.043$) (Figure 2), and an overall habituation of mean startle response was observed from BLOCK 1 compared to BLOCK 4 in the socially-reared animals receiving either saline ($p<0.0001$) or clozapine ($p=0.002$) (Figure 2A).

Similarly, in the SIR groups, a significant BLOCK*treatment interaction, was observed ($F(3,54)=1.39$ $p=0.003$) (Figure 3). Thus, clozapine treatment showed no significant overall effect on the mean startle amplitude in SIR animals compared to the SIR animals receiving saline. However, an overall habituation of mean startle response was also observed between BLOCK 1 and BLOCK 4 in the SIR animals receiving either saline ($p=0.0002$) or clozapine ($p=0.03$) (Figure 3A).

Mixed statistical modeling found the following significant interactions in the treatment cohort study in socially reared animals: %PPI*treatment ($F(3,72)=2.18$, $p=0.0004$), %PPI*housing condition ($F(3,72)=52.6$, $p<0.0001$), and treatment*housing ($F(3,72)=40.93$, $p<0.0001$) (Figure 2B). Newman-Keuls analysis indicated that the socially reared animals receiving either saline or clozapine treatment, had no significant differences in %PPI for each prepulse intensity, 72 dB ($p=0.14$), 76 dB ($p=1.0$), 80 dB ($p=1.0$) and 84 dB ($p=1.0$) (Figure 2B), suggesting no drug-related effects in healthy animals. Increased %PPI was also observed as the prepulse

intensities increased from 72 dB to 84 dB in both the saline ($p<0.0001$) and clozapine ($p<0.0001$) treated, socially reared rats (Figure 2B).

However, in the SIR animals receiving clozapine or saline treatment, post-hoc Newman-Keuls tests revealed a significant attenuation of %PPI in the clozapine treated animals compared to those receiving only saline for each prepulse intensity, 72 dB ($p<0.0001$), 76 dB ($p<0.0001$), 80 dB ($p<0.0001$) and 84 dB ($p<0.0001$) (Figure 3B). Again a significant increase in %PPI was observed as the prepulse intensities increased from 72 dB to 84 dB in the SIR animals receiving either saline ($p<0.0001$) or clozapine ($p<0.0001$) treatment (Figure 3B).

3.1.2 Social interaction studies

3.1.2.1 Social interaction in the non-treatment cohort

In the non-treatment cohort, student t-test revealed a significant decrease in social interactive behaviors in the SIR animals compared to their socially reared controls, with respect to rearing ($p<0.0001$), anogenital sniffing ($p<0.0001$), approaching ($p<0.0001$), and staying together ($p<0.0001$) (Figure 4 A-D). Student t-tests also revealed significantly increased self-directed behaviors in the SIR animals compared to their socially reared controls with respect to square crossing ($p=0.017$) and self grooming ($p<0.0001$) (Figure 5 A-B).

3.1.2.2 Social interaction changes in the drug treatment cohort

One-way factorial ANOVA revealed significant cross-group interactions with respect to social interactive behaviors in the drug treatment cohort with respect to rearing ($F(2,54)=57.28$, $p<0.0001$), anogenital sniffing ($F(2,54)=42.68$, $p<0.0001$), approaching ($F(2,54)=18.2$, $p<0.0001$), and staying together ($F(2,54)=34.14$, $p<0.0001$) (Figure 6 A-D). Post-hoc analysis with Newman-Keuls tests showed a significant decrease in social interactive behaviors in the SIR animals only receiving saline compared to their socially reared control with respect to rearing ($p<0.0001$), anogenital sniffing ($p<0.0001$), approaching ($p<0.0001$), and staying together ($p<0.0001$). These deficits in social interactive behaviors were in turn completely reversed with clozapine treatment in the SIR animals, for rearing ($p<0.0001$),

anogenital sniffing ($p<0.0001$), approaching ($p<0.0001$) and staying together ($p<0.0001$) (Figure 6 A-D). Clozapine did not have any notable effects of its own on social interactive behaviors compared to saline treated socially reared animals.

When considering self directed behaviors, one-way factorial ANOVA revealed significant cross-group interactions for square crossings ($F(2,54)=5.07$, $p=0.01$) and self grooming ($F(2,54)=58.53$, $p<0.0001$) (Figure 7 A-B). Post-hoc analysis with Newman-Keuls tests indicated significantly elevated self directed behaviors in the SIR animals only receiving saline treatment compared to their socially reared controls, for square crossing ($p<0.0001$) and self grooming ($p<0.0001$). In turn, clozapine treatment also significantly reversed the elevated self directed behaviors in SIR animals, for square crossing ($p<0.0001$) and self grooming ($p<0.0001$) (Figure 7 A-B), although did not have inherent effects of its own in healthy animals.

3.2 Neurochemical studies

3.2.1 Superoxide dismutase activity

Unpaired student t-test revealed a significant deficit in SOD activity in the non-treated SIR rats compared to their socially reared controls, in both the striatum ($p<0.0001$) and frontal cortex ($p=0.003$) (Figure 8 A).

In the drug treatment cohort, mixed statistical model analysis revealed the following significant interactions treatment*brain_region ($F(1,36)=2.1$, $p=0.006$), treatment*housing condition ($F(1,36)=3.34$, $p=0.0075$) and housing condition*brain_region ($F(1,36)=9.71$, $p=0.004$) in the treatment cohort (Figure 8 B). Post-hoc Newman-Keuls analysis indicated the same trend as observed in the non-treatment cohort (Figure 8 B, where SIR rats only receiving saline had a significantly reduced SOD activity in both the striatum ($p<0.0001$) and frontal cortex ($p<0.0001$) compared to their socially reared controls. Clozapine treatment significantly reversed this deficit in the SIR animals versus animals receiving saline in both the striatum ($p<0.0001$) and frontal cortex ($p<0.0001$). However, clozapine alone had no

significant effect on SOD activity in the socially reared rats compared to saline treated controls in both the striatum ($p=1.0$) and frontal cortex ($p=1.0$) (Figure 8 B).

3.2.2 Oxidized (GSSG) versus reduced (GSH) glutathione

Unpaired student t-test in the non-treatment cohort revealed a significant lower GSSG/GSH ratio in the SIR rats compared to their socially reared controls, in both the striatum ($p=0.0002$) and frontal cortex ($p=0.001$) (Figure 9 A), suggesting a reduction in oxidized glutathione (GSSG) and an increase in reduced glutathione (GSH).

In the treatment cohort, mixed statistical model analysis revealed the following significant interactions: treatment*brain_region ($F(1,36)=3.1$, $p=0.006$), treatment*housing condition ($F(1,36)=2.3$, $p=0.04$) and housing condition*brain_region ($F(1,36)=9.71$, $p=0.003$) (Figure 9 B). Newman-Keuls post-hoc analysis indicated the same trend as in the non-treatment cohort, with SIR rats receiving saline having a significantly reduced GSSG/GSH ratio in both the striatum ($p<0.0001$) and frontal cortex ($p<0.0001$) compared to their socially reared controls (Figure 9 B). Clozapine treatment completely reversed this deficit in the SIR animals versus saline-treated animals in both the striatum ($p<0.0001$) and frontal cortex ($p<0.0001$; Figure 9 B). However, clozapine had no significant effect alone in socially reared rats compared to saline-treated socially reared controls in the striatum ($p=0.29$) and frontal cortex ($p=0.77$) (Figure 9 B).

3.2.3 Lipid peroxidation

Unpaired student t-tests revealed a significant elevation of malondialdehyde, a measurement of lipid peroxidation, in SIR rats compared to their socially reared controls, in both the striatum ($p<0.0001$) and frontal cortex ($p<0.0001$) (Figure 10 A).

In the treatment cohort (Figure 10 B), mixed statistical model analysis revealed the following significant interactions treatment*brain_region ($F(1,36)=4.7$, $p=0.04$), treatment*housing condition ($F(1,36)=9.5$, $p<0.04$) and housing

condition*brain_region ($F(1,36)=2.7$, $p=0.006$) (Figure 10 B). Post-hoc Newman – Keuls analysis indicated the same trend as seen in the non-treatment cohort, with a significant elevation of malondialdehyde in both the striatum ($p<0.0001$) and frontal cortex ($p<0.0001$) of SIR rats receiving saline treatment compared to their socially reared controls (Figure 10 B). Clozapine treatment significantly reversed this elevation versus SIR animals receiving saline, in both the striatum ($p<0.0001$) and frontal cortex ($p<0.0001$) (Figure 10 B). However, clozapine had no significant effect alone on lipid peroxidation in socially reared rats compared to the saline-treated socially reared controls in both the striatum ($p=0.21$) and frontal cortex ($p=0.76$) (Figure 10 B).

3.3 Body weights

The mean weights of the rats during this study are as follows: PND21: 40-55 g, Day 1: 100-200 g, and Day 11: 250-300 g. One-way ANOVA with repeated measures followed by Newman-Keuls, showed no significant overall differences in weight between the different groups at the start of treatment (day 18) ($F(3,72)=1.29$, $p=0.83$), and on day 11 of treatment (day 28) ($F(3,72)=1.824$, $p=0.30$). Furthermore, all groups showed a significant and equal amount of growth over the period of the study (data not shown).

4. Discussion

Young rodents demonstrate a high sensitivity to early life manipulations of their social environment (Stevens et al., 1997; Weiss & Feldon, 2001). In humans (Walker, 1994) and rodents (Powell & Miyakawa, 2006), such events may contribute to the expression or exacerbation of a variety of physical and psychological disorders (Smith et al., 2003; Ayhan et al., 2009). Deficits in PPI are suggested to have utility as a biomarker of cognitive deficits of schizophrenia (Powell et al., 2009), while social interactive behaviours closely relate to compromised social behaviour in the illness (Jenkins et al., 2008). In rodents, PPI deficits appear to emerge only at or after puberty (Bakshi & Geyer, 1999), as is commonly seen in patients with

schizophrenia. Consequently, animal models that emphasize an early life environmental factor are important. Not only does SIR present with this latter attribute, it also demonstrates strong face and predictive validity for schizophrenia (Geyer et al., 2001; Weiss and Feldon, 2001 and Ayhan et al., 2009 for review). Although changes in social investigation and PPI needs to be viewed in the context of the complexity of the disorder, which presents with diverse positive, negative and cognitive symptoms, SIR may have special value in pharmacological studies (Powell et al., 2009).

Consistent with several previous reports in rats and mice, we found profound deficits in %PPI (Stevens et al., 1997; Geyer et al., 2001; Koch & Fendt, 2003) and social interaction (Ferdman et al., 2007), as well as hyper-locomotor activity (Del Arco et al., 2004; Heidbreder et al., 2000; Levine et al., 2007) in rats exposed to SIR. Specifically, we noted an elevation in self-directed behaviors in SIR rats, such as self grooming (Figures 5 B and 7 B), as well as a decrease in various social interactive behaviors, including rearing, anogenital sniffing, approaching and socializing behaviors (Figures 4 A-D and 6 A-D). We also noted an increase in locomotor activity in the OFT (Figures 5 A and 7 A). These behaviors, together with hyper-locomotor activity and elevated self grooming, are indicative of increased hyperactivity and anxiety, respectively (Ferdman et al., 2007; Arguello, & Gogos, 2006; Tandon et al., 2009), and are akin to symptoms typically associated with schizophrenia (Lipska & Weinberger, 2000; Braga, et al., 2005; Ventura et al., 2000). While earlier papers have studied the face validity of SIR in rodents based on changes in %PPI, this is the first paper to simultaneously look at SIR-induced changes on more than one behavior related to schizophrenia. More important, however, is that sub-chronic clozapine treatment reversed all PPI deficits and social interactive behaviors in animals reared in isolation (Figures 3 and 6 respectively). Together, these data have now provided convincing evidence of the face and predictive validity of SIR for schizophrenia.

For an animal model to realize greater relevance for the analogous human condition, it needs to be studied with respect to new hypotheses for the neurobiology of the illness. Recent work in schizophrenia has provided evidence for increased oxidative

stress (Chittiprol et al., 2009; Zhang et al., 2009) as well as changes in glutamate function (Baier et al., 2009; Stone et al., 2009; Rüscher et al., 2008; Carlsson et al., 1997; Olney et al., 1999). Likewise, pre-clinical work has brought to light evidence for altered cortical NMDA receptor binding in rats subjected to SIR (Toua et al., 2009). However, whether such a disturbance may be a driving force for changes in redox state, as well as being associated with altered behavior, has not yet been studied. In the present study, simultaneous with the above mentioned behavioral changes, we found significant deficits in SOD activity (Figure 8) and a decrease in oxidized (GSSG) versus reduced (GSH) glutathione balance (Figure 9). Thus, SIR in rats is associated with an increase in reduced glutathione (GSH), possibly in reaction to elevated levels of H_2O_2 and ROS, although this imbalance may also result from high levels of ROS that overwhelms GSH resulting in less formed oxidized glutathione (GSSG). In addition, we observed significantly elevated levels of lipid peroxidation products (TBARS) in both the striatum and frontal cortex of rats exposed to SIR (Figure 10).

Thus, SIR induced changes in social and cognitive function are associated with increased oxidative stress and evidence for oxidative cell damage (i.e. increased lipid peroxidation). That SIR has been associated with increased frontal cortical NMDA receptor binding (Toua et al., 2009), and that earlier work in cell cultures has noted that NMDA receptor antagonism enhances [^3H]MK-801 specific binding (Hu et al., 1996) and/or up-regulates NMDA receptor mRNA (Hu et al., 1996), concurs that isolation rearing may indeed evoke a state of frontal cortical *hypoglutamatergia*. In fact, NMDA receptor hypofunction has been associated with apoptosis (Olney et al., 1999; du Bois & Huang, 2007), which in itself may drive a diverse array of cellular redox disturbances (Castagne et al., 1999). That the redox disturbances described in the current study occurred together with deficits in sensory motor gating and social interactive behaviors provide for an important association with schizophrenia. Moreover, not only did sub-chronic treatment with clozapine reverse the aforementioned behavioral changes following SIR, but the drug simultaneously reversed cortico-striatal redox disturbances evident in these animals (Figures 8, 9, and 10). These observations have significant implications for novel construct and predictive validity for the SIR model of schizophrenia.

Because PPI can be studied in humans and rats using almost identical procedures and stimuli (Bubenikova-Valesova et al., 2008), it provides empirical evidence that the neurobiological mechanisms underlying deficits in PPI are similar across species. Thus, the observed changes in redox state in SIR animals may be causally related to altered sensorimotor gating described in rats, and indeed in schizophrenia. Recent studies support a strong relationship between cognitive processing and PPI, and that a disruption of PPI may be an important manifestation of dysfunctional cognitive functioning in schizophrenia (Geyer et al., 2006). The frontal cortex, especially the dorsolateral prefrontal cortex, the orbital/medial prefrontal cortex (mPFC) and the middle frontal cortex, are closely associated with sensory motor gating and PPI (Kumari et al., 2008). Since frontal cortical D₁-NMDA receptor interactions form a fundamental neural substrate for cognition, particularly in schizophrenia (Castner and Williams, 2007; Toua et al., 2009), any imbalance between D₁ and D₂ receptors, but also deficits in glutamatergic transmission, will contribute towards the symptoms of schizophrenia (Scott & Aperia, 2009).

Behavioral flexibility is mediated by the frontal lobes, but is dependent on a number of sub-cortical systems that interact with the mPFC (Bonilha et al., 2008), including the dorsal and ventral regions of the striatum, and the meso-cortico-limbic dopamine system (Tzschentke, 2001). Thus, frontal cortical hyperglutamatergia (Adams & Moghaddam, 1998) and a hyperdopaminergic condition in the mesolimbic system may be responsible for hyperlocomotor activity (Lipska & Weinberger, 2000), as well as excessive self grooming (Dall'Olio et al., 2000), as observed in this study, which in turn can be correlated with the positive symptoms in schizophrenia (Bubenikova-Valesova et al., 2008; Tandon et al., 2009). The meso-accumbens dopaminergic system is also involved in social interaction behaviors (Seillier & Giuffrida, 2009), mediating the decreased social interactive behaviors observed in the SIR rats, viz. rearing, anogenital sniffing, approaching and staying together. It is also known that prefrontal dopamine release is strongly regulated by glutamate via NMDA receptors (Del Arco & Mora, 2001). Since SIR in rats induces opposing effects on D₁-NMDA receptor binding that is further modulated following clozapine treatment (Toua et al., 2009), it is of particular significance that in the current study clozapine was found to

reverse deficits in PPI and social investigative behaviors, as well as altered cortico-striatal redox balance, in SIR animals thus strongly implicating a glutamate NMDA receptor mediated mechanism in this response.

Indeed, insufficient functioning or prolonged blockade of the NMDA receptor can evoke an increase in oxidative stress, leading to an increase in reactive oxygen and nitrogen species (RNS) *in vitro* (Wang et al., 2002) and *in vivo* (Wass et al., 2009). NMDA receptor activity is required for the expression of antioxidant enzymes (Fatokun et al., 2008). Of relevance is that increased oxidative activity and increased lipid peroxidation was observed in both the striatum and frontal cortex, which is in keeping with reduced volumes of the frontal cortical and temporal-limbic areas (Woods et al., 1996), as well as abnormalities in the cortico-striatal-thalamic-pallido-pontine circuitry (Swerdlow et al., 2001), in patients with schizophrenia. A protracted state of NMDA receptor hypofunction can trigger neuronal injury throughout many corticolimbic brain regions (Olney et al., 1989; Corso et al., 1997). That schizophrenia may be causally linked to a state of hypoglutamatergia, particularly in the cortico-striatal limbic regions (Hirsch et al., 1997), validates a basis for the altered redox state observed in the frontal cortex and striatum of SIR rats, as well as the aberrant behavior seen in SIR rats, and in schizophrenia. On the other hand, disinhibition of pyramidal glutamatergic neurons in the sub-cortical regions of the brain and a resulting increase in glutamatergic activity (Konradi & Heckers, 2003 for review), may explain the evidence for oxidative stress in the striatum of SIR rats. Early-life social isolation is therefore associated with an increase in oxidative stress that may lead to long-lasting behavioral changes that present in adulthood. Interestingly, these bio-behavioral changes are possibly linked to structural remodeling of the limbic system (Crespo-Facorro et al., 2000). In fact, magnetic resonance imaging studies in SIR animals would concur with this suggestion (Schubert et al., 2009).

When ROS and/or RNS are generated in excess, or if cellular antioxidant defense enzymes are deficient, such as reduced SOD activity and reduced GSSG:GSH balance as described here, a number of chain reactions are stimulated. The brain, which has a high lipid and iron content, is particularly sensitive to ROS-induced

damage (Akyol, 2002). ROS-mediated attack on poly-unsaturated fatty acids (PUFAs) in cell membranes will result in lipid peroxidation, as seen in the elevated malondialdehyde levels in the frontal cortex and striatum of SIR rats in this study. Decreased SOD activity, as noted in this study, can also contribute to superoxide-mediated DNA damage (Mahadik & Mukherjee, 1996), while decreased GSH peroxidation activity, which has been observed in the acute period of schizophrenia (Pavlović et al., 2002), may be insufficient to meet the demands of converting increased amounts of H_2O_2 to H_2O . A reduced GSSG:GSH ratio, as observed in this study therefore means increased reduced glutathione (GSH) as a reaction to higher levels of H_2O_2 and elevated ROS, resulting in less formed oxidized glutathione (GSSG). Together, this will lead to further free radical damage (Altuntas et al., 2000).

Of great interest is that, in addition to reversing SIR-induced decreased SOD activity and altered GSSG:GSH ratio, sub-chronic treatment with clozapine (5 mg/kg/day x 11 days) effectively reversed evidence of neuronal cell damage (increased lipid peroxidation). Thus, not only does clozapine present with putative anti-oxidant properties, a particularly novel observation, but may be neuroprotective too (e.g. Magliaro & Saldanha, 2009). An indirect antioxidant effect to modulate redox balance is possible. For example, it is well known that clozapine and similar atypical antipsychotics exerts beneficial effects on striatal function by conserving excessive dopamine turnover in response to inappropriate D_2 receptor blockade (Harvey et al., 1999). Further, the clozapine analogue, olanzapine, can reverse haloperidol-induced striatal toxicity by reversing haloperidol-induced nitric oxide synthase inhibition, thereby re-establishing an important protective mechanism against excessive superoxide production (Nel & Harvey, 2003). Nitric oxide is an important downstream messenger of glutamate NMDA activation. Thus, by conserving or bolstering NMDA-mediated events (Toua et al., 2009), or by presenting with agonist-like activity (Chen and Yang, 2002; Heresco-Levy, 2003), clozapine may successfully alter redox state in SIR rats.

Despite significant advances in the development of antipsychotic agents over the past few decades, there are still significant shortfalls in efficacy (Marder and Fenton, 2004; Cascade et al., 2008 Matrix medical communications). Troublesome side

effects and a lack of effectively treating cognitive disturbances are the most important limitations (Addington et al., 2000; Hans et al., 2000; Ellenbroek & Cools, 2002). There is thus an urgent need for well-validated analogous animal models with which to investigate and develop new treatments (Ellenbroek & Cools, 2002; Geyer & Moghaddam, 2002). This study has therefore extended the validity criteria for SIR as a model of schizophrenia, especially with respect to documenting a diverse array of behavioral manifestations that are akin to schizophrenia, namely locomotor hyperactivity, decreased social interaction, and impaired sensory motor gating. Moreover, in line with recent clinical evidence, these behaviors are causally related to altered cortico-striatal redox balance. Finally, that these bio-behavioral changes can be completely reversed by sub-chronic clozapine administration provides robust evidence for the predictive validity of SIR.

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Figure legends

Figure 1:

Sensory motor gating at prepulse intensities as indicated, in socially reared and SIR rats, in the non-treatment cohort study: (A), mean startle amplitude and (B), percent prepulse inhibition (%PPI).

Figure 2:

Sensory motor gating at prepulse intensities as indicated, in socially reared rats in the treatment cohort study: (A), mean startle amplitude and (B), percent prepulse inhibition (%PPI).

Figure 3:

Sensory motor gating at prepulse intensities as indicated, in SIR rats in the treatment cohort study: (A), mean startle amplitude and (B), percent prepulse inhibition (%PPI).

Figure 4:

Social interactive behaviors in socially reared and SIR rats in the non-treatment cohort: (A), time spent rearing; (B), time spent anogenital sniffing; (C), times approached; (D), time spent together.

Figure 5:

Self directed behaviors in socially reared and SIR rats in the non-treatment cohort: (A), squares crossed (locomotor activity); (B), time spent self grooming.

Figure 6:

Social interactive behaviors in socially reared and SIR rats in the treatment cohort: (A), time spent rearing; (B), time spent anogenital sniffing; (C), times approached; (D), time spent together.

Figure 7:

Self directed behaviors in socially reared and SIR rats in the treatment cohort: (A), squares crossed (locomotor activity); (B), time spent self grooming.

Figure 8:

Superoxide dismutase activity (U/mg protein) in both the striatum and frontal cortex of the socially reared and SIR rats in: (A), the non-treatment cohort and (B), the treatment cohort.

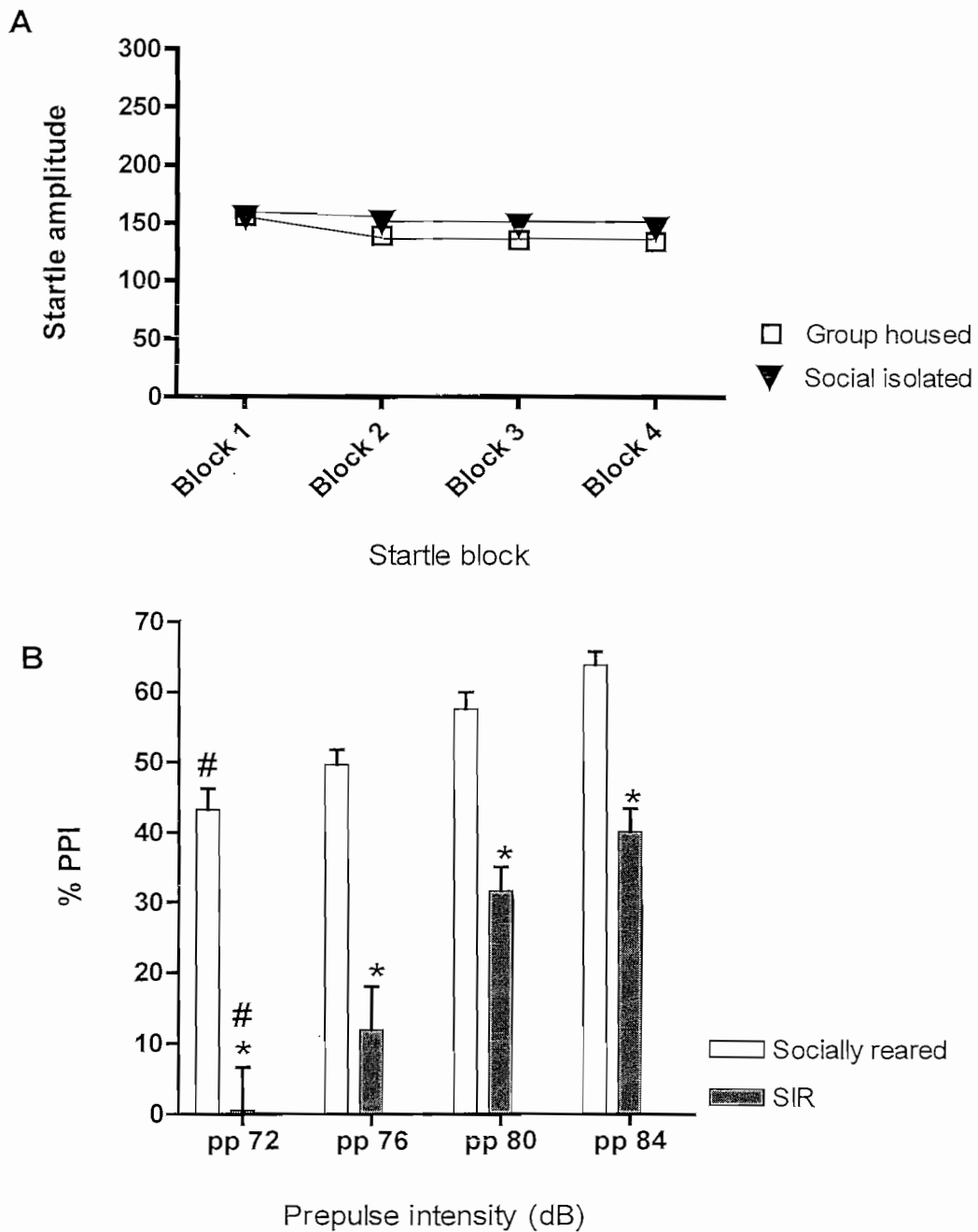
Figure 9:

Oxidized / Reduced Glutathione ratio (GSSG/GSH) in the striatum and frontal cortex of the socially reared and SIR rats in: (A), the non-treatment cohort and (B), the treatment cohort.

Figure 10:

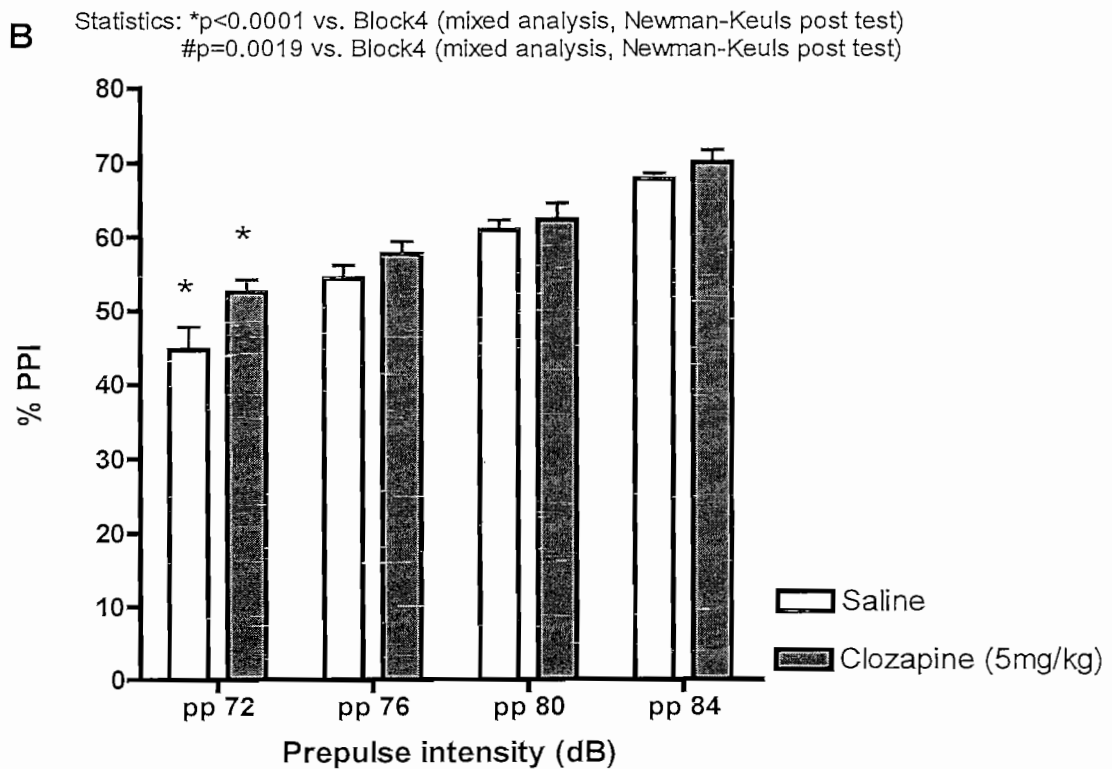
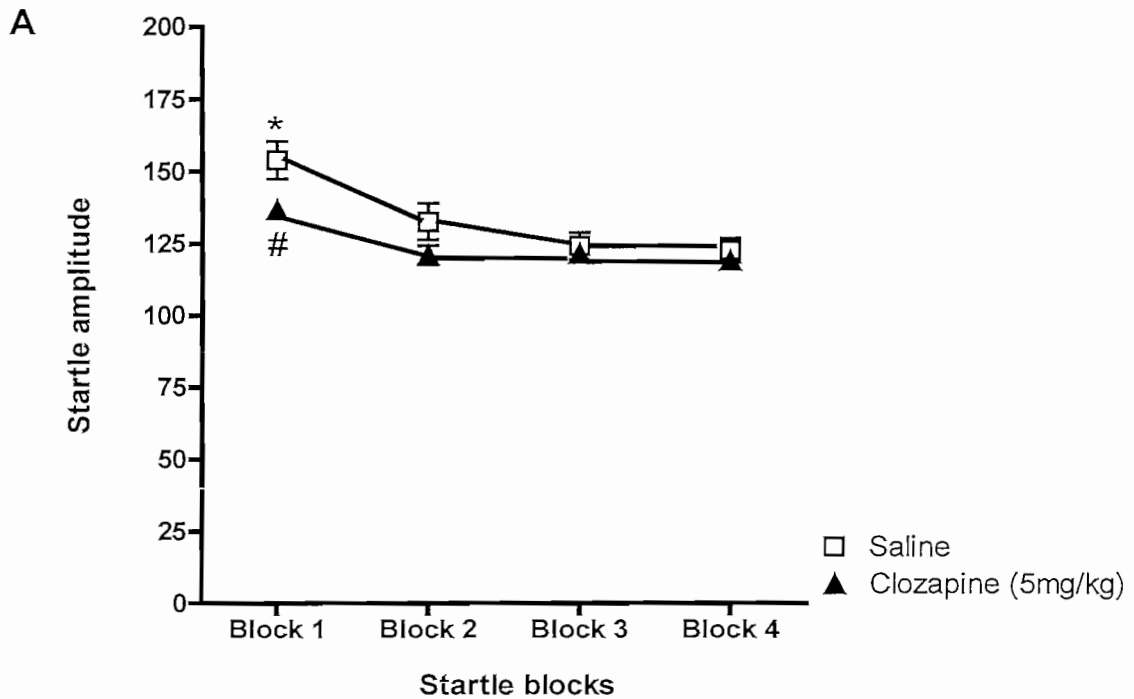
Concentration of malondialdehyde, a measurement of lipid peroxidation, in the striatum and frontal cortex of socially reared and SIR rats in: (A), the non-treatment cohort and (B), the treatment cohort.

Figures:



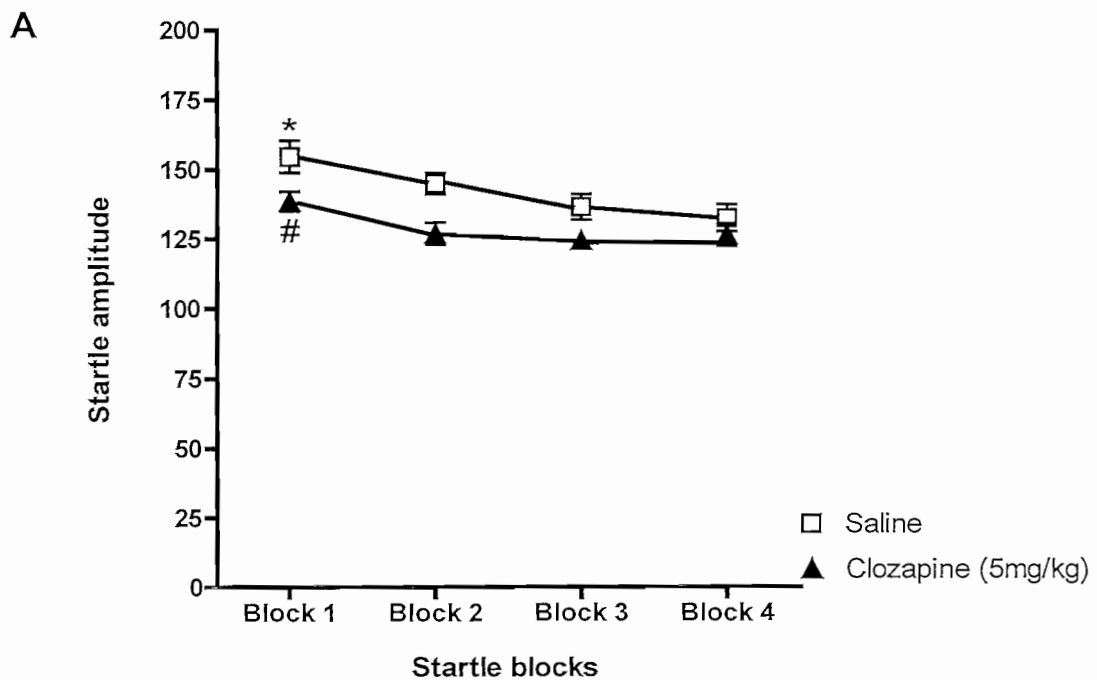
Statistics: * $p < 0.005$ vs. socially reared (mixed analysis, Newman-Keuls)
 $\#p < 0.0001$ vs. 84 dB (mixed analysis, Newman-Keuls)
see literature for precise p values

Figure 1



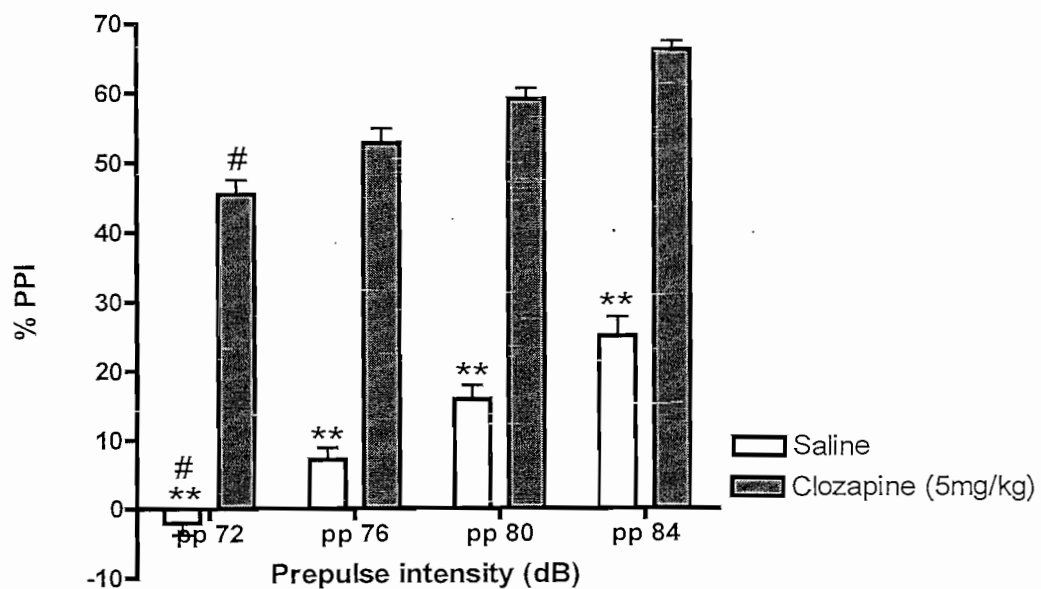
Statistics: * $p < 0.0001$ vs. 84dB (mixed analysis, Newman-Keuls posttest)

Figure 2



Statistics: * $p=0.0002$ vs. Block 4 (mixed analysis, Newman-Keuls posttest)
$p=0.032$ vs. Block 4 (mixed analysis, Newman-Keuls posttest)

B



Statistics: ** $p<0.0001$ vs. Saline (mixed analysis, Newman-Keuls posttest)
$p<0.0001$ vs. 84dB (mixed analysis, Newman-Keuls posttest)

Figure 3

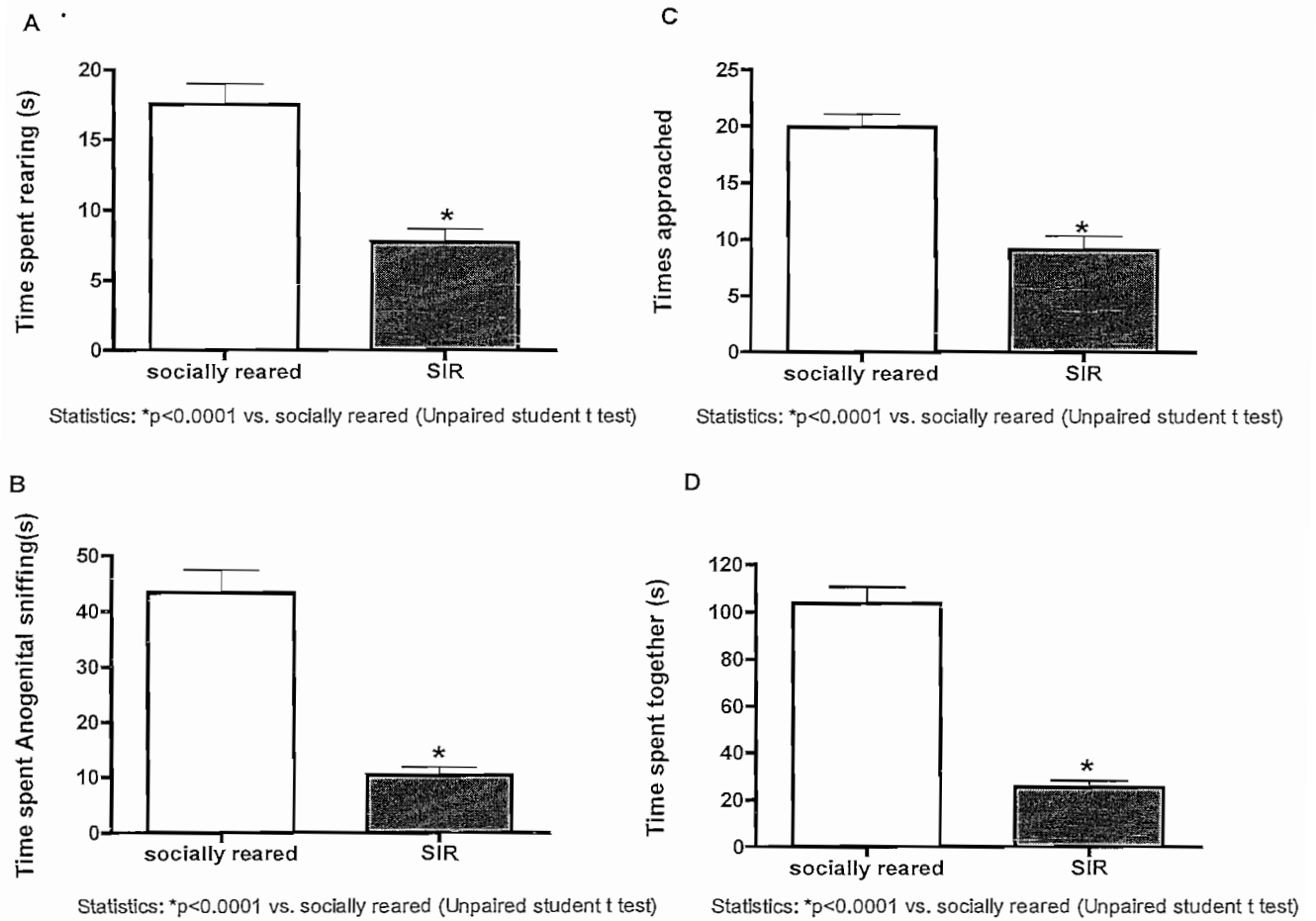
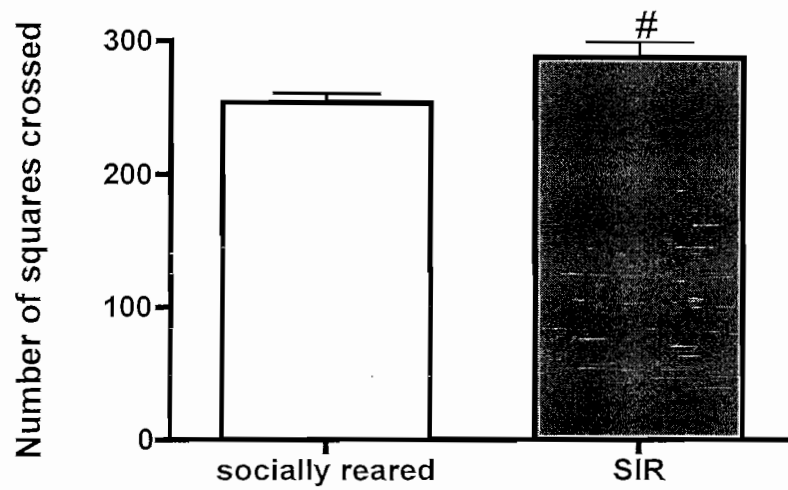


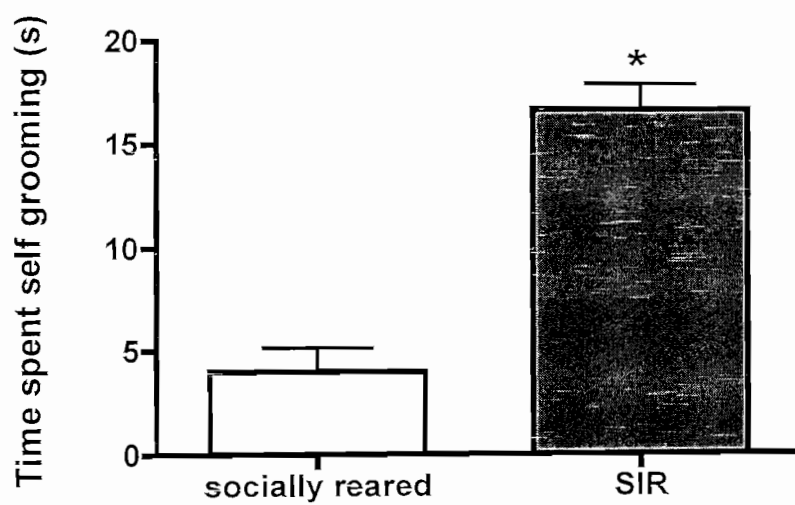
Figure 4

A



Statistics: * $p=0.0173$ vs socially reared (Unpaired student t test)

B



Statistics: * $p<0.0001$ vs. socially reared (Unpaired student t test)

Figure 5

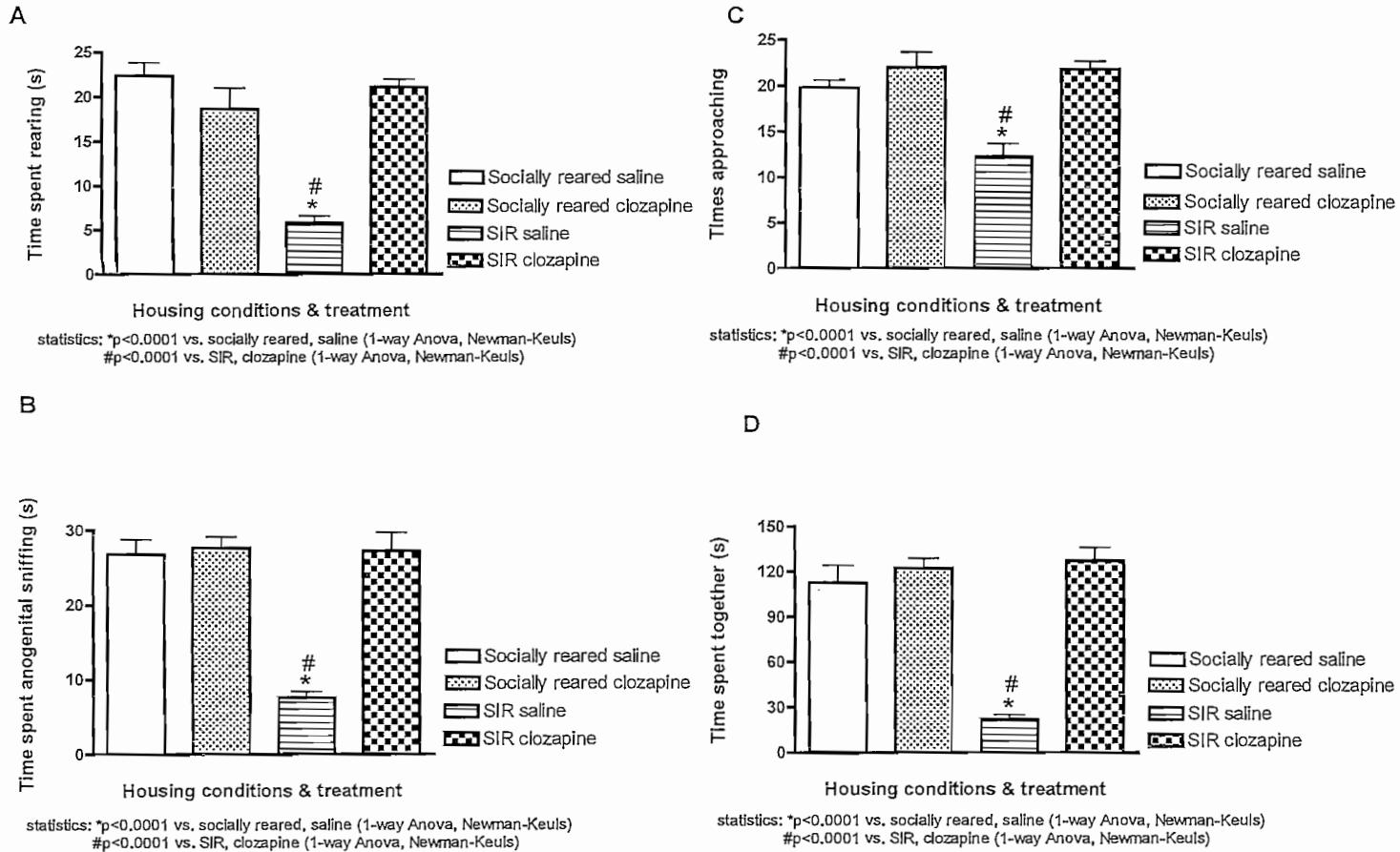
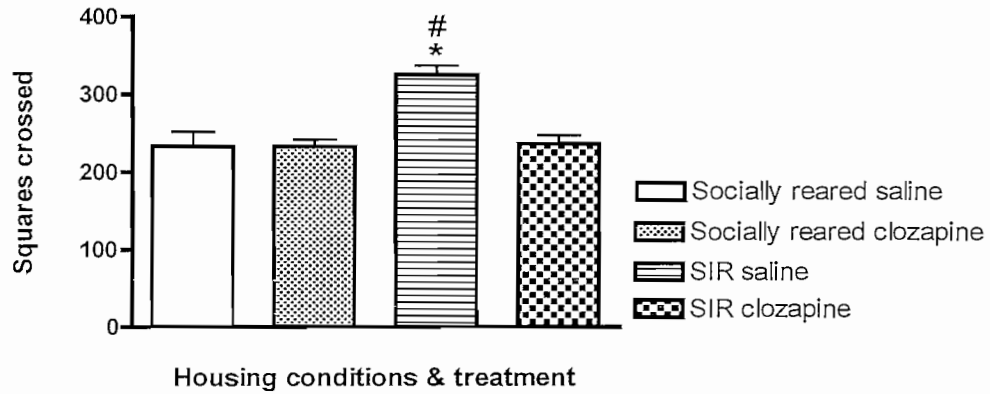


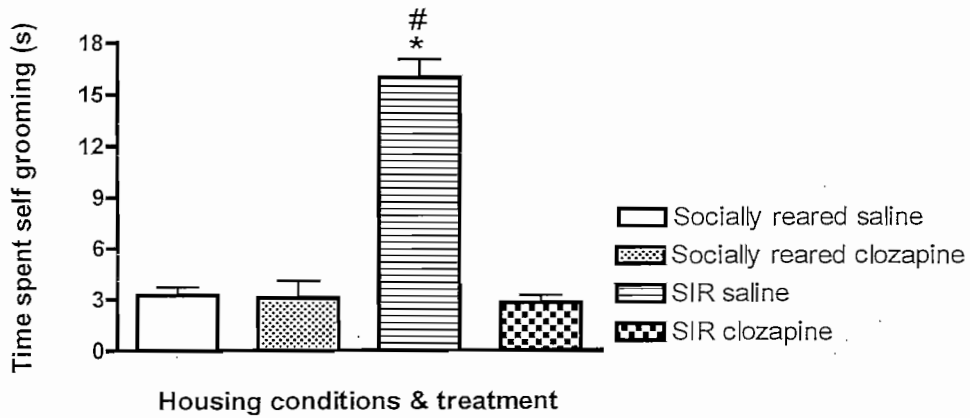
Figure 6

A



statistics: * $p < 0.0001$ vs. socially reared, saline (1-way Anova, Newman-Keuls)
$p < 0.0001$ vs. SIR, clozapine (1-way Anova, Newman-Keuls)

B



statistics: * $p < 0.0001$ vs. socially reared, saline (1-way Anova, Newman-Keuls)
$p < 0.0001$ vs. SIR, clozapine (1-way Anova, Newman-Keuls)

Figure 7

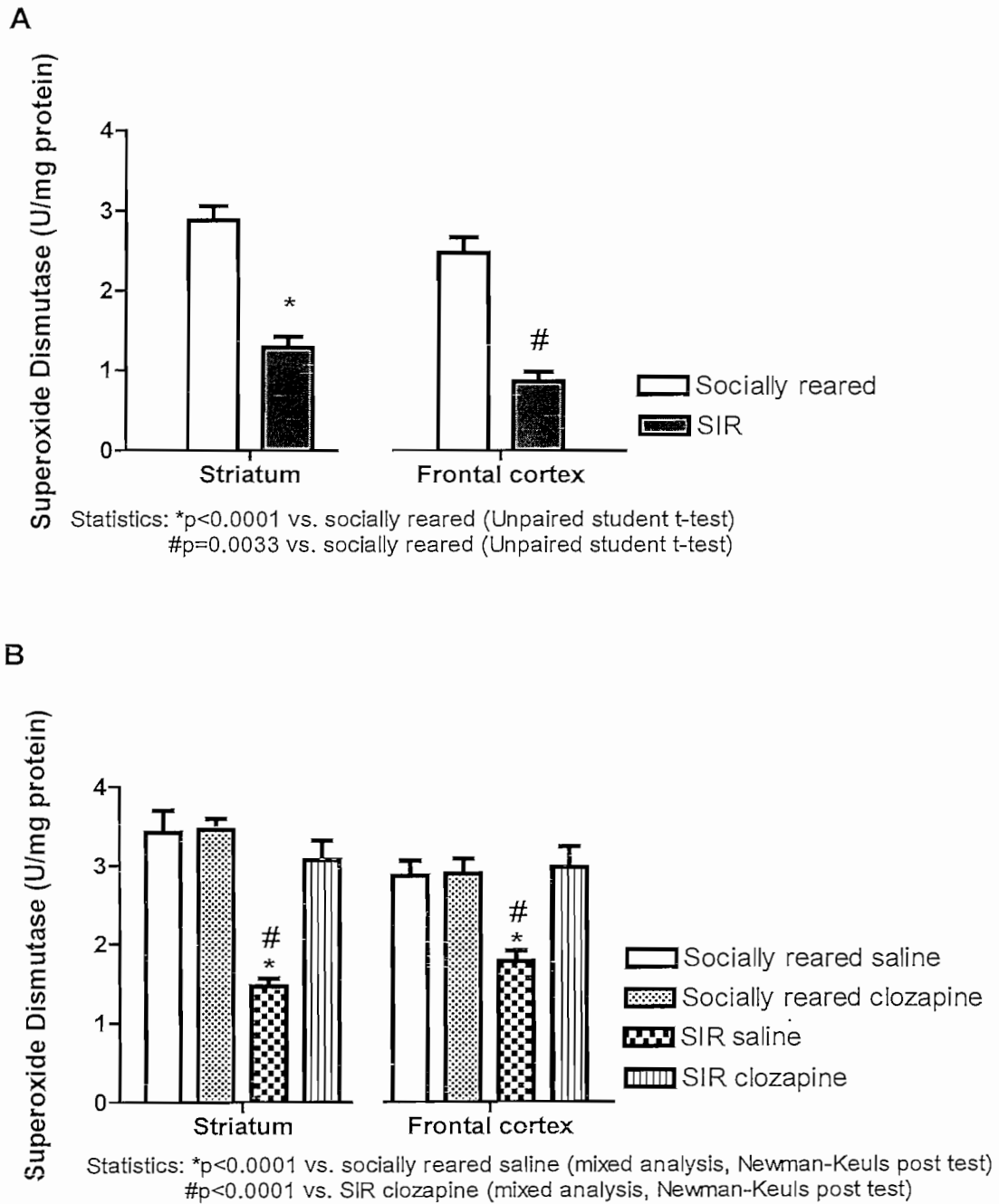
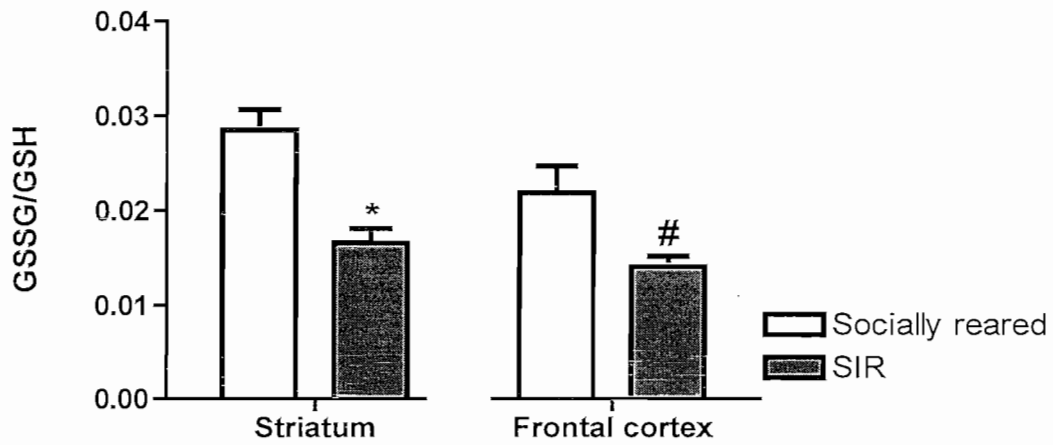


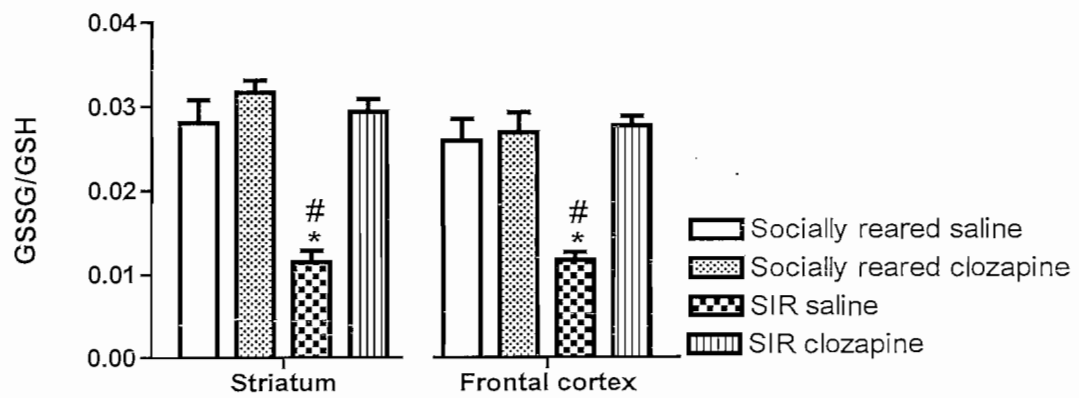
Figure 8

A



Statistics: * $p=0.0002$ vs. socially reared (Unpaired student t-test)
 # $p=0.00092$ vs. socially reared (Unpaired student t-test)

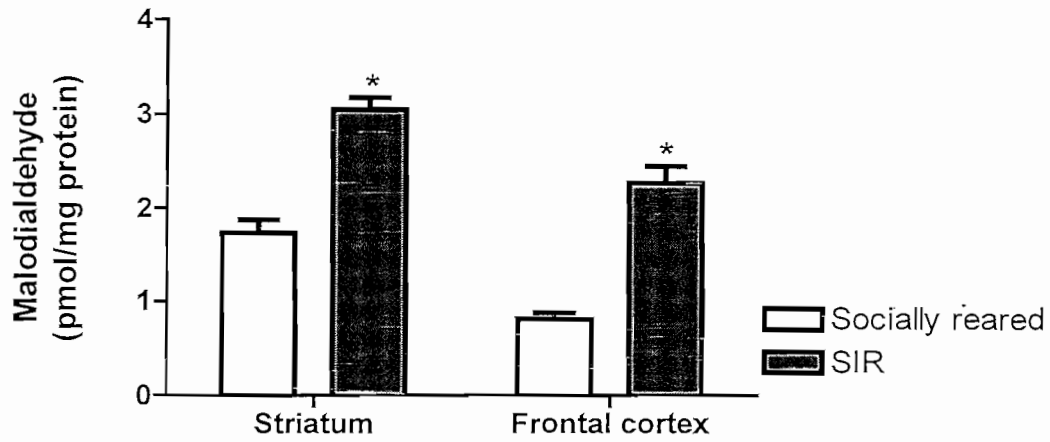
B



Statistics: * $p<0.0001$ vs. socially reared saline (mixed analysis, Newman-Keuls post test)
 # $p<0.0001$ vs. SIR clozapine (mixed analysis, Newman-Keuls post test)

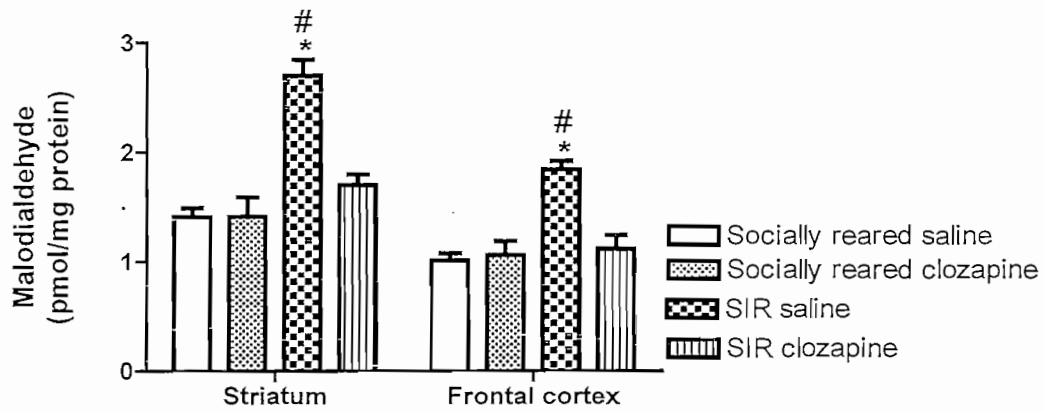
Figure 9

A



Statistics: * $p < 0.0001$ vs. socially reared (Unpaired student t-test)

B



Statistics: * $p < 0.0001$ vs. socially reared saline (mixed analysis, Newman-Keuls post test)
$p < 0.0001$ vs. SIR clozapine (mixed analysis, Newman-Keuls post test)

Figure 10

Conclusion and recommendations for future studies.

Chapter 4

Schizophrenia is a progressive degenerative illness that has been associated with poor long-term prognosis (Harvey et al., 1999). The illness has been causally linked to environmental and neurodevelopmental factors (Weiss & Feldon, 2001), as well as dysfunctional redox balance (Mukherjee et al, 1996; Kuloglu et al, 2002), that in the end are regarded as the initiators of a host of neuroanatomical and neurotransmitter changes that characterise the disease. Psychotic (positive) symptoms are the most distinctive feature of schizophrenia, although negative symptoms such as emotional flattening, social withdrawal and cognitive disturbances, are the most treatment resistant manifestation of the illness (Keefe et al., 2007; Marder and Fenton, 2004). These behavioural changes are underscored by a complex array of dopaminergic, GABA'ergic and glutamatergic disturbances in the frontal cortex and sub-cortical regions of the brain (Reynolds, 2005). Despite the advances that have been made in recent years with regard to its treatment, especially with the introduction of the atypical antipsychotic drugs, treatment remains inadequate both in terms of efficacy and side effects (Harvey et al., 1999). Consequently, there is a drive to better and quicker diagnosis of the illness in order to initiate treatment early, as well as a need to improve our understanding of schizophrenia, both of which will have lasting benefits for the effective management of the disorder. However, in order for this to be achieved, further clinical and pre-clinical research is needed. With regards to the latter, well-validated analogous animal models have a very important part to play, especially in studying the bio-behavioural factors underlying schizophrenia, and to identify novel neurobiological targets for drug development and treatment of the illness.

Social isolation rearing (SIR) has been suggested to be a useful neurodevelopmental animal model of schizophrenia (Heidbreder et al., 2000), having been found to have important face, construct and predictive validity for the human disorder, including presenting with deficits in sensorimotor gating (Varty & Geyer, 1998; Geyer & Ellenbroek, 2003), reversal of these by antipsychotic drugs (Taylor et al., 2009 for review), and presenting with changes in frontal cortical NMDA and D₁ receptor binding (Toua et al, 2009). However, the model has not been studied with respect to social interactive behaviours together with the assessment of cognitive performance using PPI, thus providing a more global view of its face validity. Moreover, the construct of increased oxidative stress, as has been established in schizophrenia, has never before been studied in this animal model.

This study has therefore investigated the effect of SIR on various parameters of oxidative stress in the rat frontal cortex and striatum. Moreover, the study relates these biochemical changes to changes in social behaviours as well as in sensory-motor gating, using the open field test (OFT) and prepulse inhibition (PPI), respectively. Finally, in order to establish whether these bio-behavioural changes following SIR are altered by known treatments for schizophrenia, i.e. predictive validity, we studied whether sub-chronic treatment with the atypical antipsychotic, clozapine, could reverse the above changes.

Primary outcomes

- Rats reared in isolation showed marked deficits in sensorimotor gating, as assessed by %PPI of the startle response, compared to socially housed rats.
- PPI disruption in SIR rats was reversed by sub-chronic treatment with the atypical antipsychotic, clozapine (5 mg/kg/day x 11 days).
- Isolation reared rats were also characterized by significant deficits in social interactive behaviors, as well as a significant increase in self directed behaviors, compared to socially housed rats.
- The deficits in social interactive and self directed behaviors in the SIR rats were reversed by clozapine treatment (5 mg/kg/day x 11 days).
- Isolation rearing was found to induce a significant decrease in superoxide dismutase (SOD) activity, altered oxidized (GSSG) versus reduced (GSH)

glutathione ratio, as well as significantly elevated markers of lipid peroxidation, compared to socially housed rats. This disturbance in redox state was observed in both the frontal cortex and striatum of SIR rats.

- Sub-chronic treatment with clozapine (5 mg/kg/day x 11 days) significantly reversed the altered redox state in SIR rats.

Secondary outcomes

- Clozapine was found not to induce any marked changes in behavior and redox state in socially reared rats.
- No significant changes in PPI, social interaction or redox state, were observed in socially reared rats with respect to injection stress (saline injection).

Recommendations for future studies:

- In order to provide further insight into the underlying mechanisms involved in schizophrenia, we would need to identify the origin of oxidative stress in the SIR model, as observed in this study, and especially how this may be involved in the progressive nature of the illness. Doing this will provide us with a novel approach to treating the illness. One such approach may be to explore the role of dopamine and/or kynurenine metabolism in SIR rats, as well as inflammatory mechanisms, as these pathways are significantly linked to a change in redox balance, with important implications for oxidative stress and neurodegeneration. Indeed, these pathways are known to be altered in patients with schizophrenia (Miller et al., 2006; Tretter et al., 2003 for review).
- Leading on from the above point, treatment in the SIR model with the antioxidant and glutathione replenisher, *N*-acetyl-L-cysteine (NAC), could be considered for future studies.
- Another important aspect in treating schizophrenia is the duration of untreated psychosis (DUP) and its association with treatment resistance (Ammeringer et al., 2002; Perkins et al., 2005). Although difficult to model in an animal, studies investigating different durations of isolation rearing, e.g. 4, 8 and 12 weeks, and how these impact on the degree of behavioral response in the

animals, as well as response to antipsychotic treatment, have not yet been performed, and would be a useful extension of the face and predictive validity of the SIR model.

- With the establishment of SIR as a non-pharmacological animal model of schizophrenia in our laboratory, new experimental compounds with novel antipsychotic properties could be tested, for example the nitric oxide synthase-guanlyl cyclase inhibitor, methylene blue.
(<http://clinicaltrials.gov/show/NCT00214877>).
- Since volumetric brain reductions have been observed in schizophrenia patients (Narre et al., 2004), reflecting not only neuronal atrophy but also loss of glia and interneurons, histological, immunohistochemical and stereological techniques could be used to quantify neurogenesis, cytomorphology, neuronal numbers and the volume of sub-cortical regions and frontal cortex in SIR versus socially reared animals.

Novel findings and conclusion

No single animal model can possibly replicate the overall symptomology of schizophrenia, which in itself is a heterogeneous, polygenetic disorder. However, this study has demonstrated that isolation rearing in rats, which also emphasises early-life adverse environment exposure, can produce a range of reproducible, long-term changes in behaviour that are closely related to some of the typical symptoms and manifestations of schizophrenia. These behaviours include significant deficits in PPI, as well as deficits in social interactive behaviours, and an increase in self directed behaviours. The latter observations are novel and have, to the best of our knowledge, not been observed during simultaneous evaluation in the SIR model, and provide robust evidence of the face validity of the model. Moreover, in line with the efficacy of clozapine to treat both positive and negative symptoms of schizophrenia, all the above behaviours were effectively reversed following sub-chronic clozapine treatment, again a novel finding and evidence for the important predictive validity of the model. We also for the first time provide robust evidence that SIR is associated

with a profound disruption of brain redox balance and increased oxidative stress in brain regions known to be intimately involved in schizophrenia. Moreover, these changes in redox state were reversed by sub-chronic clozapine treatment, thus providing novel predictive and construct validity.

Social isolation rearing therefore provides robust translational relevance to the core deficits in schizophrenia (positive and negative symptoms) as well as redox dysregulation. The model provides a useful and valid approach to investigating the neurodevelopmental aetiology of schizophrenia in order to identify longitudinal biomarkers of the illness, and to serve as a predictive screen for novel compounds with potential antipsychotic efficacy.

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The effect of acute MK-801
(dizocilpine) administration on
prepulse inhibition and social
interactive behaviors in rats.

Addendum A

Behavioural analyses form the basis upon which this study is founded. Before studying the SIR model with respect to cognitive (prepulse inhibition) and social interactive behaviours, it was imperative that we first establish whether we have set up these two behavioural paradigms correctly in our laboratory. This appendix therefore describes the validation of these behavioral protocols using a pharmacological model for assessing prepulse inhibition (PPI) and social interactive behaviours in rats. This model has two advantages: Firstly, it is an acute challenge model, and thus allows us to generate data quickly. Secondly, it follows the principle hypothesis of this study, namely that schizophrenia is a state of hypoglutamatergia, thus further validating our use of MK-801, an NMDA antagonist, as the challenge drug in this pilot study. Finally, the MK-801 model is recognized as a robust animal model of schizophrenia in its own right, and is well known to induce profound deficits in PPI and social interaction in rodents (Bubenikova-Valesova et al., 2008).

1. Introduction

Sensorimotor gating is the endogenous subconscious effort of the brain to prevent excessive extraneous sensory or cognitive stimuli from disturbing integrative mental processes (Van den Buuse et al., 2003). An operational measure of sensorimotor gating is prepulse inhibition (PPI) of the acoustic startle reflex, and is a simple measure of sensorimotor gating that is observed when a startling noise (pulse) is shortly preceded (30-800ms before) by a weaker sensory (acoustic) prepulse

(Hoffman & Ison, 1980; Koch 1999; Schwabe et al., 2005). Schizophrenia, along with other psychiatric disorders such as obsessive compulsive disorder and Huntington's disease, present with an inability to filter sensory, cognitive stimuli and as a result present with significant deficits in PPI (Braff & Geyer, 1990; Braff et al., 1992; Swerdlow et al., 1992). Another core behavioral feature of schizophrenia is that patients present with impairments in social functioning (Pinkham et al., 2003), having a marked lack of social interaction skills and social cognition (see chapter 2, section 2.2.2). This quality is one of the main reasons why these patients fail to integrate into society, placing a heavy burden on family and care providers.

One theory for explaining the deficits in PPI and social skills in schizophrenia involves dysfunctional NMDA receptors and/or its downstream effects. As has been discussed in Chapter 2, Section 5.2.3, schizophrenia has been linked to a state of hypoglutamatergia in especially the frontal cortical regions of the brain (Hirsch et al., 1997), while reduced glutamate levels have been described in the CSF of patients with schizophrenia (Kim et al., 1980). Importantly, the deficits in PPI and social function can be modelled in animals by blocking N-methyl-D-aspartate (NMDA) receptors (Levin et al., 2005), thus emulating glutamate hypofunction at these receptors. Excitatory neurotransmission in the brain is mediated predominantly by glutamate acting on either ionotropic or metabotropic glutamate receptors, although NMDA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors are the main proponents of fast excitatory transmission (du Bois & Huang, 2007). One consequence of blocking NMDA receptors with an NMDA antagonist is a profound increase in cortical excitatory activity (Pratt et al, 2008). This eventual effect is a result of complex interactions between GABA and glutamate signalling on pyramidal cells located in the cortex. Thus blockade of NMDA receptors on GABA interneurons, which in turn impact on glutamatergic pyramidal cells, will result in an insufficient suppression of cortical pyramidal cell activity culminating in an excessive release of glutamate, i.e. disinhibition of cortical glutamatergic activity (Moghaddam et al., 1997; Adams and Moghaddam, 1998). This mechanism has also been suggested to mediate an increase in cortical acetylcholine (ACh) release (Hasegawa et al., 1993; Giovannini et al., 1994; Kim et al., 1999), thus providing an overall

increase in cortical activation. It has been proposed that this excessive release of excitatory transmitters in the cortex and consequent over-stimulation of postsynaptic neurons is dependent on the dose of the NMDA antagonist (Xi et al., 2009). Thus, low dose NMDA blockade will block NMDA receptors on GABA interneurons, which in turn induces disinhibition of glutamatergic efferents, increased excitatory activity and NMDA receptor hyperactivity on pyramidal cells (Ninan et al., 2003; Olney & Farber, 1995). This process disrupts the functional integrity of the cortico-limbic circuits to cause cognitive impairment and negative symptoms. However, more severe NMDA blockade at higher doses would affect both glutamatergic pyramidal cells as well as GABA interneurons, resulting in severe dysfunction of both NMDA and AMPA receptor signalling, followed by neurotoxic cell death (Xi et al., 2009).

The above described dysfunctions in glutamate receptor regulation are associated with high, chronic doses of MK 801 administration in rats (Gorter et al., 1992), causing neuronal apoptosis and oxidative stress (Ozyurt et al., 2007; Terro et al., 2000), via severe long term NMDA blockage resulting in major hypoglutamatergia. These mechanisms could also be associated with the neurodevelopmental hypothesis of schizophrenia (Chapter 2, section 4) and possibly explaining the changes in synaptogenesis and reduced neuronal connectivity that has been linked to pre- and perinatal adversity, such as SIR, that eventually lead to oxidative stress and structural brain defects.

The central role of hyperdopaminergia in schizophrenia, especially in mediating the positive symptoms, is now well established. However, studies in the early 80's noted that psychosis is not only dependent on excessive dopamine stimulation, while new antipsychotics, such as clozapine, are relatively weak D₂ receptor blockers (Harvey et al., 1999), that favouring another explanation. Indeed, non-competitive antagonists of NMDA receptors, such as dizocilpine (MK-801), ketamine and phencyclidine (PCP), are known to induce psychotomimetic effects in humans (Snyder, 1980; Lahti et al., 1999; Krystal et al., 1999, Sharp et al., 2001 for review). In agreement with the dopamine hypothesis of schizophrenia, earlier studies demonstrated that significant impairments in PPI and startle habituation can be induced in rats following

treatment with dopamine receptor agonists, such as apomorphine (Swerdlow et al., 1996; Swerdlow et al., 1995), or with dopamine releasers such as amphetamine (Mansbach et al., 1988; Swerdlow et al., 1990). However, these same responses could also be achieved with NMDA receptor antagonists (Mansbach & Geyer 1989; Geyer et al., 2001). These studies thus provided clear indication of the behavioral overlap between dopamine agonists and NMDA antagonists, and that both pathways are involved in psychosis. NMDA-receptor antagonists produce a psychotic state that in many ways is indistinguishable from schizophrenia, including negative, positive and cognitive symptoms. Dopamine agonists, however, generally induce psychosis involving visual hallucinations (Hung et al., 2003 for review) and not the auditory hallucinations that are typical of schizophrenia (Keshavan et al., 2008 for detailed review). NMDA receptor antagonists, on the other hand, reportedly cause auditory hallucinations (Kavanagh & Mueser et al., 2007; Allen and Young, 1978). Thus, we may conclude that NMDA-receptor antagonists provide a more complete model for schizophrenia than do dopaminergic agonists (Rung et al., 2005).

The accurate setting up and evaluation of PPI and social interaction in rats form a central behavioural component of this dissertation, with the main objective being the determination of PPI and social interaction in rats as critical face validity criteria for the social isolation rearing (SIR) model used in Chapter 3. It is the setting up of the method of PPI assessment that is especially complex. Consequently, in order to confirm the accurate setting up of our behavioural methodologies for PPI and social interaction, we selected an acute pharmacological challenge model known to have robust effects on PPI and social interactive behaviours in rats. As highlighted above, the NMDA receptor antagonist model is in this regard particularly useful. We therefore chose MK-801, reputed to be the most selective non-competitive antagonist of NMDA receptors *in vivo* (Bressink et al., 1995), which binds to a site located within the NMDA ion channel, thereby blocking cation flow into the cell (Rung et al., 2005). Compared to PCP or ketamine, MK-801 also has minimal affinity at other receptors in the brain (Hustveit et al., 1995; Ault and Werling, 1999). Previous observations have indicated that a dose of 0.3 mg/kg produces “schizophrenia-like behaviours” in rats (Martin et al., 1997; Stuchlik et al., 2004). An earlier study

performed in our laboratory indicated significant PPI deficits in rats treated with 0.25 mg/kg MK-801, and which could be significantly reversed with haloperidol (0.5 mg/kg) and clozapine (5 and 10 mg/kg) treatment (Toua, 2007). The dose of the NMDA antagonist is crucial, Xi et al., 2009 indicates that MK 801 doses in rats >0.3mg/kg and <0.1mg/kg are considered high and low doses, respectively. These doses either produce severe ataxia or insufficient psychotic symptoms, so that a delicate balance is required. Thus for example, chronic administration (0.6 mg/kg) of MK-801 induces a significant increase of glutamate in the PFC of rats (Gorter et al., 1992), while lower doses (0.1 mg/kg) are incapable of evoking the psychotic and metabolic alterations similar to that observed in schizophrenia (Eyjolfsson et al., 2005). For this pilot study then, we chose a dose of 0.3 mg/kg MK-801, administered via acute intraperitoneal (i.p.) injection, in line with previous studies (Rung et al., 2005).

2. Materials and methods

2.1 Animals

Male Sprague-Dawley rats, weighing 250-300g, were provided by the Animal Research Centre of North-West University. All the animals were housed according to procedures and conditions described in Chapter 3 Section 2.1. Two different sets of animals were used for both the PPI (n=20) and OFT (n=20) tests, thus 40 rats in total. All animals were maintained according to the code of ethics in research, training, diagnosis and testing of drugs in South Africa, approved by the Ethics Committee for Research on Animals at the NWU (Ethics approval number NWU-0035-08-S5).

2.2 Study design

Prepulse inhibition (PPI) testing: Ten control rats received saline i.p., while another 10 rats were treated with MK-801 at a dose of 0.3 mg/kg i.p., as outlined in Figure 1. PPI (as described in Chapter 3, section 2.5.1) was then assessed in all the

animals, 15 min after MK-801 dosing, as described by Zhang et al (2000) and Mansbach et al (1991).

Social interaction testing: The open field test (OFT), as described in Chapter 3, section 2.5.2, (Gonzalez et al., 1996), was used for this analysis. The same protocol as described for the PPI test was used, with 10 control rats and 10 rats receiving MK-801 (0.3 mg/kg i.p.), as outlined in Figure 1. Social interaction was assessed 30 min after MK-801 administration, as previously described (Rung et al., 2005; Zou et al., 2008).

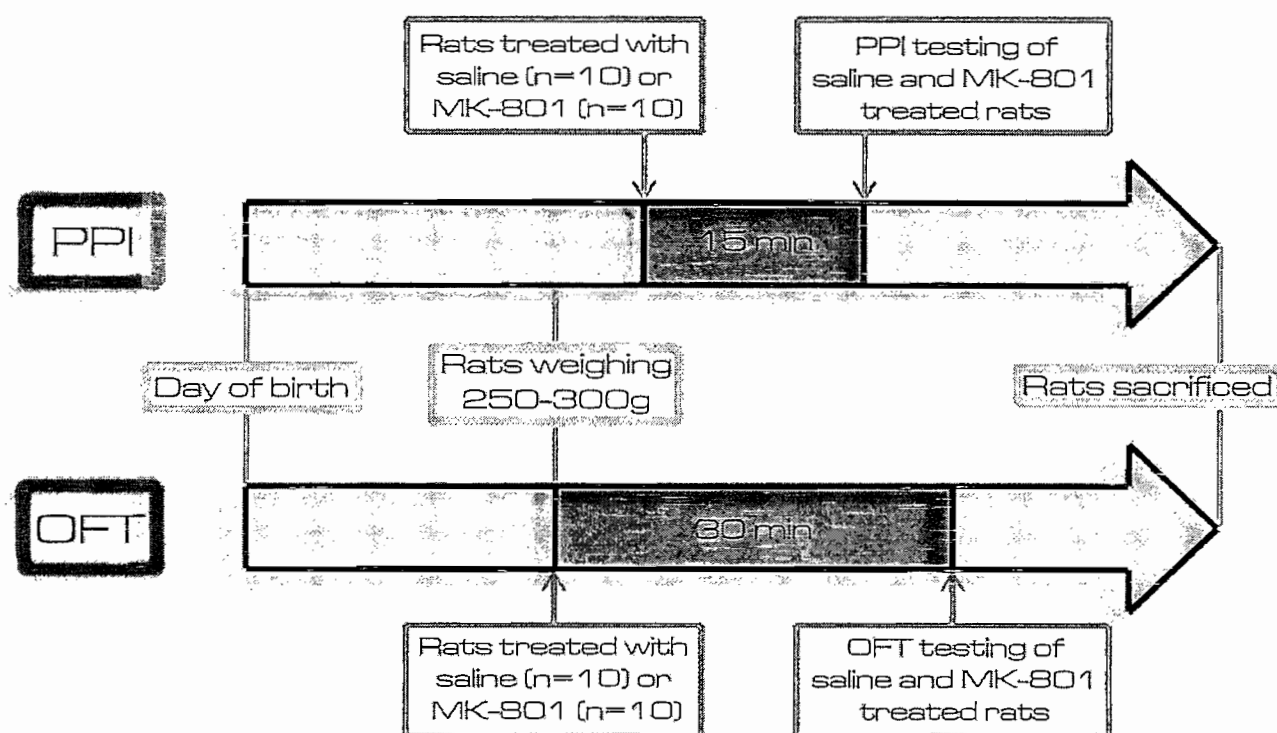


Figure 1: Study design for the validation of PPI and OFT testing in rats using the MK-801 acute challenge model.

2.3 Drug treatment

(+) MK-801 (dizocilpine) was purchased from Sigma-Adrich, Johannesburg, South Africa. The drug was freshly prepared every day by dissolving in saline and buffered with sodium hydroxide (NaOH) (pH=6) before being administered intraperitoneally. New drug was prepared each day, and stored in glass bottles covered with aluminium foil. An injection volume of 0.5 ml was used, at a dose of 0.3 mg/kg (Martin et al., 1997; Stuchlik et al., 2004).

2.4 Behavioral paradigm: PPI

2.4.1 Apparatus

PPI was assessed in two sound attenuated startle chambers (SR-LAB, San-Diego instruments, San Diego, USA), as described in Chapter 3, section 2.5.1. Self-directed and social interactive behaviors were assessed in an OFT apparatus, as described in chapter 3, section 2.5.2.

2.4.2 Method layout for PPI testing

All testing took place between 09h00-13h00. The test session consisted of a 5 min acclimatization period with 68dB white background noise. Four BLOCKS of startle stimuli, as described in chapter 3, section 2.5.1., were then applied. The first and fourth blocks consisted out of 10, 40 ms, 120dB pulse alone trials, as depicted in Figure 2. The second and third blocks consisted of 40, 120dB pulse alone trials, with no stimuli and prepulse pulse trials at 72 dB, 76dB, 80dB and 84dB presented in a random order, as depicted in Figure 2.

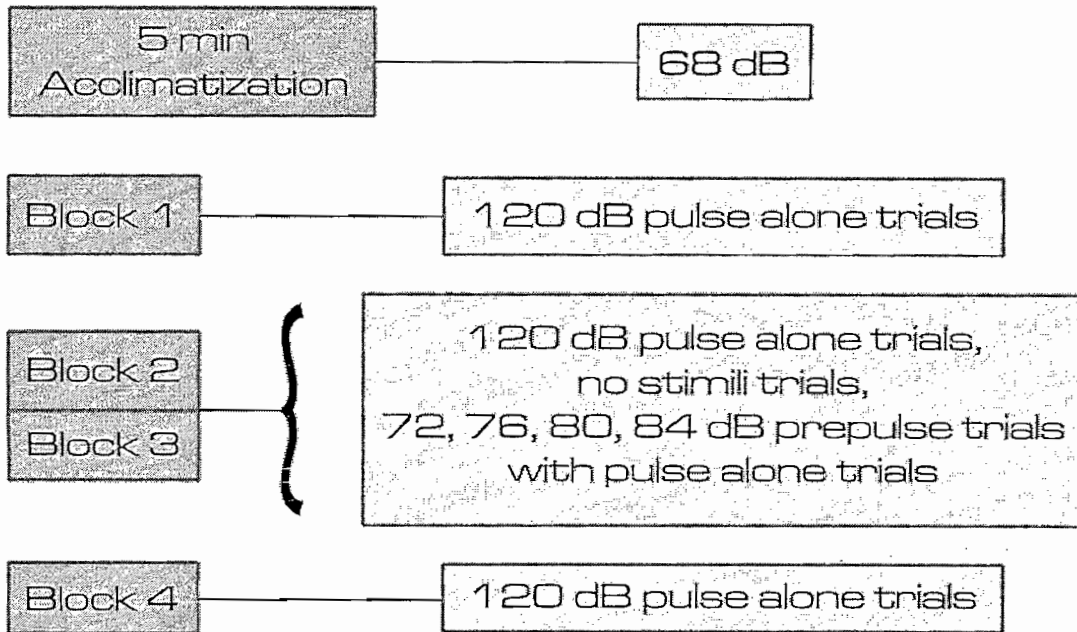


Figure 2: PPI protocol, 5 min acclimatization period and four startle blocks.

%PPI was calculated as [(startle response with the prepulse-response to the middle twenty 120 dB pulses)/response to the middle twenty 120 dB pulses $\times$ 100], as described by Van den Buuse and colleagues (2001). The total of 100 trials were delivered with an average interval of 25 s, and 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 response and habituation in response to repeated delivery of startling stimuli (Van den Buuse et al., 2001).

2.5 Behavioral paradigm: OFT

Using the OFT test for rats, as adapted from Gonzalez and colleagues (1996), and described in chapter 3, section 2.5.2., we were able to determine the extent of indulgence in self directed behaviors, such as self grooming and square crossing

(locomotor activity), and social interactive behaviors, including rearing, anogenital sniffing, approaching and staying together. The evaluation period was 10 min.

2.6 Statistical analysis

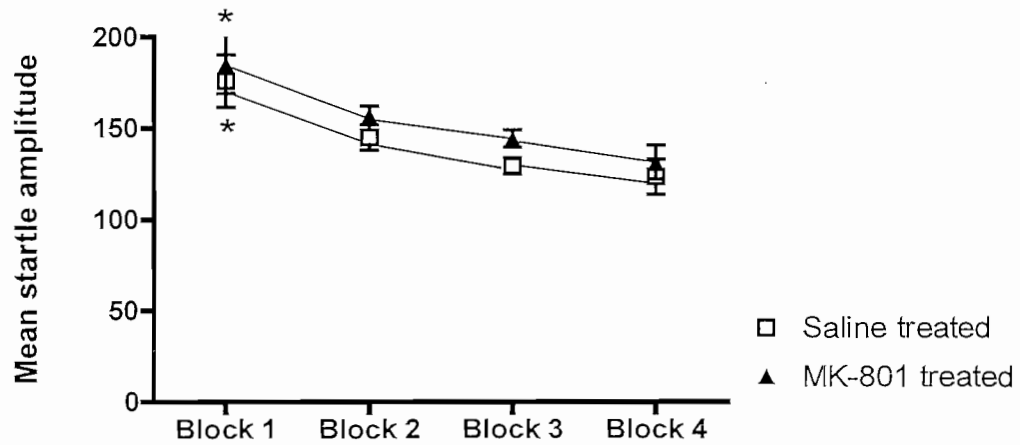
Graphpad prism version 5 for windows (Graphpad software, San Diego, USA) was used for all statistical analysis and graphical presentations. For the mean startle amplitude and PPI, a linear mixed statistical model with repeated measures for the different BLOCKS and prepulse intensities was used, followed by Newman-Keuls post-hoc test. For the evaluation OFT, saline control versus MK-801 was analyzed using an unpaired Student's T test. In all instances, statistical significance was defined as $p < 0.05$.

3. Results

3.1 Effect of acute MK-801 administration on PPI in rats.

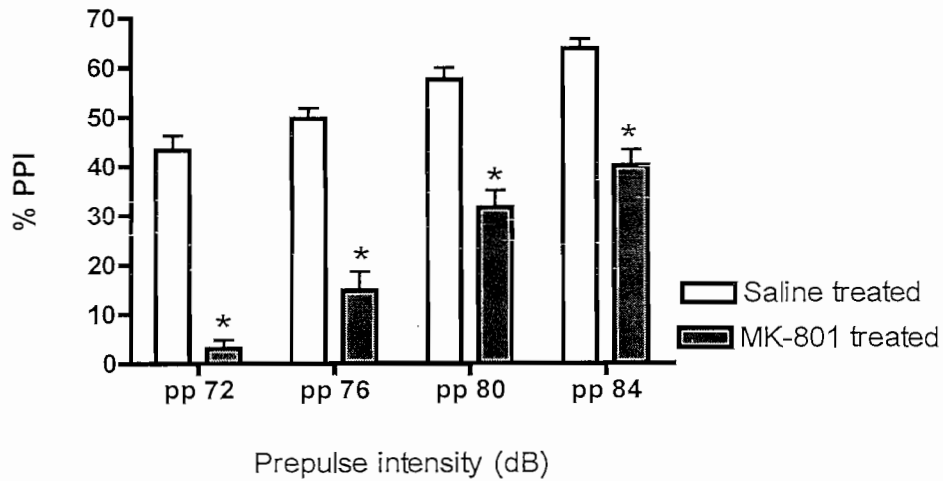
Linear mixed statistical modeling with repeated measures found no significant overall differences in the mean startle amplitude between the MK-801 treated and saline control treated rats ($F(3,54)=6.198$, $p=0.987$). However, significant habituation from BLOCK 1 to BLOCK 4 was observed in both the saline control ($p < 0.05$) and MK-801 treated rats ($p < 0.05$; Figure 3 A). Importantly, rats receiving MK-801 showed a significant deficit in %PPI compared to the saline-treated control rats, at 72dB ($p < 0.001$), 76dB ($p < 0.001$), 80dB ($p < 0.001$) and 84dB ($p < 0.001$) (Figure 3 B, 1-way ANOVA, Newman-Keuls).

A



Statistics: * $p < 0.05$ vs. BLOCK 4 (mixed analysis, Newman-Keuls)

B



Statistics: * $p < 0.001$ vs. Saline treated (mixed analysis, Newman-Keuls)

Figure 3: Sensory motor gating at the prepulse intensities indicated, in saline treated and MK-801 treated rats: (A), mean startle amplitude and (B), percent prepulse inhibition (%PPI).

3.2 Effect of acute MK-801 administration on self directed and social interactive behaviors in the OFT.

As depicted in Figure 4, MK-801 treatment evoked significant deficits in social interactive behaviors compared to saline-treated controls with respect to rearing ($p = 0.0011$; Figure 4 A), anogenital sniffing ($p < 0.0001$; Figure 4 B), approaching ($p = 0.009$; Figure 4 C) and staying together ($p < 0.0003$; Figure 4 D). When considering self-directed behaviors, MK-801 evoked a significant increase in both locomotor activity ($p = 0.0032$; Figure 5 A) and self grooming ($p < 0.0001$; Figure 5 B), compared to saline-treated controls.

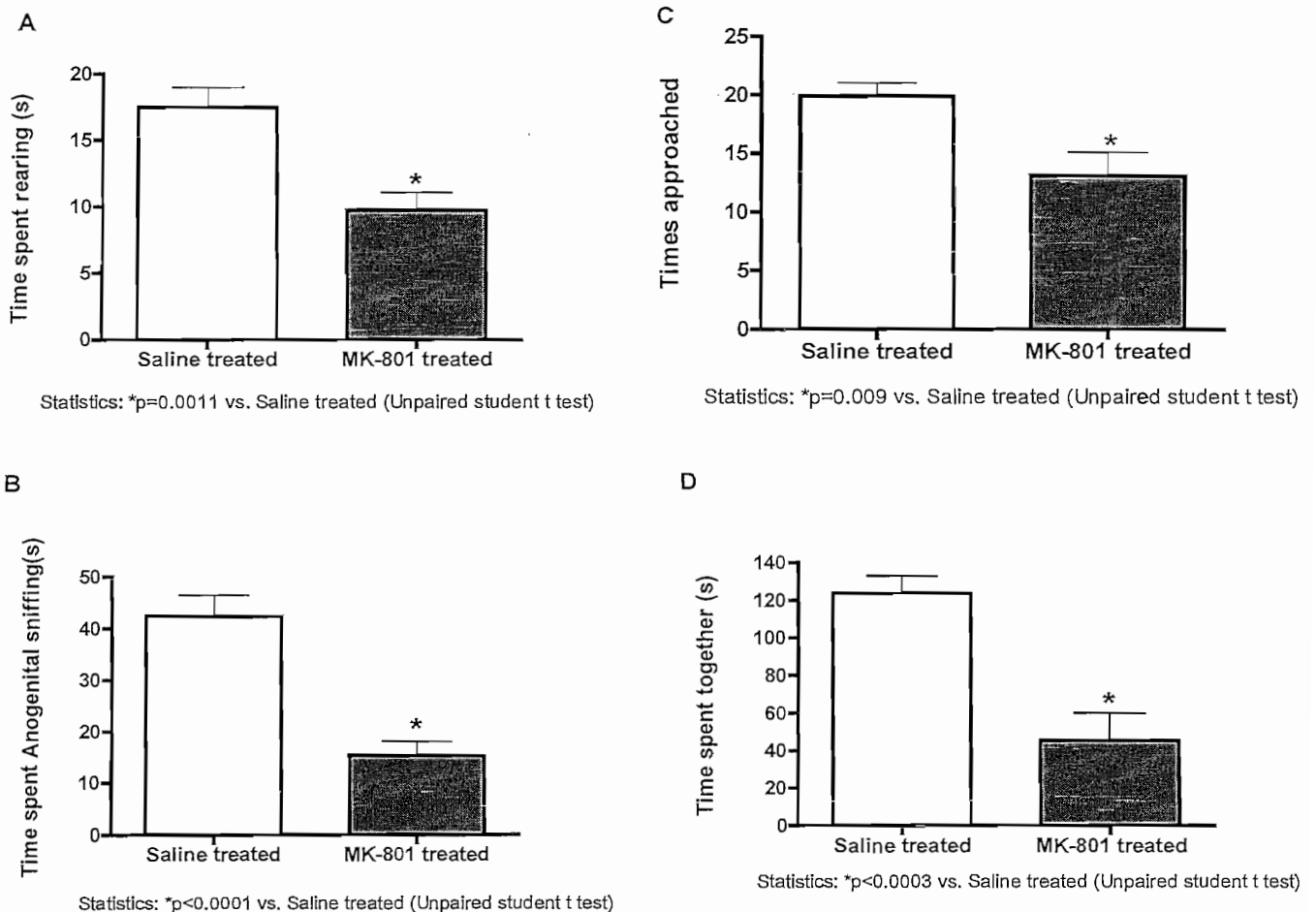


Figure 4: Social interactive behaviors in saline treated and MK-801 treated rats: (A), time spent rearing (B), time spent anogenital sniffing (C), times approached (D), time spent together.

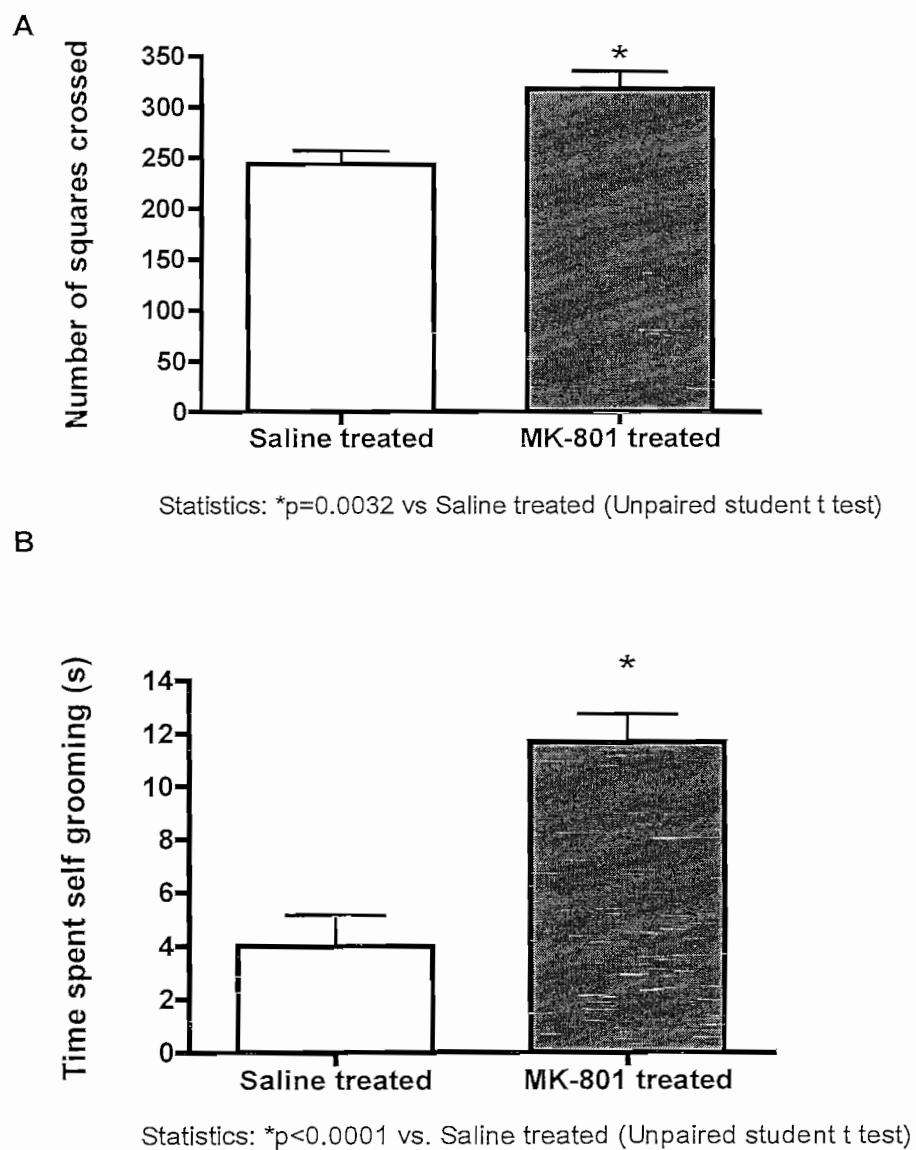


Figure 5: Self directed behaviors in saline treated and MK-801 treated rats: (A), squares crossed (locomotor activity), (B), time spent self grooming.

4. Discussion and conclusion.

The current validation study showed that the glutamate NMDA receptor antagonist, dizocilpine (MK-801), at a dose of 0.3 mg/kg, induced a significant attenuation of PPI in rats, with no significant effect on mean startle amplitude. The latter implies that MK-801 did not cause muscle fatigue or blunting of sensory receptor responsiveness in the rats treated with it. (Quednow et al., 2006 for review). However, significant habituation was observed, consistent with previous studies (eg. Kanahara et al., 2008; Uehara et al., 2009). The current PPI protocol has therefore demonstrated itself to be sensitive and effective in picking up a drug-induced change in sensorimotor gating in rodents, and supports its application in an animal model of schizophrenia, or for testing antipsychotic drugs. Both the latter qualities were applied in Chapter 3.

We also observed that a dose of 0.3 mg/kg MK-801 significantly increases locomotor activity as well as self grooming, both self-directed behaviours, while at the same time engendering a significant deficit in various social interactive behaviours, in line with earlier studies (Manahan-Vaughan et al., 2008; Karasawa et al., 2008). These data thus concur that the OFT is effective under our laboratory conditions for determining altered self directed and outwardly directed (i.e. social interactive) behaviours in rodents, and thus amenable for use in an animal model of schizophrenia, as were applied in Chapter 3.

Using MK-801 in this validation process also has direct relevance when considering the SIR model to be studied in Chapter 3. NMDA receptor hypofunction has been linked with the social and cognitive impairments observed in schizophrenia (see Chapter 2, section 5.2.3), while SIR in rats has recently been found to evoke an increase in NMDA receptor binding (Toua et al., 2009). Thus, glutamate hypofunction permeates an acute pharmacological challenge with MK-801, as well as SIR, in rats, the latter being a non-pharmacological, and neurodevelopmental model of schizophrenia. This fact instils further confidence in our hypothesis (see Chapter 1), and that we will be well positioned and prepared to observed changes in

sensorimotor gating and social interactive behaviours in animals subjected to SIR, as well as changes following drug treatment.

Blocking NMDA receptors results in an excessive release of glutamate in the cerebral cortex (Moghaddam et al., 1997), while a protracted state of NMDA receptor hypofunction can trigger neuronal injury through out many corticolimbic brain regions (Olney et al., 1989; Corso et al., 1997), which will result in numerous cognitive and social dysfunctions, as presented herein. That 8 weeks SIR is known to evoke a significant up-regulation in frontal cortical NMDA receptors (Toua et al., 2009) would imply that a chronic deficit in glutamate function is present (see Toua et al., 2009 for discussion), which may be reflected in changing redox state and cellular damage akin to that observed with high dose chronic MK-801 administration (Gorter et al., 1992). Indeed, MK-801-induced neurotoxicity is causally related to increased oxidative stress in the rat prefrontal cortex (Ozyurt et al., 2007). The validity of this suggestion and whether it has relevance for rats subjected to SIR will be presented and discussed in detail in Chapter 3.

Concluding, MK-801 induces profound deficits in social functioning, as determined using the OFT, as well as sensorimotor gating in rats. These methods can now be applied with confidence to the assessment of cognitive performance and social functioning in the SIR model of schizophrenia presented in Chapter 3.

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Addendum B

**Setting up the superoxide
dismutase (SOD) assay, using the
*SOD Assay Kit-WST®***

The following addendum is supplied to include valuable and additional information concerning the method and experimental procedures in the preparation of the brain tissues and measurement of SOD activity.

1. Introduction

Superoxide dismutase (SOD) catalyzes the dismutation of the superoxide anion (O_2^-) into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2 ; Figure 1), and is one of the most important antioxidative enzymes in the body (Cass, 1985).

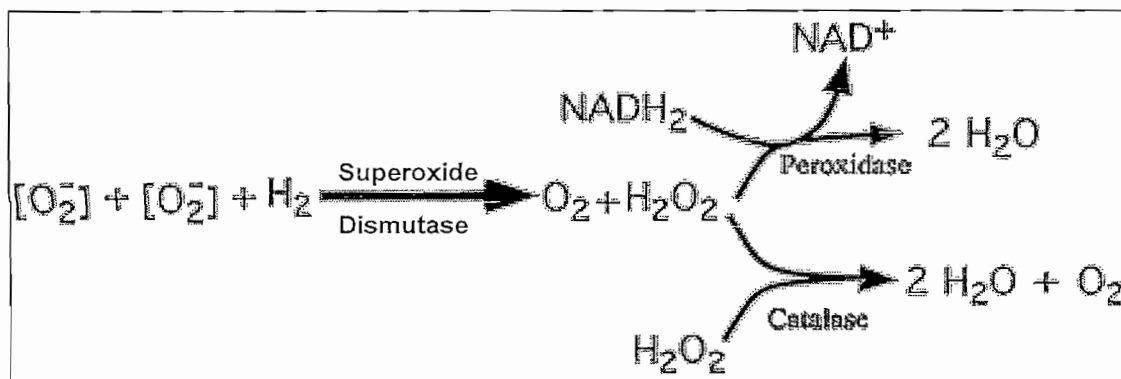


Figure 1: Dismutation of superoxide into hydrogen peroxide and molecular oxygen by the antioxidant enzyme, SOD (Adapted from Sigma-Aldrich®).

In order to determine SOD activity, several direct and indirect methods have been developed. Among these, an indirect method using nitroblue tetrazolium (NBT) is commonly used due to its convenience and ease of use. However, there are several

disadvantages to the NBT method, such as poor water solubility of the formazan dye and the interaction with the reduced form of xanthine oxidase (XO) (Tan et al., 2000).

However, the SOD Assay Kit-WST[®] allows for the convenient assay of SOD activity by utilizing Dojindo's highly water-soluble tetrazolium salt, WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt), that produces a water-soluble formazan dye upon reduction with a superoxide anion (Ukeda et al., 1999). The rate of the reduction with O_2^- is linearly related to the activity of xanthine oxidase (XO), and is inhibited by SOD (Figure 2). Therefore, the IC₅₀ (50% inhibition activity of SOD or SOD-like materials) can be determined by a colorimetric method. Since the absorbance at 440 nm is proportional to the amount of O_2^- , the activity of SOD as an inhibition activity can be quantified by measuring the decrease in the color development at 440 nm (Peskin et al., 2000).

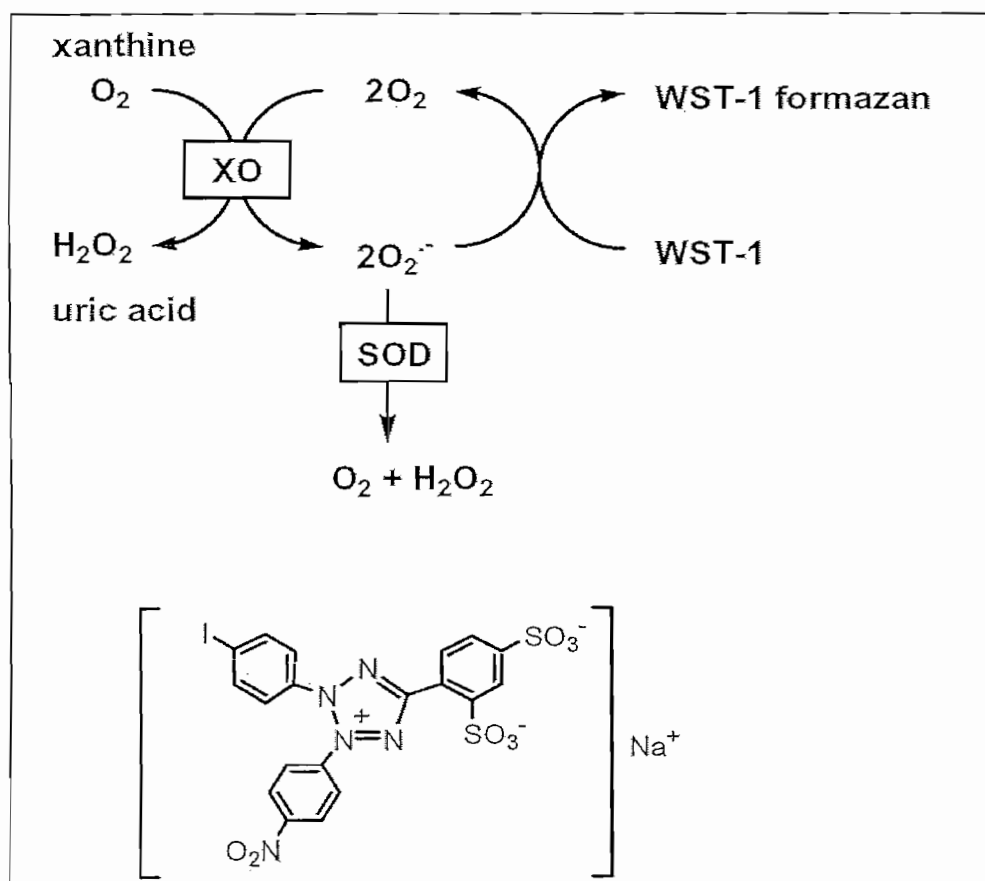


Figure 2: Principle of the SOD Assay Kit (Sigma-Aldrich®, 2004)

2. Materials and methods

2.1 Chemicals and reagents:

All chemicals and reagents used in this study were of highest grade, purchased from Sigma-Aldrich®, South-Africa, Johannesburg.

2.2 Preparation of brain homogenate:

Preparation of phosphate buffered solution:

To prepare 2.5 litres of a 100x strength solution, 200g NaCl, 5g KCl, and 22.5g Na₂HPO₄, were added to 2.5 liters of double distilled water. On the day of the assays, 1 part PBS (100x) were diluted with 9 parts double distilled water before use to obtain a 0.01 mM PBS dilution.

After rats were sacrificed and the brains quickly removed and placed on ice, the striatum and frontal cortex were dissected and immediately stored at -80°C. On the day of the SOD assay, the brain regions were removed from -80°C, allowed to thaw on ice and weighed. A 10% w/v solution was then made with the brain samples in ice-cold phosphate- buffered saline (PBS), pH 7.4.

Thereafter, the tissue samples were ultrasonically homogenized, and the resulting homogenates centrifuged at 800 ×g for 10 min, at 4°C (Pereira et al., 2009). The supernatants were used for the SOD activity assay and for the determination of protein content (Zupan et al., 2008).

2.3 Protein determination

Protein determination of the brain homogenates was carried out according to the method of Bradford (Bradford, 1976). The Bradford reagent was lightly shaken, a adequate amount removed and kept at room temperature in a dark environment, after which protein standards were prepared by dissolving 5 mg bovine serum

albumin (BSA) in 1ml double distilled water, producing a 5 mg/ml solution. A series of 100 μ l dilutions were then made, as indicated in Table 1.

Table1: Protein concentration standards

	Dilution in test tubes	
Protein concentration	Volume of 5mg/ml BSA	Volume of PBS buffer
0 mg/ml	0 μ l	100 μ l
0.5 mg/ml	10 μ l	90 μ l
1.0 mg/ml	20 μ l	80 μ l
1.75 mg/ml	35 μ l	65 μ l
2.5 mg/ml	50 μ l	50 μ l
3.5 mg/ml	70 μ l	30 μ l
5.0 mg/ml	100 μ l	0 μ l

The different protein concentrations, as well as each brain homogenate, were then added to a 96-well plate in triplicate. Thereafter, 250 μ l of the Bradford reagent was added to each well, shaken for 30 seconds and incubated at room temperature for 15 minutes. The absorbance of each well was then read at a wavelength of 560 nm and the protein concentration of the brain homogenate calculated from the plot of “net absorbance vs protein concentration of the standards”.

2.4 Preparation of working solutions for the SOD activity assay:

- *WST working solution*: Dilute 1 ml of WST Solution with 19 ml of Buffer Solution.
- *Enzyme working solution*: Vortex the Enzyme Solution tube for 5 sec. Mix by pipeting, and dilute 15 µl of Enzyme Solution with 2.5 ml of Dilution Buffer.

2.5 Determination of SOD activity in brain homogenate

Refer to Table 2

- 20 ml of the 10% brain homogenate (sample solution) was added to each well for *sample* and for *blank 2*. 20 µl of ddH₂O (double distilled water) was then added to the wells for *blank 1* and *blank 3*.
- 200 µl of WST Working Solution were then added to each well, and mixed.
- 20 µl of the Dilution Buffer were then added to *blank 2* and *blank 3* wells.
- Lastly, 20 µl of Enzyme Working Solution were added to each *sample* and *blank 1* well, and mixed thoroughly.
- The 96 well plates were then incubated at 37 °C for 20 min, and the absorbance read at 450 nm using a microplate reader.
- One unit of enzyme activity is then defined as the quantity of SOD required to cause a 50% inhibition of the absorbance change per min of the blank reaction (diluent rate).

- The % SOD activity is calculated (inhibition rate %) using the following equation: % SOD activity (inhibition rate %) = $\{[(\text{Ablank 1} - \text{Ablank 3}) - \text{Asample} - \text{Ablank 2})] / (\text{Ablank 1} - \text{Ablank 3})\} \times 100$.
- For tissue samples, as used in the present study, one unit (U) of enzyme activity is defined as the quantity of SOD required to cause a 50% inhibition of the absorbance change per min of the blank reaction (diluent rate). Units of SOD activity in the frontal cortex and striatum are expressed as U/mg protein.

Table 2: Amount of each solution for sample, blank 1, 2 and 3

	Sample (10% brain homogenate)	Blank 1	Blank2	Blank 3
Sample solution	20 µl		20 µl	
ddH₂O		20 µl		20 µl
WST Working solution	200 µl	200 µl	200 µl	200 µl
Enzyme Working solution	20 µl	20 µl		
Dilution Buffer			20 µl	20 µl

2.6 Calibration curve of SOD standards

In order to monitor the SOD activity determined in the frontal cortex and striatum, a calibration curve was set up using purified bovine erythrocyte SOD as standard, under identical conditions. This calibration curve provides the correlation of the

inhibition percentage of formazan dye formation and SOD activity. All samples were diluted with 0.01 mM phosphate buffer pH 7.0 with each assay, as follows:

- SOD standard solutions:
- 10 U/ml, 5 U/ml, 1 U/ml, 0.1 U/ml, 0.05 U/ml.

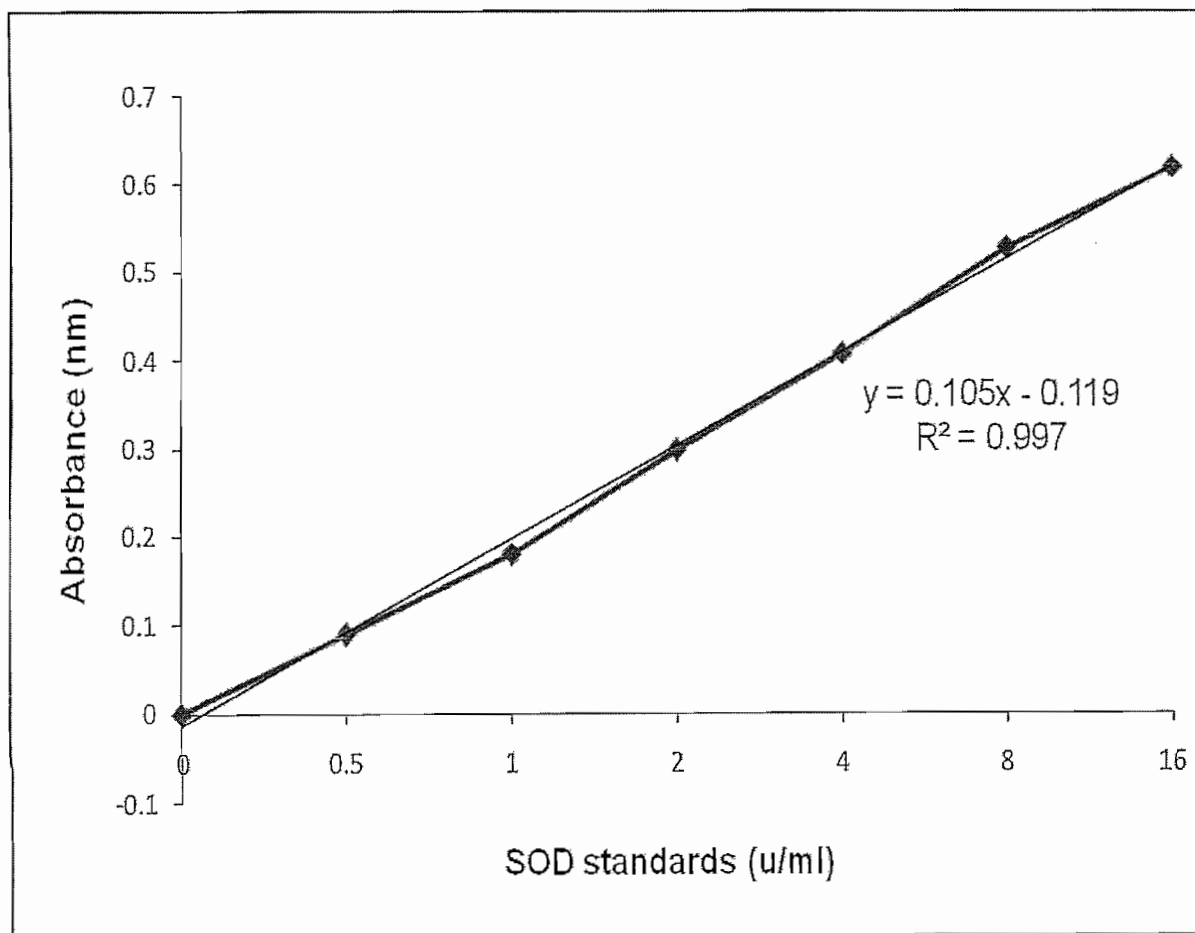


Figure 3: Calibration curve of SOD standard solutions, with a R^2 of 0.997.

2.7 Final Layout of the 96-well plate for the SOD assay

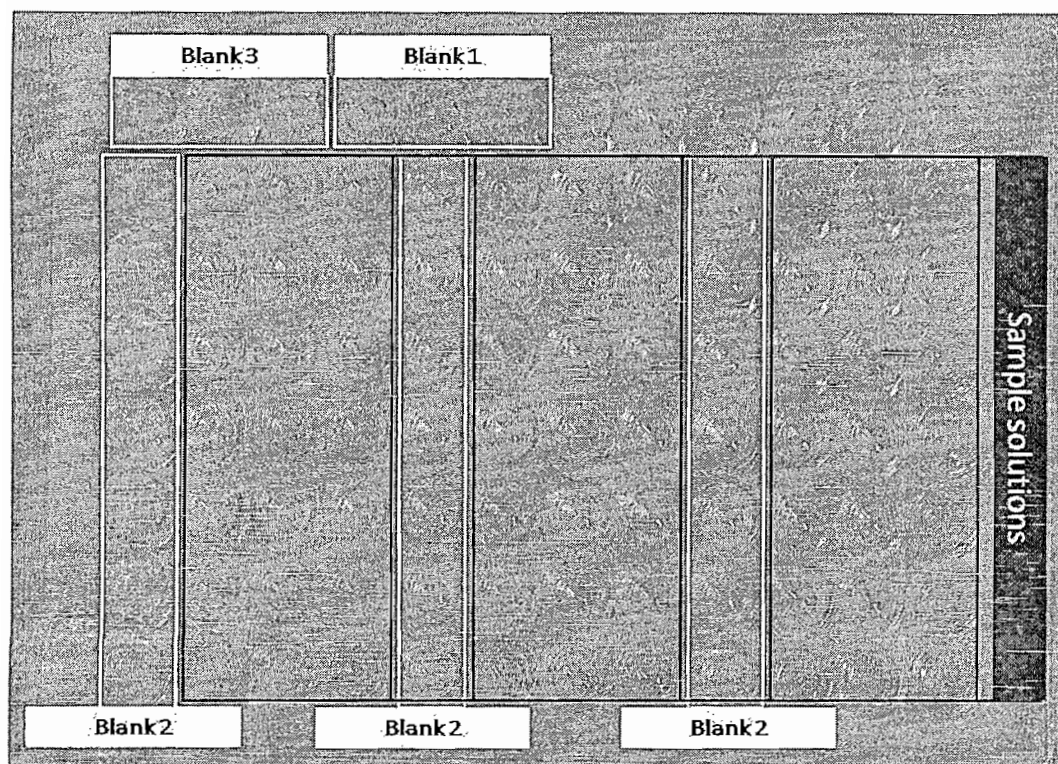


Figure 4: Layout of the 96-well plate for the SOD assay.

3. Conclusion

The above described assay procedure was subsequently applied in the current study, as presented in detail in Chapter 3.

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Addendum C

Authors' instructions

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