Chapter 5 Manuscript C

Article published in *Brain Behavior and Immunity,* entitled:

"Social isolation rearing induces immunological, neurochemical, mitochondrial and behavioural deficits in rats, and is reversed by clozapine or N-acetyl cysteine"

Introduction

This chapter presents the full-length manuscript submitted for publication in *Brain Behaviour and Immunity*, 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/S0889159112005417

Authors' contributions

- *M. Möller* designed the study along with *BH Harvey*, undertook all the behavioral, analytical and statistical analyses, 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 the analytical laboratory analysis.
- *F Viljoen* supervised the monoamine laboratory analysis.
- *M Berk* advised on the study design, the use of N-acetyl cysteine and proof read the final manuscript.
- *R Emsley* advised on the study design and proof read the final manuscript.
- *BH Harvey* supervised over 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.

ARTICLE IN PRESS

Brain, Behavior, and Immunity xxx (2013) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Brain, Behavior, and Immunity

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

Social isolation rearing induces mitochondrial, immunological, neurochemical and behavioural deficits in rats, and is reversed by clozapine or N-acetyl cysteine

Marisa Möller [a], Jan L. Du Preez [b], Francois P. Viljoen [a], Michael Berk [c,d], Robin Emsley [e], Brian H. Harvey [a,b,*]

[a] Division of Pharmacology, School of Pharmacy, North West University, Potchefstroom, South Africa
[b] Center of Excellence for Pharmaceutical Research, School of Pharmacy, North West University, Potchefstroom, South Africa
[c] School of Medicine, Deakin University, Geelong, Australia
[d] The Florey Institute of Neuroscience and Mental Health, Orygen Research Centre and the Department of Psychiatry, University of Melbourne, Parkville, Australia
[e] Department of Psychiatry, University of Stellenbosch, Cape Town, South Africa

ARTICLE INFO

Article history:
Received 27 September 2012
Received in revised form 4 December 2012
Accepted 14 December 2012
Available online xxxx

Keywords:
Schizophrenia
Social isolation rearing
Mitochondrial dysfunction
Tryptophan–kynurenine pathway
Adenosine triphosphate (ATP)
Clozapine
N-acetyl cysteine
Dopamine
Immune-inflammatory
Animal model

ABSTRACT

Apart from altered dopamine (DA) function, schizophrenia displays mitochondrial and immune-inflammatory abnormalities, evidenced by oxidative stress, altered kynurenine metabolism and cytokine release. N-acetyl cysteine (NAC), an antioxidant and glutamate modulator, is effective in the adjunctive treatment of schizophrenia. Social isolation rearing (SIR) in rats is a valid neurodevelopmental animal model of schizophrenia. This study evaluated whether SIR-induced behavioural deficits may be explained by altered plasma pro- and anti-inflammatory cytokines, kynurenine metabolism, and cortico-striatal DA and mitochondrial function (via adenosine triphosphate (ATP) release), and if clozapine or NAC (alone and in combination) reverses these changes. SIR induced pronounced deficits in social interactive behaviours, object recognition memory, and prepulse inhibition (PPI), while simultaneously increasing striatal but reducing frontal cortical accumulation of ATP as well as DA. SIR increased pro- vs. anti-inflammatory cytokine balance and altered kynurenine metabolism with a decrease in neuroprotective ratio. Clozapine (5 mg/kg/day × 14 days) as well as clozapine + NAC (5 mg/kg/day and 150 mg/kg/day × 14 days) reversed these changes, with NAC (150 mg/kg/day) alone significantly but partially effective in some parameters. Clozapine + NAC was more effective than clozapine alone in reversing SIR-induced PPI, mitochondrial, immune and DA changes. In conclusion, SIR induces mitochondrial and immune-inflammatory changes that underlie cortico-striatal DA perturbations and subsequent behavioural deficits, and responds to treatment with clozapine or NAC, with an additive effect following combination treatment. The data provides insight into the mechanisms that might underlie the utility of NAC as an adjunctive treatment in schizophrenia.

1. Introduction

Schizophrenia is characterised by positive, negative and cognitive symptoms (Schultz et al., 2007), with evidence implicating genetic predisposition, neurotransmitter abnormalities, pre-, peri- and post-natal viral infections, stress, substance abuse, vitamin D deficiency, obstetric complications and altered immune function in its pathogenesis (O'Brien et al., 2008; McGrath et al., 2010; Matheson et al., 2011). The dopamine (DA) hypothesis suggests that schizophrenia is causally associated with elevated striatal (nucleus accumbens) but reduced frontal cortical DA levels responsible for positive and negative manifestations, respectively (Harvey et al., 1999; Holcomb et al., 2004). Current drug treatments, which primarily act at DA $D_{2/3}$ receptors, are generally effective against positive symptoms, while negative and cognitive symptoms remain relatively refractory (Howes et al., 2012). Identifying new biological targets and developing treatments targeting alternative systems is therefore paramount.

Schizophrenia is associated with mitochondrial dysfunction (Ben-Shachar, 2002; Karry et al., 2004) and oxidative stress (Ng et al., 2008). Indeed, altered energy generation is perhaps the oldest biomarker in the disorder (Looney and Childs, 1934), while altered frontal lobe mitochondrial adenosine triphosphate (ATP) has been correlated with negative symptoms and neuropsychological deficits in schizophrenia (Yacubian et al., 2002). Mitochondria are a major source of reactive oxygen species (ROS), while mitochondrial diseases are associated with secondary neurotransmitter disturbances (Garcia-Cazorla et al., 2008). This is of particular relevance in schizophrenia where disturbances in DA are evident (Ben-Shachar, 2002). Moreover, mitochondrial dysfunction is linked to cognitive and memory deficits, evident in schizophrenia

* Corresponding author at: Center of Excellence for Pharmaceutical Research, School of Pharmacy, North West University, Potchefstroom, South Africa. Tel.: +27 018 299 2238; fax: +27 018 299 2225.
E-mail address: Brian.Harvey@nwu.ac.za (B.H. Harvey).

0889-1591/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbi.2012.12.011

Please cite this article in press as: Möller, M., et al. Social isolation rearing induces mitochondrial, immunological, neurochemical and behavioural deficits in rats, and is reversed by clozapine or N-acetyl cysteine. Brain Behav. Immun. (2013), http://dx.doi.org/10.1016/j.bbi.2012.12.011

(Scaglia, 2010). Importantly elevated ATP is also a trigger for the release of pro-inflammatory cytokines (Cruz et al., 2007), which in turn modulates kynurenine metabolism (see below). Thus schizophrenia presents with a pro-inflammatory state (Leonard et al., 2012), with altered blood/cerebrospinal fluid levels of interleukin-2 (IL-2), IL-6, IL-1, IL-10 and tumour necrosis factor (TNF)-α (Drzyzga et al., 2006). Mitochondrial ATP also modulates glial cytokine release, while up-regulation in DA receptors play an important role in this response (Choi et al., 2007). With DA release closely regulated by glutamate (Schwartz et al., 2012), any glutamate-redox changes will alter cortico-striatal DA function to ultimately affect behaviour.

The kynurenine pathway metabolises tryptophan via indoleamine 2,3 dioxygenase (IDO) to either kynurenic acid (KYNA), a glycine site NMDA receptor antagonist with neuroprotective properties (Stone and Darlington, 2002), or quinolinic acid (QA), an NMDA receptor agonist with neurodegenerative properties (Myint et al., 2007). Pro- (e.g. interferon-γ (INF-γ), TNF-α) and anti-inflammatory (e.g. IL-4) cytokines, respectively, activate or inhibit IDO (Myint et al., 2007). This balance determines the neuroprotective-neurodegenerative ratio that relates to the eventual downstream levels of QA following a shift in KYNA and kynurenine synthesis (Myint et al., 2007, 2011), and may explain enlarged ventricles (Wright et al., 2000) and decreased dendritic spine density (Glantz and Lewis, 2000) evident in schizophrenia (Wright et al., 2000). Schizophrenia presents with altered kynurenine metabolism (Miller et al., 2004, 2008), increased QA and decreased neuroprotective ratio (Torrey et al., 1998; Myint et al., 2011), with subsequent NMDA receptor activation releasing ROS and depleting energy stores resulting in further glutamate release and cell damage (Betzen et al., 2009). Glutamate dysfunction will also drive changes in DA release culminating in positive and negative symptoms (Schwartz et al., 2012).

It is clear that oxidative stress can be a consequence of mitochondrial dysfunction, brain DA metabolism, immune dysregulation and/or altered tryptophan metabolism. Consequently, targeting redox related mechanisms in schizophrenia represents an important therapeutic target (Ng et al., 2008; Cabungcal et al., 2012). The glutathione (GSH) precursor and antioxidant, N-acetyl cysteine (NAC) modulates glutamate activity (Bauzo et al., 2012), decreases ROS production (Kerksick and Willoughby, 2005) and prevents striatal oxidative stress in vivo (Harvey et al., 2008). Furthermore, remediation of models of mitochondrial dysfunction with NAC improves cognitive function (Sandhir et al., 2012). NAC thus presents with significant theoretical rationale for clinical application in disorders characterised by glutamate dysregulation, mitochondrial function, inflammation and oxidative stress. Although clinical studies have described its potential as an adjunctive treatment in schizophrenia (Berk et al., 2008), this response has never been validated in a neurodevelopmental animal model of schizophrenia, especially its ability to correct behavioural and biochemical changes and to bolster the response to an antipsychotic agent. In particular, knowing how these biological pathways are linked to neurobehavioural phenotypes might shed some light onto the dominant mechanisms of action of NAC, and suggest further therapeutic targets.

Post-weaning social isolation rearing (SIR) in rodents presents with robust face, construct and predictive validity for schizophrenia (Fone and Porkess, 2008). SIR increases frontal cortical glutamate N-methyl-D-aspartate (NMDA) receptor binding (Toua et al., 2010) and induces cortico-striatal oxidative stress (Möller et al., 2011), thus supportive of clinical evidence and of the glutamate hypo-function hypothesis (Coyle and Tsai, 2004). We hypothesise that the behavioural abnormalities associated with schizophrenia and SIR are founded on altered cortico-striatal mitochondrial dysfunction, followed by altered plasma pro-and anti-inflammatory cytokines and tryptophan metabolism, with subsequent changes in regional brain DA. This series of events is investigated in this study. Furthermore, we propose that sub-chronic clozapine or NAC treatment will reverse these changes, and that NAC will bolster the response to clozapine.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (160–190 g; Animal Research Centre, North West University) were randomly allocated to groups, each comprising 10 rats/group. At weaning (post-natal day 21) the animals were randomised to SIR (1 animal/cage) or social rearing (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), 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, with ethical approval for the study obtained from the North West University ethical committee: (NWU-0035-08-S5). A trained animal technologist routinely monitored the animals throughout the study for evidence of pain or distress following injections of clozapine or NAC. No untoward effects were observed using the drug preparation and treatment protocol applied in this study (see below). The number of animals used was the minimum required to obtain scientifically valid data.

2.2. Drugs and drug treatment protocol

Clozapine (Pharmaplan, Johannesburg, South Africa), dissolved in 1 M glacial acetic acid and buffered with sodium hydroxide (NaOH) (pH = 6.0), was administered as a single daily intraperitoneally (i.p.) dose of 5 mg/kg/0.5 ml/day. This dose was based on previous sub-chronic treatment studies performed in our laboratory (Toua et al., 2010; Möller et al., 2011), but also after due consultation of other studies (Bakshi et al., 1994; Zhang et al., 1999). Early antipsychotic response in schizophrenia, for example 2 weeks, is reported to be an accurate predictor of later response (Kapur et al., 2005), thus questioning the need for prolonged treatment to confirm efficacy. For this reason, but also to exclude the possible impact of injection stress evoked over extended treatment periods, the study was conducted over a 14 day treatment period. NAC (Sigma–Aldrich, Johannesburg, South Africa), dissolved in saline and buffered with 1 M glacial acetic acid and NaOH (pH = 6.0), was administered i.p at a dose of 150 mg/kg/0.5 ml/day. This dose (used in the NAC alone and CLZ + NAC studies) was determined from data described in an accompanying dose response study (i.e. vehicle, 50, 150 and 250 mg/kg NAC, see Supplement 1, Figs. 1–7). Importantly, a lower yet effective dose of NAC was selected to allow for meaningful additive effects to be observed in the combined CLZ + NAC study (see Supplement 1, Figs. 1, 2 and 4–7). This dose was also corroborated with earlier studies in rodents with similar behavioural outcomes, viz. reversal of hyper-locomotion (Fukami et al., 2004), restoration of PPI deficits (Chen et al., 2010), effects on inflammatory cytokines (Khan et al., 2004) and on altered redox status in rat brain (Harvey et al., 2008). Control groups received an equivalent volume of vehicle i.p., comprising saline and 1 M glacial acetic acid and buffered with sodium hydroxide (NaOH) (pH = 6.0). Clozapine, NAC and vehicle were administered (06–08 h 00) in the last 14 days of social/SIR rearing.

Please cite this article in press as: Möller, M., et al. Social isolation rearing induces mitochondrial, immunological, neurochemical and behavioural deficits in rats, and is reversed by clozapine or N-acetyl cysteine. Brain Behav. Immun. (2013), http://dx.doi.org/10.1016/j.bbi.2012.12.011

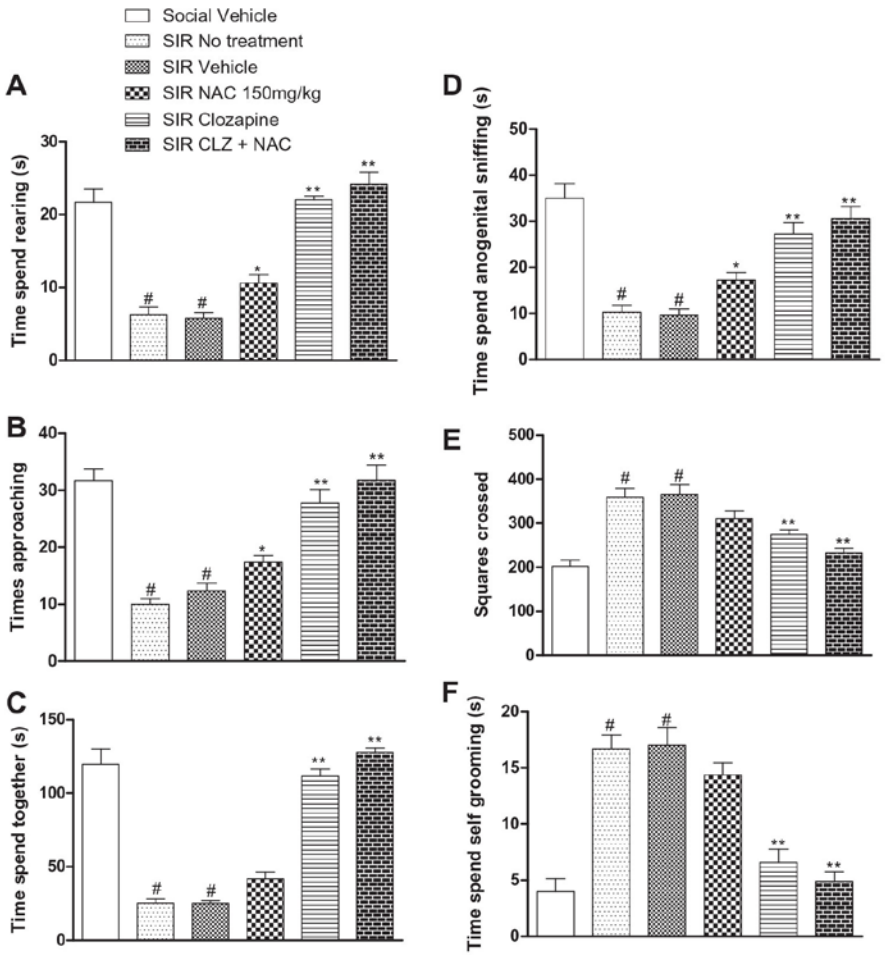


Fig. 1. Social interactive and self-directed behaviours in SIR rats following the various drug treatments, as indicated (n = 10/group). A socially reared vehicle treatment control group is included for statistical comparison (data from Supplement 1): (A) rearing; (B) approaching; (C) time together; (D) anogenital sniffing; (E) squares crossed; (F) self-grooming. Treatment had a significant main effect on (A) rearing (F (5, 54) = 16.60, p < 0.0001); (B) approaching (F (5, 54) = 7.81, p < 0.0001); (C) time together (F (5, 54) = 21.98, p < 0.0001); (D) anogenital sniffing (F (5, 54) = 6.308, p < 0.0001); (E) squares crossed (F (5, 54) = 4.6, p < 0.0001) and (F) self-grooming (F (5, 54) = 10.02, p < 0.0001) (3-way ANOVA). Rearing also indicated a significant main effect on (A) rearing (F(5, 54) = 18.9, p < 0.0001); (B) approaching (F(5, 54) = 15.00, p < 0.0001); (C) time together (F (5, 54) = 25.70, p < 0.0001); (D) anogenital sniffing (F (5, 54) = 17.50, p < 0.0001); (E) squares crossed (F (5, 54) = 18.95, p < 0.0001) and (F) self-grooming (F (5, 54) = 16.78, p < 0.0001) (3-way ANOVA). Significant cross-group interactions were observed with regards to (A) rearing (F (5, 54) = 84.64, p < 0.0001); (B) approaching (F (5, 54) = 28.10, p < 0.0001); (C) time together (F (5, 54) = 84.64, p < 0.0001); (D) anogenital sniffing (F (5, 54) = 84.64, p < 0.0001); (E) squares crossed (F (5, 54) = 16.65, p < 0.0001); (F) self-grooming (F (5, 54) = 25,12 p < 0.0001) (3-way ANOVA). #p < 0.0001 vs. social vehicle treatment, *p < 0.05 vs. SIR no treatment and vehicle, **p < 0.0001 vs. SIR no treatment and vehicle (Bonferroni post-hoc test). Refer to text for precise p values.

All animals were sacrificed by decapitation without any prior use of an anaesthetic agent.

2.3. Experimental design

In order to prevent any untoward effect of behavioural testing on the neurochemistry parameters studied, separate groups of animals were used as outlined below.

2.3.1. Behavioural study

The animals were randomly separated at weaning (21 days post-natal) into 6 groups: 1 socially reared group (3–4 rats in a cage) and 5 SIR groups (1 rat in a cage). One group of SIR animals (n = 10) and the one group of socially reared animals (n = 10), received vehicle treatment, while the remaining 4 groups (n = 10) received either of the following treatments: no treatment, NAC 150 mg/kg, clozapine 5 mg/kg or a combination of clozapine 5 mg/kg and NAC 150 mg/kg (CLZ + NAC), respectively. After 8 weeks of SIR/group rearing, social interaction, novel object recognition and sensorimotor gating were performed on days 12, 13 and 14, respectively, of the drug treatment period (18–20 h 00). All behavioural assessments were undertaken at least 12 h post-drug administration.

2.3.2. Immune-neurochemistry study

A separate 6 groups of animals (n = 10/group), randomly assigned to the same groupings as described above, followed the above protocol except that at 8 weeks the animals were sacrificed, with trunk blood collected for tryptophan metabolism and cytokine analysis. Brains (frontal cortex and striatum) were dissected for analysis of mitochondrial function and DA levels.

2.4. Body weight

Body weight was determined at PND 21 and also on days 1 and 14 of drug treatment to confirm equal development across all the treatment groups over the study period.

Please cite this article in press as: Möller, M., et al. Social isolation rearing induces mitochondrial, immunological, neurochemical and behavioural deficits in rats, and is reversed by clozapine or N-acetyl cysteine. Brain Behav. Immun. (2013), http://dx.doi.org/10.1016/j.bbi.2012.12.011

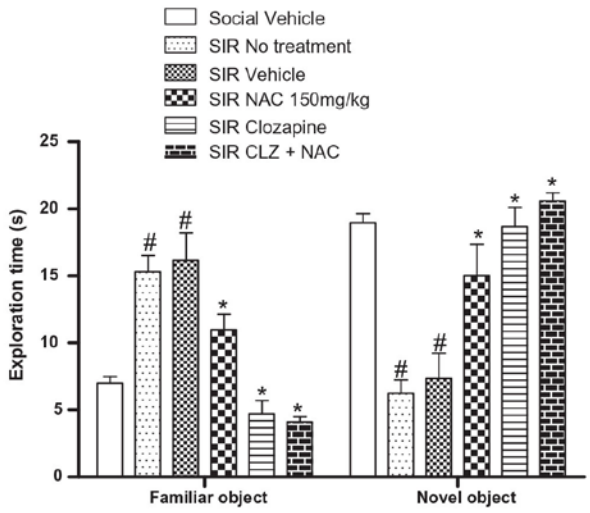


Fig. 2. Retention trial of the object recognition test in SIR rats, following the various drug treatments, as indicated ($n = 10$/group). A socially reared vehicle treatment control group is included for statistical comparison (data from Supplement 1). A significant main effect of treatment (F (5, 75) = 2.54, $p = 0.008$) and rearing (F (5, 75) = 13.36, $p < 0.001$) were observed with respect to visual recognition memory (3-way ANOVA). Significant cross-group interactions were observed with respect to visual recognition memory (F (5, 75) = 143.3, $p < 0.0001$) (3-way ANOVA). $^{\#}p < 0.0001$ vs. social vehicle treatment, $^{*}p < 0.0001$ vs. SIR no treatment and vehicle (Bonferroni post-hoc test).

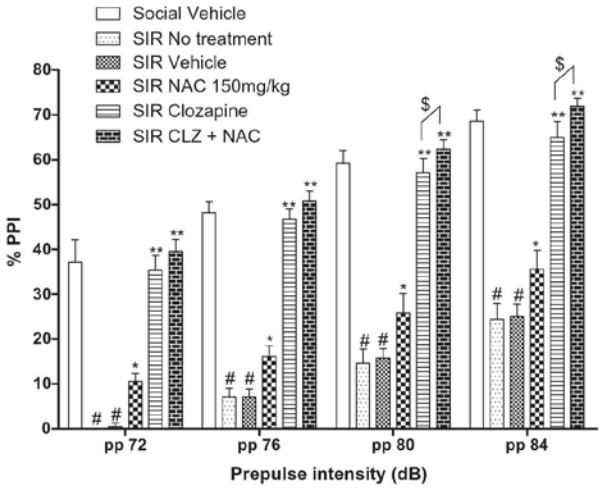


Fig. 3. Sensorimotor gating at various prepulse intensities in SIR rats, following the various drug treatments, as indicated ($n = 10$/group). A socially reared vehicle treatment control group is included for statistical comparison (data from Supplement 1). Data presented as percent prepulse inhibition (%PPI). A significant main effect of treatment (F (5, 15) = 10.08, $p < 0.0001$) and rearing (F (5, 15) = 75.8, $p < 0.0001$) were observed with respect to %PPI (3-way ANOVA). Significant cross-group interactions were observed in %PPI (F (5, 15) = 2.61, $p < 0.0001$) (3-way ANOVA). $^{\#}p < 0.0001$ vs. social vehicle treatment, $^{*}p < 0.001$ vs. SIR no treatment and vehicle, $^{**}p < 0.0001$ vs. SIR no treatment and vehicle, $^{\$}p < 0.05$ vs. SIR clozapine, at 72 dB, 76 dB, 80 dB and 86 dB, respectively (Bonferroni post-hoc test). Refer to text for precise p values.

2.5. Behavioural tests

To assure blindness throughout the behavioural testing, all animals used for the behavioural tests were labelled with permanent ink markings on the tail, assigning each group a different letter and colour. For the social interaction test and the object recognition test, behaviour was videotaped for later scoring by two trained observers, blind to treatment and rearing conditions. The mean of the two scores for each parameter were calculated and subsequently used.

2.5.1. Social interaction test

The social interaction test attempts to measure deficits in social and self-directed behaviour, as evident in schizophrenia, and was performed as previously described (Möller et al., 2011). Briefly, two rats were placed in the centre of an open field arena, illuminated with red light (40 W), and allowed 30 s for adaptation. Thereafter, time spent in active social interactive behaviours, viz. rearing, anogenital sniffing, approaching and staying with the partner, as well as self-directed behaviour, including squares crossed and self-grooming (Gonzalez et al., 1996; Möller et al., 2011), were scored for 10 min. Both members of a test-pair had received the same treatment and the same prior experience. Testing was performed between 18 h 00 and 20 h 00, with video scoring based on averaged scores by two blind independent observers who remained outside the testing room.

2.5.2. Object recognition test (ORT)

Visual recognition memory is impaired in schizophrenia (Calkins et al., 2005). The novel object recognition test (ORT) assesses declarative recognition memory in rodents and is ethologically relevant as it relies on the animal's natural tendency to explore novel environments/objects (Ennaceur and Delacour, 1988). The test was performed as described previously (Grayson et al., 2007; McLean et al., 2010). Briefly, the rats were exposed to a 5-min habituation session in a Plexiglas ORT box (70 × 70 × 40 cm), followed by the experimental trials where each rat was exposed to two identical objects (right and left object) for a period of 5 min (acquisition trial). The rats were then returned to their home cage for a 1.5 h inter-trial interval. The entire box was cleaned, both objects removed and one replaced with an identical familiar copy and one with a novel unfamiliar object. Following the 1.5 h inter-trial interval, rats were returned to explore the familiar and novel object in the test box for a 5-min retention trial. All experiments were filmed and recorded for subsequent behavioural analysis. Object exploration by the rats was defined as sniffing, licking or touching the objects with forepaws (Grayson et al., 2007). The exploration time (s) of each object in each trial was recorded manually using two stopwatches. The following factors were calculated: the total exploration time of both objects in the acquisition trial (left object and right object, respectively) and the total exploration time of both objects in the retention trial (familiar and novel object, respectively).

2.5.3. Prepulse inhibition test

Prepulse inhibition (PPI) is used to determine deficits in sensorimotor gating, a well-known abnormality in schizophrenia (Geyer et al., 1993; Möller et al., 2011). The test used two ventilated sound-attenuated startle chambers (SRLAB, San Diego Instruments, San Diego, USA). 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. The startle session started with a 5-min acclimatisation period plus a 68-dB background noise level that continued throughout the test session. Briefly, 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 was calculated according to the formula:%PPI = [100 – (startle

Please cite this article in press as: Möller, M., et al. Social isolation rearing induces mitochondrial, immunological, neurochemical and behavioural deficits in rats, and is reversed by clozapine or N-acetyl cysteine. Brain Behav. Immun. (2013), http://dx.doi.org/10.1016/j.bbi.2012.12.011

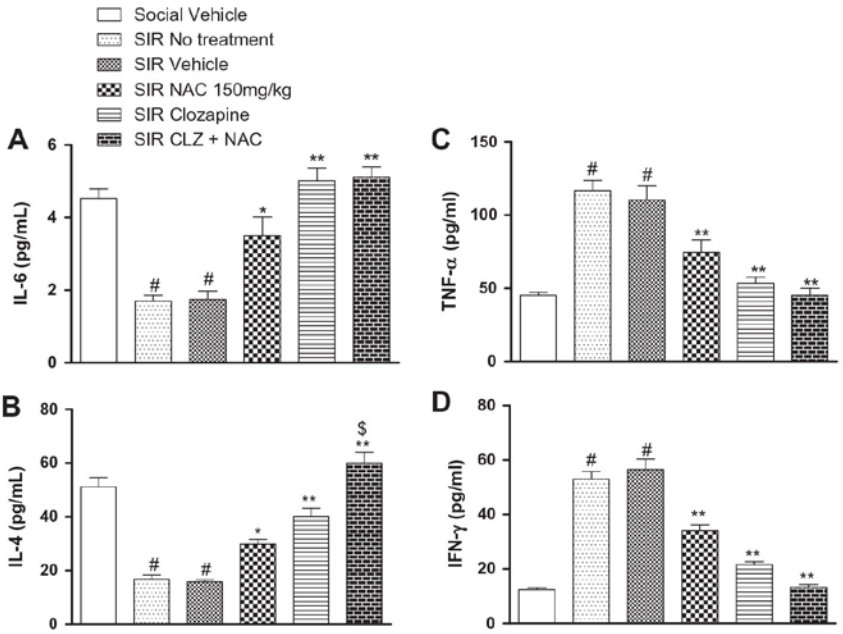


Fig. 4. Pro and anti-inflammatory cytokine concentrations. (A) IL-6; (B) IL-4; (C) TNF-α; (D) IFN-γ in SIR rats following the various drug treatments, as indicated ($n = 10$/group). A socially reared vehicle treatment control group is included for statistical comparison (data from Supplement 1). Treatment had a significant main effect with respect to (A) IL-6 ($F(5, 54) = 9.36$, $p < 0.0001$); (B) IL-4 ($F(5, 54) = 16.85$, $p < 0.0001$); (C) TNF-α ($F(5, 54) = 13.09$, $p < 0.0001$); (D) IFN-γ ($F(5, 54) = 34.87$, $p < 0.0001$) (3-way ANOVA). Rearing also indicated a significant main effect with respect to (A) IL-6 ($F(5, 54) = 34.97$, $p < 0.0001$); (B) IL-4 ($F(5, 54) = 19.58$, $p < 0.0001$); (C) TNF-α ($F(5, 54) = 14.69$, $p < 0.0001$); (D) IFN-γ ($F(5, 54) = 48.96$, $p < 0.0001$) (3-way ANOVA). Significant cross-group interactions were observed with respect to (A) IL-6 ($F(5, 54) = 23.71$, $p < 0.0001$); (B) IL-4 ($F(5, 54) = 45.10$, $p < 0.0001$); (C) TNF-α ($F(5, 54) = 24.73$, $p < 0.0001$); (D) IFN-γ ($F(5, 54) = 75.66$, $p < 0.0001$) (3-way ANOVA). $^{\#}p < 0.0001$ vs. social vehicle treatment, $^{*}p < 0.05$ vs. SIR no treatment and vehicle, $^{**}p < 0.0001$ vs. SIR no treatment and vehicle, $^{\$}p < 0.0001$ vs. SIR clozapine (Bonferroni post-hoc test). Refer to text for precise p values.

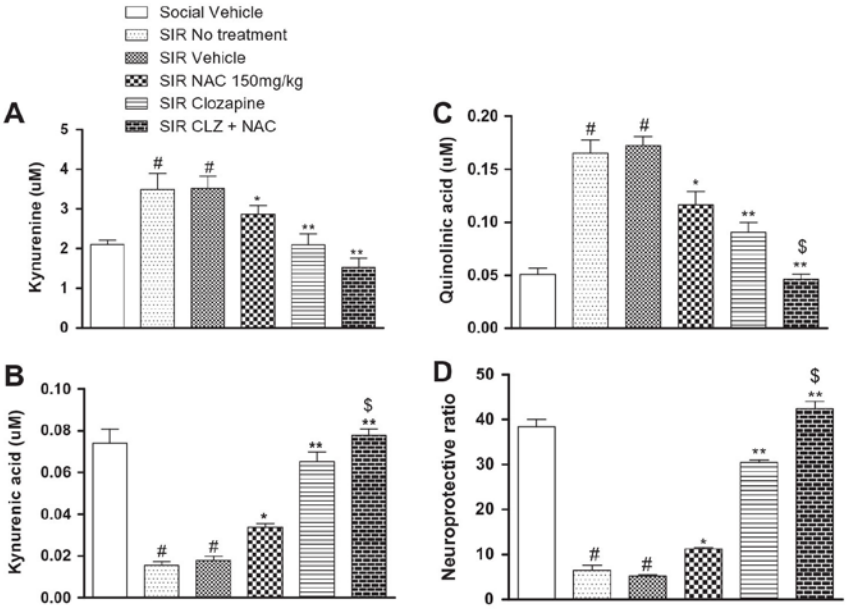


Fig. 5. Kynurenine pathway metabolites in SIR rats following the various drug treatments, as indicated ($n = 10$/group). A socially reared vehicle treatment control group is included for statistical comparison (data from Supplement 1): (A) kynurenine; (B) KYNA; (C) QA; (D) neuroprotective ratio. Treatment had a significant main effect with respect to (A) kynurenine ($F(5, 54) = 5.95$, $p < 0.0001$); (B) KYNA ($F(5, 54) = 33.83$, $p < 0.0001$); (C) QA ($F(5, 54) = 11.03$, $p < 0.0001$); (D) neuroprotective ratio ($F(5, 54) = 116.2$, $p < 0.0001$) (3-way ANOVA). Rearing also indicated a significant main effect with respect to (A) kynurenine ($F(5, 54) = 53.35$, $p < 0.0001$); (B) KYNA ($F(5, 54) = 58.28$, $p < 0.0001$); (C) QA ($F(5, 54) = 18.57$, $p < 0.0001$); (D) neuroprotective ratio ($F(5, 54) = 221.3$, $p < 0.0001$) (3-way ANOVA). Significant cross-group interactions were observed with respect to (A) kynurenine ($F(5, 54) = 9.04$, $p < 0.0001$); (B) KYNA ($F(5, 54) = 55.90$, $p < 0.0001$); (C) QA ($F(5, 54) = 21.36$, $p < 0.0001$); (D) neuroprotective ratio ($F(5, 54) = 254.7$, $p < 0.0001$) (3-way ANOVA). $^{\#}p < 0.0001$ vs. social vehicle treatment, $^{*}p < 0.05$ vs. SIR no treatment and vehicle, $^{**}p < 0.003$ vs. SIR no treatment and vehicle, $^{\$}p < 0.05$ vs. SIR clozapine (Bonferroni post-hoc test). Refer to text for precise p values.

Please cite this article in press as: Möller, M., et al. Social isolation rearing induces mitochondrial, immunological, neurochemical and behavioural deficits in rats, and is reversed by clozapine or N-acetyl cysteine. Brain Behav. Immun. (2013), http://dx.doi.org/10.1016/j.bbi.2012.12.011

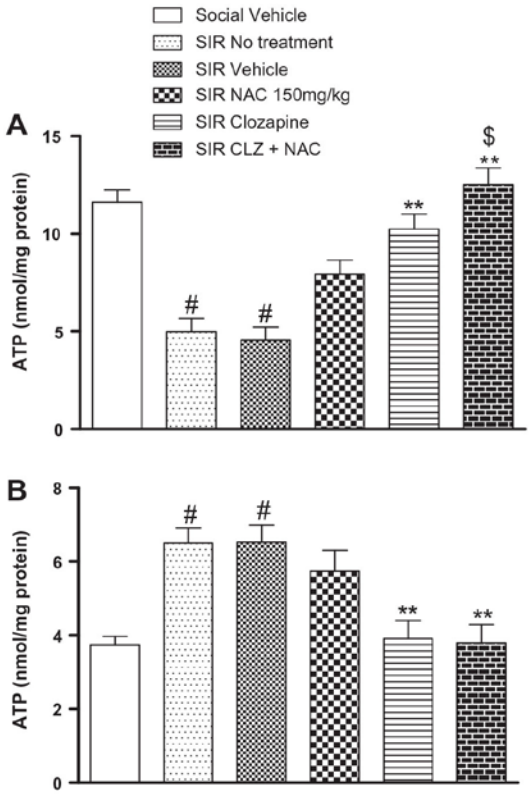


Fig. 6. ATP levels in the frontal cortex (A) and striatum (B) of SIR rats following the various drug treatments, as indicated ($n = 10$/group). A socially reared vehicle treatment control group is included for statistical comparison (data from Supplement 1). Treatment had a significant main effect on (A) the frontal cortex (F (5, 54) = 5.26, $p < 0.0001$) and (B) the striatum (F (5, 54) = 7.98, $p < 0.0001$) (3-way ANOVA). Rearing also indicated a significant main effect on (A) the frontal cortex (F (5, 54) = 5.22, $p < 0.0001$) and (B) the striatum (F (5, 54) = 8.34, $p < 0.0001$) (3-way ANOVA). Significant cross-group interactions were observed in (A) the frontal cortex (F (5, 54) = 21.39, $p < 0.0001$) and (B) the striatum (F (5, 54) = 9.051, $p < 0.0001$) (3-way ANOVA). $^{\#}p < 0.0001$ vs. social vehicle treatment, $^{**}p < 0.0001$ vs. SIR no treatment and vehicle, $^{\$}p < 0.05$ vs. SIR clozapine (Bonferroni post-hoc test). Refer to text for precise p values.

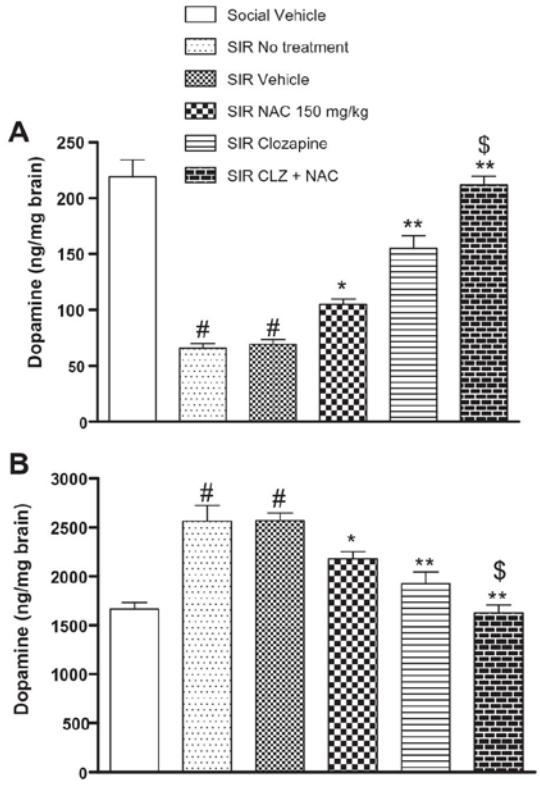


Fig. 7. DA levels in (A), the frontal cortex and (B), the striatum in SIR rats following the various drug treatments, as indicated ($n = 10$/group). A socially reared vehicle treatment control group is included for statistical comparison (data from Supplement 1). Treatment had a significant main effect on DA in (A) the frontal cortex (F (5, 54) = 14.81, $p < 0.0001$) and in (B) the striatum (F (5, 54) = 7.13, $p < 0.0001$), (3-way ANOVA). Rearing also indicated a significant main effect in (A) the frontal cortex (F (5, 54) = 39.47, $p < 0.0001$) and in (B) the striatum (F (5, 54) = 19.06, $p < 0.0001$), (3-way ANOVA). Significant cross-group interactions were observed: in (A) the frontal cortex (F (5, 54) = 58.57, $p < 0.0001$) and in (B) the striatum (F (5, 54) = 16.44, $p < 0.0001$), (3-way ANOVA). $^{\#}p < 0.0001$ vs. social vehicle treatment, $^{*}p < 0.05$ vs. SIR no treatment and vehicle, $^{**}p < 0.0001$ vs. SIR no treatment and vehicle, $^{\$}p < 0.05$ vs. SIR clozapine (Bonferroni post-hoc test). Refer to text for precise p values.

response for Prepulse + Pulse trial)/(startle response for Pulse alone trial) × 100] (Van den Buuse and Eikelis, 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 (Van den Buuse and Eikelis, 2001).

2.6. Peripheral immune-neurochemistry assays

After decapitation, trunk blood was collected in pre-chilled, 4 ml vacutainer tubes (SGVac) containing K_2EDTA solution as anti-coagulant, centrifuged at 14,000 rpm at 4 °C for 10 min, and the plasma stored at −80 °C until the day of analysis. On that day plasma samples were thawed on ice, centrifuged again, as mentioned above, and the plasma used.

2.6.1. Cytokines

Plasma anti-inflammatory cytokines (IL-4), pro-inflammatory cytokines (TNF-α, INF-γ) and multipurpose cytokine (IL-6; Rubio-Perez and Morillas-Ruiz, 2012), were measured in duplicate by sandwich enzyme-linked immunosorbent assay (ELISA) kits according to the manufacture's protocol (Invitrogen ELISA kit, Camarillo, CA). Briefly, 50 µL of a plasma sample were incubated in monoclonal antibody-coated wells in a 96 well plate. After a series of washes, a biotinylated detection antibody was added to the wells, and the reaction mixture was detected by the addition of streptavidin-horseradish peroxidase that binds to the biotinylated antibody to complete the four-member sandwich. The well sets were analysed using a microtiter plate reader at 450 nm. Unknown sample cytokine concentrations were calculated using a standard curve derived from the known reference cytokine concentrations supplied by the manufacturer. The sensitivity of the assay allowed the detection of cytokine concentrations within the following ranges: IL-6 1.5–1500 pg/mL; IL-4 0.2–100 pg/mL; IFN-γ 11.5–1400 pg/mL; TNF-α 3.5–1000 pg/mL. The final concentration of each cytokine was expressed as pg/mL of plasma used.

2.6.2. Analysis of kynurenine, kynurenic acid (KYNA) and quinolinic acid (QA)

Plasma samples were pre-purified by a solid-phase extraction (SPE) method followed by detection of tryptophan–kynurenine

Please cite this article in press as: Möller, M., et al. Social isolation rearing induces mitochondrial, immunological, neurochemical and behavioural deficits in rats, and is reversed by clozapine or N-acetyl cysteine. Brain Behav. Immun. (2013), http://dx.doi.org/10.1016/j.bbi.2012.12.011

metabolites using a novel liquid chromatography tandem mass spectrometry (LC–MS/MS) procedure developed and validated in our laboratory (Möller et al., 2012a). However, since we were primarily interested in determining those metabolites known to influence glutamatergic function and neuroprotective balance, only kynurenine, KYNA and QA were analysed. Kynurenine and KYNA were detected in positive electrospray ionisation mode, with QA detected in negative electrospray ionisation 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., 2007, 2011), hypothesised to relate to the eventual down-stream levels of QA following a shift in the relative levels of KYNA and kynurenine (Möller et al., 2012b). Neuroprotective ratio: [1000 × plasma kynurenic acid (mM)]/plasma kynurenine (mM).

2.7. Brain analysis

The frontal cortex was immediately dissected after decapitation, as described previously (Toua et al., 2010; Möller et al., 2011). The brain was then placed on its ventral side, the two cerebral hemispheres split and the striatum dissected out using the external walls of the lateral ventricles as internal limits and the corpus callosum as external limits. These brain regions were immediately fixed in liquid nitrogen and stored at −80 °C.

2.7.1. Cortico-striatal ATP

ATP accumulation was used as a measure of mitochondrial function. On the day of assay, the frontal cortical and striatal tissue were thawed on ice, weighed and a 10% tissue homogenate prepared in a Teflon homogenizer using a lysis buffer containing Triton X-100. Protein content was determined according to the Bradford method (Bradford, 1976). ATP concentrations in the frontal cortical and striatal homogenates were determined using a commercially available bioluminescence assay (Molecular Probes ATP Determination Kit; A22066) according to the manufacturer's instructions (Invitrogen ATP kit, Paisley, UK). ATP is determined with recombinant firefly luciferase and its substrate D-luciferin, with the concentration of ATP in each brain region expressed as nmol/mg protein.

2.7.2. Cortico-striatal dopamine

Quantification of cortico-striatal DA was performed using a high performance liquid chromatography (HPLC) system with electrochemical detection (HPLC-EC) (Harvey et al., 2006). Sample DA concentrations were determined by comparing the area under the peak to that of the internal standard (isoprenaline; range 5–50 ng/ml; Chemstation Rev. A 06.02 data acquisition and analysis software). Linear standard curves (regression coefficient greater than 0.99) were found in this particular range. DA concentrations were expressed as ng/mg wet weight of tissue (mean ± SEM).

2.8. Statistical analysis

Animal body weight across the groups (mean ± SEM) was analysed by two-way analysis of variance (ANOVA) with repeated factors of the respective treatment and rearing conditions, followed by Bonferroni post-hoc analyses. To model the behavioural and immune-neurochemical measurements respectively, a three-way factorial ANOVA and Bonferroni post-hoc tests was applied for the respective treatments (vehicle, clozapine, NAC, CLZ + NAC), rearing conditions (social vs. SIR) and either (a) behavioural parameter (social interaction or object recognition or prepulse intensity), with repeated measures used for the startle amplitude and different PPI intensities, or (b) immune-inflammatory parameter (cytokines or kynurenine metabolites) or (c) cortico-striatal parameters (ATP and DA). In all cases, data are expressed as the mean ± standard error of the mean (SEM), with a p value of <0.05 deemed statistically significant and NS denoting non-significance (Graphpad Prism 5; SAS/STAT® Software).

3. Results

In the drug treatment response studies, socially reared animals showed minimal changes across all parameters, and these data are presented in Supplement 1 (Figs. 1, 2 and 4–7). Only "social plus vehicle treatment" data are presented in the figures for statistical comparison. Unless specifically noted, statistical analysis of vehicle-treated vs. non-treated SIR groups was not significant, with all subsequent inter-group comparisons done vs. vehicle-treated SIR.

3.1. Body weight

Two-way repeated measures ANOVA with Bonferroni showed no significant main effect of treatment (F (2, 24) = 0.559, p = 0.87) or rearing (F (2, 24) = 0.954, p = 0.54) on weight (data not shown).

3.2. Behavioural tests

3.2.1. Social interaction test

All ANOVA data are described in the appropriate figure legends. Bonferroni post-hoc analysis showed a significant decrease (p < 0.0001) in social interactive behaviours (rearing, approaching, staying together and anogenital sniffing, Fig. 1A–D), as well as a significant (p < 0.0001) elevation in self-directed behaviours (square crossing and self-grooming, Fig. 1E and F) in SIR animals receiving no treatment or vehicle treatment compared to vehicle-treated socially reared controls. These behavioural changes were reversed by NAC, clozapine or CLZ + NAC vs. vehicle-treated SIR animals, as follows: rearing (Fig. 1A), NAC 150 mg/kg (p = 0.04), clozapine (p < 0.0001), CLZ + NAC (p < 0.0001); approaching (Fig. 1B), NAC 150 mg/kg (p = 0.02), clozapine (p < 0.0001), CLZ + NAC (p < 0.0001); staying together (Fig. 1C), NAC 150 mg/kg (NS), clozapine (p < 0.0001), CLZ + NAC (p < 0.0001); anogenital sniffing (Fig. 1D), NAC 150 mg/kg (p = 0.03), clozapine (p < 0.0001), CLZ + NAC (p < 0.0001); squares crossed (Fig. 1E), NAC 150 mg/kg (NS), clozapine (p < 0.0001), CLZ + NAC (p < 0.0001); self-grooming (Fig. 1F), NAC 150 mg/kg (NS), clozapine (p < 0.0001), CLZ + NAC (p < 0.0001).

3.2.2. Object recognition test (ORT)

All ANOVA data are described in the appropriate figure legends. In the acquisition trial, there were no significant cross-group interactions in the socially reared and SIR animals (data not shown). However, in the retention trial SIR animals receiving no treatment or vehicle treatment, Bonferroni testing revealed a significantly (p < 0.0001) increased exploration of the familiar object and a significantly (p < 0.0001) decreased exploration of the novel object compared to the vehicle-treated socially reared control groups (Fig. 2). NAC 150 mg/kg, clozapine and CLZ + NAC all significantly (p < 0.0001) reversed deficits with respect to visual recognition memory in the SIR animals (Fig. 2).

3.2.3. Sensorimotor gating test

All ANOVA data are described in the appropriate figure legends. No significant interaction on mean startle amplitude between the socially reared and SIR groups was evident (data not shown), as well as no significant interaction on %PPI in socially reared animals for each of the 4 prepulse intensities (data not shown). Bonferroni post hoc testing indicated that SIR receiving no treatment or

Please cite this article in press as: Möller, M., et al. Social isolation rearing induces mitochondrial, immunological, neurochemical and behavioural deficits in rats, and is reversed by clozapine or N-acetyl cysteine. Brain Behav. Immun. (2013), http://dx.doi.org/10.1016/j.bbi.2012.12.011

vehicle, significantly reduced %PPI vs. the corresponding vehicle-treated socially reared controls at all the prepulse intensities ($p < 0.0001$, Fig. 3). Clozapine significantly reversed the %PPI deficit in SIR animals at all 4 prepulse intensities ($p < 0.0001$) vs. the vehicle-treated SIR group, similarly for NAC 150 mg/kg ($p < 0.001$) and CLZ + NAC ($p < 0.0001$) (Fig. 3). CLZ + NAC 150 mg/kg was significantly more effective than clozapine alone at certain prepulse intensities (80 dB: $p = 0.005$, 84 dB: $p < 0.001$, Fig. 3).

3.3. Immunological assays in peripheral blood

3.3.1. Cytokines

All ANOVA data are described in the appropriate figure legends. Bonferroni post hoc testing indicated that SIR groups receiving no treatment or vehicle showed significantly decreased IL-6 (Fig. 4A, $p < 0.0001$), as well as IL-4 (Fig. 4B, $p < 0.0001$), but increased TNF-α (Fig. 4C, $p < 0.0001$) and IFN-γ (Fig. 4D, $p < 0.0001$), compared to their vehicle-treated socially reared controls. SIR-associated changes in IL-6 (Fig. 4A) was partially reversed by NAC 150 mg/kg ($p = 0.03$), and fully reversed by clozapine ($p < 0.0001$) and CLZ + NAC ($p < 0.0001$). SIR-induced changes in IL-4 (Fig. 4B) was partially reversed by NAC 150 mg/kg ($p = 0.04$) and clozapine ($p = 0.002$) and fully reversed by CLZ + NAC ($p < 0.0001$). SIR-induced changes in TNF-α (Fig. 4C) was fully reversed by NAC 150 mg/kg ($p < 0.0001$), clozapine ($p < 0.0001$) and CLZ + NAC ($p < 0.0001$). SIR-induced changes in IFN-γ (Fig. 4D) were fully reversed by NAC 150 mg/kg ($p < 0.0001$), clozapine ($p < 0.0001$) and CLZ + NAC ($p < 0.0001$). CLZ + NAC were superior to clozapine alone in reversing SIR-induced changes in IL-4 (Fig. 4B; $p < 0.0001$).

3.3.2. Tryptophan–kynurenine metabolites

All ANOVA data are described in the appropriate figure legends. Bonferroni post hoc testing indicated that SIR in animals receiving no treatment or vehicle evoked a significant ($p < 0.0001$) increase in kynurenine (Fig. 5A) and QA (Fig. 5C), and a significant ($p < 0.0001$) decrease in KYNA (Fig. 5B) and neuroprotective ratio (Fig. 5D) vs. the vehicle-treated socially reared controls. SIR-induced changes in kynurenine (Fig. 5A) was partially reversed by NAC 150 mg/kg ($p = 0.04$) and fully reversed by clozapine ($p = 0.002$) and by CLZ + NAC ($p < 0.0001$). Changes in KYNA (Fig. 5B) was partially reversed by NAC 150 mg/kg ($p = 0.03$) and fully reversed by clozapine ($p < 0.0001$) and CLZ + NAC ($p < 0.0001$). The change in QA (Fig. 5C) was partially reversed by NAC 150 mg/kg ($p = 0.002$) and fully reversed by clozapine ($p < 0.0001$) and CLZ + NAC ($p < 0.0001$). The change in neuroprotective ratio (Fig. 5D) was partially reversed by NAC 150 mg/kg ($p = 0.04$) and fully reversed by clozapine ($p < 0.0001$) and CLZ + NAC ($p < 0.0001$). CLZ + NAC treatment was superior to clozapine alone in reversing SIR-associated changes in KYNA ($p < 0.05$), QA ($p < 0.05$) and neuroprotective ratio ($p < 0.0001$) (Fig. 5B–D, respectively).

3.4. Brain analyses

3.4.1. Cortico-striatal ATP

All ANOVA data are described in the appropriate figure legends. Bonferroni post hoc testing indicated a significant ($p < 0.0001$) decrease in the frontal cortical ATP (Fig. 6A) and a significant ($p < 0.0001$) increase in the striatal ATP (Fig. 6B) in SIR animals receiving no treatment or vehicle treatment, compared to the vehicle-treated socially reared controls. In the frontal cortex, clozapine ($p < 0.0001$) and CLZ + NAC ($p < 0.0001$) fully reversed SIR-induced deficits in ATP level in SIR animals, while CLZ + NAC was superior ($p = 0.02$) to clozapine treatment alone (Fig. 6A). In the striatum, clozapine ($p < 0.0001$) and CLZ + NAC ($p < 0.0001$) fully reversed SIR induced increases in ATP levels (Fig. 6B).

3.4.2. Cortico-striatal dopamine levels

All ANOVA data are described in the appropriate figure legends. Bonferroni post hoc testing indicated a significant decrease in frontal cortical DA ($p < 0.0001$, Fig. 7A) in SIR animals receiving either no treatment or vehicle treatment, compared to vehicle-treated socially reared controls. SIR also induced a significant increase in striatal DA ($p < 0.0001$, Fig. 7B).

In the frontal cortex, the SIR-induced decrease in DA (Fig. 7A) was partially reversed by NAC 150 mg/kg ($p = 0.04$) and fully reversed by clozapine ($p < 0.0001$) and CLZ + NAC ($p < 0.0001$). In the striatum, SIR-induced increases in DA (Fig. 7B) was partially reversed by 150 mg/kg NAC ($p = 0.009$), and fully reversed by clozapine ($p < 0.0001$) and CLZ + NAC ($p < 0.0001$). Importantly CLZ + NAC was superior to clozapine treatment alone with respect to reversing SIR-induced changes in both frontal cortical and striatal DA levels (Fig. 7A and B; $p = 0.0008$; $p = 0.04$, respectively).

4. Discussion

This study has reaffirmed the validity of SIR in rats as a model for schizophrenia, including the reduction of %PPI (Fig. 3; Geyer et al., 1993), effects on social interactive behaviours (Fig. 1; Möller et al., 2011), elevated kynurenine and QA vs. KYNA and reduced neuroprotective ratio (Fig. 5; Möller et al., 2012b), and their reversal by clozapine (Möller et al., 2011, 2012b). We now show that SIR reduces plasma IL-4 and IL-6 (Fig. 4A and B) and increases TNF-α and IFN-γ (Fig. 4C and D), reduces frontal cortical ATP (Fig. 6A) but increases striatal ATP (Fig. 6B), with attenuated novel object recognition memory (Fig. 2). Frontal cortical DA is reduced (Fig. 7A) but elevated in the striatum (Fig. 7B). SIR associated bio-behavioural changes are reversed by clozapine or NAC (Figs. 1–5 and 7), with clozapine + NAC more effective than clozapine on a number of parameters.

4.1. Mechanisms and consequences of SIR-mediated immune dysregulation

Early life adversity, especially via inflammation and oxidative stress, increases the risk of schizophrenia (Sorensen et al., 2009). The mitochondrial respiratory chain is a major source of ROS that is maintained by the GSH and other endogenous antioxidant systems (Bains and Shaw, 1997; Johnson and Giulivi, 2005), failure of which results in disruption of ATP synthesis and oxidative damage (Smith et al., 2012). Schizophrenia presents with oxidative stress (Ng et al., 2008) as well as reduced mitochondrial function in the frontal and temporal lobes (Fukuzako et al., 1999; Volz et al., 2000; Jensen et al., 2006). SIR reduced frontal cortical ATP, in line with the clinical evidence, and is also associated with frontal cortical and striatal oxidative stress (Schiavone et al., 2009; Möller et al., 2011). This response seems to involve increased expression of nicotinamide adenosine dinucleotide phosphate (NADPH) oxidase 2 (NOX2; Schiavone et al., 2009), an innate immune enzyme responsible for superoxide production. NOX2 controls glutamate release in the prefrontal cortex (Sorce et al., 2010; Schiavone et al., 2012) and compromises cortical parvalbumin-positive γ-amino butyric acid (GABA) inter-neurons (Schiavone et al., 2009; Powell et al., 2012). Unstable excitatory-inhibitory pathways and oxidative stress thus play a pathological role in SIR and by implication, schizophrenia.

ATP mediates the release of pro-inflammatory cytokines (Cruz et al., 2007), while schizophrenia is characterised by immune dysfunction (Drzyzga et al., 2006) and a pro-inflammatory state (Leonard et al., 2012), e.g. decreased plasma/serum anti-inflammatory cytokines (IL-4; Kim et al., 2004) and elevated pro-inflammatory cytokines (IFN-γ and TNF-α; Kim et al., 2004; Drzyzga et al.,

Please cite this article in press as: Möller, M., et al. Social isolation rearing induces mitochondrial, immunological, neurochemical and behavioural deficits in rats, and is reversed by clozapine or N-acetyl cysteine. Brain Behav. Immun. (2013), http://dx.doi.org/10.1016/j.bbi.2012.12.011

2006). The SIR-induced increase in plasma TNF-α and IFN-γ and a decrease in IL-4 confirm the presence of a pro-inflammatory state.

The kynurenine pathway occurs primarily in astrocytes and microglia, with 60% of cerebral kynurenine contributed from the periphery (Heyes et al., 1997). Pro- vs. anti-inflammatory cytokines and their activation/inhibition of IDO links immune-inflammatory pathways with redox balance (Stone and Darlington, 2002). SIR increased plasma QA and reduced the neuroprotective ratio. Elevated KYNA (Schwarcz et al., 2001; Erhardt et al., 2001), kynurenine and QA (Torrey et al., 1998; Miller et al., 2008) are described in schizophrenia. However, decreased plasma KYNA in medication-naïve/free schizophrenia patients (Myint et al., 2011) confirms findings in SIR rats described here and elsewhere (Möller et al., 2012b).

The DA hypothesis proclaims increased sub-cortical DA but reduced frontal cortical DA activity in schizophrenia (Guillin et al., 2007), corresponding to elevated striatal but reduced frontal cortical DA in SIR animals. The latter coincides with hypo-frontality in schizophrenia (Riehemann et al., 2001) manifesting here as deficits in social behaviour (increased self-directed, reduced social behaviours) and memory (decreased novel object recognition) akin to that observed in schizophrenia (Russell et al., 2000; Barch et al., 2002). Increased striatal DA, however, coincides with the positive symptoms of the illness, depicted here by increased square crossings and self-grooming (self-directed behaviours) and deficits in %PPI. SIR-induced schizophrenia-like behaviours therefore relate to the DA hypothesis via altered PPI (increased striatal DA; Zhang et al. 2000), deficits in cognition (reduced cortical DA; Ventura et al., 2004) and social behaviour (reduced cortical DA; Fernandez, 2003). The effects of SIR on DA also support the social defeat model of schizophrenia (Selten and Cantor-Graae, 2005). Whether SIR-induced change in DA metabolism follows on from mitochondrial dysfunction, altered cytokines, kynurenine metabolism, or a combination of these is now discussed.

4.2. Mitochondria, immune-inflammatory dysregulation and dopamine

Mitochondrial ATP occupies a central role in glial cytokine release, with DA involved in this response (Choi et al., 2007). Moreover, DA release in the nucleus accumbens is associated with an increase in ATP (Krügel et al., 1999, 2001). Deficits in PPI are related to GSH-DA interactions (Dean et al., 2010) while glutamate-dependent striatal DA release involves hydrogen peroxide (H_2O_2) activation of ATP-sensitive K^+ channels (Avshalumov and Rice, 2003). Thus mitochondrial ATP and redox state are associated with positive symptoms. Indeed SIR increased striatal ATP and DA, increased self-directed behaviours and reduced %PPI. Conversely, altered frontal lobe mitochondrial ATP is correlated with negative symptoms and neuropsychological deficits in schizophrenia (Yacubian et al., 2002), congruent with SIR-induced reduction in frontal cortical ATP, DA and social interactive behaviours.

DA dysfunction and schizophrenia progression is related to cytokines via inflammation, oxidative stress and apoptosis (Meyer and Feldon, 2009). Indeed the multifunctional cytokine, IL-6, that is altered in the disorder (Rubio-Perez and Morillas-Ruiz, 2012), is linked to NOX2 activation (Behrens et al., 2008), while it also alters frontal cortical DA activity (Zalcman et al., 1994). Interestingly prenatal immune challenge, like SIR, is an early life neurodevelopmental trauma that facilitates postnatal DA dysfunction (Meyer and Feldon, 2009; Vuillermot et al., 2010), while TNF-α is neurotoxic to DA neurons during foetal brain development (Pauly and Slotkin, 2008).

Frontal-cortical glutamate transmission influences cortical and sub-cortical DA release (Schwartz et al., 2012), while its modulation via the kynurenine pathway may have similar effects (Wonodi and Schwarcz, 2010). Although QA has minimal effects on striatal DA release (Pittaluga et al., 2001), DA is reduced by an elevation in KYNA (Okuno et al., 2011). Reduced KYNA, as our SIR data confirms, will thus increase striatal DA release to illicit positive behavioural symptoms. KYNA bi-directionally modulates frontal cortical glutamate and DA release, implicating KYNA-DA interactions in cognition (Wonodi and Schwarcz, 2010). KYNA-DA interactions also influence survival of nigrostriatal DAergic neurons (Miranda et al., 1997).

4.3. Response to clozapine and NAC and relevance for schizophrenia

Clozapine reversed SIR-induced frontal cortical DA deficits, as well as self-directed social and memory deficits. This coincides with its known effects on cortical DA release (Moghaddam and Bunney, 1990; Kuroki et al., 1999), and explains its positive influence on frontal lobe deficits in schizophrenia (Harvey et al., 1999). Similarly its reversal of elevated striatal DA and social and PPI deficits coincides with elevated DA levels purported to underlie positive symptom schizophrenia (Harvey et al., 1999). However, clozapine modulates proinflammatory cytokine and ROS release (Hu et al., 2012), reverses oxidative stress (Möller et al., 2011) and corrects aberrant kynurenine metabolism (Möller et al., 2012b), suggesting that its effect on DA may involve immunological actions. Although such an action is evident in clinical studies (Monteleone et al., 1997; Hinze-Selch et al., 1998), precisely how clozapine re-establishes redox homeostasis is uncertain. Regulation of mitochondrial activity, inhibition of microglial-dependent inflammatory responses (Hu et al., 2012), modulating IDO activity (Wang et al., 2010) and inhibiting pro-inflammatory cytokines (Sugino et al., 2009) seems evident. Clozapine reversed SIR-induced changes in cortico-striatal ATP, KYNA, QA and neuroprotective ratio, and re-established pro- vs. anti-inflammatory cytokine balance. Since cytokines influence IDO activity, a primary action regulating cytokine release is more likely, followed thereafter by effects on IDO and kynurenine metabolism.

A GSH active antioxidant and glutamate modulator such as NAC may therefore offer clinical utility in treating schizophrenia and may bolster the above-mentioned effects of clozapine. NAC 150 mg/kg/day partially yet significantly reversed SIR induced deficits in memory, PPI and social interactive behaviours, as well as changes in cytokines, kynurenine metabolites and DA. Although NAC failed to reverse SIR-induced cortico-striatal ATP changes and self-directed social behaviours, a higher dose (250 mg/kg see Supplement 1, Figs. 1 and 6) proved effective. Thus NAC effectively reverses the bio-behavioural effects of SIR, albeit less so than clozapine. Since clozapine reverses SIR-induced GSH imbalance (Möller et al., 2011), both NAC and clozapine share actions on the GSH system, although their beneficial interaction extends to other redox-active systems as demonstrated in this paper. NAC also affects cortico-striatal DA release via actions on the cystine-glutamate transporter (Bauzo et al., 2012), while clozapine too affects glutamate pathways (Toua et al., 2010). Combined effects on NOX2 (Hu et al., 2012) and redox impaired parvalbumin interneurons (Cabungcal et al., 2012) also warrant consideration. This commonality in action possibly explains NAC's efficacy as adjunctive therapy to improve negative symptoms, social-interaction and auditory sensory processing amongst others (Berk et al., 2008; Dean et al., 2011; Lavoie et al., 2008), and concurs with our behavioural data (Figs. 1–3 and Supplement 1 Figs. 1–3). Although NAC reversal of mitochondrial toxicity is not new (e.g. Singh et al., 2010), its ability to reverse reduced cortical ATP in a model of social isolation is noteworthy. Interestingly CLZ + NAC was superior to clozapine with respect to changes in PPI, KYNA, QA and neuroprotective ratio, frontal cortical ATP and DA, all parameters targeted by each drug separately. By re-establishing homeostasis in frontal cortical mitochondrial function, correcting aberrant immune-inflammatory cascades and stabilization of

Please cite this article in press as: Möller, M., et al. Social isolation rearing induces mitochondrial, immunological, neurochemical and behavioural deficits in rats, and is reversed by clozapine or N-acetyl cysteine. Brain Behav. Immun. (2013), http://dx.doi.org/10.1016/j.bbi.2012.12.011

kynurenine/glutamatergic activity, CLZ + NAC more effectively stabilizes cortico-striatal DA circuits to improve treatment outcome.

5. Conclusion

SIR-induced behavioural deficits in social interaction, memory and PPI are associated with altered cortico-striatal ATP levels, an increase and decrease in pro- and anti-inflammatory cytokines, respectively, leading to altered plasma tryptophan metabolites, a reduction in the neuroprotective ratio and cell damage. These effects combine to initiate striatal DA changes expressed as positive- or negative-like behavioural symptoms. Moreover, these changes are reversed by sub-chronic clozapine or NAC treatment with evidence for an additive effect for CLZ + NAC on selected parameters. This study confirms mitochondrial and immune-inflammatory involvement in schizophrenia using a neurodevelopmental animal model (SIR), and provides insight into the mechanisms underlying the utility of NAC as an adjunctive treatment in schizophrenia.

Author disclosures

Sources of funding: The authors declare that 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: The authors declare that over the past three years, 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. We also declare that Brian Harvey has participated in speakers/advisory boards and received honoraria from Organon, Pfizer and Servier, and has received research funding from Lundbeck. The authors declare that, except for income from the primary employer and research funding to BHH from the South African Medical Research Council, no financial support or compensation has been received from any individual or corporate entity over the past three years for research or professional services, and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest. Michael Berk has received Grant/Research Support from the NIH, Cooperative Research Centre, Simons Autism Foundation, Cancer Council of Victoria, Stanley Medical Research Foundation, MBF, NHMRC, Beyond Blue, Rotary Health, Geelong Medical Research Foundation, Bristol Myers Squibb, Eli Lilly, Glaxo SmithKline, Organon, Novartis, Mayne Pharma and Servier, has been a speaker for Astra Zeneca, Bristol Myers Squibb, Eli Lilly, Glaxo SmithKline, Janssen Cilag, Lundbeck, Merck, Pfizer, Sanofi Synthelabo, Servier, Solvay and Wyeth, and served as a consultant to Astra Zeneca, Bristol Myers Squibb, Eli Lilly, Glaxo SmithKline, Janssen Cilag, Lundbeck Merck and Servier. Dr. Berk is co-inventor of two provisional patents regarding the use of NAC and related compounds for psychiatric indications, which, while assigned to the Flory Institute of Neuroscience and Mental Health, could lead to personal remuneration upon a commercialization event.

Acknowledgments

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.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbi.2012.12.011.

References

Avshalumov, M.V., Rice, M.E., 2003. Activation of ATP-sensitive K^+ (K(ATP)) channels by H_2O_2 underlies glutamate-dependent inhibition of striatal dopamine release. Proc. Natl. Acad. Sci. U.S.A. 100, 11729–11734.

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.

Bains, J.S., Shaw, C.A., 1997. Neurodegenerative disorders in humans: the role of glutathione in oxidative stress-mediated neuronal death. Brain Res. Rev. 25, 335–358.

Barch, D.M., Csernansky, J.G., Conturo, T., Snyder, A.Z., 2002. Working and long-term memory deficits in schizophrenia: is there a common prefrontal mechanism? J. Abnorm. Psychol. 111, 478–494.

Bauzo, R.M., Kimmel, H.L., Howell, L.L., 2012. The cystine-glutamate transporter enhancer N-acetyl-L-cysteine attenuates cocaine-induced changes in striatal dopamine but not self-administration in squirrel monkeys. Pharmacol. Biochem. Behav. 101, 288–296.

Behrens, M.M., Ali, S.S., Dugan, L.L., 2008. Interleukin-6 mediates the increase in NADPH-oxidase in the ketamine model of schizophrenia. J. Neurosci. 28, 13957–13966.

Ben-Shachar, D., 2002. Mitochondrial dysfunction in schizophrenia: a possible linkage to dopamine. J. Neurochem. 83, 1241–1251.

Berk, M., Copolov, D., Dean, O., Lu, K., Jeavons, S., Schapkaitz, I., 2008. N-acetyl cysteine as a glutathione precursor for schizophrenia—a double-blind, randomized, placebo-controlled trial. Biol. Psychiatry 64, 361–368.

Betzen, C., White, R., Zehendner, C.M., Pietrowski, E., Bender, B., Luhmann, H.J., Kuhlmann, C.R.W., 2009. Oxidative stress upregulates the NMDA receptor on cerebrovascular endothelium. Free Radical Biol. Med. 47, 1212–1220.

Bradford, M.M., 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein dye-binding. Anal. Biochem. 72, 248–254.

Cabungcal, J.H., Steullet, P., Kraftsik, R., Cuenod, M., Do, K.Q., 2012. Early-life insults impair parvalbumin interneurons via oxidative stress: reversal by N-acetylcysteine. Biol. Psychiatry. http://dx.doi.org/10.1016/j.biopsych.2012.09.020. [Epub ahead of print]

Calkins, M.E., Gur, R.C., Ragland, J.D., Gur, R.E., 2005. Face recognition memory deficits and visual object memory performance in patients with schizophrenia and their relatives. Am. J. Psychiatry 162, 1963–1966.

Chen, H., Stoker, A., Markou, A., 2010. The glutamatergic compounds sarcosine and N acetylcysteine ameliorate prepulse inhibition deficits in metabotropic glutamate 5 receptor knockout mice. Psychopharmacology 209, 343–350.

Choi, H.B., Ryu, J.K., Kim, S.U., McLarnon, J.G., 2007. Modulation of the purinergic P2X7 receptor attenuates lipopolysaccharide-mediated microglial activation and neuronal damage in inflamed brain. J. Neurosci. 27, 4957–4968.

Coyle, J.T., Tsai, G., 2004. The NMDA receptor glycine modulatory site: a therapeutic target for improving cognition and reducing negative symptoms in schizophrenia NMDA receptor and schizophrenia. Psychopharmacology 174, 32–38.

Cruz, C.M., Rinna, A., Forman, H.J., Ventura, A.L., Persechini, P.M., Ojcius, D.M., 2007. ATP activates a reactive oxygen species-dependent oxidative stress response and secretion of proinflammatory cytokines in macrophages. J. Biol. Chem. 282, 2871–2879.

Dean, O., Bush, A.I., Berk, M., Copolov, D.L., van den Buuse, M., 2010. Interaction of glutathione depletion and psychotropic drug treatment in prepulse inhibition in rats and mice. Pharmacol. Biochem. Behav. 97, 293–300.

Dean, O., Giorlando, F., Berk, M., 2011. N-acetylcysteine in psychiatry: current therapeutic evidence and potential mechanisms of action. J. Psychiatry Neurosci. 36, 78–86.

Drzyzga, Ł., Obuchowicz, E., Marcinowska, A., Herman, Z.S., 2006. Cytokines in schizophrenia and the effects of antipsychotic drugs. Brain Behav. Immun. 20, 532–545.

Ennaceur, A., Delacour, J., 1988. A new one-trial test for neurobiological studies of memory in rats. Behav. Brain Res. 31, 47–59.

Erhardt, S., Blennow, K., Nordin, C., Skogh, E., Lindstrom, L.H., Engberg, G., 2001. Kynurenic acid levels are elevated in the cerebrospinal fluid of patients with schizophrenia. Neurosci. Lett. 313, 96–98.

Fernandez, E., 2003. Prefrontocortical dopamine loss in rats delays long-term extinction of contextual conditioned fear, and reduces social interaction without affecting short-term social interaction memory. Neuropsychopharmacology 28, 490–498.

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.

Fukami, G., Hashimoto, K., Koike, K., Okamura, N., Shimizu, E., Iyo, M., 2004. Effect of antioxidant N-acetyl-L-cysteine on behavioural changes and neurotoxicity in rats after administration of methamphetamine. Brain Res. 1016, 90–95.

Fukuzako, H., Fukuzako, T., Hashiguchi, T., Kodama, S., Takigawa, M., Fujimoto, T., 1999. Changes in levels of phosphorus metabolites in temporal lobes of drug naive schizophrenic patients. Am. J. Psychiatry 156, 1205–1208.

Garcia-Cazorla, A., Duarte, S., Serrano, M., Nascimento, A., Ormazabal, A., Carrilho, I., et al., 2008. Mitochondrial diseases mimicking neurotransmitter defects. Mitochondrion 8, 273–278.

Please cite this article in press as: Möller, M., et al. Social isolation rearing induces mitochondrial, immunological, neurochemical and behavioural deficits in rats, and is reversed by clozapine or N-acetyl cysteine. Brain Behav. Immun. (2013), http://dx.doi.org/10.1016/j.bbi.2012.12.011

Geyer, M.A., Wilkinson, L.S., Humby, T., Robbins, T.W., 1993. Isolation rearing of rats produces a deficit in prepulse inhibition of acoustic startle similar to that in schizophrenia. Biol. Psychiatry 34, 361–372.

Glantz, L.A., Lewis, D.A., 2000. Decreased dendritic spine density on prefrontal cortical pyramidal neurons in schizophrenia. Arch. Gen. Psychiatry 57, 65–73.

Gonzalez, L.E., Andrews, N., File, S.E., 1996. 5-HTIA and benzodiazepine receptors in the basolateral amygdala modulate anxiety in the social interaction test, but not in the elevated plus-maze. Brain Res. 732, 145–153.

Grayson, B., Idris, N.F., Neill, J.C., 2007. Atypical antipsychotics attenuate a sub-chronic PCP-induced cognitive deficit in the novel object recognition task in the rat. Behav. Brain Res. 184, 31–38.

Guillin, O., Abi-Dargham, A., Laruelle, M., 2007. Neurobiology of dopamine in schizophrenia. Int. Rev. Neurobiol. 78, 1–39.

Harvey, B.H., Brand, L., Jeeva, Z., Stein, D.J., 2006. Cortical/hippocampal monoamines, HPA-axis changes and aversive behavior following stress and restress in an animal model of post-traumatic stress disorder. Physiol. Behav. 87, 881–890.

Harvey, B.H., Joubert, C., Preez, J.L., Berk, M., 2008. Effect of chronic N-acetyl cysteine administration on oxidative status in the presence and absence of induced oxidative stress in rat striatum. Neurochem. Res. 33, 508–517.

Harvey, B.H., Stein, D.J., Emsley, R.A., 1999. The new-generation anti-psychotics: integrating the neuro-pathology and pharmacology of schizophrenia. SAMJ 89, 661–672.

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, 351–356.

Hinze-Selch, D., Becker, E.W., Stein, G.M., Berg, P.A., Mullington, J., Holsboer, F., Pollmächer, T., 1998. Effects of clozapine on in vitro immune parameters: a longitudinal study in clozapine-treated schizophrenic patients. Neuropsychopharmacology 19, 114–122.

Holcomb, H.H., Rowland, L.M., Tagamets, M.A., 2004. Cognitive dysfunction in schizophrenia: glutamatergic hypoactivity and dopaminergic failure. Drug Discov. Today: Dis. Mech. 1, 435–439.

Howes, O.D., Kambeitz, J., Kim, E., Stahl, D., Slifstein, M., Abi-Dargham, A., Kapur, S., 2012. The nature of dopamine dysfunction in schizophrenia and what this means for treatment: meta-analysis of imaging studies. Arch. Gen. Psychiatry (Epub ahead of print).

Hu, X., Zhou, H., Zhang, D., Yang, S., Qian, L., Wu, H.M., Chen, P.S., Wilson, B., Gao, H.M., Lu, R.B., Hong, J.S., 2012. Clozapine protects dopaminergic neurons from inflammation-induced damage by inhibiting microglial overactivation. J. Neuroimmune. Pharmacol. 7, 187–201.

Jensen, J.E., Miller, J., Williamson, P.C., Neufeld, R.W., Menon, R.S., Malla, A., et al., 2006. Grey and white matter differences in brain energy metabolism in first episode schizophrenia: ^{31}P-MRS chemical shift imaging at 4 Tesla. Psychiatry Res. 146, 127–135.

Johnson, F., Giulivi, C., 2005. Superoxide dismutases and their impact upon human health. Mol. Aspects Med. 26, 340–352.

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.

Karry, R., Klein, E., Ben-Shachar, D., 2004. Mitochondrial complex I subunits expression is altered in schizophrenia: a postmortem study. Biol. Psychiatry 55, 676–684.

Kerksick, C., Willoughby, D., 2005. The antioxidant role of glutathione and N-acetyl-cysteine supplements and exercise-induced oxidative stress. J. Int. Soc. Sports Nutr. 2, 38–44.

Khan, M., Sekhon, B., Jatana, M., Giri, S., Gilg, A.G., Sekhon, C., Singh, I., Singh, A.K., 2004. Administration of N-acetylcysteine after focal cerebral ischemia protects brain and reduces inflammation in a rat model of experimental stroke. J. Neurosci. Res. 76, 519–527.

Kim, Y., Myint, A., Lee, B., Han, C., Lee, H., Kim, D., Leonard, B.E., 2004. Th1, Th2 and Th3 cytokine alteration in schizophrenia. Prog. Neuro-Psychopharmacol. Biol. Psychiatry 28, 1129–1134.

Krügel, U., Kittner, H., Illes, P., 1999. Adenosine 5′-triphosphate-induced dopamine release in the rat nucleus accumbens in vivo. Neurosci. Lett. 265, 49–52.

Krügel, U., Kittner, H., Illes, P., 2001. Mechanisms of adenosine 5′-triphosphate-induced dopamine release in the rat nucleus accumbens in vivo. Synapse 39, 222–232.

Kuroki, T., Meltzer, H.Y., Ichikawa, J., 1999. Effects of antipsychotic drugs on extracellular dopamine levels in rat medial prefrontal cortex and nucleus accumbens. J. Pharmacol. Exp. Ther. 288, 774–778.

Lavoie, S., Murray, M.M., Deppen, P., Knyazeva, M.G., Berk, M., Boulat, O., 2008. Glutathione precursor, N-acetyl-cysteine, improves mismatch negativity in schizophrenia patients. Neuropsychopharmacology 33, 2187–2199.

Leonard, B.E., Schwarz, M., Myint, A.M., 2012. The metabolic syndrome in schizophrenia: is inflammation a contributing cause? J. Psychopharm. 26, 33–41.

Looney, J.M., Childs, H.M., 1934. The lactic acid and glutathione content of the blood of schizophrenia patients. J. Clin. Invest. 13, 963–968.

Matheson, S.L., Shepherd, A.M., Laurens, K.R., Carr, V.J., 2011. A systematic metareview grading the evidence for non-genetic risk factors and putative antecedents of schizophrenia. Schizophr Res. 133, 133–142.

McGrath, J.J., Eyles, D.W., Pedersen, C.B., Anderson, C., Ko, P., Burne, T.H., Norgaard-Pedersen, B., Hougaard, D.M., Mortensen, P.B., 2010. Neonatal vitamin D status and risk of schizophrenia: a population-based case-control study. Arch. Gen. Psychiatry 67, 889–894.

McLean, S., Grayson, B., Harris, M., Protheroe, C., Woolley, M., Neill, J., 2010. Isolation rearing impairs novel object recognition and attentional set shifting performance in female rats. J. Psychopharmacology 24, 57–63.

Meyer, U., Feldon, J., 2009. Prenatal exposure to infection: a primary mechanism for abnormal dopaminergic development in schizophrenia. Psychopharmacol 206, 587–60.

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.

Miller, C.L., Llenos, I.C., Dulay, J.R., Barillo, M.M., Yolken, R.H., Weis, S., 2004. Expression of the kynurenine pathway enzyme tryptophan 2,3-dioxygenase is increased in the frontal cortex of individuals with schizophrenia. Neurobiol. Dis. 15, 618–629.

Miranda, A.F., Boegman, R.J., Beninger, R.J., Jhamandas, K., 1997. Protection against quinolinic acid-mediated excitotoxicity in nigrostriatal dopaminergic neurons by endogenous kynurenic acid. Neuroscience 78, 967–975.

Moghaddam, B., Bunney, B.S., 1990. Acute effects of typical and atypical antipsychotic drugs on the release of dopamine from prefrontal cortex, nucleus accumbens, and striatum of the rat: an in vivo microdialysis study. J. Neurochem. 54, 1755–1760.

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., 2012a. Development and validation of a single analytical method for the determination of tryptophan, and its kynurenine metabolites in rat plasma. J. Chromatogr. B 898, 121–129.

Möller, M., Du Preez, J.L., Emsley, R., Harvey, B.H., 2012b. Social isolation rearing in rats alters plasma tryptophan metabolism and is reversed by sub-chronic clozapine treatment. Neuropharmacology 62, 2499–2506.

Monteleone, P., Fabrazzo, M., Tortorella, A., Maj, M., 1997. Plasma levels of interleukin-6 and tumor necrosis factor alpha in chronic schizophrenia: effects of clozapine treatment. Psychiatry Res. 71, 11–17.

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.

Myint, A., Kim, Y.K., Verkerk, R., Scharpé, S., Steinbusch, H., Leonard, B., 2007. Kynurenine pathway in major depression: evidence of impaired neuroprotection. J. Affect. Disord. 98, 143–151.

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.

O'Brien, S.M., Scully, P., Dinan, T.G., 2008. Increased tumor necrosis factor-alpha concentrations with interleukin-4 concentrations in exacerbations of schizophrenia. Psychiatry Res. 160, 256–262.

Okuno, A., Fukuwatari, T., Shibata, K., 2011. High tryptophan diet reduces extracellular dopamine release via kynurenic acid production in rat striatum. J. Neurochem. 118, 796–805.

Pauly, J.R., Slotkin, T.A., 2008. Maternal tobacco smoking, nicotine replacement and neurobehavioural development. Acta. Paediatr. 97, 1331–1337.

Pittaluga, A., Pattarini, R., Feligioni, M., Raiteri, M., 2001. N-Methyl-d-aspartate receptors mediating hippocampal noradrenaline and striatal dopamine release display differential sensitivity to quinolinic acid, the HIV-1 envelope protein gp120, external pH and protein kinase C inhibition. J. Neurochem. 76, 139–148.

Powell, S.B., Sejnowski, T.J., Behrens, M.M., 2012. Behavioral and neurochemical consequences of cortical oxidative stress on parvalbumin-interneuron maturation in rodent models of schizophrenia. Neuropharmacology 62, 1322–1331.

Riehemann, S., Volz, H., Stützer, P., Smesny, S., Gaser, C., Sauer, H., 2001. Hypofrontality in neuroleptic-naive schizophrenic patients during the Wisconsin card sorting test – a fMRI study. Eur. Arch. Psychiatry Clin. Neurosci. 251, 66–71.

Rubio-Perez, J.M., Morillas-Ruiz, J.M., 2012. A review: inflammatory process in Alzheimer's disease, role of cytokines. Sci. World J. 2012, 756357.

Russell, T.A., Rubia, K., Bullmore, E.T., Soni, W., Suckling, J., Brammer, M.J., et al., 2000. Exploring the social brain in schizophrenia: left prefrontal underactivation during mental state attribution. Am. J. Psychiatry 157, 2040–2042.

Sandhir, R., Sood, A., Mehrotra, A., Kamboj, S.S., 2012. N-Acetylcysteine reverses mitochondrial dysfunctions and behavioral abnormalities in 3-nitropropionic acid-induced Huntington's disease. Neurodegener. Dis. 9, 145–157.

Scaglia, F., 2010. The role of mitochondrial dysfunction in psychiatric disease. Dev. Disabil. Res. Rev. 16, 136–143.

Schiavone, S., Jaquet, V., Sorce, S., Dubois-Dauphin, M., Hultqvist, M., Bäckdahl, L., et al., 2012. NADPH oxidase elevations in pyramidal neurons drive psychosocial stress-induced neuropathology. Transl. Psychiatry 2, e111.

Schiavone, S., Sorce, S., Dubois-Dauphin, M., Jaquet, V., Colaianna, M., Zotti, M., Cuomo, V., Trabace, L., Krause, K.H., 2009. Involvement of NOX2 in the development of behavioral and pathologic alterations in isolated rats. Biol. Psychiatry 66, 384–392.

Schwartz, T.L., Sachdeva, S., Stahl, S.M., 2012. Glutamate neurocircuitry: theoretical underpinnings in Schizophrenia. Front. Pharmacol. 3, 195. http://dx.doi.org/10.3389/fphar.2012.00195.

Please cite this article in press as: Möller, M., et al. Social isolation rearing induces mitochondrial, immunological, neurochemical and behavioural deficits in rats, and is reversed by clozapine or N-acetyl cysteine. Brain Behav. Immun. (2013), http://dx.doi.org/10.1016/j.bbi.2012.12.011

Schultz, S., North, S., Shields, C., 2007. Schizophrenia: a review. Am. Fam. Physician 75, 1821–1829.
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.
Selten, J.P., Cantor-Graae, E., 2005. Social defeat: risk factor for schizophrenia? Br. J. Psychiatry 2005 (187), 101–102.
Singh, S., Greene, R.M., Pisano, M.M., 2010. Arsenate-induced apoptosis in murine embryonic maxillary mesenchymal cells via mitochondrial-mediated oxidative injury. Birth Defects Res. A Clin. Mol. Teratol. 88, 25–34.
Smith, R.A., Hartley, R.C., Cochemé, H.M., Murphy, M.P., 2012. Mitochondrial pharmacology. Trends Pharmacol. Sci. 33, 341–352.
Sorce, S., Schiavone, S., Tucci, P., Colaianna, M., Jaquet, V., Cuomo, V., et al., 2010. The NADPH oxidase NOX2 controls glutamate release: a novel mechanism involved in psychosis-like ketamine responses. J. Neurosci. 30, 11317–11325.
Sorensen, H.J., Mortensen, E.L., Reinisch, J.M., Mednick, S.A., 2009. Association between prenatal exposure to bacterial infection and risk of schizophrenia. Schizophr. Bull. 35, 631–637.
Stone, T.W., Darlington, L.G., 2002. Endogenous kynurenines as targets for drug discovery and development. Nat. Rev. Drug Discov. 1, 609.
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. Neuro-Psychopharmacol. Biol. Psychiatry 33, 303–307.
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. Neuroscience 165, 492–499.
Van den Buuse, M., Eikelis, N., 2001. Estrogen increases prepulse inhibition of acoustic startle in rats. Eur. J. Pharmacol. 425, 33–41.
Ventura, R., Pascucci, T., Catania, M.V., Musumeci, S.A., Puglisi-Allegra, S., 2004. Object recognition impairment in Fmr1 knockout mice is reversed by amphetamine: involvement of dopamine in the medial prefrontal cortex. Behav. Pharmacol. 15, 433–442.
Volz, H.R., Riehemann, S., Maurer, I., Smesny, S., Sommer, M., Rzanny, R., et al., 2000. Reduced phosphodiesters and high-energy phosphates in the frontal lobe of schizophrenic patients: a 31P chemical shift spectroscopic-imaging study. Biol. Psychiatry 47, 954–961.
Vuillermot, S., Weber, L., Feldon, J., Meyer, U., 2010. A longitudinal examination of the neurodevelopmental impact of prenatal immune activation in mice reveals primary defects in dopaminergic development relevant to schizophrenia. J. Neurosci. 30, 1270–1287.
Wang, Y., Lawson, M.A., Dantzer, R., Kelley, K.W., 2010. LPS-induced indoleamine 2,3-dioxygenase is regulated in an interferon-γ-independent manner by a JNK signaling pathway in primary murine microglia. Brain Behav. Immun. 24, 201–209.
Wonodi, I., Schwarcz, R., 2010. Cortical kynurenine pathway metabolism: a novel target for cognitive enhancement in schizophrenia. Schizophr. Bull. 36, 211–218.
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.
Yacubian, J., de Castro, C.C., Ometto, M., Barbosa, E., de Camargo, C.P., Tavares, H., 2002. ^{31}P-spectroscopy of frontal lobe in schizophrenia: alterations in phospholipid and high-energy phosphate metabolism. Schizophr. Res. 58, 117–122.
Zalcman, S., Green-Johnson, J.M., Murray, L., Nance, D.M., Dyck, D., Anisman, H., Greenberg, A.H., 1994. Cytokine-specific central monoamine alterations induced by interleukin-1, -2 and -6. Brain Res. 643, 40–49.
Zhang, J., Forkstam, C., Engel, J.A., Svensson, L., 2000. Role of dopamine in prepulse inhibition of acoustic startle. Psychopharmacology 149, 181–188.
Zhang, W., Pouzet, B., Jongen-Rêlo, A., 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. NeuroReport 10, 1–6.

Please cite this article in press as: Möller, M., et al. Social isolation rearing induces mitochondrial, immunological, neurochemical and behavioural deficits in rats, and is reversed by clozapine or N-acetyl cysteine. Brain Behav. Immun. (2013), http://dx.doi.org/10.1016/j.bbi.2012.12.011

Supplement 1, Möller et al., 2012

1. Drugs and drug treatment protocol

Clozapine (Pharmaplan, Johannesburg, South Africa), dissolved in 1M glacial acetic acid and buffered with sodium hydroxide (NaOH) (pH = 6.0), was administered as a single daily intraperitoneally (i.p) dose of 5 mg/kg/0.5 ml /day (Toua et al., 2010; Möller et al., 2011). NAC (Sigma-Aldrich, Johannesburg, South Africa), dissolved in saline and buffered with 1M glacial acetic acid and NaOH (pH = 6.0), was administered i.p over a dose range of 50, 150, 250 mg/kg/0.5ml/day (Fukami et al., 2004; Khan et al., 2004; Chen et al., 2010). Control groups received an equivalent volume of vehicle i.p., comprising saline and 1 M glacial acetic acid and buffered with sodium hydroxide (NaOH) (pH = 6.0). Clozapine, NAC and vehicle was administered between 06-08h00 in the last 14 days of social/SIR rearing. All animals were sacrificed by decapitation without any prior use of an anaesthetic agent.

2. Socially reared cohort

This group was used to evaluate the effects of the various drug treatments in healthy animals.

Behavioural studies: The animals were randomly separated at weaning (21 days post-natal) into 7 groups of socially reared rats (3-4 rats in a cage). One group of animals (n = 10) received no treatment, while the remaining 6 groups (n = 10) received either of the following treatments: vehicle, NAC (50, 150, 250 mg/kg), clozapine (5 mg/kg) or a combination of clozapine (5 mg/kg) and NAC (150 mg/kg)

(CLZ + NAC), respectively. A NAC dose of 150 mg/kg was found in the SIR cohort (see below) to be best suited for use in the main manuscript (see manuscript for further explanation). After 8 weeks of SIR/group rearing, social interaction, novel object recognition and sensorimotor gating (see below) were performed on days 12, 13 and 14, respectively, of the drug treatment period (18-20h00). All behavioural assessments were undertaken at least 12 hours post-drug administration.

Immune-neurochemistry study: A separate 7 groups of animals (n=10/group), randomly assigned to the same groupings as described above, followed the above protocol except that at 8 weeks the animals were sacrificed, trunk blood collected for tryptophan metabolism and cytokine analysis and brains (frontal cortex and striatum) dissected for analysis of mitochondrial function and dopamine (DA) levels (see below).

3. SIR cohort

This group was used to determine a suitable dose for NAC for application in the main study (see manuscript).

The SIR cohort was divided into 4 groups for the respective behavioural and immune-neurochemistry studies as outlined above, viz. SIR + vehicle, SIR + NAC (50, 150 and 250 mg/kg). On PND 21 the rats were place in isolation (1 rat in a cage) for 8 weeks. The NAC dose response study was used to determine an appropriate dose of NAC for the clozapine (5 mg/kg) plus NAC combination in SIR animals presented in the main study (see manuscript) as well as in the drug-treated social control groups described here. Our findings established NAC 150 mg/kg as the preferred dose.

4. Behavioural tests

Social interaction test: The social interaction was performed as previously described by Möller et al., (2011). Briefly, two rats were placed in the centre of an open field arena, illuminated with red light (40 W), and allowed 30 s for adaptation. Thereafter, time spent in active social interactive behaviours, namely rearing, anogenital sniffing, approaching and staying with the partner, as well as self-directed behaviour, including squares crossed and self-grooming (Gonzalez et al., 1996; Möller et al., 2011), were scored for 10 min. Both members of a test-pair had received the same treatment and the same prior experience. Testing was performed between 18h00 and 20h00, with video scoring based on averaged scores by two blind independent observers who remained outside the testing room.

Object recognition test (ORT): The test was performed as described previously (Grayson et al., 2007; McLean et al., 2010). Briefly, the rats were exposed to a 5-min habituation session in a Plexiglas ORT box (70cm x 70cm x 40cm), followed by the experimental trials where each rat was exposed to two identical objects (right and left object) for a period of 5 min (acquisition trial). The rats were then returned to their home cage for a 1.5 hr inter-trial interval. The entire box was cleaned, both objects removed and one replaced with an identical familiar copy and one with a novel unfamiliar object. Following the 1.5 hr inter-trial interval, rats were returned to explore the familiar and novel object in the test box for a 5-min retention trial. All experiments were filmed and recorded for subsequent behavioural analysis. The total exploration time of both objects in the retention trial (familiar and novel object respectively) were calculated.

Prepulse inhibition test: Prepulse inhibition (PPI) was assessed, as described previously (Van den Buuse and Eikelis, 2001; Möller et al., 2011). Briefly, 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. Percent PPI (% PPI) for the four prepulse intensities was calculated according to the formula: % PPI = [100−(startle response for PREPULSE+PULSE trial)/(startle response for PULSE ALONE trial)×100] (Van den Buuse and Eikelis, 2001).

5. Peripheral immune-neurochemistry assays

After decapitation, 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 that day plasma samples were thawed on ice, centrifuged again, as mentioned above, and the plasma used.

Cytokine analysis: Plasma anti-inflammatory cytokines (IL-4), pro-inflammatory cytokines (TNF-α, INF-γ) and multipurpose cytokine (IL-6), were measured in duplicate by sandwich enzyme-linked immunosorbent assay (ELISA) kits according to the manufacture's protocol (Invitrogen ELISA kit, Camarillo, CA). The sensitivity of the assay allowed the detection of cytokine concentrations within the following ranges: IL-6 1.5–1500 pg/mL; IL-4 0.2-100 pg/mL; IFN-γ 11.5-1400 pg/mL; TNF-α 3.5–1000 pg/mL. The final concentration of each cytokine was expressed as pg/mL of plasma used.

Analysis of tryptophan-kynurenine metabolism: Plasma samples were pre-purified by a solid phase extraction (SPE) method followed by detection using a novel liquid chromatography-tandem mass spectrometry (LC-MS/MS) procedure developed and validated in our laboratory (Möller et al., 2012a). Kynurenine, KYNA and QA were quantified, and plasma KYNA:kynurenine ratio used to determine the neuroprotective ratio (Myint et al., 2007; Myint et al., 2011). The neuroprotective ratio is hypothesized to relate to the eventual down-stream levels of QA following a shift in the relative levels of KYNA and kynurenine (Möller et al., 2012b). Neuroprotective ratio: [1000 x plasma kynurenic acid (mM)]/plasma kynurenine (mM).

6. Brain analysis

The frontal cortex was immediately dissected after decapitation, as described previously (Toua et al., 2010; Möller et al., 2011). The brain was then placed on its ventral side, the two cerebral hemispheres split and the striatum dissected out using the external walls of the lateral ventricles as internal limits and the corpus callosum as external limits. These brain regions were immediately fixed in liquid nitrogen and stored at −80°C.

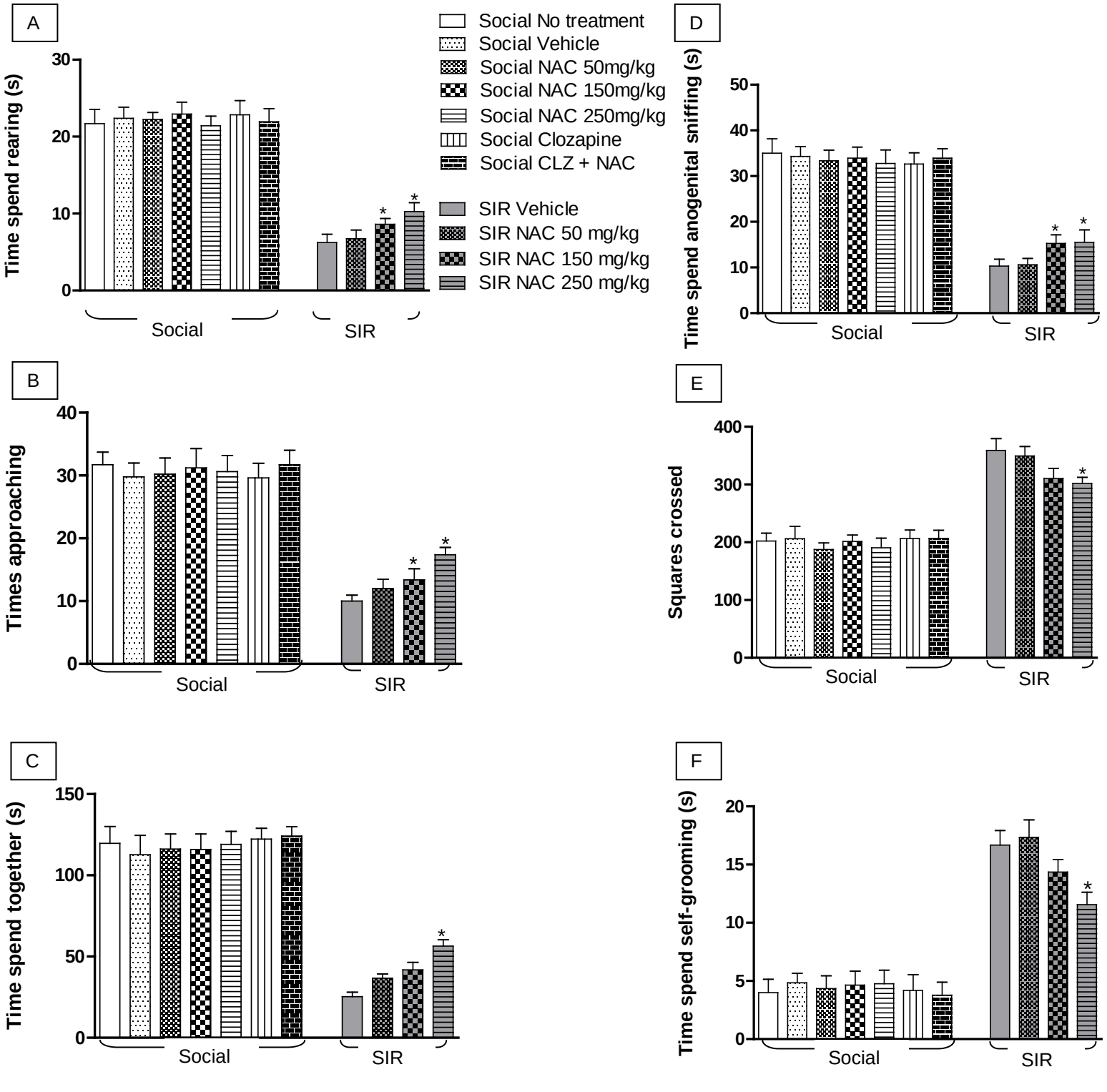
Cortico-striatal ATP analysis: ATP accumulation was determined in the frontal cortex and striatum tissue using a commercially available bioluminescence assay (Molecular Probes ATP Determination Kit; A22066) according to the manufacturer's instructions (Invitrogen ATP kit, Paisley, UK). ATP is determined with recombinant firefly luciferase and its substrate D-luciferin, with the concentration of ATP in each brain region expressed as nmol/mg protein.

Cortico-striatal DA analysis: Quantification of cortico-striatal DA was performed using a HPLC system with electrochemical detection (HPLC-EC) (Harvey et al., 2006). DA concentration was expressed as ng/mg wet weight of tissue (mean ± SEM).

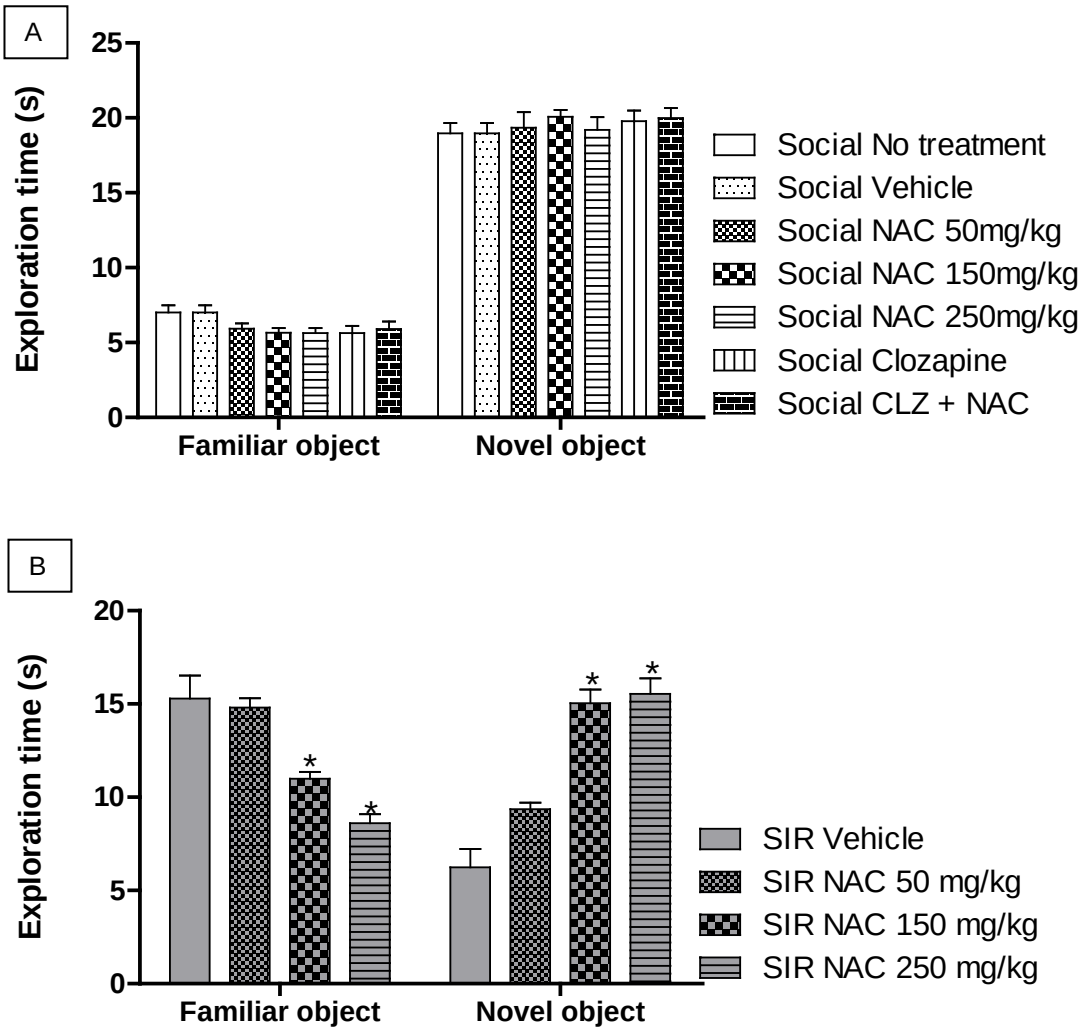
7. Statistical analysis

To model the behavioural and immune-neurochemical measurements respectively, a three-way factorial ANOVA and Bonferroni post-hoc tests was applied for the respective treatments (vehicle, clozapine, NAC, clozapine + NAC), rearing conditions (social vs. SIR) and either (a) behavioural parameter, either social interaction, object recognition or prepulse intensity (repeated measures was used for the startle amplitude and different PPI intensities) or (b) immune-inflammatory parameter (cytokines or kynurenine metabolites) or (c) cortico-striatal parameters (ATP, DA). In all cases, data are expressed as the mean ± standard error of the mean (SEM), with a p value of <0.05 deemed statistically significant (Graphpad Prism 5; SAS/STAT® Software).

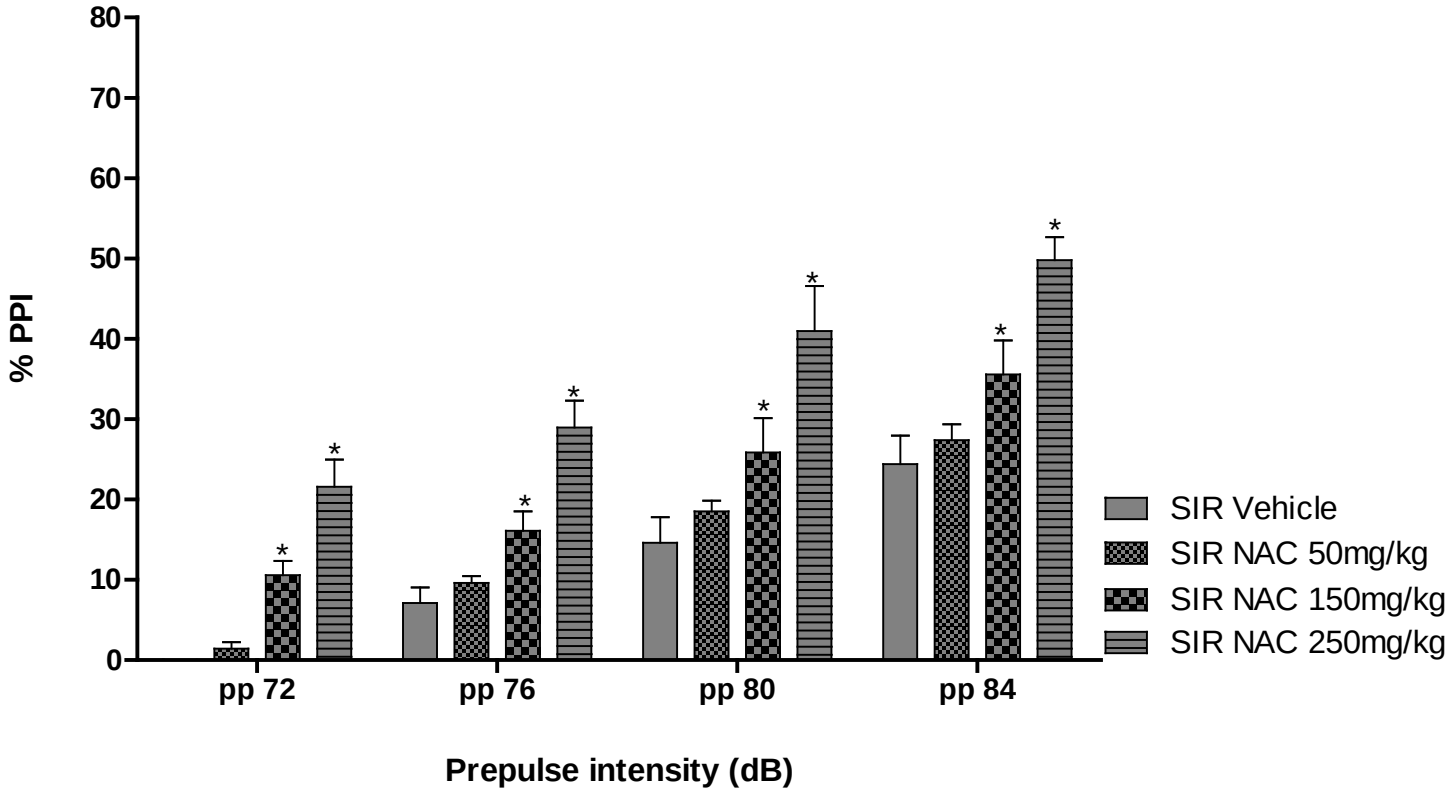
Supplementary Fig. 1, Möller et al., 2012



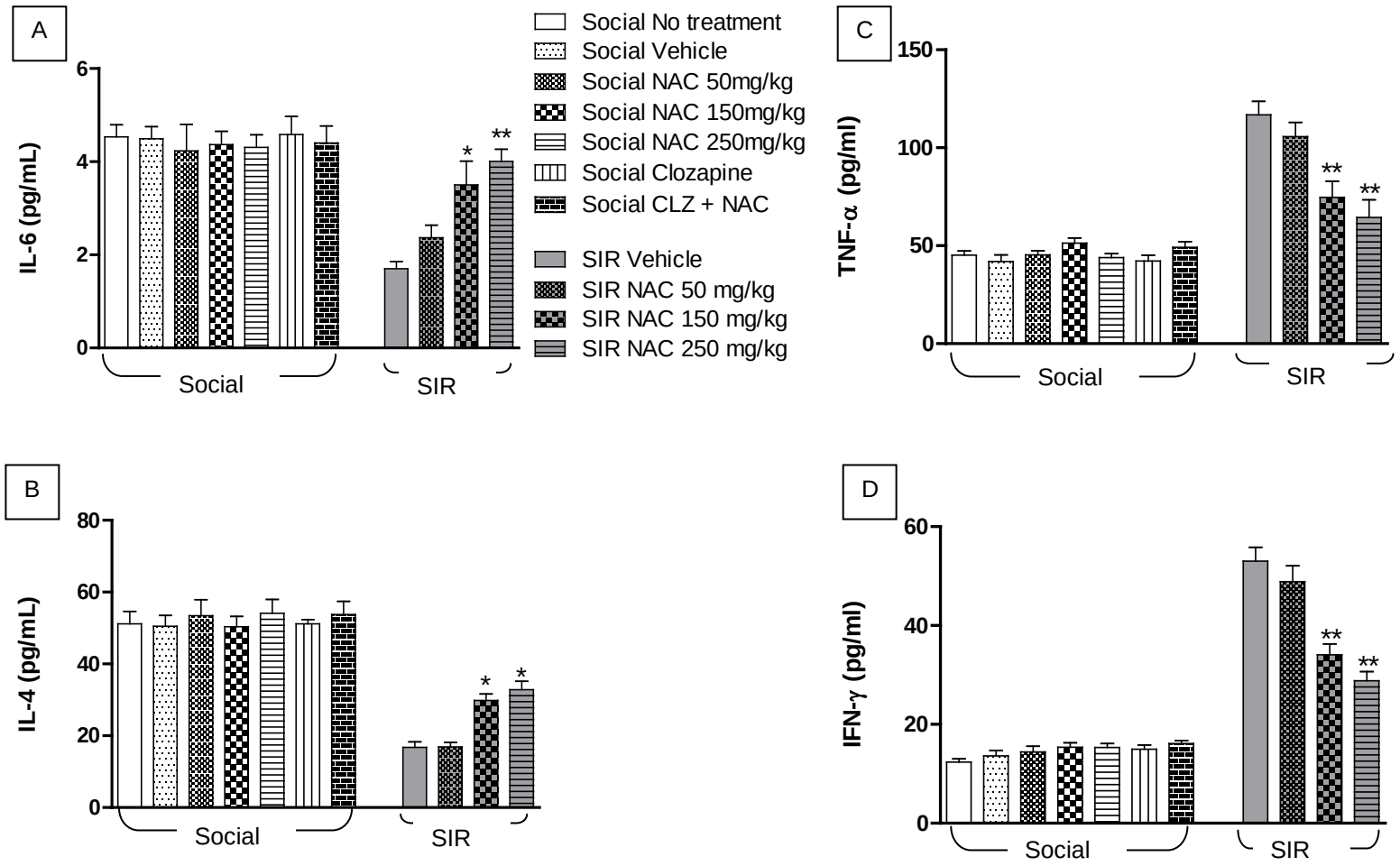
Supplementary Fig. 2, Möller et al., 2012



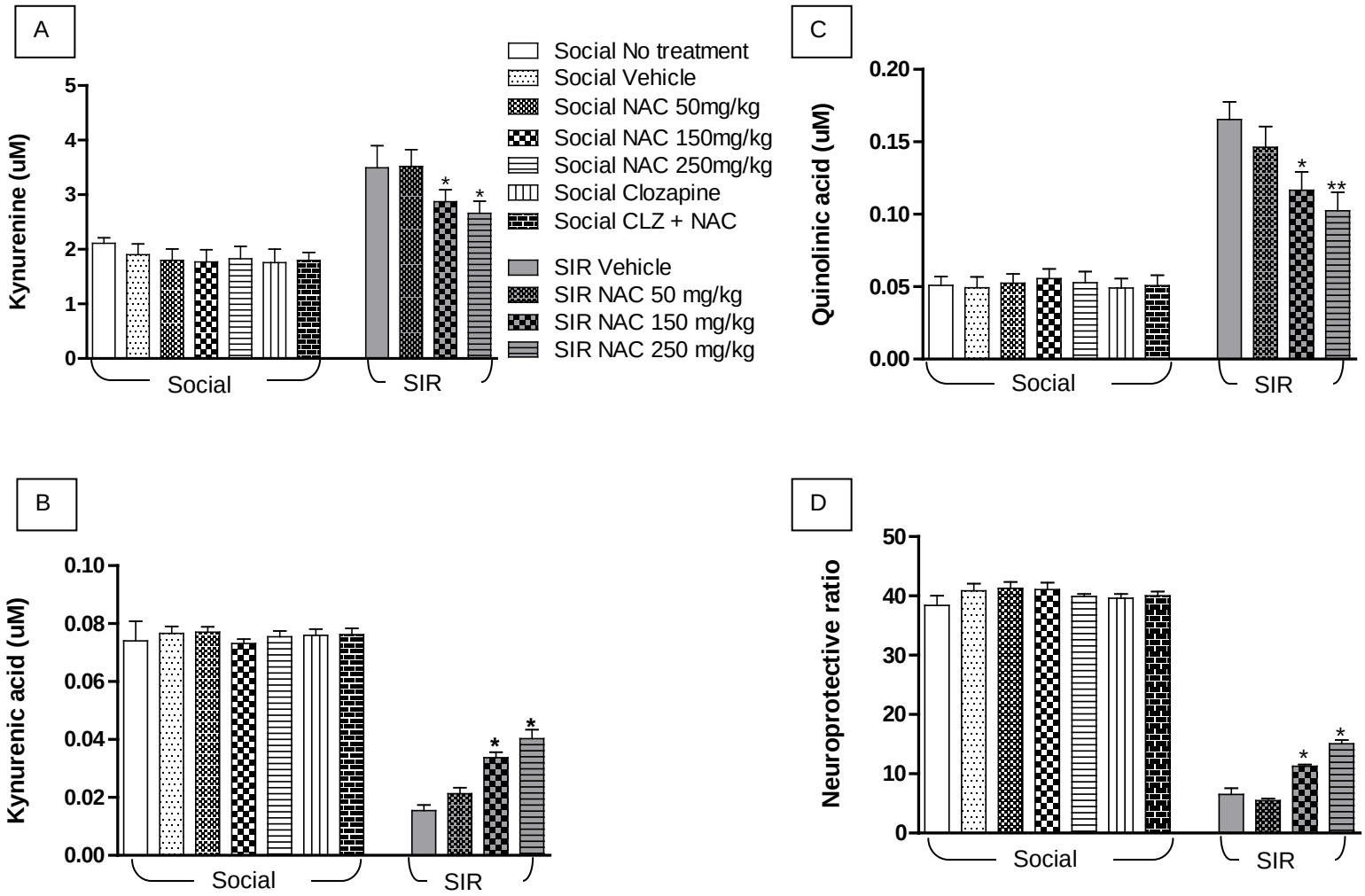
Supplementary Fig. 3, Möller et al., 2012



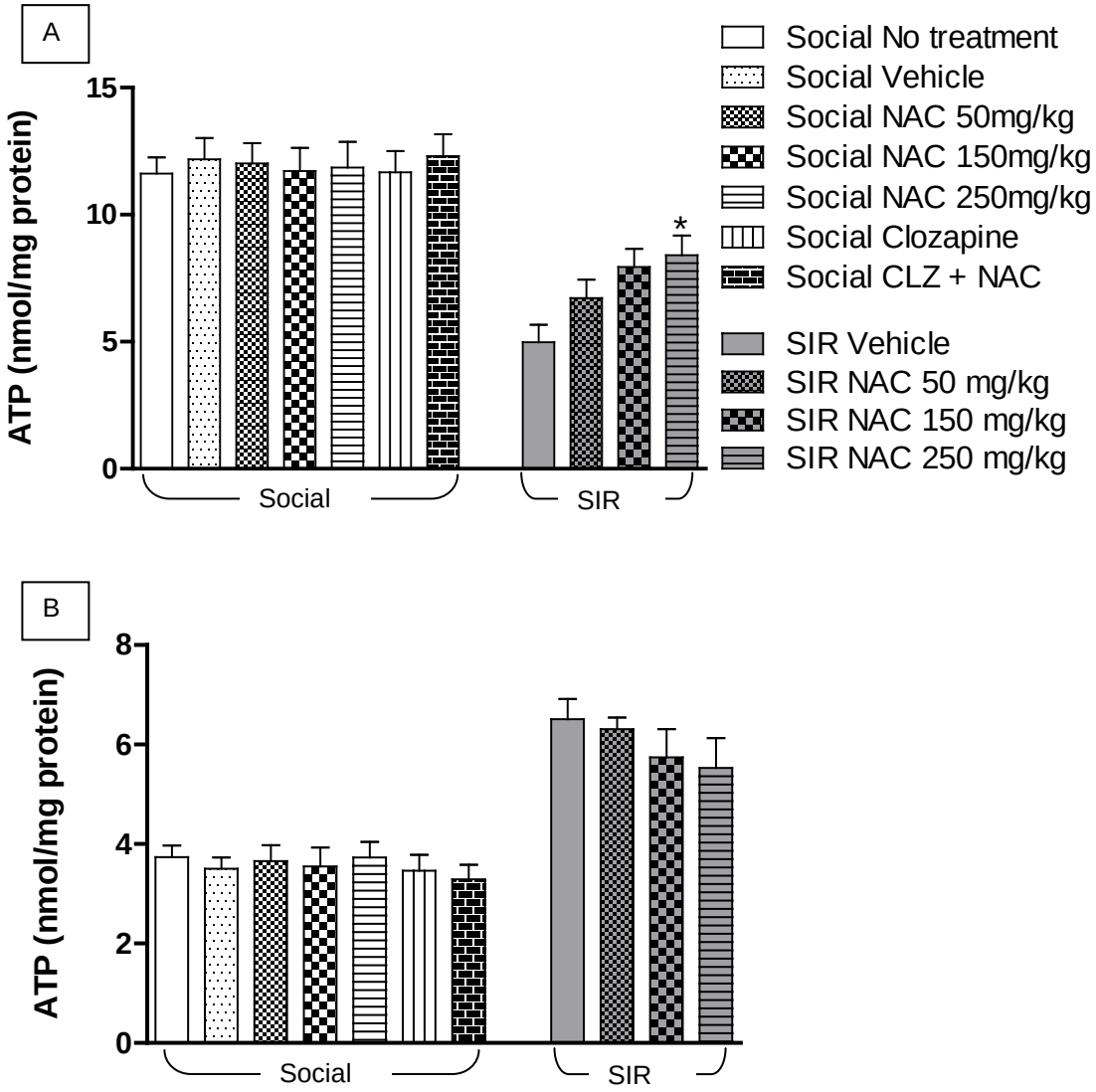
Supplementary Fig. 4, Möller et al., 2012



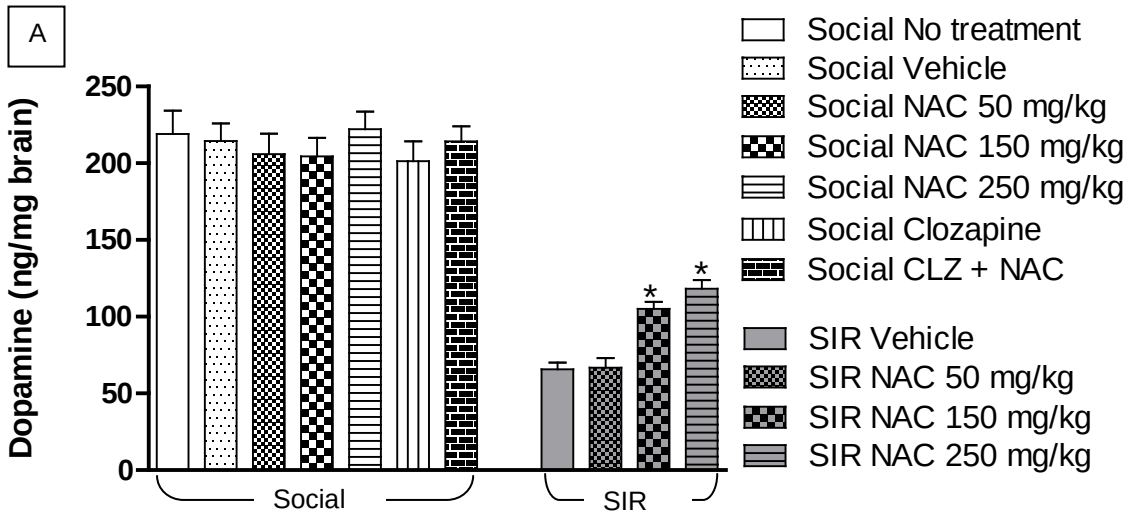
Supplementary Fig. 5, Möller et al., 2012

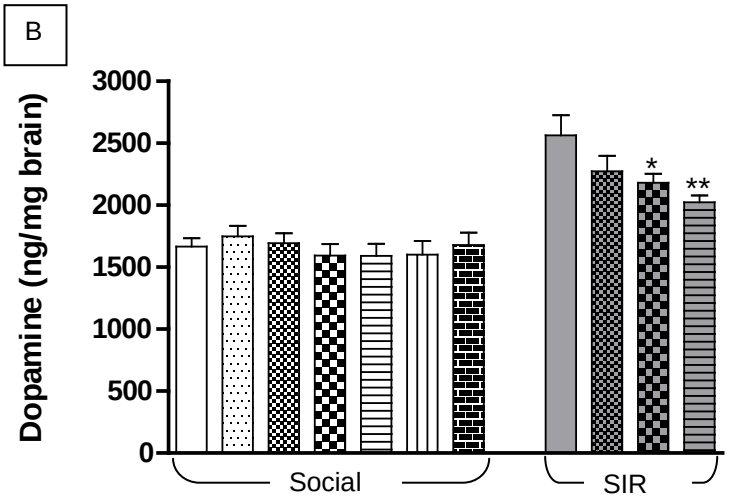


Supplementary Fig. 6, Möller et al., 2012



Supplementary Fig. 7, Möller et al., 2012





Supplementary Figure Legends, Möller et al., 2012

Supplementary Fig. 1 Social interactive and self-directed behaviours in socially reared rats following the various drug treatments, and SIR rats following the NAC dose response study, as indicated (n = 10/group): (A) rearing; (B) approaching; (C) time together; (D) anogenital sniffing; (E) squares crossed; (F) self-grooming. A SIR vehicle treatment group is included for statistical comparison in the NAC dose response study. Treatment had a significant main effect with respect to (A) rearing (F (10, 99) = 17.41, $p < 0.0001$); (B) approaching (F (10, 99) = 41.51, $p < 0.0001$); (C) time together (F (10, 99) = 51.4, $p < 0.0001$); (D) anogenital sniffing (F (10,99) = 18.19, $p < 0.0001$); (E) squares crossed (F (10,99) = 22.36, $p < 0.0001$); (F) self-grooming (F (10,99) = 12.70 $p < 0.0001$) (3-way ANOVA). Rearing also indicated a significant main effect with respect to (A) rearing (F (10, 99) = 41.50, $p < 0.0001$); (B) approaching (F (10, 99) = 29.67, $p < 0.0001$); (C) time together (F (10, 99) = 41.93, $p < 0.0001$); (D) anogenital sniffing (F (10,99) = 31.96, $p < 0.0001$); (E) squares crossed (F (10,99) = 14.49, $p < 0.0001$); (F) self-grooming (F (10,99) = 26.53 $p < 0.0001$) (3-way ANOVA). Significant cross-group interactions were observed with regards to (A) rearing (F (10, 99) = 27.86, $p < 0.0001$); (B) approaching (F (10, 99) = 17.94, $p < 0.0001$); (C) time together (F (10, 99) = 29.03, $p < 0.0001$); (D) anogenital sniffing (F (10,99) = 20.71, $p < 0.0001$); (E) squares crossed (F (10,99) = 18.82, $p < 0.0001$); (F) self-grooming (F (10,99) = 22,27 $p < 0.0001$) (3-way ANOVA). SIR induced a significant ($p < 0.0001$) decrease in rearing (A), approaching (B), time together (C), anogenital sniffing (D), and a significant ($p < 0.0001$) increase in squares crossed (E) and self-grooming (F), vs. social vehicle. No significant treatment differences were observed between the socially reared groups (A-F)

(Bonferroni post hoc test). In the SIR treatment groups, NAC 150 mg/kg significantly attenuated alterations in (A) rearing ($p = 0.04$), (B) times approaching ($p = 0.02$) and (D) anogenital sniffing ($p = 0.03$); NAC 250 mg/kg significantly reversed alterations in (A) rearing ($p = 0.008$), (B) times approaching ($p = 0.02$), (C) time together ($p = 0.01$), (D) anogenital sniffing ($p = 0.03$), (E) squares crossed ($p = 0.02$) and (F) self-grooming ($p = 0.002$). *$p < 0.05$ vs. SIR vehicle treatment (Bonferroni post-hoc test).

Supplementary Fig. 2 Retention trial of the object recognition test in (A) socially reared rats following the various drug treatments, and (B) SIR rats following the NAC dose response study, as indicated (n = 10/group). A SIR vehicle treatment group is included for statistical comparison in the NAC dose response study. Treatment had a significant main effect with respect to visual recognition memory in (A) the socially reared rats (F (10, 98) = 20.34, $p = 0.0039$) and in (B) the SIR rats (F (3, 72) = 30.78, p = 0.0032) (3-way ANOVA). Rearing also indicated a significant main effect with respect to visual recognition memory in (A) the socially reared rats (F (10, 98) = 83.29, $p < 0.0001$) and in (B) the SIR rats (F (3, 72) = 28.11, p = 0.0098) (3-way ANOVA). Significant cross-group interactions were observed with respect to visual recognition memory in (A) the socially reared rats (F (10, 98) = 78.70, $p < 0.0001$) and in (B) the SIR rats (F (3, 72) = 51.65, p < 0.0001) (3-way ANOVA). No significant treatment differences were observed in (A) the socially reared groups (Bonferroni post hoc test). NAC 150 mg/kg and NAC 250 mg/kg significantly ($p < 0.0001$) reversed the visual recognition memory deficits in (B) the SIR group. *$p < 0.0001$ vs. SIR vehicle treatment (Bonferroni post-hoc test).

Supplementary Fig. 3 Sensorimotor gating at various prepulse intensities in SIR rats, following the NAC dose response study, as indicated (n = 10/group), presented as percent prepulse inhibition (%PPI). A SIR vehicle treatment group at each

respective prepulse is included for statistical comparison. A significant main effect of treatment (F (3, 9) = 33.91, $p < 0.0001$) and rearing (F (3, 9) = 38.12, $p < 0.0001$) was observed in % PPI (3-way ANOVA). Significant cross-group interactions were observed in % PPI (F (3, 9) = 45.1, $p < 0.0001$) (3-way ANOVA). NAC 150 mg/kg ($p < 0.001$) and NAC 250 mg/kg ($p < 0.001$) significantly reversed the PPI deficit observed vs. SIR vehicle treatment group, at each prepulse inhibition intensity. *$p < 0.001$ vs. SIR vehicle treatment at 72 dB, 76 dB, 80 dB and 86 dB respectively (Bonferroni post-hoc test).

Supplementary Fig. 4 Pro and anti-inflammatory cytokine concentrations, (A) IL-6; (B) IL-4; (C) TNF-α; (D) IFN-γ in socially reared rats following the various drug treatments, and in SIR rats following the NAC dose response study, as indicated (n = 10/group). A SIR vehicle treatment group is included in the NAC dose response study for statistical comparison. Treatment had a significant main effect with respect to (A) IL-6 (F (10,99) = 9.31, $p < 0.0001$); (B) IL-4 (F (10,99) = 22.24, $p < 0.0001$); (C) TNF-α (F (10,99) = 38.70, $p < 0.0001$); (D) IFN-γ (F (10,99) = 70.75, $p < 0.0001$) (3-way ANOVA). Rearing also indicated a significant main effect with respect to (A) IL-6 (F (10,99) = 12.08, $p < 0.0001$); (B) IL-4 (F (10,99) = 26.78, $p < 0.0001$); (C) TNF-α (F (10,99) = 27.13, $p < 0.0001$); (D) IFN-γ (F (10,99) = 75.22, $p < 0.0001$) (3-way ANOVA). Significant cross-group interactions were observed with respect to (A) IL-6 (F (10,99) = 7.71, $p < 0.0001$); (B) IL-4 (F (10,99) = 26.58, $p < 0.0001$); (C) TNF-α (F (10,99) = 25.94, $p < 0.0001$); (D) IFN-γ (F (10,99) = 76.93, $p < 0.0001$) (3-way ANOVA). No significant treatment changes on IL-6, IL-4, TNF-α and IFN-γ (A-D), were observed in the socially reared groups (Bonferroni post hoc). SIR induced a significant ($p < 0.0001$) decrease in IL-6 (A) and IL-4 (B), and a significant ($p < 0.0001$) increase in TNF-α (C) and IFN-γ (D) vs. social vehicle. SIR-induced deficits

in: (A) IL-6, was partially reversed by NAC 150 mg/kg (p = 0.03), and fully reversed by NAC 250 mg/kg (p < 0.0001); (B) IL-4 was partially reversed by NAC 150 mg/kg (p = 0.04) and NAC 250 mg/kg (p = 0.009). SIR-induced elevations in: (C) TNF-α was fully reversed by NAC 150 mg/kg and NAC 250 mg/kg (p < 0.0001); (D) IFN-γ was fully reversed by NAC 150 mg/kg and NAC 250 mg/kg (p < 0.0001). *p < 0.05 vs. SIR vehicle treatment, **p < 0.0001 vs. SIR vehicle treatment (Bonferroni post-hoc test).

Supplementary Fig. 5 Kynurenine pathway metabolites in socially reared rats following the various drug treatments, and in SIR rats following the NAC dose response study, as indicated (n = 10/group): (A) kynurenine; (B) KYNA; (C) QA; (D) neuroprotective ratio. A SIR vehicle treatment group is included for statistical comparison in the NAC dose response study. Treatment had a significant main effect with respect to (A) kynurenine (F (10, 99) = 8.90, p < 0.0001); (B) KYNA (F (10, 99) = 74.68, p < 0.0001); (C) QA (F (10, 99) = 27.26, p < 0.0001); (D) neuroprotective ratio (F (10, 99) = 292.40, p < 0.0001) (3-way ANOVA). Rearing also indicated a significant main effect with respect to (A) kynurenine (F (10, 99) = 5.22, p < 0.0001); (B) KYNA (F (10, 99) = 75.32, p < 0.0001); (C) QA (F (10, 99) = 22.92, p < 0.0001); (D) neuroprotective ratio (F (10, 99) = 271.9, p < 0.0001) (3-way ANOVA). Significant cross-group interactions were observed with respect to (A) kynurenine (F (10, 99) = 8.54, p < 0.0001); (B) KYNA (F (10, 99) = 75.67, p < 0.0001); (C) QA (F (10, 99) = 20.92, p < 0.0001); (D) neuroprotective ratio (F (10, 99) = 284.8, p < 0.0001) (3-way ANOVA). No significant drug effects were evident in the socially reared treatment groups (A-D) (Bonferroni post hoc test). SIR induced a significant (p < 0.0001) increase in kynurenine (A) and QA (C), and a significant (p < 0.0001) decrease in KYNA (B) and neuroprotective ratio (D) vs. social vehicle. The SIR-

induced elevations in kynurenine (A) was partially reversed by NAC 150 mg/kg (p = 0.04) and NAC 250 mg/kg (p = 0.03); in QA (C), was partially reversed by NAC 150 mg/kg (p = 0.002) and fully reversed by NAC 250 mg/kg (p < 0.0001). SIR-induced deficits in KYNA (B), was partially reversed by NAC 150 mg/kg (p = 0.03) and NAC 250 mg/kg (p = 0.001); in neuroprotective ratio (D), partially reversed by NAC 150 mg/kg (p = 0.04) and NAC 250 mg/kg (p = 0.02). *p < 0.05 vs. SIR vehicle treatment, **p < 0.0001 vs. SIR vehicle treatment. (Bonferroni post-hoc test).

Supplementary Fig. 6 ATP levels in (A) the frontal cortex and (B) striatum in socially reared rats following the various drug treatments, and in SIR rats following the NAC dose response study, as indicated (n = 10/group). A SIR vehicle treatment group is included for statistical comparison in the NAC dose response study. Treatment had a significant main effect in (A) the frontal cortex (F (10, 99) = 12.70, p < 0.0001) and (B) the striatum (F (10, 99) = 12.49, p < 0.0001) (3-way ANOVA). Rearing also indicated a significant main effect in (A) the frontal cortex (F (10, 99) = 14.97, p < 0.0001) and (B) the striatum (F (10, 99) = 14.24, p < 0.0001) (3-way ANOVA). Significant cross-group interactions were observed in (A) the frontal cortex (F (10, 99) = 10.51, p < 0.0001) and (B) the striatum (F (10, 99) = 11.63, p < 0.0001) (3-way ANOVA). SIR induced a significant (p < 0.0001) decrease in frontal cortex ATP (A) and a significant (p < 0.0001) increase in striatal ATP (B) vs. social vehicle. No significant treatment changes were observed in (A) and (B) in the socially reared treatment groups (Bonferroni post hoc test). NAC 250 mg/kg partially reversed (p = 0.01) the SIR-induced ATP deficits in (A) the frontal cortex, however, no significant differences were observed in (B) the striatum between the SIR treatment groups. *p < 0.05 vs. SIR vehicle treatment (Bonferroni post-hoc test).

Supplementary Fig. 7 DA in (A) the frontal cortex and (B) the striatum in socially reared rats following the various drug treatments, and in SIR rats following the NAC dose response study, as indicated (n = 10/group). A SIR vehicle treatment group is included for statistical comparison in the NAC dose response study. Treatment had a significant main effect in the frontal cortex (A) (F (10, 99) = 34.60, $p < 0.0001$) and in the striatum (B) (F (10, 99) = 12.20, $p < 0.0001$) (3-way ANOVA). Rearing also indicated a significant main effect in the frontal cortex (A) (F (10, 99) = 38.64, $p < 0.0001$) and in the striatum (B) F (10, 99) = 18.12, $p < 0.0001$) (3-way ANOVA). Significant cross-group interactions were observed: in the frontal cortex (A) F (10, 99) = 37.60, $p < 0.0001$) and in the striatum (B) F (10, 99) = 11.22, $p < 0.0001$) (3-way ANOVA). SIR induced a significant ($p < 0.0001$) decrease in frontal cortical DA (A) and a significant ($p < 0.0001$) increase in striatal DA (B) vs. social vehicle. No significant treatment changes of DA levels in (A) the frontal cortex and (B) the striatum were observed in the socially reared groups (Bonferroni post hoc test). In the frontal cortex (A), SIR-induced decrease in DA was partially reversed by NAC 150 mg/kg ($p = 0.04$) and NAC 250 mg/kg ($p < 0.008$). In the striatum (B), SIR-induced increases in DA was partially reversed by NAC 150 mg/kg ($p = 0.009$), and fully reversed by NAC 250 mg/kg ($p < 0.0001$). *$p < 0.05$ vs. SIR vehicle treatment, **$p < 0.0001$ vs. SIR vehicle treatment (Bonferroni post-hoc test).

Supplement 1, References

Chen, H., Stoker, A., Markou, A., 2010. The glutamatergic compounds sarcosine and *N* acetylcysteine ameliorate prepulse inhibition deficits in metabotropic glutamate 5 receptor knockout mice. Psychopharmacol 209, 343-350.

Fukami, G., Hashimoto, K., Koike, K., Okamura, N., Shimizu, E., Iyo, M., 2004. Effect of antioxidant N-acetyl-l-cysteine on behavioral changes and neurotoxicity in rats after administration of methamphetamine. Brain Res 1016, 90-95.

Gonzalez, L.E., Andrews, N., File, S.E., 1996. 5-HTIA and benzodiazepine receptors in the basolateral amygdala modulate anxiety in the social interaction test, but not in the elevated plus-maze. Brain Res. 732, 145-153.

Grayson, B., Idris, N.F., Neill, J.C., 2007. Atypical antipsychotics attenuate a sub-chronic PCP-induced cognitive deficit in the novel object recognition task in the rat. Behav Brain Res 184, 31-38.

Harvey, B.H., Brand, L., Jeeva, Z., Stein, D.J., 2006. Cortical/hippocampal monoamines, HPA-axis changes and aversive behavior following stress and restress in an animal model of post-traumatic stress disorder. Physiol. Behav 87, 881–890.

Khan, M., Sekhon, B., Jatana, M., Giri, S., Gilg, A. G., Sekhon, C., Singh, I., Singh, A. K., 2004. Administration of N-acetylcysteine after focal cerebral ischemia protects brain and reduces inflammation in a rat model of experimental stroke. J Neurosci Res 76, 519-527.

McLean, S., Grayson, B., Harris, M., Protheroe, C., Woolley, M., Neill, J., 2010. Isolation rearing impairs novel object recognition and attentional set shifting performance in female rats. J Psychopharmacology 24, 57-63.

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., 2012a. Development and validation of a single analytical method for the determination of tryptophan, and its kynurenine metabolites in rat plasma. J Chromatogr B 898, 121-129.

Möller, M., Du Preez, J. L., Emsley, R., Harvey, B. H., 2012b. Social isolation rearing in rats alters plasma tryptophan metabolism and is reversed by sub-chronic clozapine treatment. Neuropharmacol 62, 2499-2506.

Myint, A., Kim, Y. K., Verkerk, R., Scharpé, S., Steinbusch, H., Leonard, B., 2007. Kynurenine pathway in major depression: Evidence of impaired neuroprotection. J Affect Disorders 98, 143-151.

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.

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.

Van den Buuse, M., Eikelis, N., 2001. Estrogen increases prepulse inhibition of acoustic startle in rats. Eur J Pharmacol 425, 33-41.