
Chapter 4

Manuscript B

Article published in *Neuropharmacology*, entitled:

“Social isolation rearing in rats alters plasma tryptophan metabolism and is reversed by sub-chronic clozapine treatment”

Introduction

This chapter presents the full-length manuscript published in *Neuropharmacology*, published by Elsevier. The manuscript is presented as published by the publisher, and can be viewed or accessed on-line via the journal website:

<http://www.sciencedirect.com/science/article/pii/S0028390812000822>

Authors' contributions

- *M. Möller* designed the study along with *BH Harvey*, undertook the entire analytical laboratory and statistical analyses as well as the animal study, wrote the first draught of the manuscript, and edited the manuscript after receiving comments from co-authors for publication.
- *JL du Preez* supervised all aspects of analytical laboratory analysis.
- *R Emsley* advised on the study design and proof read the final manuscript.
- *BH Harvey* supervised the study design and assisted in the interpretation of the study data, as well as finalized the manuscript for publication.

All co-authors provided permission to use this manuscript as part of M. Möller's Ph.D. thesis.



Contents lists available at SciVerse ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

Social isolation rearing in rats alters plasma tryptophan metabolism and is reversed by sub-chronic clozapine treatment

Marisa Möller^a, Jan L. Du Preez^b, Robin Emsley^c, Brian H. Harvey^{b,*}

^a Division of Pharmacology, School of Pharmacy, North West University, Potchefstroom, South Africa

^b Research Unit, Drug Research and Development Focus Area, School of Pharmacy, North West University, Potchefstroom, South Africa

^c Department of Psychiatry, University of Stellenbosch, Cape Town, South Africa

ARTICLE INFO

Article history:

Received 16 September 2011

Received in revised form

8 February 2012

Accepted 25 February 2012

Keywords:

Tryptophan metabolism

Social isolation rearing

Clozapine

Schizophrenia

Quinolinic acid

Kynurenic acid

Anti-inflammatory

Antioxidant

ABSTRACT

Schizophrenia is associated with increased oxidative stress, although the source of this redox disequilibrium requires further study. Altered tryptophan metabolism has been described in schizophrenia, possibly linked to inflammation and glutamate-directed excitotoxicity. Social isolation rearing (SIR) in rats induces various behavioural manifestations akin to schizophrenia, as well as altered frontal cortical glutamate N-methyl-D-aspartate (NMDA) receptor binding and increased oxidative stress, all reversed by antipsychotic treatment. Tryptophan is catabolized via the kynurenine pathway to kynurenine, 3-hydroxykynurenine, quinolinic acid (QA), kynurenic acid (KYNA), anthranilic acid and 3-hydroxyanthranilic acid (3-OHAA), ultimately contributing to neuronal integrity and redox balance in the brain. We studied tryptophan metabolism and neuroprotective-neurodegenerative balance in post-natal SIR rats, and its response to clozapine treatment. Male Sprague-Dawley (SD) rats (10 rats/group) were exposed to SIR or social rearing for 8 weeks, whereupon they received either sub-chronic vehicle or clozapine (5 mg/kg i.p.) treatment. Plasma tryptophan metabolites were analysed by liquid-chromatography electrospray ionization tandem mass spectrometry. Plasma tryptophan, kynurenine, anthranilic acid, 3-OHAA and QA were significantly elevated in SIR vs. socially housed rats. KYNA and the neuroprotective ratio were significantly decreased. The latter implies a decrease in KYNA (neuro-protective) but an increase in QA (neurodegenerative) directed components of the pathway. Clozapine significantly reversed all the above alterations in SIR animals. Concluding, SIR in rats significantly disrupts tryptophan metabolism via the kynurenine pathway with increased risk for neurodegenerative changes in the brain. These changes are reversed by clozapine, emphasising the importance of these findings for the neurobiology and treatment of schizophrenia.

© 2012 Elsevier Ltd. All rights reserved.

1. Introduction

Schizophrenia is a devastating psychiatric disorder with a broad range of behavioural and biological manifestations. Although schizophrenia was once seen as a disease affecting only a few key brain regions and regionally discrete neurotransmitter systems such as dopamine, more recent findings implicate a multifactorial

view of cortical and subcortical dysfunction. Indeed, the primary lesion(s) in schizophrenia may not directly involve certain neurotransmitters but rather be representative of a more general defect in connectivity, perhaps of neurodevelopmental origin (Carlsson et al., 2001).

Social isolation rearing (SIR) in rats, regarded as a neurodevelopmental animal model of schizophrenia, demonstrates strong face and predictive validity for the human disorder (see (Fone and Porkess, 2008) and (Weiss and Feldon, 2001) for review). Recently we established that SIR induces discrete dopamine (DA) D₁ and glutamate N-methyl-D-aspartate (NMDA) receptor changes in the frontal cortex of rats, with a selective response to first vs. second generation antipsychotics. This not only suggests that the cognitive dysfunction observed in schizophrenia involves a disturbance of cortical D₁-NMDA receptor function, but may also explain how new generation “atypical”

Abbreviations: SIR, social isolation rearing; DA, dopamine; ROS, reactive oxygen species; KYNA, kynurenic acid; QA, quinolinic acid; 3-OHAA, 3-hydroxyanthranilic acid; NMDA, N-methyl-D-aspartate; 5-HT, serotonin; IDO, indoleamine-2,3-dioxygenase; SPE, solid-phase extraction; LCMS, liquid chromatography mass spectrometry; NAD, nicotinamide adenine dinucleotide; CSF, cerebrospinal fluid; TNF- α , tumour necrosis factor- α ; GABA, γ -amino-butyric acid.

* Corresponding author. Tel.: +27 018 299 2238; fax: +27 018 299 2225.

E-mail address: Brian.Harvey@nwu.ac.za (B.H. Harvey).

antipsychotic drugs selectively target these processes for improved response of negative symptoms (Toua et al., 2010). Altered glutamate activity is well described in patients with schizophrenia (Carlsson et al., 2001; Javitt, 2010; Kantrowitz and Javitt, 2010) while there is also increasing evidence that NMDA receptor active agents, such as D-cycloserine and N-acetyl cysteine, have therapeutic potential in the disorder (Berk et al., 2008; Evins et al., 2002; Goff et al., 1999; Lavoie et al., 2008). There is also evidence that schizophrenia may be causally related to altered redox balance, especially increased oxidative stress (Akyol et al., 2002; Ng et al., 2008). Indeed, we have recently demonstrated the important association between cortico-striatal oxidative stress and schizophrenia-related behaviours in rats subjected to SIR (Möller et al., 2011).

Under excitotoxic conditions, excessive release of stored synaptic glutamate into the extracellular space is followed by an overstimulation of NMDA receptors. This causes a massive calcium influx and a subsequent rise in the production of reactive oxygen species (ROS). Cellular energy stores are depleted, causing a further release of glutamate as well as cellular damage or apoptosis (Betzen et al., 2009). However, the proposed dysfunctional glutamatergic state in schizophrenia may also involve a disturbance in other endogenous excitatory transmitters in the brain (Schwarcz et al., 2001), one in particular being the tryptophan-kynurenine pathway.

Tryptophan is catabolized into kynurenine by two haem-dependent enzymes, namely tryptophan-2,3-dioxygenase (TDO) in the liver and indoleamine-2,3-dioxygenase (IDO) in the central nervous system, lungs and placenta. As illustrated in Fig. 1 (left panel), tryptophan is catabolized via the kynurenine pathway to kynurenine, then to either to kynurenic acid (KYNA) or 3-hydroxykynurine, following then to anthranilic acid, 3-hydroxyanthranilic acid (3-OHAA) and quinolinic acid (QA). This highly regulated pathway accounts for the metabolism of approximately 80% of non-protein-bound tryptophan, the essential amino acid needed for the synthesis of serotonin (5-HT) (Allegrri et al., 2003). TDO specifically metabolizes tryptophan, while IDO is responsible for the oxidative metabolism of tryptophan, serotonin and melatonin (Stone and Darlington, 2002). In the brain, tryptophan catabolism occurs in astrocytes and microglia albeit with 60% of cerebral kynurenine contributed from the periphery (Heyes et al., 1997). QA is a recognized NMDA receptor agonist and excitotoxin capable of inducing neurodegenerative changes in the brain (Schwarcz, 2004; Stone, 2001). KYNA, on the other hand, is an antagonist at the facilitatory glycine site on the NMDA receptor ion channel, thus possessing potential neuroprotective properties (Guillemin et al., 2007). IDO is also a major component of the antioxidant forces of the cell in conjunction with superoxide dismutase (reviewed in Stone and Darlington, 2002), so that any abnormalities in cellular redox may be intimately linked to disturbances in the kynurenine pathway. Together these metabolites contribute significantly to the neuroprotective-neurodegenerative balance in the brain (Myint et al., 2007a, 2007b). It is not surprising then that diverse changes in tryptophan metabolism has been described in patients with schizophrenia (Issa et al., 1994; Torrey et al., 1998; Ravikumar et al., 2000; Schwarcz et al., 2001; Miller et al., 2008; Myint et al., 2011).

The plasma KYNA: kynurenine ratio, also referred to as the neuroprotective ratio (Myint et al., 2007a, 2007b, 2011), is hypothesized to relate to the eventual downstream levels of QA following a shift in the relative levels of KYNA and kynurenine (see Fig. 1, left panel). We therefore studied whether peripheral tryptophan metabolism and changes in the plasma KYNA: kynurenine ratio is altered in post-natal SIR rats, and whether these changes could be reversed following sub-chronic treatment with the new generation antipsychotic clozapine.

2. Experimental procedures

2.1. Animals

Male Sprague-Dawley rats (160–190 g; Animal Research Centre, North West University) were randomly allocated to groups of 10 rats/group. At weaning (post-natal day 21) the animals were randomized to SIR (1 animal/cage) or socially reared (3–4 rats/cage) for 8 weeks (day 77). The rats were reared under identical conditions: cages (230(h) × 380(w) × 380(l) mm) with sawdust (Möller et al., 2011; Weiss and Feldon, 2001), temperature (21 ± 0.5 °C), humidity (50 ± 10%), white light (350–400 lux), 12 h light/dark cycle and free access to food and water. SIR and socially reared animals experienced minimal handling and no environmental enrichment. Sawdust was changed weekly. The animals were handled according to the code of ethics in research, training and testing of drugs in South Africa (Ethical approval was obtained by the North West University ethical committee: NWU-0035-08-S5). No distressful effects of clozapine were observed at the dosage used in this study and the number of animals used was the minimum required to obtain scientifically valid data.

2.2. Drugs and drug treatment protocol

Clozapine (Sigma-Aldrich, Johannesburg, South Africa), dissolved in 1 M glacial acetic acid and buffered with sodium hydroxide (NaOH) (pH = 6.0), was administered intraperitoneally (i.p.) at a dose of 5 mg/kg in 0.5 ml. This single dose challenge with clozapine was carefully chosen based on previous studies in our laboratory where clozapine was administered to SIR animals using the same sub-chronic treatment protocol (Toua et al., 2010; Möller et al., 2011), but also after due consultation of other studies (Bakshi et al., 1994; Zhang et al., 1999) and considering approximate therapeutic equivalence (Atkins et al., 1997). Control groups received vehicle injections, comprising saline and 1 M glacial acetic acid and buffered with sodium hydroxide (NaOH) (pH = 6.0). Early antipsychotic response in schizophrenia, at for example 2 weeks, is reported to be an accurate predictor of later response (Kapur et al., 2005; Ascher-Svanum et al., 2008) thus questioning the need for prolonged treatment to confirm efficacy. Furthermore, since earlier studies have demonstrated efficacy for clozapine to reverse sensory motor gating deficits, oxidative stress and neuroreceptor changes in SIR animals following an 11 day treatment period (Toua et al., 2010; Möller et al., 2011), and to exclude the possible impact of injection stress evoked over extended treatment periods, this study was conducted over a similar treatment period. Clozapine, freshly prepared each day as previously described (Toua et al., 2010), and vehicle were therefore administered in the last 11 days of social/SIR rearing. All animals were sacrificed by decapitation without any prior use of an anaesthetic agent.

2.3. Experimental design

This study consisted of a non-treatment and a treatment cohort. Non-treatment cohort: animals were randomly separated at weaning (21 days post-natal) into two groups. One group of animals ($n = 10$) were placed into 8 weeks SIR, while a parallel socially reared control group ($n = 10$) was run concurrently. Treatment cohort: animals were randomly separated at weaning (21 days post-natal) into four groups. Two groups ($n = 10$ each) received 8 weeks SIR or social rearing plus vehicle treatment, while the remaining two groups ($n = 10$ each) received 8 weeks SIR or social rearing plus clozapine treatment. After the respective 8 weeks rearing, the animals were sacrificed and trunk blood collected for tryptophan metabolite analysis.

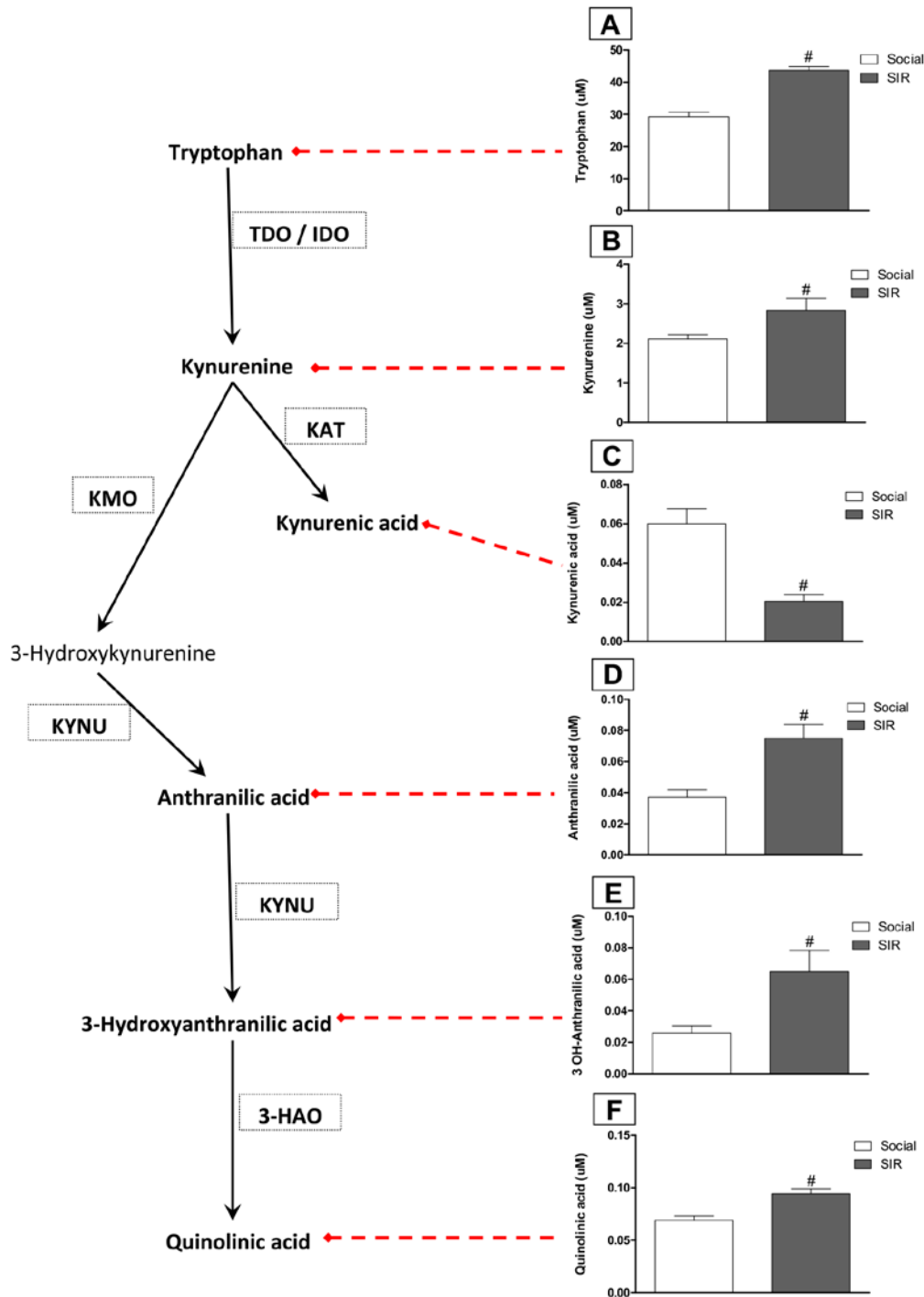


Fig. 1. The kynurenine pathway (left panel), adapted from Stone (2001), illustrating the major intermediates and component enzymes (boxed). The right panel (A–F) describes the experimental results for the kynurenine precursor (tryptophan) and metabolites kynurenine, kynurenic acid (KYNA), anthranilic acid, 3-OH-anthranilic acid (3-OHAA) and quinolinic acid (QA) as analysed by SPE-LCMS in the socially reared and SIR animals in the non-treatment cohort ($n = 10/\text{group}$). IDO = indoleamine-2,3-dioxygenase; TDO = tryptophan-2,3-dioxygenase; KAT = kynurenine aminotransferase; KMO = kynurenine mono-oxygenase; KYNU = kynureninase; 3-HAO = 3-hydroxyanthranilic acid oxidase. [#] $p < 0.05$ vs. socially reared (unpaired Student's t -test). Refer to text for precise p values.

2.4. Tryptophan metabolite analysis

Trunk blood was collected in pre-chilled, 4 ml vacutainer tubes (SGVac) containing K_2EDTA solution as anticoagulant, centrifuged at 14,000 rpm at 4 °C for 10 min, and the plasma stored at –80 °C until

the day of analysis. On the day of analysis the plasma samples were thawed on ice, centrifuged again, as mentioned above, and the plasma used. Samples were pre-purified by a solid-phase extraction method followed by detection using a novel liquid chromatography mass spectrometry (LCMS) procedure (Möller et al., submitted for

publication) based on a modification of existing methods (Amirkhani et al., 2002; de Jong et al., 2009). Briefly, plasma samples were pre-purified by solid-phase extraction (SPE) using a polymeric water-wettable reversed-phase sorbent (Oasis HLB 3cc, Waters, Milford, MA part no. WAT094226). Plasma (800 μ l) was treated with 1% formic acid (in water v/v) (1 ml) and loaded on an extraction column, conditioned, washed, dried for 10 min and subsequently eluted with 1% NH_4OH : 50% methanol (50:50 v/v) (2 ml). The eluted samples were evaporated under nitrogen, dissolved in 1% NH_4OH : 50% methanol (150 μ l), and injected on the LCMS. Chromatographic separation of the analytes occurred by C18 reversed phase chromatography (Ultra Aqueous C18, 5 μ m, 150 $\times$ 2.1 mm, Restek, Bellefonte, PA). Mass spectrometric detection was performed using a mass spectrometer in positive and negative electrospray ionization, with a flow rate of 0.2 ml/min and an injection volume of 10 μ l. Total run time including sample clean-up was 15 min.

Multiple reaction monitoring (MRM) tuning was carried out to optimize fragmentation conditions and to identify the best precursor/product transition for quantitation. Tryptophan, kynurenine, KYNA and 3-OHAA were detected in positive electrospray ionization mode, while anthranilic acid and QA were detected in negative electrospray ionization mode. The ratio between plasma KYNA and plasma kynurenine, from which KYNA and QA are formed, enabled the determination of the neuroprotective ratio (Myint et al., 2007a, 2007b, 2011). Neuroprotective ratio: $[1000 \times \text{plasma kynurenine acid } (\mu\text{M})]/\text{plasma kynurenine } (\mu\text{M})$.

2.5. Body weight

Body weight was determined at PND 21 and also on days 1 and 11 of drug treatment to confirm equal development across all the treatment groups over the study period.

2.6. Statistical analysis

For the non-treatment cohort an unpaired Student's *t*-test was used and for the treatment cohort a one-way ANOVA was used followed by Newman-Keuls post-hoc test (Graphpad Prism 5; SAS/STAT[®] Software). In all cases, data are expressed as the mean $\pm$ standard error of the mean (SEM), with a *p* value of <0.05 deemed statistically significant. Animal body weight (mean $\pm$ SEM) was analysed by one-way ANOVA with repeated factors of treatment (vehicle and clozapine) and rearing conditions to compare body weight across the groups, followed by Newman-Keuls post-hoc analyses.

3. Results

3.1. Non-treatment cohort

In the non-treatment cohort, unpaired Student's *t*-test revealed a significant increase in plasma tryptophan ($p < 0.0001$), kynurenine ($p = 0.04$), anthranilic acid ($p = 0.002$), 3-OHAA ($p = 0.014$) and QA ($p = 0.0009$) (Fig. 1 A, B, D, E, and F respectively) in SIR vs. group-housed animals. Moreover, a significant decrease in plasma KYNA ($p = 0.0002$) (Fig. 1C) was observed in SIR animals compared to their socially reared controls.

3.2. Treatment cohort

One-way factorial ANOVA revealed significant cross-group interactions with respect to tryptophan metabolism in the drug treatment cohort with respect to tryptophan ($F = 54.8$, $p < 0.0001$), kynurenine ($F = 4.96$, $p = 0.006$), KYNA ($F = 9.69$, $p < 0.0001$), anthranilic acid ($F = 16.44$, $p < 0.0001$), 3-OHAA ($F = 7.413$,

$p = 0.0005$) and QA ($F = 9.046$, $p < 0.0001$) (Fig. 2A–F respectively). Post-hoc analysis with Newman–Keuls tests indicated a significant increase in tryptophan ($p < 0.0001$) (Fig. 2A); kynurenine ($p = 0.007$) (Fig. 2B); anthranilic acid ($p < 0.0001$) (Fig. 2D); 3-OHAA ($p = 0.001$) (Fig. 2E); and QA ($p < 0.0001$) (Fig. 2F), as well as a significant decrease in KYNA ($p = 0.002$) (Fig. 2C) in the SIR animals only receiving vehicle compared to vehicle-treated socially reared controls. These alterations in tryptophan metabolism were in turn completely reversed by clozapine treatment in the SIR animals, with respect to tryptophan ($p < 0.0001$), kynurenine ($p = 0.005$), anthranilic acid ($p < 0.0001$), 3-OHAA ($p < 0.0001$), QA ($p < 0.0001$) as well as KYNA ($p < 0.0001$) (Fig. 2A–F). Clozapine did not have any notable effects of its own on tryptophan metabolism compared to vehicle-treated socially reared animals.

3.3. Neuroprotective ratio

After calculation of the neuroprotective ratio for each animal, one-way factorial ANOVA revealed significant cross-group interactions with respect to the neuroprotective ratio ($F = 49.56$, $p < 0.0001$) (Fig. 3). Post-hoc analysis with Newman–Keuls tests indicated a significant decrease in both SIR animals receiving no treatment ($p < 0.0001$) and receiving only vehicle ($p < 0.0001$), compared to all the other groups. We also found that sub-chronic clozapine treatment significantly reversed this deficit in the SIR animals compared to SIR animals receiving vehicle ($p < 0.0001$), with no effect in socially housed controls (Fig. 3).

3.4. Body weight

The mean weights of the rats during this study were as follows: PND 21: 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 drug treatment (day 67) ($F = 1.29$, $p = 0.83$), and on day 11 of treatment (day 78) ($F = 1.824$, $p = 0.30$). Furthermore, all groups showed a significant and equal amount of growth over the period of the study (data not shown).

4. Discussion

SIR in rats, a putative neurodevelopmental animal model of schizophrenia, induced a profound increase in plasma tryptophan, kynurenine, anthranilic acid, 3-OHAA as well as QA, and a significant decrease in plasma KYNA. SIR also induced a significant decrease in the neuroprotective ratio, thus supporting the hypothesis that in the event of increased QA concentrations, the kynurenine pathway will shift towards QA production and away from KYNA production, therefore decreasing the neuroprotective properties of QA. Importantly, sub-chronic clozapine treatment reversed all SIR-evoked changes in tryptophan metabolism.

Schizophrenia demonstrates disturbances in neuronal plasticity and survival mechanisms, particularly involving immune-inflammatory processes (Maes et al., 1994; Lin et al., 1998; Potvin et al., 2008), altered redox status (Ng et al., 2008; Akyol et al., 2002) and excitotoxicity (Deutsch et al., 2001). Moreover, these changes appear to originate as a result of early life adversity during pre-, peri- or post-natal stages of neuronal development (Willinger et al., 2001; Meyer and Feldon, 2010), and modelled by SIR in rats. The kynurenine pathway is regulated by all of the above processes and results in the production of several neuroactive intermediates that are immune-active, albeit with opposing neurotoxic and neuroprotective actions (Stone, 2001). Indeed, increased IDO activity not only increases tryptophan conversion to QA but also

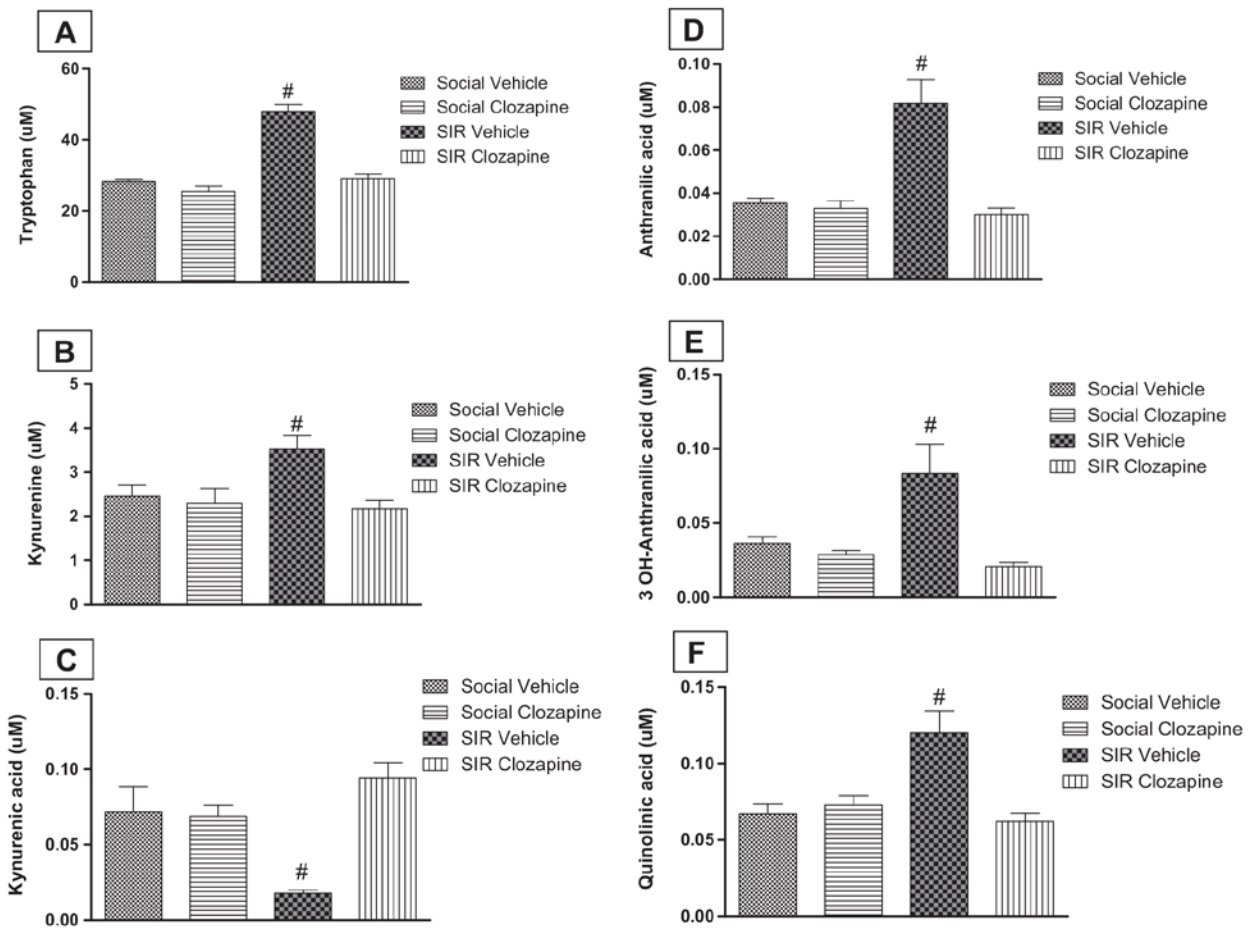


Fig. 2. The kynurenine pathway metabolites in the socially reared and the SIR rats in the treatment cohort ($n = 10/\text{group}$): (A) tryptophan; (B) kynurenine; (C) KYNA; (D) anthranilic acid; (E) 3-OHAA and (F) QA. $\#p < 0.01$ vs. SIR clozapine, vs. social vehicle, and vs. social clozapine (one-way ANOVA and Newman–Keuls post-hoc test). Refer to text for precise p values.

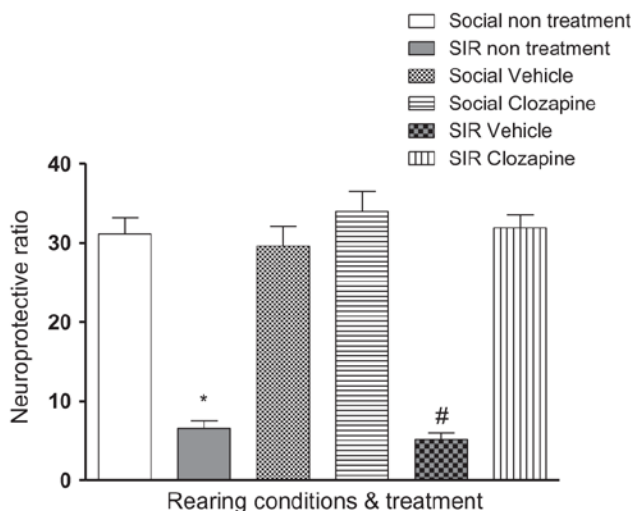


Fig. 3. Neuroprotective ratio, calculated with the KYNA and kynurenine concentrations for both the non-treatment cohort and the treatment cohort, socially reared and SIR rats ($n = 10/\text{group}$). $^*p < 0.0001$ vs. social non-treatment, $\#p < 0.0001$ vs. SIR clozapine, vs. social vehicle, and vs. social clozapine (one-way ANOVA and Newman–Keuls post-hoc test).

effectively diverts tryptophan metabolism away from 5-HT synthesis. This change (loss) in 5-HT may effectively drive the development of depressive behaviour, which also is a prominent feature of schizophrenia (Stone et al., 2003). The metabolites of the kynurenine pathway are thus important neuro-regulators that normally have beneficial effects, yet if altered by illness of other exogenous means can have deleterious effects on the functioning of the central nervous system.

Altered tryptophan metabolism has been suggested to link to psychosis via an altered neuroprotective-neurotoxic balance through disruption of the equilibrium between KYNA and QA (Barry et al., 2009). Following its synthesis from tryptophan (Fig. 1, left panel), kynurenine is metabolised either via the toxic quinolinic pathway, in which anthranilic acid, 3-OHAA, and finally QA are produced, or into the kynureninic pathway, in which neuroprotective KYNA is the final metabolite (Schwarcz et al., 1983; Myint et al., 2007a, 2007b). SIR induced a significant increase in plasma tryptophan (Figs. 1A and 2A), kynurenine (Figs. 1B and 2B), anthranilic acid (Figs. 1D and 2D), 3-OHAA (Figs. 1E and 2E) and QA (Figs. 1F and 2F), and a significant decrease in KYNA (Figs. 1C and 2C). Since tryptophan and its kynurenine metabolites are important regulators of immune function (Miller et al., 2008), these observations emphasise the epiphenomena of inflammation, and tryptophan flux to QA, as a consequence of SIR in rats. Indeed, patients with schizophrenia have been found to have elevated levels of tryptophan, 3-OHAA, kynurenine and QA in various brain regions (Torrey

et al., 1998; Miller et al., 2008). Moreover, unmedicated and medicated individuals with schizophrenia also have increased CSF and plasma levels of tryptophan (Issa et al., 1994; Ravikumar et al., 2000). Although elevated KYNA levels have been described in post-mortem brain tissue of medicated patients with schizophrenia (Schwarcz et al., 2001), Myint and colleagues have described a significant decrease in plasma KYNA concentrations in medication-naïve and medication-free schizophrenia patients (Myint et al., 2011), thus attesting to the validity of our findings in SIR rats. Schizophrenia also presents with pronounced up-regulation of TDO (Miller et al., 2006), supporting the view that schizophrenia presents with an increased production of tryptophan as well as increased turnover towards the synthesis of QA. Although as yet unsubstantiated in SIR animals, up-regulation of IDO in schizophrenia has been explained by an increase in pro-inflammatory cytokines, such as interferon- γ and tumour necrosis factor- α (TNF- α) (reviewed in Stone et al., 2003), which may have relevance in SIR animals. Further work in this regard is warranted.

Neuro-imaging studies have confirmed the presence of structural brain changes and degenerative pathology in patients with schizophrenia, eg. enlarged ventricles (Wright et al., 2000; Theberge et al., 2007), decreased dendritic spine density (Glantz and Lewis, 2000; Byne et al., 2006) and abnormalities in frontal cortical blood flow (Andreassen et al., 1996). In sufficient quantities, and if not adequately countered by endogenous NMDA receptor antagonists such as KYNA, or if inadequately tempered by endogenous inhibitory pathways such as γ -amino-butyric acid (GABA), QA-mediated activation of NMDA receptors, together with the increase in 3-OHAA (a free radical generator), will shift the QA vs. KYNA balance away from a favourable neuroprotective profile. This will contribute to oxidative stress and neuronal apoptosis that may underlie the above-mentioned degenerative pathology. A shift away from a favourable neuroprotective balance did indeed occur in SIR rats (see Fig. 3). Importantly, a significant decrease in the neuroprotective ratio is also evident in the plasma of medication-naïve and medication-free schizophrenia patients (Myint et al., 2011). Indeed, we have earlier observed a significant increase in lipid peroxidation in cortico-striatal brain regions in SIR rats, together with marked changes in schizophrenia-like behaviours (Möller et al., 2011), suggesting that increased oxidative stress-related damage and associated behavioural changes following SIR in rats can be directly linked to a disruption of the kynurenine pathway, as described here.

Predictive validity of an animal model, especially reversal of bio-behavioural phenomena by drugs that are effective in the human disorder, is generally regarded as the definitive criterion for validation of an animal model (Young et al., 2010). Since elevated levels of tryptophan, 3-OHAA, kynurenine and QA (Schwarcz et al., 2001; Miller et al., 2008; Torrey et al., 1998), as well as decreased KYNA levels (Myint et al., 2011), have been described in patients with schizophrenia, an effective treatment for schizophrenia (such as clozapine) should ideally reverse these changes. In agreement with this statement, reduced plasma KYNA levels in medication-naïve and medication-free schizophrenia patients are reversed by antipsychotic treatment (Myint et al., 2011). Sub-chronic treatment with clozapine significantly reversed SIR-induced increases in plasma tryptophan, kynurenine, anthranilic acid, 3-OHAA as well as QA, as well as reversed the decrease in KYNA (Fig. 2A–F). Clozapine treatment also reversed decreases in the neuroprotective ratio (Fig. 3). Interestingly, decreased KYNA levels have been found to potentiate the excitatory effects of clozapine on DA neurons in the ventral tegmental area (Schwielar and Erhardt, 2003; Schwielar et al., 2008), ultimately leading to increased DA activity in the frontal cortex and possibly explaining clozapine's efficacy in ameliorating the negative symptoms of schizophrenia.

Clozapine treatment has been found to significantly decrease tryptophan plasma levels in schizophrenia patients (Meltzer, 1989). To the best of our knowledge its effects on plasma levels of kynurenine, 3-OHAA, anthranilic acid or QA have not been studied in schizophrenia patients or, for that matter, in a translational animal model. This study is therefore the first to describe the effects of clozapine treatment on the kynurenine pathway in an animal model of relevance for schizophrenia. Our study argues that increased availability of neuroprotective KYNA in the periphery and in the brain following effective drug treatment may result in greater protection against the neurotoxic actions of 3-OHAA and QA. Clozapine may reverse SIR-induced changes in tryptophan metabolism through reduction in inflammatory cytokines (Sugino et al., 2009), suppression of microglial-dependent inflammatory responses (Hu et al., 2011) and/or re-establishing redox homeostasis (Magliaro and Saldanha, 2009; Möller et al., 2011). Circumstantial evidence for immunological nitric oxide (NO) synthase (iNOS) (Ganzinelli et al., 2010) and neuronal NOS (Bernstein et al., 2005) involvement in schizophrenia has been suggested. iNOS-dependent NO can inhibit IDO (Wang et al., 2010), thereby affecting the accumulation of kynurenine and its downstream metabolites. Interestingly, clozapine and other antipsychotics are known to variably target NOS (Hu et al., 1994; Nel and Harvey, 2003; Boussey et al., 2005; Zhang et al., 2010).

In conclusion, our results confirm that SIR in rats induces an imbalance in the kynurenine pathway with an increase in activity of the QA pathway and reduced activity of the KYNA pathway, culminating in a reduction in the neuroprotective balance. Furthermore, these changes are fully reversed by sub-chronic clozapine treatment, thus supporting the validity of these findings for schizophrenia. This study not only extends the validity of the SIR model for schizophrenia, but also reveals more of the role of the kynurenine pathway in schizophrenia and emphasizes its therapeutic potential as an avenue for novel antipsychotic drug development.

Sources of funding

This work has been funded by the South African Medical Research Council (BHH). The funder has no other role in this study.

Conflicts of interest

Robin Emsley has participated in speakers/advisory boards and received honoraria from AstraZeneca, Bristol-Myers Squibb, Janssen, Lilly, Lundbeck, Organon, Pfizer, Servier, Otsuka and Wyeth. He has received research funding from Janssen, Lundbeck and AstraZeneca. Brian Harvey has participated in speakers/advisory boards and received honoraria from Bristol-Myers Squibb, Organon, Pfizer and Servier, and has received research funding from Lundbeck.

Acknowledgements

The authors would like to thank Mr. Cor Bester, Me. Antoinette Fick and Mr. Petri Bronkhorst for their assistance in the breeding and welfare of the animals.

References

- Akyol, O., Herken, H., Uz, E., Fadillioglu, E., Unal, S., Sogut, S., Ozyurt, H., Savas, H.A., 2002. The indices of endogenous oxidative and antioxidative processes in plasma from schizophrenic patients. the possible role of oxidant/antioxidant imbalance. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 26, 995–1005.
- Allegri, G., Costa, C.V.L., Bertazzo, A., Biasiolo, M., Ragazzi, E., 2003. Enzyme activities of tryptophan metabolism along the kynurenine pathway in various species of animals. *Il Farmaco* 58, 829–836.

- Amirkhani, A., Heldin, E., Markides, K.E., Bergquist, J., 2002. Quantitation of tryptophan, kynurenine and kynurenic acid in human plasma by capillary liquid chromatography–electrospray ionization tandem mass spectrometry. *J. Chromatogr. B* 780, 381–387.
- Andreasen, N.C., O'Leary, D.S., Cizadlo, T., Arndt, S., Rezaei, K., Ponto, L.L., Watkins, G.L., Hichwa, R.D., 1996. Schizophrenia and cognitive dysmetria: a positron-emission tomography study of dysfunctional prefrontal-thalamic-cerebellar circuitry. *Proc. Natl. Acad. Sci. U. S. A.* 93, 9985–9990.
- Atkins, M., Burgess, A., Bottomly, C., Riccio, M., 1997. Chlorpromazine equivalents: a consensus of opinion for both clinical and research applications. *Psychiatr. Bull.* 21, 224–226.
- Ascher-Svanum, H., Nyhuis, A.W., Faries, D.E., Kinon, B.J., Baker, R.W., Shekhar, A., 2008. Clinical, functional, and economic ramifications of early nonresponse to antipsychotics in the naturalistic treatment of schizophrenia. *Schizophr. Bull.* 34, 1163–1171.
- Barry, S., Clarke, G., Scully, P., Dinan, T., 2009. Kynurenine pathway in psychosis: evidence of increased tryptophan degradation. *J. Psychopharmacol.* 23, 287–294.
- Bakshi, V.P., Swerdlow, N.R., Geyer, M.A., 1994. Clozapine antagonizes phencyclidine-induced deficits in sensorimotor gating of the startle response. *J. Pharmacol. Exp. Ther.* 271, 787–794.
- Berk, M., Copolov, D., Dean, O., et al., 2008. N-acetyl cysteine as a glutathione precursor for Schizophrenia – a double-blind, randomized, placebo-controlled trial. *Biol. Psychiatry* 64, 361–368.
- Bernstein, H.G., Bogerts, B., Keilhoff, G., 2005. The many faces of nitric oxide in schizophrenia. A review. *Schizophr. Res.* 78, 69–86.
- Betzen, C., White, R., Zehndner, C.M., Pietrowski, E., Bender, B., Luhmann, H.J., Kuhlmann, C.R.W., 2009. Oxidative stress upregulates the NMDA receptor on cerebrovascular endothelium. *Free Radic. Biol. Med.* 47, 1212–1220.
- Boussery, K., Lambrecht, S., Delaey, C., Van de Voorde, J., 2005. Clozapine directly relaxes bovine retinal arteries. *Curr. Eye Res.* 30, 139–146.
- Byne, W., Kiddard, S., Tatusov, A., Yiannoulos, G., Buchsbaum, M.S., Haroutunian, V., 2006. Schizophrenia-associated reduction of neuronal and oligodendrocyte numbers in the anterior principal thalamic nucleus. *Schizophr. Res.* 85, 245–253.
- Carlsson, A., Waters, N., Holm-Waters, S., Tedroff, J., Nilsson, M., Carlsson, M.L., 2001. Interactions between monoamines, glutamate, and GABA in schizophrenia: new evidence. *Annu. Rev. Pharmacol. Toxicol.* 41, 237.
- de Jong, W.H.A., Smit, R., Bakker, S.J.L., de Vries, E.G.E., Kema, I.P., 2009. Plasma tryptophan, kynurenine and 3-hydroxykynurenine measurement using automated on-line solid-phase extraction HPLC – tandem mass spectrometry. *J. Chromatogr. B* 877, 603–609.
- Deutsch, S.I., Rosse, R.B., Schwartz, B.L., Mastropaulo, J., 2001. A revised excitotoxic hypothesis of schizophrenia: therapeutic implications. *Clin. Neuropharmacol.* 24, 43–49.
- Evins, A.E., Amico, E., Posever, T.A., Tokar, R., Goff, D.C., 2002. D-cycloserine added to risperidone in patients with primary negative symptoms of schizophrenia. *Schizophr. Res.* 56, 19–23.
- Fone, K.C.F., Porkess, M.V., 2008. Behavioural and neurochemical effects of post-weaning social isolation in rodents – relevance to developmental neuropsychiatric disorders. *Neurosci. Biobehav. Rev.* 32, 1087–1102.
- Ganzinelli, S., Borda, E., Sterin-Borda, L., 2010. Autoantibodies from schizophrenia patients induce cerebral cox-1/iNOS mRNA expression with NO/PGE2/MMP-3 production. *Int. J. Neuropsychopharmacol.* 13, 293–303.
- Glantz, L.A., Lewis, D.A., 2000. Decreased dendritic spine density on prefrontal cortical pyramidal neurons in schizophrenia. *Arch. Gen. Psychiatry* 57, 65–73.
- Goff, D.C., Tsai, G., Levitt, J., Amico, E., Manoach, D., Schoenfeld, D.A., Hayden, D.L., McCauley, R., Coyle, J.T., 1999. A placebo-controlled trial of D-cycloserine added to conventional neuroleptics in patients with schizophrenia. *Arch. Gen. Psychiatry* 56, 21–27.
- Guillemin, G.J., Cullen, K.M., Lim, C.K., Smythe, G.A., Garner, B., Kapoor, V., Takikawa, O., Brew, B.J., 2007. Characterization of the kynurenine pathway in human neurons. *J. Neurosci.* 27, 12884–12892.
- Heyes, M.P., Chen, C.Y., Major, E.O., Saito, K., 1997. Different kynurenine pathway enzymes limit quinolinic acid formation by various human cell types. *Biochem. J.* 326 (Pt. 2), 351–356.
- Hu, J., Lee, J.H., el-Fakahany, E.E., 1994. Inhibition of neuronal nitric oxide synthase by antipsychotic drugs. *Psychopharmacology (Berl)* 114, 161–166.
- Hu, X., Zhou, H., Zhang, D., et al., 2011. Clozapine protects dopaminergic neurons from inflammation-induced damage by inhibiting microglial overactivation. *J. Neuroimmune Pharmacol.* doi:10.1007/s11481-011-9309-0.
- Issa, F., Gerhardt, G.A., Bartko, J.J., Suddath, R.L., Lynch, M., Gamache, P.H., Freedman, R., Wyatt, R.J., Kirch, D.G., 1994. A multidimensional approach to analysis of cerebrospinal fluid biogenic amines in schizophrenia: I. comparisons with healthy control subjects and neuroleptic-treated/unmedicated pairs analyses. *Psychiatry Res.* 52, 237–249.
- Javitt, D.C., 2010. Glutamate theories of schizophrenia. *Isr. J. Psychiatry Relat. Sci.* 47, 4–16.
- Kantrowitz, J.T., Javitt, D.C., 2010. Thinking glutamatergically: changing concepts of schizophrenia based upon changing neurochemical models. *Clin. Schizophr. Relat. Psychoses* 4, 189–200.
- Kapur, S., Arenovich, T., Agid, O., Zipursky, R., Lindborg, S., Jones, B., 2005. Evidence for onset of antipsychotic effects within the first 24 hours of treatment. *Am. J. Psychiatry* 162, 939–946.
- Lavoie, S., Murray, M.M., Deppen, P., et al., 2008. Glutathione precursor, N-acetyl-cysteine, improves mismatch negativity in schizophrenia patients. *Neuropsychopharmacology* 33, 2187–2199.
- Lin, A., Kenis, G., Bignotti, S., Tura, G., De Jong, R., Bosmans, E., Pioli, R., Altamura, C., Scharpé, S., Maes, M., 1998. The inflammatory response system in treatment-resistant schizophrenia: increased serum interleukin-6. *Schizophr. Res.* 32, 9–15.
- Maes, M., Meltzer, H.Y., Bosmans, E., 1994. Immune-inflammatory markers in schizophrenia: comparison to normal controls and effects of clozapine. *Acta Psychiatrica. Scand.* 89, 346–351.
- Magliaro, B.C., Saldanha, C.J., 2009. Clozapine protects PC-12 cells from death due to oxidative stress induced by hydrogen peroxide via a cell-type specific mechanism involving inhibition of extracellular signal-regulated kinase phosphorylation. *Brain Res.* 1283, 14–24.
- Meltzer, H.Y., 1989. Clinical studies on the mechanism of action of clozapine: the dopamine-serotonin hypothesis of schizophrenia. *Psychopharmacology* 99 (Suppl.), S18–S27.
- Meyer, U., Feldon, J., 2010. Epidemiology-driven neurodevelopmental animal models of schizophrenia. *Prog. Neurobiol.* 90, 285–326.
- Miller, C.L., Llenos, I.C., Dulay, J.R., Weis, S., 2006. Upregulation of the initiating step of the kynurenine pathway in postmortem anterior cingulate cortex from individuals with schizophrenia and bipolar disorder. *Brain Res.* 1073–1074, 25–37.
- Miller, C.L., Llenos, I.C., Cwik, M., Walkup, J., Weis, S., 2008. Alterations in kynurenine precursor and product levels in schizophrenia and bipolar disorder. *Neurochem. Int.* 52, 1297–1303.
- Möller, M., Du Preez, J.L., Emsley, R., Harvey, B.H., 2011. Isolation rearing-induced deficits in sensorimotor gating and social interaction in rats are related to cortico-striatal oxidative stress, and reversed by sub-chronic clozapine administration. *Eur. Neuropsychopharmacol.* 21, 471–483.
- Möller, M., Du Preez, J. L., & Harvey, B. H., submitted for publication. Development and validation of a single analytical method for the determination of tryptophan, and its kynurenine metabolites in rat plasma. *J. Chromatogr. B*.
- Myint, A., Kim, Y.K., Verkerk, R., Scharpé, S., Steinbusch, H., Leonard, B., 2007a. Kynurenine pathway in major depression: evidence of impaired neuroprotection. *J. Affect. Disord.* 98, 143–151.
- Myint, A.M., Kim, Y.K., Verkerk, R., Park, S.H., Scharpé, S., Steinbusch, H.W., Leonard, B.E., 2007b. Tryptophan breakdown pathway in bipolar mania. *J. Affect. Disord.* 102, 65–72.
- Myint, A.M., Schwarz, M.J., Verkerk, R., Mueller, H.H., Zach, J., Scharpé, S., Steinbusch, H.W.M., Leonard, B.E., Kim, Y.K., 2011. Reversal of imbalance between kynurenic acid and 3-hydroxykynurenine by antipsychotics in medication-naïve and medication-free schizophrenic patients. *Brain Behav. Immun.* 25, 1576–1581.
- Nel, A., Harvey, B.H., 2003. Haloperidol-induced dyskinesia is associated with striatal NO synthase suppression: reversal with olanzapine. *Behav. Pharmacol.* 14, 251–255.
- Ng, F., Berk, M., Dean, O., Bush, A.I., 2008. Oxidative stress in psychiatric disorders: evidence base and therapeutic implications. *Int. J. Neuropsychopharmacol.* 11, 851.
- Potvin, S., Stip, E., Sepehry, A.A., Gendron, A., Bah, R., Kouassi, E., 2008. Inflammatory cytokine alterations in schizophrenia: a systematic quantitative review. *Biol. Psychiatry* 63, 801–808.
- Ravikumar, A., Deepadevi, K.V., Arun, P., Manojkumar, V., Kurup, P.A., 2000. Tryptophan and tyrosine catabolic pattern in neuropsychiatric disorders. *Neurol. India* 48, 231–238.
- Schwarcz, R., Whetsell Jr., W.O., Mangano, R.M., 1983. Quinolinic acid: an endogenous metabolite that produces axon-sparing lesions in rat brain. *Science* 219, 316–318.
- Schwarcz, R., Rassoulpour, A., Wu, H.Q., Medoff, D., Tamminga, C.A., Roberts, R.C., 2001. Increased cortical kynurenate content in schizophrenia. *Biol. Psychiatry* 50, 521–530.
- Schwarcz, R., 2004. The kynurenine pathway of tryptophan degradation as a drug target. *Curr. Opin. Pharmacol.* 4, 12–17.
- Schwieler, L., Erhardt, S., 2003. Inhibitory action of clozapine on rat ventral tegmental area dopamine neurons following increased levels of endogenous kynurenic acid. *Neuropsychopharmacology* 28, 1770–1777.
- Schwieler, L., Linderholm, K.R., Nilsson-Todd, L.K., Erhardt, S., Engberg, G., 2008. Clozapine interacts with the glycine site of the NMDA receptor: electrophysiological studies of dopamine neurons in the rat ventral tegmental area. *Life Sci.* 83, 170–175.
- Stone, T.W., 2001. Kynurenes in the CNS: from endogenous obscurity to therapeutic importance. *Prog. Neurobiol.* 64, 185–218.
- Stone, T.W., Darlington, L.G., 2002. Endogenous kynurenes as targets for drug discovery and development. *Nat. Rev. Drug Discov.* 1, 609.
- Stone, T.W., Mackay, G.M., Forrest, C.M., Clark, C.J., Darlington, L.G., 2003. Tryptophan metabolites and brain disorders. *Clin. Chem. Lab. Med.* 41, 852–859.
- Sugino, H., Futamura, T., Mitsumoto, Y., Maeda, K., Marunaka, Y., 2009. Atypical antipsychotics suppress production of proinflammatory cytokines and up-regulate interleukin-10 in lipopolysaccharide-treated mice. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 33, 303–307.
- Theberge, J., Williamson, K.E., Aoyama, N., et al., 2007. Longitudinal grey-matter and glutamatergic losses in first-episode schizophrenia. *Brit. J. Psychiatry* 191, 325–334.
- Torrey, E.F., Yolken, R.H., Zito, M., Heyes, M., 1998. Increased CSF and brain quinolinic acid in schizophrenia and bipolar disorder. *Schizophr. Res.* 29, 91–92.

- Toua, C., Brand, L., Möller, M., Emsley, R.A., Harvey, B.H., 2010. The effects of sub-chronic clozapine and haloperidol administration on isolation rearing induced changes in frontal cortical N-methyl-D-aspartate and D1 receptor binding in rats. *Neurosci* 165, 492–499.
- Wang, Y., Lawson, M.A., Kelley, K.W., Dantzer, R., 2010. Primary murine microglia are resistant to nitric oxide inhibition of indoleamine 2,3-dioxygenase. *Brain Behav. Immun.* 24, 1249–1253.
- Weiss, I.C., Feldon, J., 2001. Environmental animal models for sensorimotor gating deficiencies in schizophrenia: a review. *Psychopharmacology* 156, 305–326.
- Willinger, U., Heiden, A.M., Meszaros, K., Formann, A.K., Aschauer, H.N., 2001. Neurodevelopmental schizophrenia: obstetric complications, birth weight, premorbid social withdrawal and learning disabilities. *Neuropsychobiology* 43, 163–169.
- Wright, I.C., Rabe-Hesketh, S., Woodruff, P.W., David, A.S., Murray, R.M., Bullmore, E.T., 2000. Meta-analysis of regional brain volumes in schizophrenia. *Am. J. Psychiatry* 157, 16–25.
- Young, J.W., Zhou, X., Geyer, M.A., 2010. Animal models of schizophrenia. *Curr. Top. Behav. Neurosci.* 4, 391–433.
- Zhang, W., Pouzet, B., Jongen-Rêlo, A.L., Weiner, I., Feldon, J., 1999. Disruption of prepulse inhibition following N-methyl-D-aspartate infusion into the ventral hippocampus is antagonized by clozapine but not by haloperidol: a possible model for the screening of atypical antipsychotics. *Neuropharm. Neurotoxicol.* 10, 2533–2538.
- Zhang, X.R., Zhang, Z.J., Jenkins, T.A., Cheng, W.R., Reynolds, G.P., 2010. The effect of chronic antipsychotic drug administration on nitric oxide synthase activity and gene expression in rat penile tissues. *Eur. Neuropsychopharmacol.* 20, 211–217.