

Addendum A

Additional Methodology and Results

In this Addendum, additional methodology and results from experiments not incorporated in the manuscripts (Chapters 3, 4, 5 & 6) are provided, such as method validation and results that were not considered appropriate for inclusion in an article. The experiments will be presented separately, with a short description of the aims, methods and results provided for each experiment, as well as a short discussion that indicates the significance of the data.

1. Evaluation of % prepulse inhibition (PPI) in socially reared controls and mean startle amplitude in SIR and socially reared animals, with selected drug treatments.

1.1 Aims

- Socially reared rats were used in all experiments throughout the study as controls for the social isolation reared (SIR) rats; the aim of this experiment was to establish that there are no drug effects on %PPI in the socially reared controls receiving selected drug treatments.
- The startle amplitude is a measure of reflexive responses that can be reliably elicited; these responses can be modulated by non-associative learning processes such as habituation and sensitization (Koch, 1999; Fendt and Yeomans, 2001). The aim was to evaluate if there are any drug effects in the SIR and socially reared rats on the habituation and sensitization of the startle amplitude in the PPI paradigm.

1.2 Methods

1.2.1 PPI and mean startle amplitude test

Animals used in the behavioural cohort, as described in Chapter 5 were used. After 8 weeks of either social rearing or SIR and 14 days of selected treatments (NAC 50, 150 or 250 mg/kg, clozapine 5 mg/kg or the combination of clozapine and NAC 150 mg/kg (CLZ + NAC)), described in Chapter 5, animals were subjected to the PPI test, as described in Chapter 5, to obtain the %PPI in the socially reared controls. The startle amplitude was calculated as follows: 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).

1.2.2 Statistical analysis

All statistical analysis were done by a three-way factorial ANOVA and Bonferroni post-hoc tests, applied for the respective treatments. In all cases, data are expressed as the mean $\pm$ standard error of the mean (SEM), with a p value of < 0.05 deemed statistically significant (Graphpad Prism 5; SAS/STAT® Software).

1.3 Results

No significant interaction on %PPI in socially reared animals for each of the 4 prepulse intensities (3-way ANOVA; $F(6, 18) = 0.10$, $p = 0.81$) were observed, as indicated in figure 1. As presented in figure 2A, there was also no significant interaction on mean startle amplitude between the socially reared treatment groups (3-way ANOVA; $F(6, 18) = 1.60$, $p = 0.06$), as well as no significant group interaction was evident on mean startle amplitude (3-way ANOVA; $F(6, 18) = 3.54$, $p = 0.07$) in the SIR rats, figure 2B

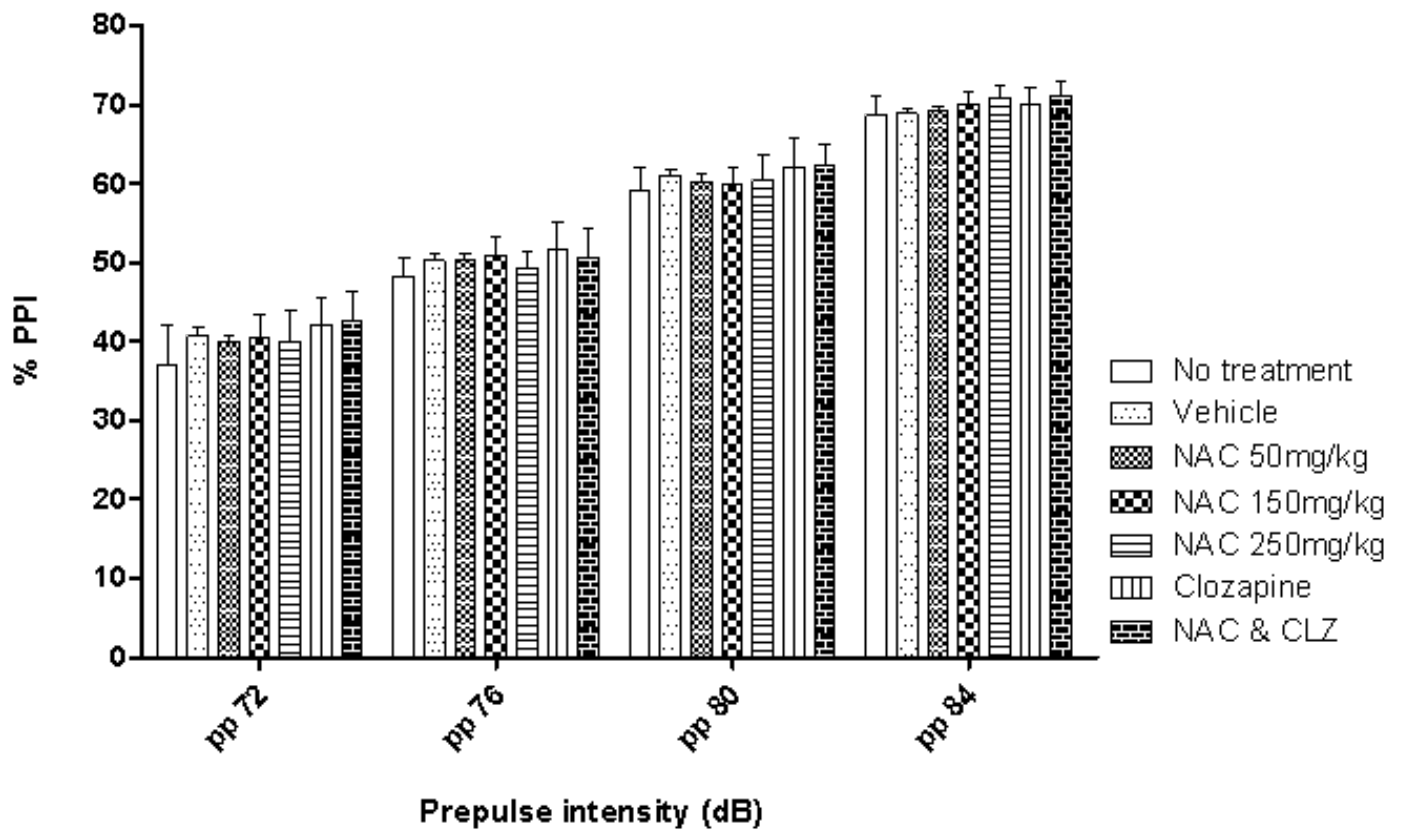


Figure 1: % Prepulse inhibition at various prepulse intensities in socially reared rats following the various drug treatments, as indicated (n = 10/group).

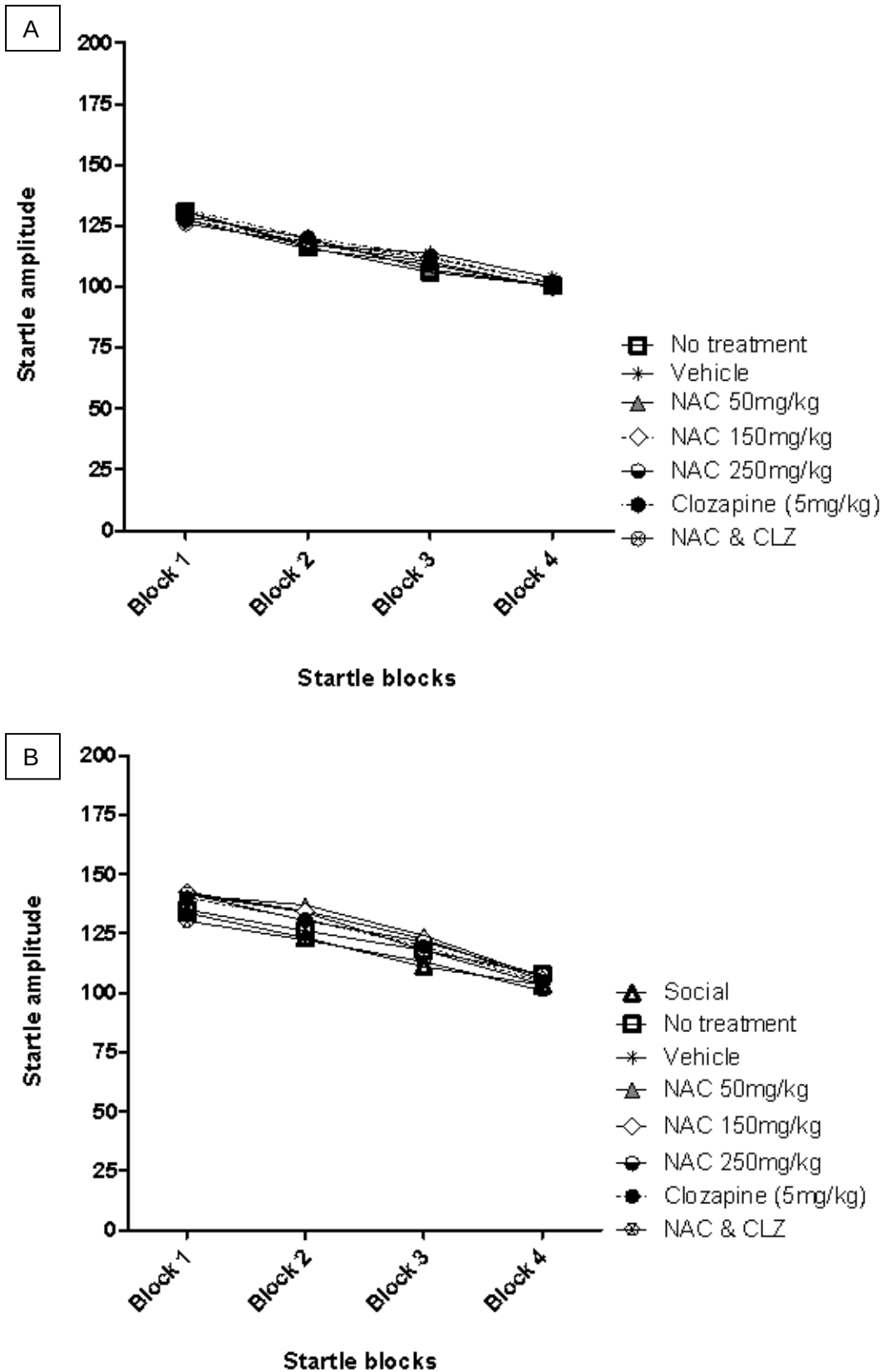


Figure 2: Mean startle amplitude at various prepulse intensities in (A) socially reared and (B) SIR rats following the various drug treatments, as indicated (n = 10/group).

1.4 Discussion

The fact that there were no significant differences in % PPI in all treatments in the socially reared animals, figure 1, indicates that NAC and clozapine as well as the combination treatment (CLZ + NAC) had little to no effect on the ability of the healthy animal to filter sensory information, and can therefore be used as valid controls for the SIR treatment groups, described in Chapter 5.

As indicated in our results, there were also no significant differences in startle amplitude in the socially reared (figure 2A), as well as in the SIR (figure 2B) animals in all the different treatment groups, indicating that habituation and sensitization of the startle amplitude were not affected in these animals. However, a decrease (not significant) in startle amplitude (block 1 – block 4) can be observed in the socially reared (figure 2A) as well as the SIR (figure 2B) animals, indicating a trend towards habituation. Habituation is a form of non-associative learning; it can also be viewed as a form of sensory filtering, since it reduces the animal's response to a non-threatening stimulus. Habituation decreases the startle response amplitudes, thus opposite to sensitization which increases startle response upon repeated presentation (Davis, 1974).

In conclusion, habituation and sensitization are two independent processes affecting the same behaviour (Groves and Thompson, 1970) and which can complicate interpretation of our results. However, habituation does not appear to play a role, or is affected by the various treatments, in both the socially reared (figure 2A) as well as the SIR (figure 2B) animals in this study. Drug treatment responses can therefore be regarded as being accurate and unaffected by habituation to the startle stimulus.

2. Tryptophan metabolite analysis in socially reared and SIR rats with selected drug treatment.

2.1 Aims

To investigate the effect of selected drug treatments on plasma levels of tryptophan, anthranilic acid and 3-hydroxy-anthranilic acid (3-OHAA) in socially reared and SIR rats. Plasma tryptophan, anthranilic acid and 3-OHAA were determined together with kynurenine,

KYNA and QA in Chapter 5, article 4, although here the former three were excluded from Chapter 5, article 4 in order to contain the length of the paper and will be presented here.

2.2 Methods

2.2.1 Tryptophan metabolite analysis

The same animals used in the peripheral immune-neurochemistry cohort, described in Chapter 5, were used. After 8 weeks of either social rearing or SIR and 14 days of selected treatments (NAC 50, 150 or 250 mg/kg, clozapine 5 mg/kg or the combination of clozapine and NAC 150 mg/kg (CLZ + NAC)) described in Chapter 5, animals were decapitated and trunk blood collected in 4 ml vacutainer tubes (SGVac) containing K₂EDTA solution as anticoagulant, centrifuged at 14,000 rpm at 4°C for 10 min. The plasma was stored at -80°C until the day of analysis. On the day of analysis the plasma samples were thawed on ice, centrifuged again, as mentioned above, and the plasma used.

Plasma tryptophan, anthranilic acid and 3-OHAA were determined in plasma using a solid-phase extraction (SPE) - liquid chromatography mass spectrometry (LC-MS/MS) procedure developed and validated in our laboratory (Chapter 3, Möller *et al.*, 2012b).

2.2.2 Statistical analysis

All statistical analysis were done by a three-way factorial ANOVA and Bonferroni post-hoc tests applied across all the respective treatments. In all cases, data are expressed as the mean $\pm$ standard error of the mean (SEM), with a *p* value of < 0.05 deemed statistically significant (Graphpad Prism 5; SAS/STAT® Software).

2.3 Results

Significant treatment-group interactions was evident with respect to tryptophan (3-way ANOVA; $F(6, 26) = 35.51$, $p < 0.0001$), anthranilic acid ($F(6, 26) = 23.95$, $p < 0.0001$) and 3-OHAA ($F(6, 26) = 24.23$, $p < 0.0001$) (figure 3A-C). Bonferroni post hoc test indicated no significant drug effects in the socially reared animals (figure 3A-C). However, SIR evoked a significant ($p < 0.0001$) increase in tryptophan (figure 3A), anthranilic acid (figure 3B) and 3-OHAA (figure 3C) vs. socially reared controls. SIR-induced elevations in tryptophan (figure 3A), anthranilic acid (figure 3B) and 3-OHAA (figure 3C) were reversed by clozapine ($p <$

0.0001) and CLZ + NAC ($p < 0.0001$). Only sub-chronic NAC 250 mg/kg effectively reversed increased 3-OHAA (figure 3C) in SIR rats ($p < 0.0001$).

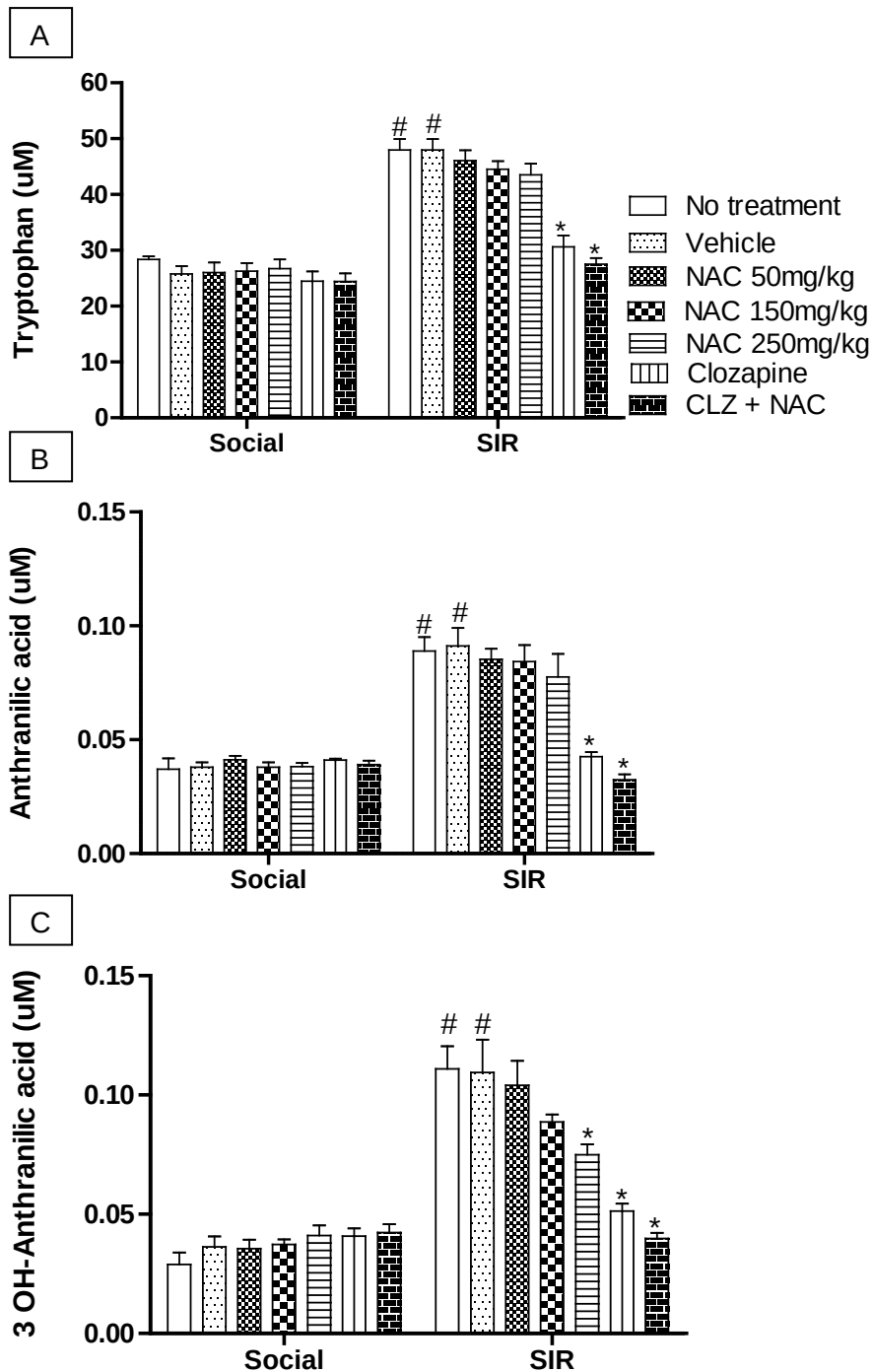


Figure 3: Kynurenine pathway metabolites in socially reared and SIR rats following the various drug treatments, as indicated ($n = 10/\text{group}$): (A) tryptophan; (B) anthranilic acid; (C) 3-OHAA. # $p < 0.0001$ vs. social no treatment and vehicle, * $p < 0.0001$ vs. SIR no treatment and vehicle, (Bonferroni post-hoc test).

2.4 Discussion

That the SIR rats presented with elevations in plasma tryptophan, anthranilic acid and 3-OHAA compared to socially reared controls confirms previous results (Chapter 4, Möller *et al.*, 2012a). Sub-chronic NAC 150 mg/kg had no effect on tryptophan and anthranilic acid elevations. However, NAC at 250 mg/kg significantly reversed the 3-OHAA elevations in the SIR rats. Since 3-OHAA is a well-known free radical (Schwarcz, 2004) and hence a pro-oxidant, these actions can possibly be explained by NAC's antioxidant properties (Kerksick and Willoughby, 2005). Clozapine and the combination CLZ + NAC significantly reversed the tryptophan, anthranilic acid and 3-OHAA elevations in the SIR rats, congruent with my previous findings (Chapter 4, Möller *et al.*, 2012a).

In conclusion, SIR significantly alters tryptophan metabolism that can be reversed by clozapine treatment, thereby emphasizing a possible new mechanism of action for clozapine. This mechanism involves immune-inflammatory components and is described in more detail in Chapter 4. Another important observation was that 3-OHAA elevation in SIR rats could be reversed with NAC 250 mg/kg alone, emphasizing the anti-oxidant properties of NAC, and at the same time re-affirming that both NAC and clozapine may be exerting a similar mode of action (see Chapter 4 for further discussion) responsible for their therapeutic potential in psychiatric disorders characterized by oxidative stress (Berk *et al.*, 2008a, b). Indeed, both clozapine (Möller *et al.*, 2011) and NAC target the GSH system (Kerksick and Willoughby, 2005).

3. Cortico-striatal monoamine analysis in socially reared and SIR rats with selected drug treatment.

3.1 Aims

To evaluate the effects of clozapine treatment and the combination of clozapine and NAC (CLZ + NAC) on SIR-induced changes of cortico-striatal 3,4-dihydroxyphenylacetic acid (Dopac), homovanillic acid (HVA), serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), noaradrenaline (NA) and 3-methoxy-4-hydroxyphenylglycol (MHPG). This data were excluded from Chapter 6, article 4 to contain the focus of article 4 on the effects of NAC on

regional brain monoamines and its therapeutic potential in not only schizophrenia, but also depression and anxiety disorders.

3.2 Methods

3.2.1 Monoamine analysis

The same animals used in the peripheral immune-neurochemistry cohort, as described in Chapter 5 and 6, were used. After 8 weeks of either social rearing or SIR and 14 days of selected treatments (vehicle treatment (for statistical comparison), clozapine 5 mg/kg or the combination of clozapine and NAC 150 mg/kg (CLZ + NAC)), described in Chapter 5, animals were decapitated; brains rapidly dissected into the frontal cortex and striatum and snap frozen. Brains were stored at -80 °C until the day of analysis. On the day of analysis the quantification of cortico-striatal Dopac, HVA, 5-HT, 5-HIAA, NA and MHPG was performed using a high performance liquid chromatography (HPLC) system with electrochemical detection (HPLC-EC) (Harvey *et al.*, 2006, 2010). Dopac, HVA, 5-HT, 5-HIAA, NA and MHPG concentrations were expressed as ng/mg wet weight of tissue (mean $\pm$ SEM).

3.2.2 Statistical analysis

All statistical analysis were done by a three-way factorial ANOVA and Bonferroni post-hoc tests, applied for the respective treatments. In all cases, data are expressed as the mean $\pm$ standard error of the mean (SEM), with a *p* value of < 0.05 deemed statistically significant (Graphpad Prism 5; SAS/STAT® Software).

3.3 Results

3.3.1 Frontal cortical monoamines

3-way ANOVA revealed significant treatment group interactions with regards to frontal cortical Dopac ($F(2, 54) = 19.26$, $p < 0.0001$), HVA ($F(2, 54) = 46.57$, $p < 0.0001$), 5-HT ($F(2, 54) = 35.27$, $p < 0.0001$) and 5-HIAA ($F(2, 54) = 34.29$, $p < 0.0001$) (figure 4A-D). Bonferroni post hoc tests indicated no significant treatment effect in the socially reared rats for frontal cortical Dopac, HVA, 5-HT, 5-HIAA, NA and MHPG (figure 4A-F). However, the SIR vehicle group induced deficits in frontal cortical Dopac (figure 4A), HVA (figure 4B), 5-HT (figure 4C), 5-HIAA (figure 4D) and MHPG (figure 4F) as well as an elevation in NA

(figure 4E) (explained in Chapter 6, article 4) were significantly ($p < 0.0001$) reversed with clozapine and CLZ + NAC. An important observation was that the combination treatment, CLZ + NAC, was significantly more effective than clozapine alone in reversing the frontal cortical deficits in Dopac ($p = 0.005$), HVA ($p = 0.0007$), 5-HT ($p = 0.036$) and 5-HIAA ($p = 0.002$) (figure 4A-D).

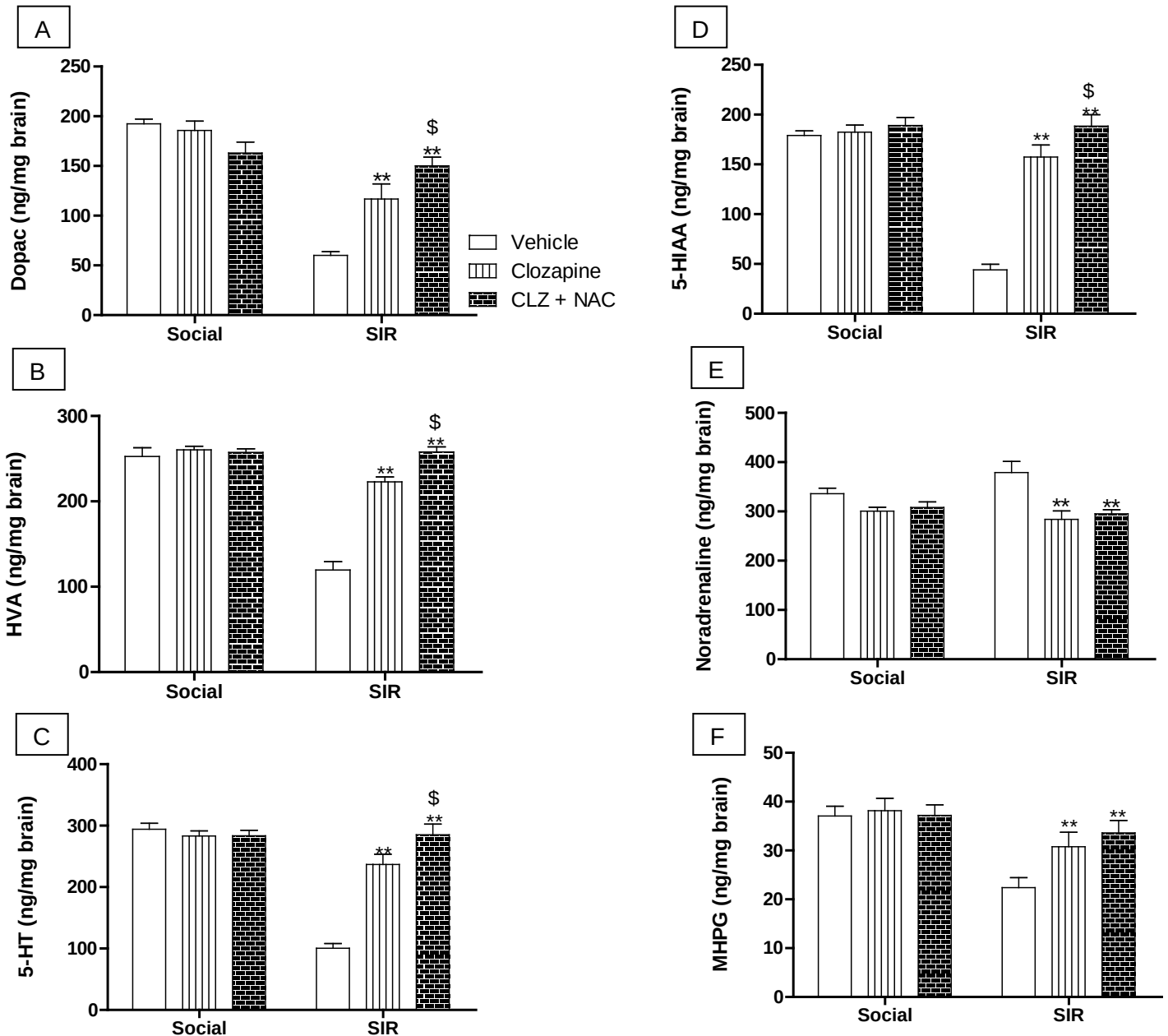


Figure 4: Frontal cortical monoamines in socially reared and SIR rats following the various drug treatments, as indicated ($n = 10/\text{group}$): (A) Dopac, (B) HVA, (C) 5-HT, (D) 5-HIAA, (E) NA, (F) MHPG. ** $p < 0.0001$ vs. SIR vehicle, \$ $p < 0.05$ vs. SIR clozapine treatment. (Bonferroni post-hoc test).

3.3.2 Striatal monoamines

Significant treatment group interactions were evident with 3-way ANOVA in striatal Dopac, ($F(2, 54) = 12.30, p < 0.0001$), HVA ($F(2, 54) = 16.93, p < 0.0001$), 5-HT ($F(2, 54) = 17.21, p < 0.0001$), 5-HIAA ($F(2, 54) = 19.66, p < 0.0001$), NA ($F(2, 54) = 47.74, p < 0.0001$) and MHPG ($F(2, 54) = 7.477, p = 0.0014$) (figure 5A-F). Bonferroni post hoc tests indicated no significant treatment effect in the socially reared rats for striatal Dopac, HVA, 5-HT, 5-HIAA, NA and MHPG (figure 5A-F). However, the SIR vehicle group induced significant elevations in striatal Dopac (figure 5A), HVA (figure 5B), 5-HT (figure 5C), 5-HIAA (figure 5D), NA (figure 5E) and MHPG (figure 5F) (explained in Chapter 6, article 4), were significantly ($p < 0.0001$) reversed with clozapine and CLZ + NAC (figure 5A-F). An important observation was that the combination treatment, CLZ + NAC, was significantly more effective than clozapine alone in reversing the striatal elevations in Dopac ($p = 0.038$), HVA ($p = 0.041$), 5-HT ($p = 0.008$) and 5-HIAA ($p = 0.041$) (figure 5A-D).

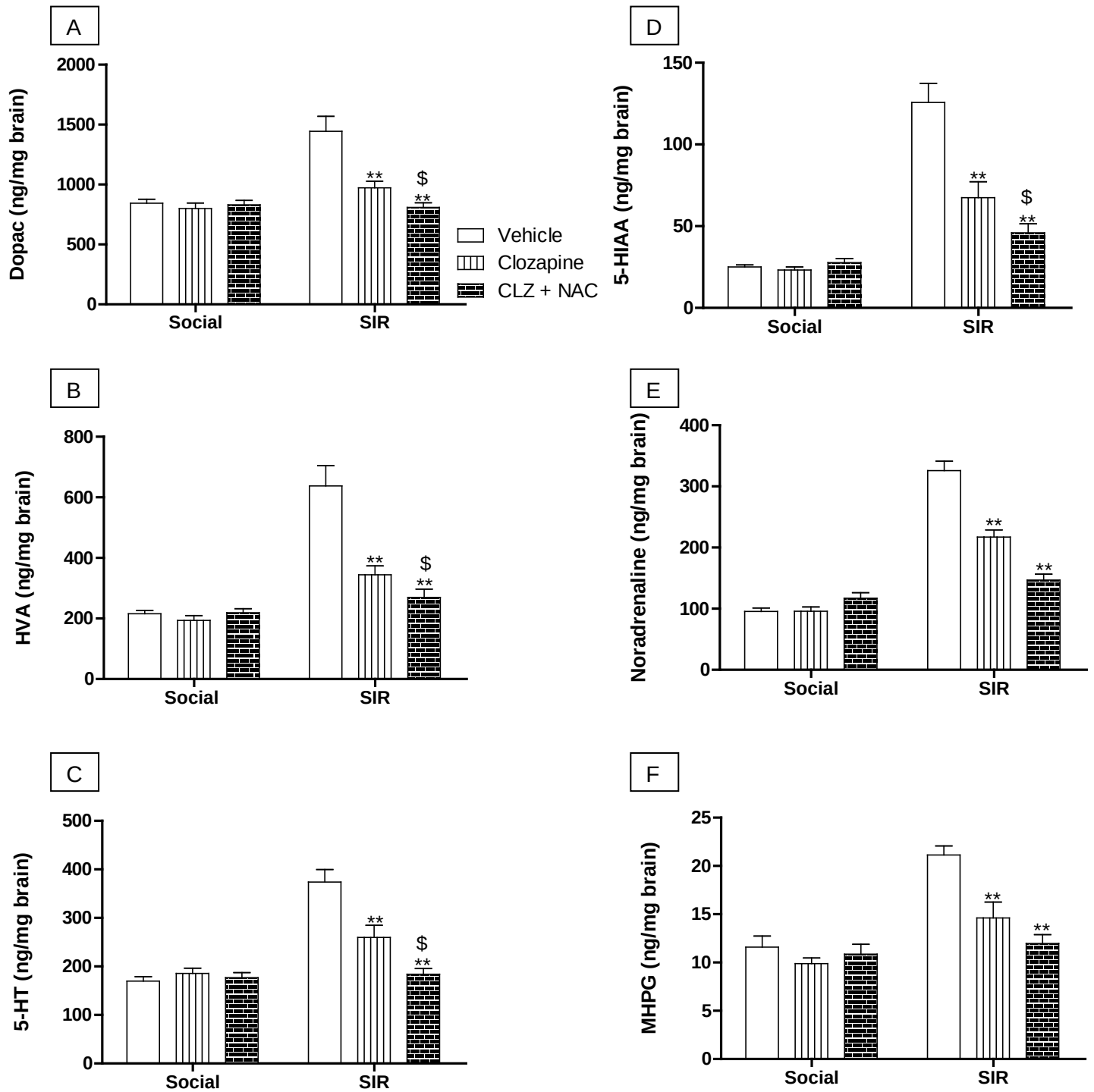


Figure 5: Striatal monoamines in socially reared and SIR rats following the various drug treatments, as indicated (n = 10/group): (A) Dopac, (B) HVA, (C) 5-HT, (D) 5-HIAA, (E) NA, (F) MHPG. **p < 0.0001 vs. SIR vehicle, \$p < 0.05 vs. SIR clozapine treatment. (Bonferroni post-hoc test).

3.4 Discussion

SIR induced significant deficits in frontal cortical Dopac, HVA, 5-HT, 5-HIAA and MHPG with elevations in NA (figure 4A-F), as well as significant elevations in striatal Dopac, HVA, 5-HT, 5-HIAA, NA and MHPG (figure 5A-F) (described in Chapter 6, article 4). Moreover, these changes were all reversed with clozapine and CLZ + NAC treatment. An important observation was that the combination treatment (CLZ + NAC) was significantly more effective than clozapine alone in reversing the frontal cortical and striatal Dopac, HVA, 5-HT and 5-HIAA alterations.

Clozapine's ability to increase frontal cortical Dopac, HVA, 5-HT and 5-HIAA levels could possibly be explained via an indirect mechanism: clozapine antagonises 5-HT₂ receptors, thereby increasing 5-HT and associated 5-HIAA release in the frontal cortex, in turn increasing DA and associated metabolites, Dopac and HVA release in the frontal cortex (reviewed in Di Matteo *et al.*, 2008, explained in Chapter 5 and 6). Indeed, a previous study indicated that clozapine increased DA and 5-HT levels in the frontal cortical dialysates of rats (Nomikos *et al.*, 1994). Other studies also indicated that clozapine dose-dependently elevates Dopac and HVA concentrations in the rat prefrontal cortex (Cartmell *et al.*, 2000; Kuroki *et al.*, 1999) and chronic clozapine treatment increased prefrontal cortical 5-HT levels (Yamamoto *et al.*, 1994). All these studies are in line with our frontal cortical findings (DA metabolites, Dopac and HVA, figure 4A, B) and (5-HT, 5-HIAA, figure 4C, D) in the SIR rat receiving clozapine treatment.

With regards to clozapine's ability to reverse the elevated striatal Dopac, HVA, 5-HT and 5-HIAA levels, could be explained via indirect excitatory glutamate and inhibitory gamma-aminobutyric acid (GABA) pathways projecting from the frontal cortex to the striatal regions (Stahl, 2007). Thus, the increase in frontal cortical Dopac, HVA, 5-HT and 5-HIAA levels (mediated by clozapine) will stimulate glutamate/GABA pathways to inhibit striatal release of these monoamines (Stahl, 2007). Indeed, a previous microdialysis study in rats indicated that cocaine-induced release of striatal 5-HT was decreased with clozapine treatment (Broderick *et al.*, 2004) and chronic clozapine decreased 5-HT levels in the striatum of rats (Yamamoto *et al.*, 1994) in line with my data (figure 5C, D). A previous microdialysis study also indicated that clozapine not only increased the release of DA in the mesolimbic regions, but also in the striatum (Moghaddam and Bunney, 1990) in line with our striatal Dopac and HVA data (figure 5A, B).

Clozapine's mechanism to decrease the frontal cortical NA and increase the MHPG, as well as its ability to decrease striatal NA and MHPG, in the SIR rats, could possibly be explained

via an indirect mechanism on the glutamate cortico-striatal pathways. Various forms of stress are known to provoke the release of glutamate (Musazzi *et al.*, 2011; Swanson *et al.*, 2005) which in turn stimulates the release of NA (Pittaluga *et al.*, 2001; Harvey *et al.*, 2006; Stahl, 2007). Such changes underlie the neurobiology of stress-related conditions and how SIR may evoke NA release in the frontal cortex and striatum. Indeed, a previous study indicated that clozapine inhibited glutamate release from rat pre-frontocortical nerve terminals (Yang and Wang, 2005); this inhibition of glutamate release could down-regulate NA release in the frontal cortex and striatum, as observed in our cortico-striatal NA, SIR clozapine results (figure 4E and 5E, F). Interestingly, our elevated NA data also relates to a previous microdialysis study in rats, indicating that clozapine enhance the release of NA not only in the frontal cortex but also in striatal regions and the hippocampus, possibly mediated via the antagonism of 5-HT₂ receptors (Westerink *et al.*, 2001).

More importantly, NAC bolstered the effects of clozapine in reversing the cortico-striatal Dopac, HVA, 5-HT and 5-HIAA alterations. One explanation could be clozapine's ability to reverse SIR-induced glutathione (GSH) imbalance (Möller *et al.*, 2011) and NAC's maintenance of GSH (Kerksick and Willoughby, 2005) contributing to their anti-oxidant properties and extending to regulate other redox-active systems as observed in Chapter 5, article 3, all contributing to cortico-striatal monoamine release. In addition, NAC and clozapine in combination could re-establish cortico-striatal Dopac, HVA, 5-HT and 5-HIAA release via its actions on the cysteine-glutamate transporter (NAC, Bauzo *et al.*, 2012) and regulation of glutamate pathways via NMDA receptors (clozapine, Toua *et al.*, 2010; Schwieler and Erhardt, 2003), thereby decreasing striatal and increasing frontal cortical Dopac, HVA, 5-HT and 5-HIAA release.

Thus, the mechanism whereby NAC bolstered the effect of clozapine in regulating cortico-striatal monoamines in this study could be explained by clozapine's and NAC's anti-oxidant properties along with their effects on the glutamatergic system.

In conclusion, this study indicates possible new mechanisms of clozapine as well as CLZ + NAC in regulating cortico-striatal 5-HT, 5-HIAA, NA and MHPG release in the SIR model, emphasizing NAC's use as adjunctive treatment in psychiatric disorders. However, these findings should not be viewed in isolation but together with the CLZ + NAC findings in Chapter 5, article 3 and Chapter 6, article 4.

4. Bioluminescence adenosine triphosphate (ATP) assay.

3.1 Aims

To formulate a standard calibration curve for ATP standard solutions, read by bioluminescence, in order to calculate brain homogenate ATP concentrations (described in Chapter 5) according to the protein content of each sample. Brain ATP values will be used as an expression of mitochondrial function in the study (see Chapter 5).

3.2 Methods

3.2.1 Brain homogenate preparation

Preparation of lysis buffer:

To prepare 500 mL of a 20x strength solution, 200 mM Tris buffer (pH 7.5), 2 M NaCl, 20 mM EDTA and 0.2 % Triton x-100 were mixed with deionized water to 500 mL. On the day of the assays, 1 part lysis buffer (20x) was diluted with 19 parts deionized water before use to obtain a 1x strength lysis buffer (Molecular Probes TM Invitrogen technologies).

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

Thereafter, the tissue samples were homogenized by ultrasonification, and the resulting homogenates centrifuged at 800 ×g for 10 min, at 4°C. The supernatants were used for the ATP assay and for the determination of protein content.

3.2.2 Protein determination

Protein determination of the brain homogenates was carried out according to the method of Bradford (Bradford, 1976). The Bradford reagent was lightly shaken, an adequate amount removed and kept at room temperature in a dark environment, after which protein standards were prepared by dissolving 5 mg bovine serum albumin (BSA) in 1 mL double distilled water, producing a 5 mg/mL solution. A series of 100 µL dilutions were then made, as indicated in Table 1 and the absorbance read at 560 nm. The protein content of each brain

homogenate was determined from the net absorbance against the protein content of the standards, illustrated in figure 6A.

Table1: Protein concentration standards

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

3.2.3 ATP kit reagent preparation

- **Reaction buffer** was prepared by adding 50 µL of the 20x Reaction buffer (component E, provided by the kit), to 950 µL of deionized water.
- **D-luciferin stock solution** (10 mM) was prepared by adding 1mL of 1x Reaction buffer to one vial of D-luciferin (component A, provided by the kit).
- **Dithiothreitol (DTT) stock solution** (0.1 M) was prepared by adding 1.62 mL of deionized water to the bottle containing 25 mg DTT.
- **Low-concentration ATP standard solutions** was prepared by diluting the 5 mM solution (component D, provided by the kit) in deionized water and preparing the following ATP serial dilutions: 50 nM, 25 nM, 12.5 nM, 6.25 nM, 3.13 nM, 1.56 nM, 0.781 nM.

3.2.4 Standard reaction solution

The contents of the following solutions were combined to prepare the standard reaction solution.

- 8.9 mL deionized water
- 0.5 mL 20x reaction buffer (component E)
- 0.1 mL 0.1 M DTT
- 0.5 mL of 10 mM D-luciferin
- 2.5 μ L of firefly luciferase 5 mg/mL stock solution

3.2.5 Standard curve

- The reaction was started by adding 100 μ L of dilute ATP standard solution to the wells of a black 96 well plate and the background read in the bioluminescence meter.
- 10 μ L of each ATP standard were then added to the ATP standard solution, the luminescence read and the background subtracted to generate the standard curve as illustrated in figure 6B.

3.2.6 Brain homogenate ATP analysis

The same directions were used as explained for the standard curve, except that the ATP standard solutions were substituted with the brain homogenate samples. The amount of ATP in the brain homogenate was calculated from the standard curve generated in figure 6B and divided by the protein content of each sample as determined in section 3.2.2. The ATP concentration in each brain homogenate, as described in Chapter 5, was expressed as nmol/mg protein.

3.3 Results

3.3.1 Bradford protein standard calibration curve

The Bradford protein standard curve, determined with Bovine serum albumin (mg/mL), had a linear regression of 0.9942, with the following formula: $y = 0.574x$ (see Figure 6A). This formula was used to calculate the protein content in each 10% brain homogenate sample (see Chapter 5).

3.3.2 ATP standard calibration curve

The ATP standard curve indicated a linear regression of 0.999, with the following formula: $y = 3195x$ (see figure 6B). This formula was used to calculate the ATP content in each brain homogenate sample (see Chapter 5).

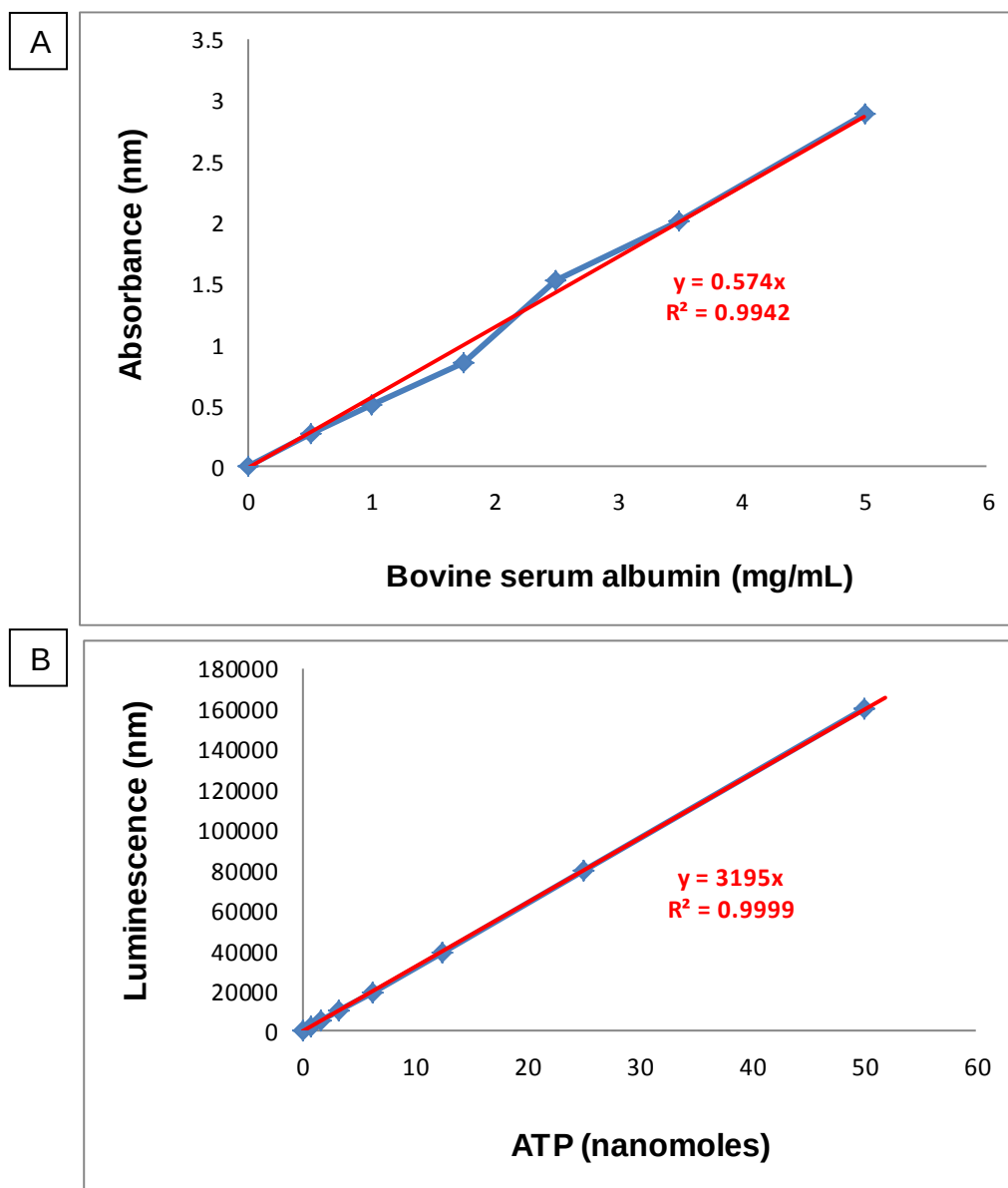


Figure 6: Standard calibration curves for (A) Bovine serum albumin in the Branford protein assay and (B) the ATP determination kit.

3.4 Discussion

Molecular Probes' ATP Determination kit (A22066) offers a convenient bioluminescence assay for quantitative determination of ATP with recombinant firefly luciferase and its

substrate D-luciferin. The assay is based on luciferase's requirement for ATP producing light (emission maximum -560 nm at pH 7.8) from the reaction, illustrated in figure 7.

In conclusion, the ATP assay along with the Bradford protein assay was subsequently applied in brain homogenate in the current study, presented in detail in Chapter 5.

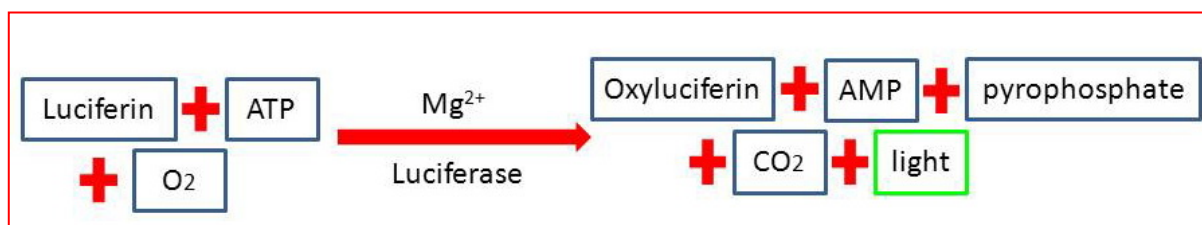


Figure 7: An illustration of the luciferin and ATP reaction, the principle of the ATP kit, producing light and detected as bioluminescence (Adapted from Molecular Probes TM Invitrogen technologies).

5. Enzyme-Linked-Immuno-Sorbent Assay (ELISA) cytokine assays.

4.1 Aims

The aim of this study was to formulate standard calibration curves for anti-inflammatory (IL-4), pro-inflammatory (TNF- α , IFN- γ) and dual action (IL-6) cytokines respectively, in order to calculate each cytokine concentration in the plasma of socially reared and SIR animals, described in Chapter 5.

4.2 Methods

4.2.1 Plasma sample preparation

After animals were sacrificed, trunk blood was collected in pre-chilled, 4 ml vacutainer tubes (SGVac) containing K₂EDTA solution as anticoagulant, centrifuged at 14,000 rpm at 4°C for 10 min, and the plasma stored at -80°C until the day of analysis. On the day of analysis the plasma samples were thawed on ice, centrifuged again, and 120 μ L of the supernatant added to 120 μ L Standard diluent buffer (provided by the kit, Invitrogen TM, Camarillo, CA), in order to make a 50% dilution. The 50% diluted plasma followed the method illustrated in figure 8.

4.2.2 Standard cytokine preparation

The rat (IL-4, TNF- α , IFN- γ and IL-6) cytokine standards as provided by the kit (Invitrogen™, Camarillo, CA) were prepared separately at the concentrations indicated in each calibration curve (figure 9A-D). Briefly, each rat cytokine standard was reconstituted in the Standard diluent buffer, vortexed for 30 seconds and left to stand for 10 min at room temperature. Serial dilutions were then in turn prepared and mixed thoroughly between each step at the concentrations indicated in figure 9A-D. The standard dilutions in turn followed the method illustrated in figure 8.

4.2.3 Solutions preparations

The Streptavidin-HRP (SAV-HRP) concentrate:

SAV-HRP (100x, provided by the kit) was prepared 15 min before usage in 50% glycerol. Since the latter is viscous, to ensure accurate dilution it was left to reach room temperature and gently vortexed. For each 8-well strips used, 10 μ L of this 100x concentrated solution was diluted with 1 ml of Streptavidin-HRP Diluent (provided by the kit, Invitrogen™, Camarillo, CA).

The dilution of wash buffer:

The Wash Buffer Concentrate (25x, provided by the kit) was allowed to reach room temperature and vortexed to ensure that any precipitated salts had dissolved. The Wash Buffer Concentrate was diluted with deionized water as follows: 1 volume of the Wash Buffer Concentrate (25x) with 24 volumes of deionized water (e.g. 100 mL may be diluted up to 2.5 L). The diluted Wash Buffer was put into a squirt bottle in order to flood the plate with the diluted Wash Buffer, completely filling all the wells during the washing steps (figure 8). After the washing procedure, the plate was inverted and tapped dry on absorbent tissue.

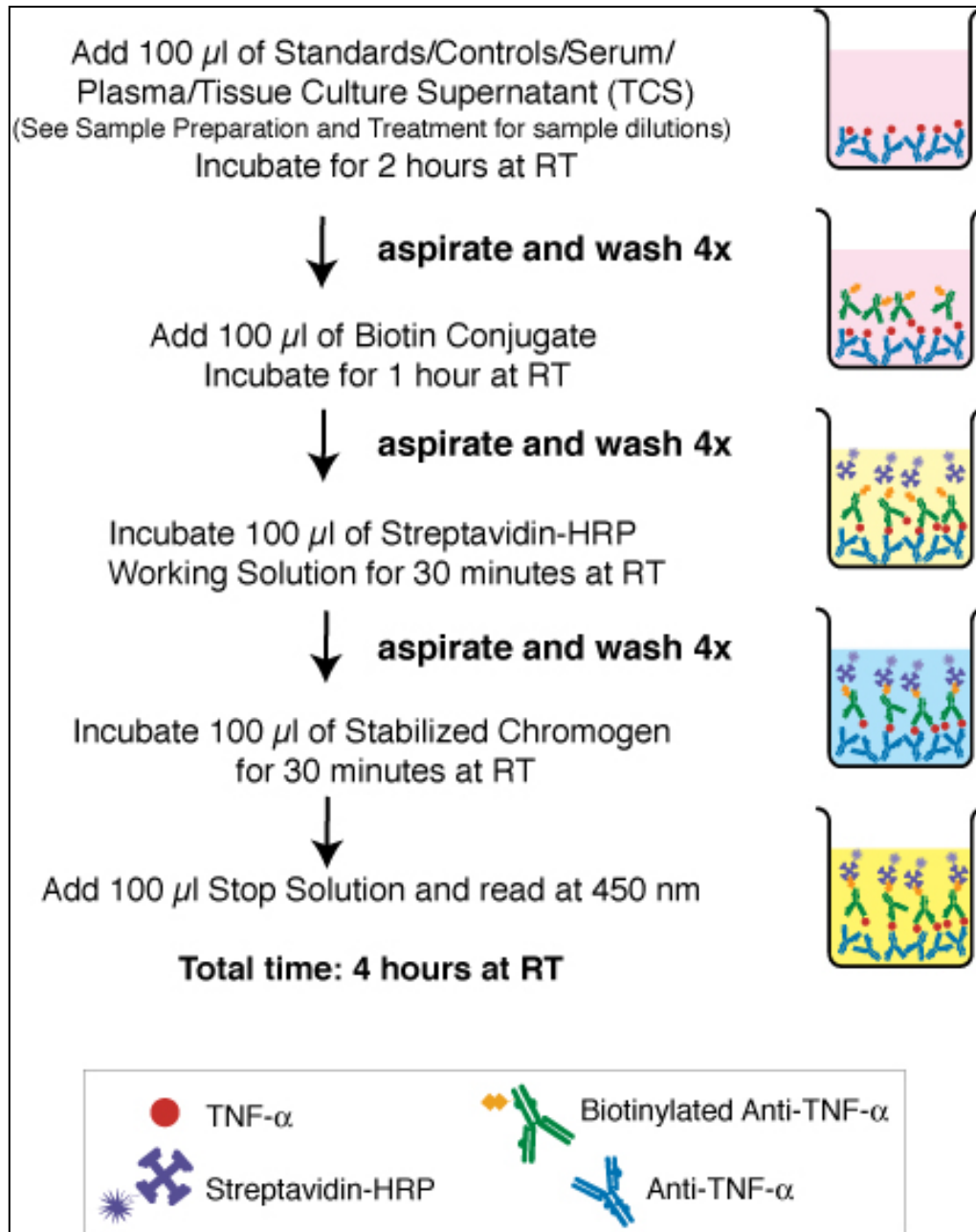


Figure 8: Principle and method of the rat cytokine sandwich ELISA kits to formulate a standard calibration curve in order to determine the concentration of IL-4, TNF- α , IFN- γ and IL-6 in unknown rat plasma samples (Invitrogen™, Camarillo, CA.).

4.3 Results

The Standard calibration curve for each cytokine was as follows:

Rat IL-4 showed a linear regression of 0.9899, with a formula of $y = 0.0304x$ (figure 9A).

Rat TNF- α showed linear regression of 0.9959, with a formula of $y = 0.005x$ (figure 9B).

Rat IFN- γ showed a linear regression of 0.9834, with a formula of $y = 0.0023x$ (figure 9C).

Rat IL-6 showed a linear regression of 0.9911, with a formula of $y = 0.0022x$ (figure 9D).

All these formulas were used to calculate the respective rat cytokine concentrations in rat plasma used in Chapter 5.

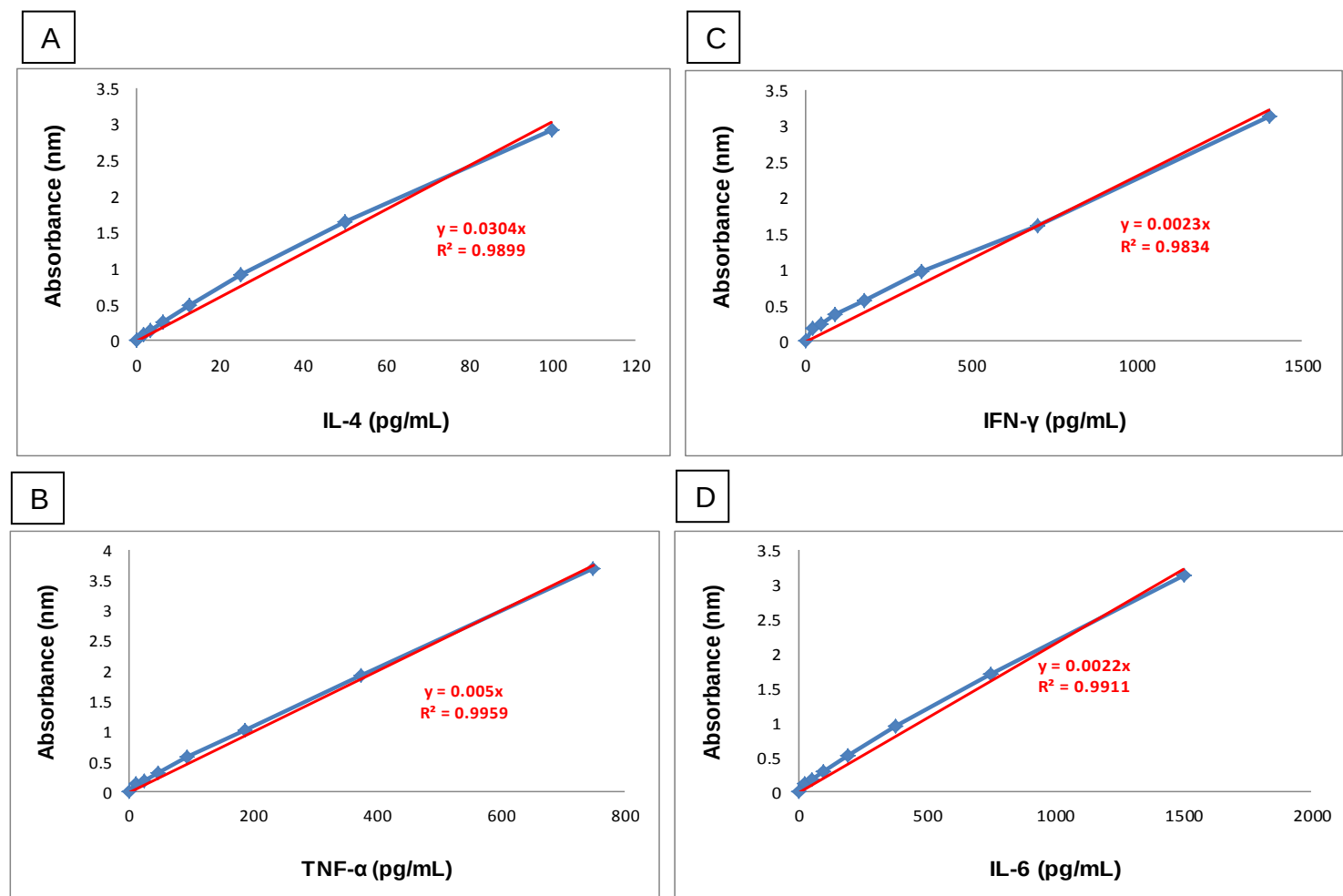


Figure 9: Standard calibration curves for the following rat cytokines: (A) IL-4, (B) TNF- α , (C) IFN- γ and (D) IL-6, determined by sandwich ELISA.

4.4 Discussion

The Invitrogen rat IL-4, TNF- α , IFN- γ and IL-6 assay kits are solid phase sandwich ELISA. The principle of the assay, illustrated in figure 7, is that monoclonal antibody specific for rat IL-4, TNF- α , IFN- γ and IL-6 is coated onto the wells of the microtiter strips provided by the kit manufacturers (Invitrogen TM, Camarillo, CA). Samples, including standards of known rat cytokine content, control specimens, and unknowns, are pipetted into these wells. During the first incubation, the rat cytokine antigens bind to the immobilized (capture) antibody on one

site. After washing, a biotinylated monoclonal antibody specific for rat cytokine is added. During the second incubation, this antibody binds to the immobilized rat cytokine captured during the first incubation. After removal of excess second antibody, Streptavidin-Peroxidase (enzyme) is added. This binds to the biotinylated antibody to complete the four-member sandwich. After a second incubation and washing to remove all of the unbound enzymes, a substrate solution is added, which is acted upon by the bound enzyme to produce colour. The intensity of this coloured product is directly proportional to the concentration of rat cytokine present in the original specimen.

In conclusion, these ELISA cytokine (IL-4, TNF- α , IFN- γ and IL-6) assay kits provide easy usage with excellent standard calibration curve regressions, and were therefore applied to determine the respective cytokine concentration in the plasma of the rats used in Chapter 5.

Addendum B

Toua et al., 2010

In this Addendum, a manuscript describing work undertaken during the candidates MSc is presented, and was central in setting the course for the current PhD study. This manuscript provides background information on the SIR model, evaluating dopamine D₁ and N-methyl-D-aspartate (NMDA) receptor binding characteristics with/without chronic haloperidol (a typical antipsychotic) or clozapine (an atypical antipsychotic) treatment.

Article published in *Neuroscience*, entitled:

“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.”

<http://www.sciencedirect.com/science/article/pii/S0306452209017230>

Reference

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.

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

C. TOUA,^a L. BRAND,^a M. MÖLLER,^a R. A. EMSLEY^b
AND B. H. HARVEY^{a*}

^aUnit for Drug Research and Development, Division of Pharmacology, School of Pharmacy, Hofman Street, Potchefstroom Campus, North-West University, Private Bag X6001, Potchefstroom, 2520, South Africa

^bDepartment of Psychiatry, University of Stellenbosch, PO Box 19063, Tygerberg, Cape Town, 7505, South Africa

Abstract—Glutamate and dopamine disturbances are implicated in frontal cortical dysfunction in schizophrenia. Little, however, is known about the nature of dopamine D₁ and *N*-methyl-D-aspartate (NMDA) receptor interactions in the illness, nor of the extent of their co-involvement in antipsychotic drug response. It is well known that early life adversity may pre-date the development of schizophrenia. Using a neurodevelopmental model of schizophrenia, namely post weaning social isolation rearing (SIR), we studied the effect of SIR (post natal day 21–61) on frontal cortical NMDA and D₁ receptor binding characteristics with/without chronic haloperidol (0.1 mg/kg/day i.p.) or clozapine (5 mg/kg/day i.p.) treatment, undertaken from post-natal day 50–60. SIR increased frontal cortical NMDA-density, with decreased affinity (decreased pK_D), but reduced D₁ receptor density (without effects on pK_D). In socially reared animals, clozapine but not haloperidol increased NMDA receptor density without effects on pK_D. Neither drug markedly affected D₁ receptor density, although clozapine increased D₁ affinity. Increased NMDA density in SIR animals was unaffected by haloperidol, but further increased by clozapine. However, SIR-associated decrease in NMDA affinity remained unaltered despite drug treatment. Reduced D₁ receptor density in SIR animals was exacerbated by haloperidol, but unaltered by clozapine, without changes in pK_D. SIR thus induces opposing effects on frontal cortical NMDA and D₁ radio-receptor binding characteristics, which has direct bearing on the mutual interplay of these receptors in schizophrenia. The ability of SIR to affect NMDA receptor affinity warrants deeper study. Furthermore, at the doses examined, in contrast to haloperidol, clozapine bolsters frontal cortical glutamatergic but stabilizes D₁ dopaminergic pathways in a neurodevelopmental animal model of schizophrenia, possibly explaining the atypical clinical characteristics of this drug. © 2010 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: social isolation rearing, NMDA receptor, D₁ receptor, clozapine versus haloperidol, frontal cortex, schizophrenia.

*Corresponding author. Tel: +27-17-299-2238; fax: +27-18-299-2225. E-mail address: Brian.Harvey@nwu.ac.za (B. H. Harvey).

Abbreviations: ANOVA, analysis of variance; B_{max}, receptor density; D₁, dopamine-1 receptor; D₂, dopamine-2 receptor; NMDA, *N*-methyl-D-aspartate; pK_D, -log K_D (receptor affinity); SIR, social isolation rearing.

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Cognitive dysfunction in schizophrenia remains an unresolved problem in the successful management of the illness, so much so that the Matrics initiative (Measurement and Treatment Research to Improve Cognition in Schizophrenia) has been set up to facilitate the development of better treatments targeted at cognition (Marder and Fenton, 2004). Although second-generation antipsychotics, previously referred to as “atypical,” have been lauded for their improved negative symptom efficacy over earlier first-generation agents (Lidow, 2000), this has recently been challenged by the Clinical Antipsychotic Trials of Intervention Effectiveness (CATIE) study, which supports the view that first- and second-generation antipsychotic drugs are extensively similar in mechanism of action, efficacy for psychotic symptoms, and lack of efficacy for avolition and impaired cognition, yet have diverse adverse effect profiles (Keefe et al., 2007). What was evident, however, was that the archetypal atypical antipsychotic, clozapine, was confirmed as the most effective drug for individuals with a poor symptom response to previous antipsychotic drug trials (Swartz et al., 2008).

Dopamine-1 (D₁) receptors are especially concentrated in the frontal cortex, (Lidow et al., 1998), while dopamine-2 (D₂) receptors are expressed in sub-cortical regions (Guillin et al., 2007). The frontal cortex controls behavior and adaptation to environmental change and decision-making by mediating selection and perception of relevant information and working memory (Javitt et al., 2000). While traditional D₂ receptor antagonists are effective in positive symptoms management, they are ineffective for negative symptom schizophrenia (Harvey et al., 1999). Deficient prefrontal D₁ signaling is an important neurobiological correlate of cognitive deficits in schizophrenia (Goldman-Rakic et al., 2004). Indeed, the D₁ receptor has now been highlighted as an important neurobiological target for the treatment of schizophrenia (Gray and Roth, 2007; Mu et al., 2007). Despite these convincing observations, however, neuroimaging studies in schizophrenia have reported either reduced (Okubo et al., 1997), no change (Karlsson et al., 2002), or increased (Abi-Dargham et al., 2002) D₁ binding in this brain region, prompting deeper investigation.

An imbalance between D₁ and D₂ receptors, as well as deficits in glutamatergic transmission, has been suggested to contribute towards the symptoms of schizophrenia (Scott and Aperia, 2009). Prefrontal dopamine is elevated

during working memory tasks, acting through D₁ but not D₂ receptors, to modulate glutamate neural activity (Scott and Aperia, 2009). Dopamine D₁ receptor activation also improves cognitive deficits induced by NMDA receptor antagonists (McLean et al., 2009) while interestingly, D₁ agonists can also reverse the cognitive deficits induced by haloperidol (Castner et al., 2000). Conversely, prefrontal dopamine release is strongly regulated by glutamate via *N*-methyl-D-aspartate (NMDA) receptors (Del Arco and Mora, 2001), with various non-competitive NMDA antagonists mimicking the positive and negative characteristics of schizophrenia (Steinpreis, 1996; Holcomb et al., 2004). Prefrontal D₁-NMDA receptor interactions therefore appear to form a fundamental neural substrate for cognition, particularly in schizophrenia (Castner and Williams, 2007). However, the preferential role, if any, of these two receptors in the development and presentation of schizophrenia, as well as which are targeted following successful drug treatment, remain unanswered.

Adverse early life environmental conditions profoundly affects neuronal growth and differentiation (Murray, 1994; Weiss and Feldon, 2001) and is deemed an important risk factor for the later development of schizophrenia (Weiss and Feldon, 2001). In rats, NMDA receptor expression in the brain is critical for developmental synaptogenesis in the first 2 weeks after birth (Ikonomidou et al., 1999), with early life stress suggested to increase the susceptibility of the cortex to neurodevelopmental disturbances, such as NMDA receptor hypofunction-induced apoptosis (du Bois and Huang, 2007). Considering then that schizophrenia likely results from disturbances in normal development (Ayhan et al., 2009), models that emphasize an environmental factor are important. Building on this, post-weaning isolation rearing in rodents is suggested to lead to an “environmental lesion” that produces perturbations in behavior and neurochemistry thought to resemble schizophrenia (Ayhan et al., 2009). At the same time, the model demonstrates strong face and predictive validity for schizophrenia (Geyer et al., 2001; Weiss and Feldon, 2001 and Ayhan et al., 2009 for review). Social isolation rearing (SIR) in particular displays deficits in prepulse inhibition (PPI), a sensory motor gating abnormality that is associated with schizophrenia and suggested to have utility as a biomarker of cognitive deficits of the disorder (Powell et al., 2009). Although changes in PPI needs to be viewed in the context of the complexity of the disorder, which presents with positive, negative and cognitive symptoms, SIR may have special value in pharmacological studies (Powell et al., 2009). In the present study, we have studied the differential effect of SIR in rats on frontal cortical NMDA and D₁ receptor radioligand binding. In order to relate these changes to antipsychotic action, we studied the response to sub-chronic treatment with two different classes of antipsychotic, viz. haloperidol (typical) and clozapine (atypical), and their ability to modify the ligand-binding characteristics of these receptors during grouped (social) and isolation rearing.

EXPERIMENTAL PROCEDURES

Animal housing

Weaned Sprague–Dawley rats were reared in sawdust cages (230(h)×380(w)×380(l) cm³) as previously described (Weiss and Feldon, 2001) and kept at constant conditions of temperature (21±5 °C), relative humidity (50±5%) with free access to food and water. Full spectrum cold white light (350–400 lx) was provided over a 12 h light/dark cycle. Positive air pressure was maintained in the facility with air filtration 99.7% effective for a particle size of 2 µm and 99.9% for a particle size of 5 µm. All animals were handled according to the code of ethics in research, training and testing of drugs as laid down by the Animal Ethics Committee of the North-West University (Ethics approval number 06D08).

Drugs

Haloperidol and clozapine were purchased from Sigma–Aldrich, Johannesburg, South Africa. The latter two drugs were first dissolved in 50 µl glacial acetic acid and buffered with sodium hydroxide (NaOH) (pH=6.0) before being administered intraperitoneally. In both cases, an injection volume of 0.5 ml was used. Drugs were freshly prepared each day before experimental testing and stored in glass bottles covered with aluminium foil.

Experimental design

Social isolation rearing (SIR). Weaned male pups and their pregnant dams were bred in-house in the Animal Research Center of North-West University (NWU). Dams were not time pregnant, with 21 day-old pups obtained from an on-going breeding program. The litters in the breeding colony were not culled. According to previously described methods (Weiss et al., 2000; Varty et al., 1999), the weaned animals (weighing 40–55 g) were randomly assimilated on post-natal day 21 into socially reared (three rats/cage) or SIR (one rat/cage) groups. Social or isolation rearing was maintained for 8 weeks, at which time the animals weighed between 250 and 300 g (socially reared and SIR animals did not differ significantly in this respect).

Drug treatments included chronic haloperidol (0.1 mg/kg/day i.p.) or clozapine (5 mg/kg/day i.p.) administered from post-natal days 50–60. Doses of the two drugs were carefully based on the animal literature (Keith et al., 1991; Bakshi et al., 1994; Feifel and Priebe, 1999; Bast et al., 2000) and approximate therapeutic equivalence (Atkins et al., 1997). Socially-reared animals (*n*=21) received saline by i.p. injection, while the SIR animals received either clozapine (*n*=15), haloperidol (*n*=17) or saline (*n*=15) in the last 11 days of isolation rearing. Separate drug treatment studies alone in socially-reared animals received either clozapine (*n*=10), haloperidol (*n*=10) or saline (*n*=10) for 11 days. This duration of treatment was selected, as motivated below.

On a relative scale, 7 days in the life span of a rat is approximately equivalent to 6 months in the human life span (Adamec et al., 1997), and can be regarded as being equivalent to chronic treatment in humans, and indeed adequate for psychotropic drug-induced receptor changes to become evident (Brink et al., 2008). However, increasing clinical evidence points to an early response at 2 weeks as being an accurate and specific predictor of subsequent response (Kapur et al., 2005; Emsley et al., 2007; Ascher-Svanum et al., 2008), thus questioning the need for prolonged treatment to confirm efficacy. Bearing the above in mind, but also to exclude the possible impact of injection stress evoked over extended treatment periods, the current study applied an 11 day treatment period as opposed to 28 days or longer used in other studies (e.g. Tarazi et al., 1996, 2003; Ossowska et al., 1999). Animals thus received an i.p. injection of drug or saline by a skilled veterinary technician, leaving housing condition and drug treatment as the only variables. This design allowed us to be more conservative in our use of animals, while the reduced treatment duration and skilled drug administration

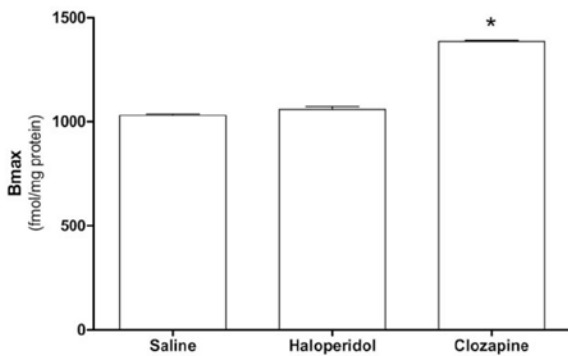


Fig. 1. The effect of chronic treatment with saline, haloperidol (0.1 mg/kg i.p.) or clozapine (5 mg/kg i.p.) in socially-reared rats on frontal cortex NMDA receptor density (B_{max} ; $n=3$ assays). Refer to text for full description. * $P<0.05$ versus saline (Dunnetts test).

was deemed to be the more ethically sound approach. A 24 h wash-out period was instated following the last dose of antipsychotic before the animals were sacrificed for the neuro-receptor binding studies. All animals were sacrificed by decapitation without any prior use of an anesthetic agent.

Receptor binding studies

Dopamine D_1 receptor (Billard et al., 1984) and glutamate NMDA receptor (Harvey et al., 2004) binding were performed as previously described. Group-housed rats and SIR rats receiving chronic drug treatments (i.e. haloperidol, clozapine or saline) were sacrificed by decapitation and the frontal cortex rapidly dissected on ice. The tissue was snap-frozen in liquid nitrogen and stored at -86°C until neuro-receptor analysis. In order to obtain adequate amounts of protein (0.1–0.3 mg/ml) per assay, two cortices were pooled for the determination of receptor density (B_{max}) and affinity (pK_D). Frontal cortical tissue was dissected following the removal of the olfactory bulb from the cortex and cutting around the anterior tip of the corpus callosum. The tissue was homogenized in either 50 volumes of cold HTS buffer (5 mM HEPES/4.5 mM Tris buffer, pH 7.4) or Tris buffer (Tris-HCL (50 mM), NaCl (120 mM), KCl_2 (5 mM), CaCl_2 (2 mM), MgCl_2 (1 mM), ascorbic acid (0.1%) pH 7.4) for NMDA and D_1 receptor analysis respectively with a Brinkman Polytron PT 10 homogenizer (setting 6, 10 s). The homogenate was centrifuged at $48\,000\times g$ for 15 min at 4°C and the resulting supernatant was decanted whereafter 30 ml of the appropriate buffer was added and the centrifugation step repeated as above. The final membrane suspension was made up in 60 volumes of the respective buffer solutions and kept on ice.

NMDA receptor binding was measured by incubating 300 μl tissue homogenate, 50 μl 300 μM glycine, 50 μl 100 μM L-glutamate, 50 μl buffer or MK801 and 50 μl [^3H]-MK-801 (specific activity 27.5 Ci/mmol) (0.7–25 nM) for 90 min in a shaking waterbath at 25°C . D_1 receptor binding was measured by incubating 300 μl homogenate, 50 μl buffer or SCH23390, 100 μl 0.5 μM ketanserin and 50 μl of [^3H]-SCH 23390 (specific activity 85.0 Ci/mmol) (0.1–10 nM) in a shaking waterbath for 15 min at 37°C . Drug-receptor binding reaction was terminated by rapid vacuum filtration through Whatman GF/B filters pre-soaked with the respective ice-cold buffers. The filters were rinsed twice with 4 ml ice-cold buffer and placed in polypropylene counting vials containing 3 ml Filter Count® scintillation cocktail and radioactivity determined by liquid scintillation spectrometry (Packard Tri-Carb 2100 TR, Packard, Downers Grove, IL, USA). Specific binding was defined as the total binding minus binding in the presence of either 25 μM MK-801 or 10 μM SCH23390. Receptor binding data were analyzed by non-linear regression analysis using Prism from

GraphPad Software, Inc. (<http://www.graphpad.com>) to give affinity (pK_D) and receptor density (B_{max}) values. Receptor density and affinity data were expressed in fmol/mg protein and nM, respectively, with protein routinely assayed using the Bradford assay (Bradford, 1976).

Statistical analysis

Graphpad Prism version 4 for windows (Graphpad Software, San Diego, USA) was used for the statistical analysis and graphical presentations. Since measured K_D values do not necessarily adhere to a normal distribution, whereas these values on the logarithmic scale (expressed as $-\log$ or pK_D) do display normal distribution (McKinney and Raddatz, 2006), all K_D data (nM) were first converted to pK_D before statistical analyses. Drug related effects on frontal cortical receptor binding in socially-reared animals were evaluated with one-way analysis of variance (ANOVA) followed by Dunnetts test, while receptor changes associated with SIR with/without antipsychotic treatment were analysed using one-way ANOVA followed by Newman–Keuls test. In all instances, statistical significance was defined as $P<0.05$.

RESULTS

Effect of chronic clozapine and haloperidol treatment in socially-reared animals on frontal cortical NMDA and D_1 receptors

In healthy, socially-reared animals receiving the various drug treatments, one-way ANOVA of the NMDA receptor B_{max} data revealed significant group differences ($F(2,6)$; 409.3, $P<0.0001$), although no differences were evident in NMDA receptor pK_D data ($F(2,6)$; 2.57, $P=0.16$). One-way ANOVA of D_1 receptor B_{max} data failed to reveal any significant group differences ($F(2,6)$; 0.122, $P=0.89$), although significant group differences were evident in the D_1 receptor pK_D data ($F(2,6)$; 6.92, $P=0.03$).

Haloperidol and clozapine treatment in healthy socially-reared animals revealed that clozapine, but not haloperidol treatment, evoked a significant increase in NMDA receptor density versus saline treated groups ($P<0.01$, Dunnetts; Fig. 1). Neither drug alone affected NMDA receptor affinity (Table 1). Neither drug altered frontal cortical D_1 receptor density (Fig. 2), although clozapine ($P<0.05$, Dunnetts; Table 1), but not haloperidol, showed a small but significant increase in receptor affinity (increased pK_D) compared to saline treated animals (Table 1).

Social isolation rearing (SIR) effects on frontal cortex glutamate NMDA receptor binding and response to clozapine or haloperidol treatment

For SIR animals receiving saline or the various drug treatments, one-way ANOVA of the NMDA receptor B_{max} data

Table 1. Effect of drug treatment, as indicated, on the pK_D values of frontal cortical NMDA and D_1 receptors in socially reared animals ($n=3$ assays). Refer to the text for full description

Treatment	NMDA (Mean $\pm$ SEM)	D_1 (Mean $\pm$ SEM)
Saline	8.56 $\pm$ 0.11	8.15 $\pm$ 0.03
Haloperidol	8.57 $\pm$ 0.04	8.47 $\pm$ 0.12
Clozapine	8.75 $\pm$ 0.01	8.60 $\pm$ 0.10*

* $P<0.05$ versus saline-treated animals (Dunnetts test).

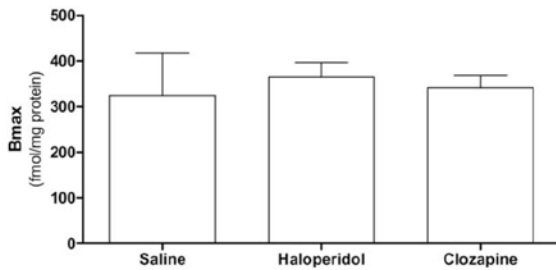


Fig. 2. The effect of chronic treatment with saline, haloperidol (0.1 mg/kg i.p.) or clozapine (5 mg/kg i.p.) in socially-reared rats on frontal cortex D₁ receptor density ($B_{\max}$; $n=3$ assays). Refer to text for full description.

revealed significant group differences ($F(3,8)$; 15.58, $P=0.001$). Similarly, one-way ANOVA of the NMDA receptor pK_D data indicated significant differences across these groups ($F(3,12)$; 9.41, $P=0.002$).

SIR significantly increased NMDA $B_{\max}$ values ($P<0.05$; Fig. 3, Newman–Keuls), thereby confirming a marked increase in NMDA receptor density. Chronic treatment with 0.1 mg/kg haloperidol failed to reverse the isolation-induced increase in NMDA receptor density, which remained significantly higher than that of socially-reared animals ($P<0.05$; Fig. 3, Newman–Keuls) and no different to the SIR groups ($P>0.05$; Fig. 3, Newman–Keuls). Chronic treatment with 5 mg/kg clozapine, on the other hand, resulted in a sustained and further increase in NMDA receptor density that was significantly higher than that of socially housed animals ($P<0.05$; Fig. 3; Newman–Keuls) but also significantly higher than that of the SIR animals ($P<0.05$; Fig. 3; Newman–Keuls).

Interestingly, in the presence of either saline, haloperidol or clozapine treatment, SIR in all instances was associated with significantly decreased pK_D ($P<0.05$; Table 2, Newman–Keuls) compared to socialized animals, suggest-

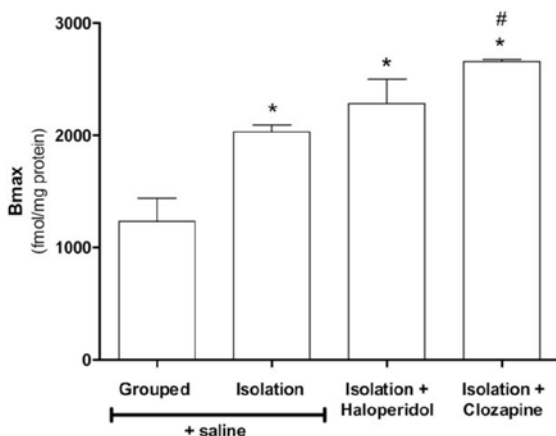


Fig. 3. The effect of 8 weeks social rearing (grouped) compared to isolation rearing of rats on frontal cortex NMDA receptor density ($B_{\max}$), and response to chronic saline, haloperidol (0.1 mg/kg i.p.) or clozapine (5 mg/kg i.p.) treatment ($n=3$ –5 assays). Refer to text for full description. * $P<0.05$ versus grouped (Newman–Keuls test). # $P<0.05$ versus isolation (Newman–Keuls test).

Table 2. Effect of drug treatment, as indicated, on the pK_D -values of frontal cortical NMDA and D₁ receptors in SIR and socially reared animals ($n=3$ –5 assays). Refer to the text for full description

Treatment	NMDA (Mean $\pm$ SEM)	D ₁ (Mean $\pm$ SEM)
Social+Saline	8.61 $\pm$ 0.06	8.27 $\pm$ 0.09
SIR+Saline	8.38 $\pm$ 0.08*	8.37 $\pm$ 0.11
SIR+Haloperidol	8.25 $\pm$ 0.06*	8.51 $\pm$ 0.13
SIR+Clozapine	8.15 $\pm$ 0.03*	8.38 $\pm$ 0.01

* $P<0.05$ versus socially reared animals (Newman–Keuls test).

ing a small associated decrease in receptor affinity following SIR. However, the response to saline or either drug treatment in SIR animals were not different to one another ($P>0.05$; Table 2, Newman–Keuls), suggesting a pronounced effect of SIR on receptor affinity despite drug treatment.

Social isolation rearing effects on frontal cortex dopamine D₁ receptor binding and response to clozapine or haloperidol treatment

One-way ANOVA of the D₁ receptor $B_{\max}$ data in isolated rats receiving saline or the various drug treatments indicated significant group differences, viz. ($F(3,10)$; 18.31, $P=0.0002$), although no differences with respect to pK_D values were noted across the groups ($F(3,10)$; 0.92, $P=0.47$).

SIR evoked a significant reduction in $B_{\max}$ for the D₁ receptor compared to group-housed animals ($P<0.05$; Fig. 4, Newman–Keuls), thereby confirming a marked decrease in D₁ receptor density. Chronic treatment of SIR animals with 0.1 mg/kg haloperidol failed to reverse SIR-associated effects on D₁ receptor density, in fact evoking a further and significant downregulation of already reduced

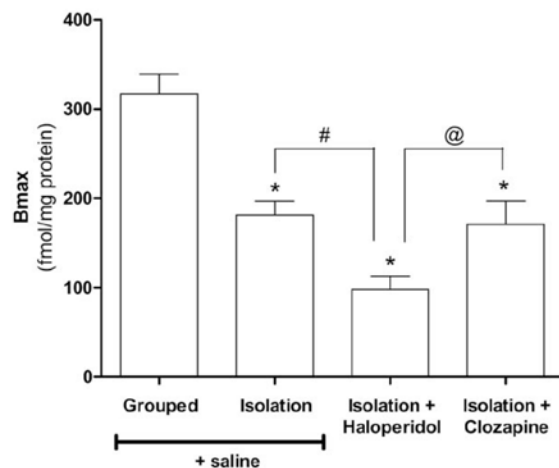


Fig. 4. The effect of 8 weeks social rearing (grouped) compared to isolation rearing of rats on frontal cortex D₁ receptor density ($B_{\max}$), and response to chronic saline, haloperidol (0.1 mg/kg i.p.) or clozapine (5 mg/kg i.p.) treatment ($n=3$ –5 assays). Refer to text for full description. * $P<0.05$ versus grouped (Newman–Keuls test). # $P<0.05$ versus isolation (Newman–Keuls test). @ $P<0.05$ versus isolation plus clozapine (Newman–Keuls test).

cortical dopamine D₁ receptor density compared to isolation-reared animals ($P < 0.05$; Fig. 4; Newman–Keuls), and compared to group-housed controls ($P < 0.05$; Fig. 4; Newman–Keuls). In contrast, however, chronic treatment with 5 mg/kg clozapine did not alter isolation-induced suppression of D₁ receptor density, remaining similar to that observed in SIR-animals, but remaining significantly lower than that in group-housed animals ($P < 0.05$; Fig. 4; Newman–Keuls).

With regard to D₁ receptor affinity, SIR alone and with saline or either drug treatment failed to evoke any noteworthy effects on receptor affinity (pK_D) ($P > 0.05$; Table 2; Newman–Keuls).

DISCUSSION

Substantial evidence supports D₁-NMDA interactions in the rat prefrontal cortex (Kruse et al., 2009; Scott and Aperia, 2009). Because of their pathological role in schizophrenia (Castner and Williams, 2007), as well as in determining cognitive performance (Tseng and O'Donnell, 2004; Castner and Williams, 2007), D₁-NMDA receptor interactions are important considerations in antipsychotic drug action.

Earlier studies have noted a reduction in the expression of NMDA NR1 receptor subunit mRNA in the hippocampus of schizophrenia patients (Gao et al., 2000; Law and Deakin, 2001). However, Bressan and colleagues (2005) found no evidence for differences in NMDA receptor binding between drug-free patients with schizophrenia and healthy controls using single photon emission tomography (SPET). In the present study, SIR was associated with an upregulation of frontal cortical NMDA receptors (Fig. 3) with an associated reduction in affinity (pK_D ; Table 2). In support of these findings, Turnock-Jones and colleagues (2009) have most recently described increased expression of the NR2A NMDA receptor subunit in the prefrontal cortex of rats reared in isolation, although this has not always been reproducible (e.g. Hall et al., 2002). Due to the lack of direct functional analysis of the NMDA receptor in the present study, interpretation of the observed receptor binding changes is inherently speculative. Nevertheless, earlier work in cell cultures has noted that functional events that follow administration of an NMDA receptor antagonist enhance [³H]MK-801 specific binding (Hu and Ticku, 1995), while chronic treatment with NMDA antagonists selectively upregulates NMDA receptor mRNAs and polypeptides in cultured mouse cortical neurons (Follesa and Ticku, 1996). Based on these latter studies, it is reasonable to propose that conditions conducive to increased frontal cortical NMDA B_{max} and decreased affinity, as observed in the present study, implies that SIR as a neurodevelopmental model underscores a state of reduced frontal cortical glutamatergic activity that culminates in a reactive increase in NMDA receptor binding.

When considering response to drug treatment, pre-clinical studies using long-term clozapine (Tarazi et al., 1996), or other atypical antipsychotic treatments (Tarazi et al., 2003), have found a reduction in NMDA receptor bind-

ing in rat forebrain regions. However, some authors have described an upregulation of NMDA receptors in cortical regions following either clozapine or haloperidol treatment (Ossowska et al., 1999). In the current study, clozapine but not haloperidol markedly bolstered frontal cortical NMDA receptor density in socially reared animals (Fig. 1). Similarly, in SIR animals, 11 days of clozapine treatment, but not with haloperidol, further increased frontal cortical NMDA receptor density versus both SIR and socialized animals (Fig. 3). That clozapine and haloperidol, both effective antipsychotics, exert differing effects on NMDA B_{max} , with clozapine seemingly acting to further increase NMDA B_{max} , highlights the complexity of modeling schizophrenia and its response to drug treatment in a single analogous animal model. However, at its simplest level it does concur that clozapine and haloperidol affect NMDA signaling differently, possibly explaining the cognitive benefits that are well-described for clozapine (Harvey et al., 1999).

Interestingly, clozapine and olanzapine (Chen and Yang, 2002; Heresco-Levy, 2003) but not haloperidol (Ninan et al., 2003), have mild agonistic actions on NMDA receptors. Over long-term treatment, as was applied in the current study, this action may engender diverse auto-regulatory effects on glutamate release via the actions of its down-stream messenger, nitric oxide (Prast and Philippu, 2001). Nitric oxide strongly inhibits the activity of excitatory amino acid-utilizing neurons (Prast and Philippu, 2001) which may lead to increased NMDA receptor expression, and the increased NMDA density observed here. Congruent with a glutamatergic action for clozapine is that chronic haloperidol treatment decreases striatal nitric oxide synthase activity, but which is reversed by the clozapine analogue, olanzapine (Nel and Harvey, 2003). Thus, first- and second-generation agents (typified by clozapine), differ in their effects on glutamatergic signaling, with clozapine promoting NMDA receptor signaling and haloperidol inhibiting this function.

Neither drug affected NMDA affinity in socially reared animals (Table 1). However, the marked decrease in NMDA receptor affinity (i.e. decreased pK_D) following SIR (Table 2), and its sustained decrease following treatment with saline or either clozapine or haloperidol (Table 2) is especially noteworthy. Saline or either drug treatment did not significantly alter pK_D with respect to one another, akin to that noted in socially reared animals (Table 1), implying that SIR induced a lasting reduction in NMDA receptor affinity irrespective of drug treatment. Although speculative until studied in more depth, this phenomenon may possibly occur via post-translational modification and/or phosphorylation following sustained adverse environmental effects on glutamatergic transmission (Crowe et al., 2006).

Okubo and colleagues (1997) have earlier reported reduced D₁ binding in schizophrenia, although more recently Karlsson and colleagues (2002) and Abi-Dargham and colleagues (2002) have reported no change or increased D₁ binding in this brain region. In the present study, SIR significantly decreased cortical D₁ receptor density without changes in affinity (Fig. 4, Table 2). Func-

tional interactions between D₁ and NMDA receptors are implicated in the maintenance of normal brain activity and in neurological dysfunction (Abekawa et al., 2006; Pei et al., 2004), with NMDA receptor activation proposed to upregulate D₁ receptors via a direct protein–protein interaction (Pei et al., 2004). Consequently, the observed decrease in D₁ receptor density following SIR suggest altered cortical function, and may primarily be attributed to the above-described NMDA receptor changes.

Earlier studies have noted that several types of antipsychotic drug downregulate prefrontal cortical D₁ sites (see Lidow et al., 1998 for review). However, we found no change in this regard with respect to haloperidol- or clozapine-treatment in socially reared animals (Fig. 2). This discrepancy may reflect the differences in duration of treatment used in the aforementioned and present studies (6 months v s 11 days). However, what is noteworthy is that similar changes to that observed above were observed in SIR rats, with both clozapine and haloperidol treatment in isolated rats associated with reduced D₁ density compared to that of socially reared animals (Fig. 4). On closer scrutiny, however, clozapine treatment maintained, but did not reverse cortical D₁ receptor density changes evoked by SIR while haloperidol further and significantly reduced D₁ receptor density compared to the SIR group (Fig. 4). The opposing effects of clozapine and haloperidol on D₁ receptor density during SIR are very likely related to how these drugs provoke the release of frontal cortical dopamine (Gessa et al., 2000; Kuroki et al., 1999). This of course is congruent with the ability of clozapine to increase dopamine levels in the medial prefrontal cortex (Kuroki et al., 1999) and to enhance D₁ receptor mediated neurotransmission (Ahlenius, 1999). Indeed, the observed differences on D₁ receptor binding described here may be critical in explaining how clozapine, as opposed to haloperidol, may benefit cortical function.

From a functional perspective, various studies (e.g. Didriksen et al., 2007; Beraki et al., 2008) have demonstrated that deficits in spatial learning induced by hypoglutamatergia (using NMDA antagonists) are effectively reversed by clozapine and related second generation antipsychotics, but not haloperidol. Furthermore, Castner et al. (2000) show that D₁ receptor activation can reverse haloperidol-induced cognitive deficits, highlighting that haloperidol may indeed worsen cognition (Artaloytia et al., 2006) by compromising D₁-dependent signaling. Interestingly, D₁ receptor binding studies found that clozapine but not haloperidol evoked a small but significant increase in cortical D₁ receptor affinity (increased pK_D) in socially reared animals (Table 1), although no effects on affinity for either drug were observed in SIR animals (Table 2). The practical significance of this finding is unknown. With dopamine release in the prefrontal cortex being regulated by NMDA receptors (Del Arco and Mora, 2001), differences in NMDA receptor involvement may underlie why second generation antipsychotics, and clozapine included, increase extra-cellular dopamine release in the medial prefrontal cortex whereas only small increments are evident with haloperidol (Gessa et al., 2000). Acute treatment with clozapine,

but not haloperidol, has been found to induce a significant increase in frontal cortical dopamine in socially-reared rats, and hyper-responsiveness in isolates (Heidbreder et al., 2001), a response that may evoke long-term downregulation of D₁ receptor binding sites as has been described here (Fig. 4).

Concluding, rodents reared in isolation (SIR) present with an upregulation of frontal cortical NMDA receptors and downregulation of D₁ receptors. Considering the similarity of receptor changes in animals exposed to SIR, as described here, with that in schizophrenia highlights the possible construct validity of SIR. At the doses examined, chronic treatment with clozapine but not haloperidol bolsters frontal cortical NMDA receptor density in SIR animals. Haloperidol further reduces cortical D₁ receptor density in SIR animals, while clozapine does not. Thus, SIR evokes a state of frontal cortical D₁-NMDA receptor imbalance that shows a differential response to clozapine compared to haloperidol. Reducing schizophrenia to the confines of observations made from a single analogous animal model such as SIR has distinct limitations. Nevertheless, this study has demonstrated that at the neuroreceptor level and following chronic treatment, clozapine and haloperidol differ in how they target frontal cortical NMDA and D₁ signaling. Moreover, these results reinforce the predictive validity of the SIR model, as well as possibly explaining why first-generation agents and clozapine (and possibly other second generation antipsychotics) differ markedly in how they may address the frontal cortical disturbances of schizophrenia. The ability of environmental challenge to alter NMDA receptor affinity is of interest and requires further study in order to establish its possible significance.

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Addendum C

Möller et al., 2011

In this Addendum, a manuscript describing work undertaken during the candidates MSc is presented, and was central in setting the course for the current PhD study. This manuscript provides background information on the SIR model, evaluating cortico-striatal redox, social and cognitive behaviors with/without chronic clozapine treatment.

Article published in *European Neuropsychopharmacology*, entitled:

“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.”

<http://www.sciencedirect.com/science/article/pii/S0924977X1000194X>

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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

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

^a Unit for Drug Research and Development, Division of Pharmacology, School of Pharmacy, North West University (Potchefstroom campus), Hofman street, Private bag X6001, Potchefstroom, South Africa, 2520

^b Unit for Drug Research and Development, School of Pharmacy, North West University (Potchefstroom campus), Hofman street, Private bag X6001, Potchefstroom, South Africa, 2520

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

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Abstract

Social isolation rearing (SIR) in rats induces behavioral and glutamatergic changes akin to schizophrenia. We studied the effects of 8 weeks SIR on cortico-striatal redox and social and cognitive behaviors in rats. SIR increased superoxide dismutase activity, decreased oxidized: reduced glutathione ratio and increased lipid peroxidation in both brain regions, and induced deficits in prepulse inhibition and social and self-directed interactive behaviors. Both behavioral and cortico-striatal redox disturbances were corrected by clozapine (5 mg/kg/day×11 days). Behavioral changes evoked by SIR are associated with cortico-striatal oxidative stress that is reversed by clozapine treatment, providing novel insight into the neurobiology and treatment of schizophrenia.

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1. Introduction

Early life manipulations in both humans (Walker, 1994) and rodents (Powell and Miyakawa, 2006) “shape” a pre-existing genetic vulnerability to stress that will determine the

development of a psychological illness (Smith et al., 2003; Heim and Nemeroff, 2001). Schizophrenia has been causally linked to genetic, environmental and neurodevelopmental factors (Weiss and Feldon, 2001), with increased incidence of the illness in patients subjected to different forms of pre- and perinatal stress (Van den Buuse et al., 2003). Schizophrenia is characterized by positive symptoms (hallucinations, delusions, thought disorder), negative symptoms (affective flattening, social isolation) and cognitive deficits in areas such as attention, learning, memory and behavioral flexibility

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

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

(Lewis and González-Burgos, 2008). Anxiety is a prominent symptom early in the course of the illness, although is more perceived as a comorbidity than a clinical expression of schizophrenia (Tandon et al., 2009). While first and second generation antipsychotics are effective in reducing positive symptoms, negative and cognitive symptoms are relatively refractory to treatment (Keefe et al., 2007).

Early life adversity is a recognised risk factor for the development of schizophrenia (Lipska and Weinberger, 2000). In rodents, post-natal social isolation rearing (SIR) engenders long-lasting aberrant behaviors akin to those observed in schizophrenia (Heidbreder et al., 2000), including neophobia (Fone, 2008), locomotor hyperactivity (Levine et al., 2007; Weiss and Feldon, 2001), learning and attentional shifting (Andreas et al., 2004), and sensorimotor gating deficits such as in prepulse inhibition (PPI; Schubert et al., 2009; Geyer and Ellenbroek, 2003). PPI reflects the basic inhibitory process of sensorimotor gating that regulates the input of sensory stimuli to the brain, thus preventing cognitive fragmentation and sensory overload that is otherwise typical of schizophrenia (Young et al., 2009). Earlier studies show that disruption of PPI in schizophrenic patients is related to several typical positive or negative symptoms (Braff et al., 1999; Li et al., 2009). Of note is that deficits in PPI in rodents, as in patients with schizophrenia, appear only at or after puberty (Bakshi and Geyer, 1999). PPI thus has important utility as a biomarker of cognitive deficits of schizophrenia (Powell et al., 2009). Another important behavioral component of schizophrenia is deficits in social drive and cognition (Tandon et al., 2009). Interestingly, SIR also induces altered anxiety and social interactive behaviors that closely emulate that seen in schizophrenia (Ferdman et al., 2007; Koike et al., 2009). These behavioral characteristics, and that antipsychotics reverse SIR-induced attenuation of PPI (Pen and Moreau, 2002), supports the face, construct and predictive validity of SIR for schizophrenia (Geyer et al., 2001; Weiss and Feldon, 2001), as well as its usefulness for studying antipsychotic drug action (Andreas et al., 2004; Geyer and Ellenbroek, 2003). Nevertheless, despite these benefits caution needs to prevail when viewing SIR associated changes within the context of the complexity of the human disorder.

Glutamate N-methyl-D-aspartate (NMDA) receptor dysfunction and excitotoxicity has become a focus of attention in attempts to explain the neurobiology of schizophrenia (Krystal et al., 2005; Olney et al., 1999; Krivoy et al., 2008). Glutamatergic neurons are present in both the striatum and cortex (Amadio et al., 2004; Haroutunian et al., 2003), where excessive release of glutamate, activation of NMDA receptors and increased calcium influx not only alter dopamine release (Moore et al., 1999) but also impact upon cellular redox balance (Smythies, 1999). Indeed, an altered redox state is evident in schizophrenia, including reduction in both reduced and oxidized glutathione, reduced superoxide dismutase (SOD), reduced catalase and glutathione peroxidase, as well as increased lipid peroxidation (Mukherjee et al., 1996; Kuloglu et al., 2002; Kunz et al., 2008; Ng et al., 2008). Since SIR in rats induces changes in frontal cortical NMDA receptor binding (Toua et al., 2010) and increases NR2A NMDA expression (Turnock-Jones et al., 2009), this will have distinct implications for altered redox state, especially since oxidative stress may upregulate the NMDA

receptor (Betzen et al., 2009). While altered redox state has been studied in drug-induced models of schizophrenia (e.g. Radonjic et al., 2010), this has not been studied in a neurodevelopmental model nor related to aberrant behaviors akin to that observed in schizophrenia.

In the current study we investigated the association between social interaction and PPI in rats subjected to 8 weeks SIR, and changes in frontal cortical and striatal levels of SOD activity, oxidized versus reduced glutathione ratio (GSSG/GSH) and malondialdehyde (MDA) accumulation (a marker of lipid peroxidation). To relate these bio-behavioral changes to schizophrenia and with antipsychotic response, we also investigated the above with respect to response to sub-chronic treatment with the antipsychotic agent, clozapine.

2. Experimental procedures

2.1. Animals

Male Sprague–Dawley rats (160–190 g; Animal Research Centre, North West University) were randomly allocated to groups of 10 rats/group. At weaning (post-natal day 21) they were randomized to SIR (1 animal/cage) or socially reared (3–4 rats/cage) groups for 8 weeks (day 77). The rats were reared under identical conditions: Cages (230(h) × 380(w) × 380(l) cm) with sawdust (Weiss and Feldon, 2001), temperature (21 ± 0.5 °C), humidity (50 ± 10%), free access to food and water, white light (350–400 lux), 12 hour light/dark cycle. SIR and socially reared animals experienced minimal handling and no environmental enrichment. Sawdust was changed weekly. Behavioural testing was carried out between 08:00 and 17:00 h. The animals were handled according to the code of ethics in research, training and testing of drugs in South Africa (NWU-0035-08-S5).

2.2. Drugs and drug treatment protocol

Clozapine (Sigma-Aldrich, Johannesburg, South Africa) was administered intraperitoneally (i.p) at a dose of 5 mg/kg in 0.5 ml (Toua et al., 2010) while control groups received saline injection (pH 5). Previous studies indicated that clozapine (5.0 mg/kg) antagonized PPI deficits in rats without itself having effects on PPI (Bakshi et al., 1994). Early antipsychotic response at 2 weeks is suggested to be an accurate predictor of later response (Kapur et al., 2005; Emsley et al., 2007; Ascher-Svanum et al., 2008) thus questioning the need for prolonged treatment to confirm efficacy. Bearing this in mind, but also to exclude the possible impact of injection stress evoked over extended treatment periods, we have conducted this study over an 11 day treatment period (see Toua et al., 2010). Clozapine, freshly prepared each day as previously described (Toua et al., 2010), and saline were therefore administered in the last 11 days of housed/isolation rearing. All animals were sacrificed by decapitation without any prior use of an anesthetic agent.

2.3. Experimental design

In order to prevent any untoward effect of behavioral testing on the neurochemical parameters studied, we have used separate groups of animals for the behavioral and neurochemical analyses, as outlined below.

2.3.1. Non-treatment cohort

Behavioral study: The animals were randomly separated at weaning (21 days post-natal) into two groups. One group of animals (n=10) were placed into 8 weeks SIR, while a parallel socially reared control group (n=10) was run concurrently. After 8 weeks, social and

sensorimotor gating behaviors were studied in both groups with 1 day between the two tests, and thereafter sacrificed by decapitation without the prior use of an anesthetic agent.

Neurochemical (redox) study: A separate two groups of animals, randomly assigned to the same groupings as described above, followed the above protocol except that at 8 weeks the animals were sacrificed and brains harvested for regional brain neurochemical analysis.

2.3.2. Drug-treatment cohort

Behavioral study: Animals were randomly separated at weaning (21 days post-natal) into four groups. Two groups (n=10 each) received 8 weeks SIR or social rearing plus saline treatment. The remaining two groups (n=10) received 8 weeks SIR or social rearing plus clozapine treatment. After 8 weeks, the animals were tested for social and sensorimotor behaviors (PPI test) on day 9 and day 11 of treatment, respectively. Social and sensorimotor gating behaviors were studied 30 min after final injection. Thereafter the animals were sacrificed by decapitation without the prior use of an anesthetic agent.

Neurochemical (redox) study: A separate two groups of animals, randomly allocated as described above, received saline or clozapine treatment. At 8 weeks the animals were sacrificed and the brains harvested for neurochemical analysis.

2.3.3. Acute drug treatment study

To confirm the effects of clozapine on SIR-induced bio-behavioral changes to be sub-chronic treatment dependent, similar to the typical treatment response in schizophrenia, we undertook an acute treatment study where clozapine and saline were administered as per the dosing procedure on the final day of the chronic treatment protocol. This would also assist in distinguishing an acute drug treatment effect following the last dose of the sub-chronic drug treatment regimen.

Behavioral study: The animals were randomly separated at weaning (21 days post-natal) into two groups. One group of animals received saline treatment (n=10) while the other group received clozapine treatment (5 mg/kg) (n=10). Social and sensorimotor gating behaviors were studied, 30 min after each injection, in both these groups with 1 day between the two tests, and thereafter sacrificed by decapitation without the prior use of an anesthetic agent.

Neurochemical study: An additional two groups of animals, randomly assigned to the same groupings as described above, followed the above protocol except that after 30 min, the animals were sacrificed and brains harvested for regional brain neurochemical analysis.

2.4. Body weight

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

2.5. Behavioral analyses

2.5.1. Social interaction test

An open field arena (70 cm×70 cm×40 cm), consisting of a black square divided into 35 cm×35 cm equal squares (Sherif and Orelund, 1995), was illuminated with red light (40 W) and monitored with a digital video camera. For the treatment cohort, rats received clozapine or saline 30 min prior to each session (day 9). Two rats were placed in the centre of the floor and allowed 30 s for adaptation. Thereafter, time spent in active social interactive behaviors, namely rearing, anogenital sniffing, approaching and staying with the partner (Gonzalez et al., 1996), as well as self-directed behavior, including squares crossed and self grooming (Gonzalez et al., 1996), were scored for 10 min. Of the two rats tested, one of these animals was tested for social interaction with an

unknown test partner that did not differ by more than 10 g in weight. Both members of a test-pair had received the same treatment and the same prior experience. Testing was performed between 08.00 and 12.00 h, with video scoring based on averaged scores by two blind independent observers who remained outside the testing room. The arena was cleaned with 10% ethanol solution after each test.

2.5.2. Prepulse inhibition testing

PPI was assessed according to Geyer and Swerdlow (1998) and Wang et al. (2003) in ventilated sound-attenuated startle chambers (SR-LAB, San Diego Instruments, San Diego, USA) consisting of a transparent Plexiglas cylinder (diameter 8.2 cm, length 20 cm) mounted on a Plexiglas frame (the stabilimeter). The acoustic startle responses, induced by a speaker mounted 24 cm above the cylinder, were transduced and detected by a piezoelectric accelerometer mounted below the frame and processed by SR-LAB software. 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 30 min after the final injection (day 11), with a 5-min acclimatization period plus a 68-dB background noise level that continued throughout the test session. To evaluate the basal startle response, 10 trials consisting of a single 40 ms 120 dB white-noise startle stimulus was presented to the rat. Next, the test session continued with 80 trials consisting of a random delivery of twenty 120 dB pulse-alone trials, 10 trials during which no stimuli was delivered, and 50 prepulse trials. Prepulse trials included a single 120 dB pulse preceded by a 20 ms non-startling prepulse-stimulus with intensities of 72, 76, 80 or 84 dB. The time interval between the prepulse offset and the pulse onset was 80 ms. Percent PPI (% PPI) for the four prepulse intensities 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). The last 10 trials were single 40 ms 120 dB pulse-alone startle stimuli. The total of 100 trials was delivered with an average interval of 25 s. The first and last 10 pulse-alone stimuli (BLOCK 1 and BLOCK 4, respectively) and the 20 pulse-alone stimuli included in the PPI block itself (BLOCK 2 and BLOCK 3), were used to obtain a measure of mean startle amplitude indicative of habituation in response to repeated delivery of startling stimuli (Wang et al., 2003; Van den Buuse and Eikelis, 2001).

2.6. Neurochemical (redox) analyses

2.6.1. Preparation of brain tissue

After decapitation, the frontal cortex was immediately dissected according to Toua et al. (2010). 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 were immediately fixed in liquid nitrogen and stored at -72 °C. On the day of assay, the tissue was thawed on ice, weighed and a 10% tissue homogenate prepared in a Teflon homogenizer using phosphate buffered saline (PBS). Protein content was determined according to the Bradford method (Bradford, 1976).

2.6.2. Assessment of redox state

2.6.2.1. Superoxide dismutase (SOD) activity. Percentage SOD activity was determined in brain homogenates using a commercially available colorimetric assay kit (SOD Assay Kit-WST (Sigma-Aldrich)). One unit (U) of enzyme activity was defined as the quantity of SOD required to cause a 50% inhibition of the absorbance change per min of the blank reaction (diluent rate), and expressed as U/mg protein.

2.6.2.2. Oxidized (GSSG) versus reduced (GSH) glutathione. GSSG and GSH were analysed utilizing a liquid chromatography/mass spectrometry (LC/MS) method described previously (Harvey et al.,

2008). Briefly, a derivatization solution was added to brain extracts together with internal standard (200 ng/ml of gabapentin) and sulfosalicylic acid solution (10% w/v) to precipitate proteins. The supernatant was transferred to 200 μ l inserts, in autosampler vials, and analysed using an Applied Biosystems API 2000 triple quadrupole LC/MS. Data (nmol/mg tissue) are presented as GSSG/GSH.

2.6.2.3. Lipid peroxidation. Lipid peroxidation was determined by thiobarbituric acid (TBA) assay with butanol extraction using tetramethoxypropane as standard and a 10% protein concentration (Harvey et al., 2008). Data are expressed as pmol malondialdehyde formed/mg protein.

2.7. Statistical analysis

To model % PPI and mean startle amplitude three-way analysis of variance (ANOVA) were used for treatment (clozapine vs. vehicle) and rearing conditions, plus repeated measures for the startle amplitude and different PPI intensities. For the non-treatment cohort and the acute treatment study, two-way ANOVA were used, plus repeated measures, as above. Bonferroni post-hoc test were subsequently applied (Graphpad Prism 5; SAS/STAT® Software). In the social interaction tests, unpaired Student's t-test was used in the non-treatment cohort and the acute treatment study. Regional brain analysis of redox parameters was analysed by unpaired Student's t-test in the non-treatment cohort and the acute drug treatment study. In the drug treatment cohort, three-way factorial ANOVA with repeated measures and Bonferroni post-hoc tests was applied. In all cases, data are expressed as the mean $\pm$ standard error of the mean (SEM), with a p value of <0.05 deemed statistically significant. Animal body weight (mean $\pm$ SEM) was analysed by one-way ANOVA with repeated factors of treatment (saline and clozapine) and housing conditions to compare body weight across the groups, followed by Bonferroni post-hoc analyses.

3. Results

3.1. Behavioral studies: prepulse inhibition (PPI)

3.1.1. PPI in the non-treatment cohort

The mean startle amplitudes of the socially-housed controls and isolated rats during four consecutive blocks of ten 120 dB stimuli are shown in Fig. 1A. There were no significant overall differences in mean startle amplitude between the group-housed controls and SIR rats. Furthermore, no significant habituation was observed in the group-housed or SIR rats with repeated stimulation between BLOCK 1 and BLOCK 4 of startle (Fig. 1A).

Two-way ANOVA revealed a significant PPI*housing condition interaction ($F(3,72)=2.81$, $p=0.046$) (Fig. 1B). %PPI was significantly reduced in the SIR rats compared to their socially reared controls, with a significant overall effect at each prepulse intensity: 72 dB ($p<0.0001$), 76 dB ($p<0.0001$), 80 dB ($p<0.0003$) and 84 dB ($p=0.001$) (Fig. 1B).

3.1.2. PPI in the drug treatment cohort

Using two-way ANOVA, we found an overall significant BLOCK*treatment interaction in socially reared and SIR rats ($F(3,54)=2.91$, $p=0.04$, Fig. 2A). This suggests a minor decrease in startle following clozapine treatment, particularly in the first blocks, as is seen in both the non-treatment (Fig. 1A) and the drug treatment cohort (Fig. 2A). However, the startle reduction is rather small and is unlikely to explain drug and housing effects on PPI.

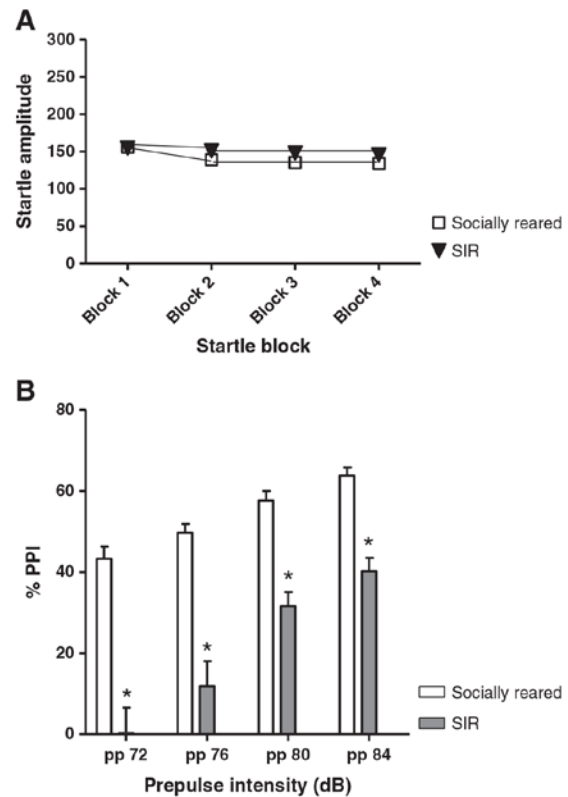


Figure 1 Sensorimotor gating at prepulse intensities as indicated, in socially reared and SIR rats, in the non-treatment study ($n=10$ /group): (A) mean startle amplitude and (B) percent prepulse inhibition (%PPI). * $p<0.005$ vs. socially reared (two-way ANOVA and Bonferroni post-hoc test). Refer to text for exact p values.

Using three-way ANOVA, we found the following significant interactions in the drug treatment study in SIR and socially reared animals: %PPI*treatment ($F(3,72)=2.18$, $p=0.0004$), %PPI*housing condition ($F(3,72)=52.6$, $p<0.0001$), and treatment*housing ($F(3,72)=77.34$, $p<0.0001$) (Fig. 2B). Bonferroni post-hoc analysis indicated no significant differences in %PPI for each prepulse intensity in socially reared animals receiving either saline or clozapine treatment (i.e. 72 dB, 76 dB, 80 dB, 84 dB; Fig. 2B), suggesting no drug-related effects in socially reared animals. However, in the SIR animals receiving clozapine or saline treatment, post-hoc Bonferroni tests revealed a significant increase in %PPI in clozapine treated animals compared to those receiving only saline for each prepulse intensity (i.e. 72 dB ($p<0.0001$), 76 dB ($p<0.0001$), 80 dB ($p<0.0001$) and 84 dB ($p<0.0001$), Fig. 2B).

3.2. Behavioral studies: social interaction studies

3.2.1. Social interaction in the non-treatment cohort

In the non-treatment cohort, Student t-test revealed a significant decrease in social interactive behaviors in the SIR animals compared to their socially reared controls, with respect to rearing ($p<0.0001$), anogenital sniffing ($p<0.0001$), ap-

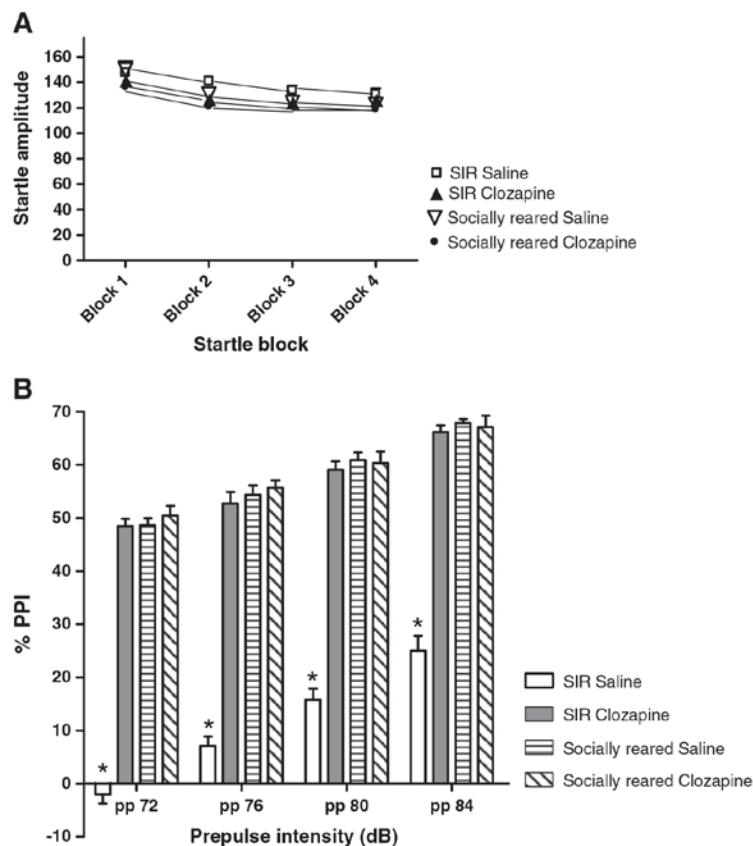


Figure 2 Sensorimotor gating at prepulse intensities as indicated, in socially reared and SIR rats (A–B) in the drug treatment study ($n=10/\text{group}$): (A) mean startle amplitude and (B) percent prepulse inhibition (%PPI). * $p<0.0001$ vs. SIR clozapine, vs. socially reared saline, vs. socially reared clozapine, at 72 dB, 76 dB, 80 dB and 86 dB respectively (three-way ANOVA and Bonferroni post-hoc test).

proaching ($p<0.0001$), and staying together ($p<0.0001$) (Fig. 3A–D). Student t-tests also revealed significantly increased self-directed behaviors in the SIR animals compared to their socially reared controls with respect to square crossing ($p=0.03$) and self grooming ($p<0.0001$) (Fig. 3E–F).

3.2.2. Social interaction changes in the drug treatment cohort

Three-way factorial ANOVA revealed significant cross-group interactions in the drug treatment cohort with respect to time spent rearing ($F(2,54)=57.28$, $p<0.0001$), anogenital sniffing ($F(2,54)=42.68$, $p<0.0001$), approaching ($F(2,54)=18.2$, $p<0.0001$), and staying together ($F(2,54)=34.14$, $p<0.0001$) (Fig. 4A–D). Post-hoc analysis with Bonferroni tests showed a significant decrease in social interactive behaviors in the SIR animals receiving saline compared to their socially reared control with respect to rearing ($p<0.0001$), anogenital sniffing ($p<0.0001$), approaching ($p<0.0001$), and staying together ($p<0.0001$). These deficits in social interactive behaviors were in turn completely reversed with clozapine treatment in the SIR animals, for rearing ($p<0.0001$), anogenital sniffing ($p<0.0001$), approaching ($p<0.0001$) and staying together ($p<0.0001$) (Fig. 4A–D). Clozapine did not have any notable effects of its

own on social interactive behaviors compared to saline-treated socially reared animals (Fig. 4A–D).

When considering self-directed behaviors, three-way factorial ANOVA revealed significant cross-group interactions for square crossings ($F(2,54)=5.07$, $p=0.01$) and self grooming ($F(2,54)=58.53$, $p<0.0001$) (Fig. 4E–F). Post-hoc analysis with Bonferroni tests indicated significantly elevated self-directed behaviors in the SIR animals only receiving saline treatment compared to their socially reared controls, for square crossing ($p<0.0001$) and self grooming ($p<0.0001$). In turn, clozapine treatment also significantly reversed the elevated self-directed behaviors in SIR animals, for square crossing ($p<0.0001$) and self grooming ($p<0.0001$) versus SIR saline (Fig. 4E–F), although did not have inherent effects on these parameters of its own in socially reared animals (Fig. 4E–F).

3.3. Neurochemical (redox) studies

3.3.1. Superoxide dismutase activity

Unpaired Student t-test revealed a significant deficit in SOD activity in the non-treated SIR rats compared to their socially reared controls, in both the striatum ($p<0.0001$) and frontal cortex ($p<0.0001$) (Fig. 5A). In the drug treatment cohort,

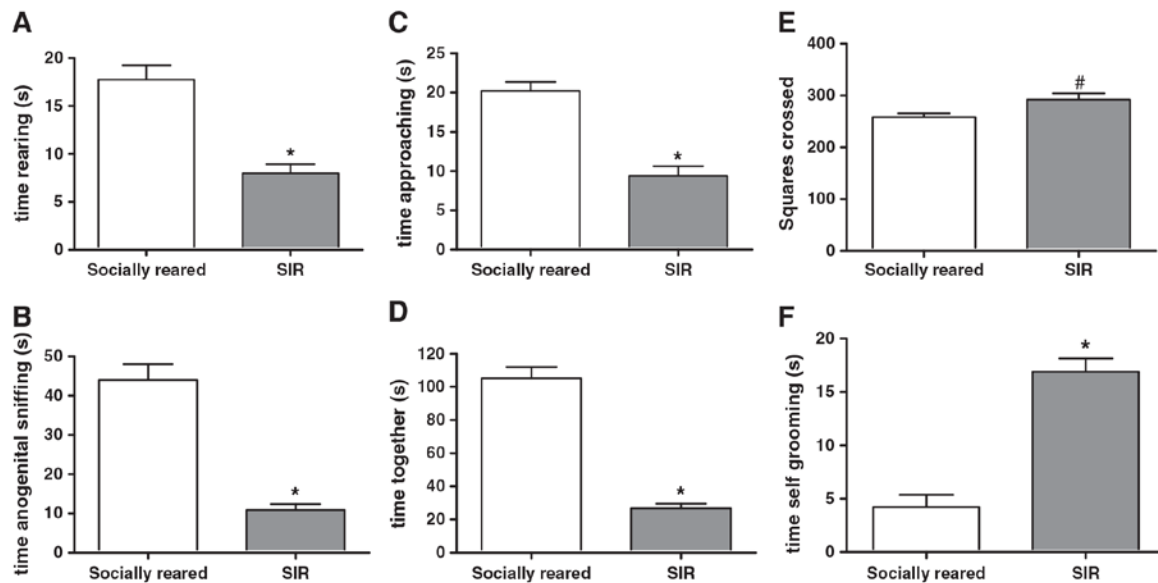


Figure 3 Social interactive behaviors (A–D), and self-directed behaviors (E–F), in socially reared and SIR rats in the non-treatment study ($n=10/\text{group}$): (A) time spent rearing ($*p<0.0001$ vs. socially reared); (B) time spent anogenital sniffing ($*p<0.0001$ vs. socially reared); (C) times approached ($*p<0.0001$ vs. socially reared); (D) time spent together ($*p<0.0001$ vs. socially reared); (E) squares crossed (locomotor activity) ($\#p=0.03$ vs. socially reared); and (F) time spent self grooming ($*p<0.0001$ vs. socially reared) (Student's *t*-test).

three-way ANOVA analysis revealed the following significant interaction: treatment*brain region ($F(1,36)=2.1$, $p=0.006$), treatment*housing condition ($F(1,36)=3.34$, $p=0.008$) and housing condition*brain region ($F(1,36)=9.71$, $p=0.004$) (Fig. 5B). Post-hoc Bonferroni analysis indicated the same as observed in the non-treatment cohort (Fig. 5A), with SIR rats receiving saline having a significantly reduced SOD activity in both the striatum ($p<0.0001$) and frontal cortex ($p<0.0001$) compared to their socially reared controls (Fig. 5B). Clozapine treatment significantly reversed this deficit in the SIR animals versus animals receiving saline in both the striatum ($p<0.0001$) and frontal cortex ($p<0.0001$, Fig. 5B). However, clozapine alone had no significant effect on SOD activity in the socially reared rats compared to saline-treated controls in both the striatum and frontal cortex (Fig. 5B).

3.3.2. Oxidized (GSSG) versus reduced (GSH) glutathione

Unpaired Student *t*-test in the non-treatment cohort revealed a significantly lower GSSG/GSH ratio in the SIR rats compared to their socially reared controls, in both the striatum ($p=0.0003$) and frontal cortex ($p=0.002$) (Fig. 6A), suggesting a reduction in oxidized glutathione (GSSG) and an increase in reduced glutathione (GSH) in these brain regions. In the drug treatment cohort, three-way ANOVA analysis revealed the following significant interaction: treatment*brain region ($F(1,36)=3.1$, $p=0.006$), treatment*housing condition ($F(1,36)=2.3$, $p=0.008$) and housing condition*brain region ($F(1,36)=9.71$, $p=0.003$) (Fig. 6B). Bonferroni post-hoc analysis indicated the same as in the non-treatment cohort (Fig. 6A), with SIR rats receiving saline having a significantly reduced GSSG/GSH ratio in both the striatum ($p<0.0001$) and frontal cortex ($p<0.0001$) compared to their socially reared controls (Fig. 6B). Clozapine treatment completely reversed this deficit in the SIR animals

versus saline-treated animals in both the striatum ($p<0.0001$) and frontal cortex ($p<0.0001$; Fig. 6B). However, clozapine had no significant effect alone in socially reared rats compared to saline-treated socially reared controls in the striatum and frontal cortex (Fig. 6B).

3.3.3. Lipid peroxidation

Unpaired Student *t*-tests revealed a significant elevation of malondialdehyde, a product of lipid peroxidation, in SIR rats compared to their socially reared controls, in both the striatum ($p<0.0001$) and frontal cortex ($p<0.0001$) (Fig. 7A). In the drug treatment cohort (Fig. 7B), three-way ANOVA analysis revealed the following significant interactions: treatment*brain region ($F(1,36)=4.7$, $p=0.04$), treatment*housing condition ($F(1,36)=9.5$, $p=0.04$) and housing condition*brain region ($F(1,36)=2.7$, $p=0.006$) (Fig. 7B). Post-hoc Bonferroni analysis indicated the same as seen in the non-treatment cohort (Fig. 7A), with a significant elevation of malondialdehyde in both the striatum ($p<0.0001$) and frontal cortex ($p<0.0001$) of SIR rats receiving saline treatment compared to their socially reared controls (Fig. 7B). Clozapine treatment significantly reversed this elevation versus SIR animals receiving saline, in both the striatum ($p<0.0001$) and frontal cortex ($p<0.0001$) (Fig. 7B). However, clozapine had no significant effect alone on lipid peroxidation in socially reared rats compared to the saline-treated socially reared controls in both the striatum and frontal cortex (Fig. 7B).

3.4. Affect of acute clozapine versus saline treatment on behavior and redox status

Affect of acute drug treatment on PPI: Two-way ANOVA with Bonferroni post-hoc tests revealed no significant overall differences in mean startle amplitude between the acute

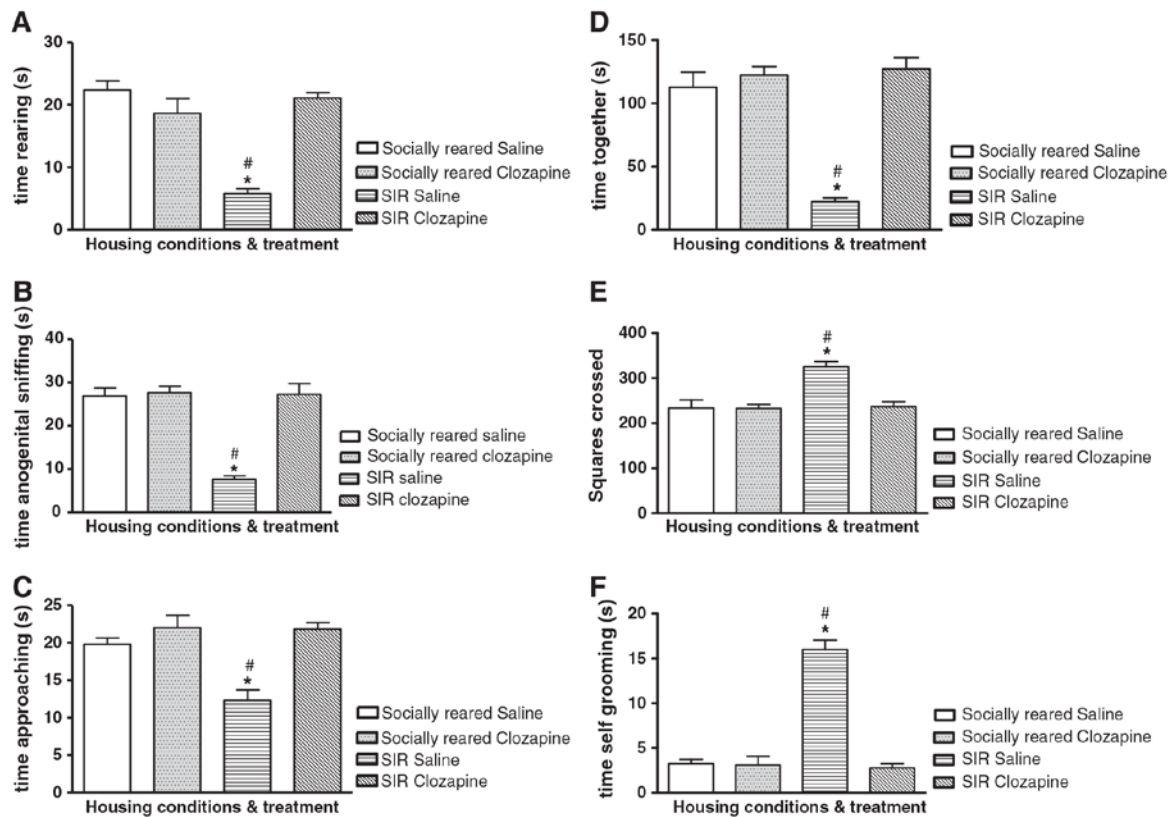


Figure 4 Social interactive behaviors (A–D), and self-directed behaviors (E–F), in socially reared and SIR rats in the drug treatment study ($n=10/\text{group}$): (A) time spent rearing ($*p<0.0001$ vs. socially reared saline; $\#p<0.0001$ vs. SIR clozapine); (B) time spent anogenital sniffing ($*p<0.0001$ vs. socially reared saline; $\#p<0.0001$ vs. SIR clozapine); (C) times approached ($*p<0.0001$ vs. socially reared saline; $\#p<0.0001$ vs. SIR clozapine); (D) time spent together ($*p<0.0001$ vs. socially reared saline; $\#p<0.0001$ vs. SIR clozapine); (E) squares crossed (locomotor activity) ($*p<0.0001$ vs. socially reared saline; $\#p<0.0001$ vs. SIR clozapine); and (F) time spent self grooming ($*p<0.0001$ vs. socially reared saline; $\#p<0.0001$ vs. SIR clozapine) (three-way ANOVA and Bonferroni post-hoc test).

saline and clozapine treated rats (Table 1). Furthermore, no significant habituation was observed in the saline or clozapine treated rats with repeated stimulation between BLOCK 1 and BLOCK 4 of startle, although a slight decrease in startle was observed in the first blocks (Table 1). Bonferroni post-hoc analysis also indicated no significant effect on %PPI, in clozapine versus saline-treated animals for each prepulse intensity (72 dB, 76 dB, 80 dB and 84 dB; Table 1).

Affect of acute drug treatment on social interaction: Student t-test revealed no significant differences in social interactive behaviors (rearing, anogenital sniffing, approaching, staying together) in saline versus clozapine treated animals (Table 1), as well as with regard to self-directed behaviors (square crossing, self grooming) in the saline versus clozapine treated animals (Table 1).

Affect of acute drug treatment on redox markers: Student t-test revealed no significant differences in SOD activity, GSSG/GSH ratio and malondialdehyde accumulation between acute saline versus clozapine treated rats (Table 1).

3.5. Body weights

The mean weights of the rats over all the groups used during this study are as follows: PND 21: 47.10 ± 1.95 g (mean $\pm$ SEM), day 1 of treatment: 169.90 ± 9.97 g (mean $\pm$ SEM), and day 11 of treatment: 276.40 ± 6.39 g (mean $\pm$ SEM). One-way ANOVA with repeated measures followed by Bonferroni, indicated no significant overall differences in weight between the different groups at the start of treatment, and on day 11 of treatment, suggesting that rearing condition or drug treatment did not adversely affect weight gain or development. Furthermore, all SIR and socially reared animals showed a significant and equal amount of growth over the period of the study (data not shown).

4. Discussion

Consistent with several previous reports in rats and mice, this study has concurred that SIR is capable of inducing profound

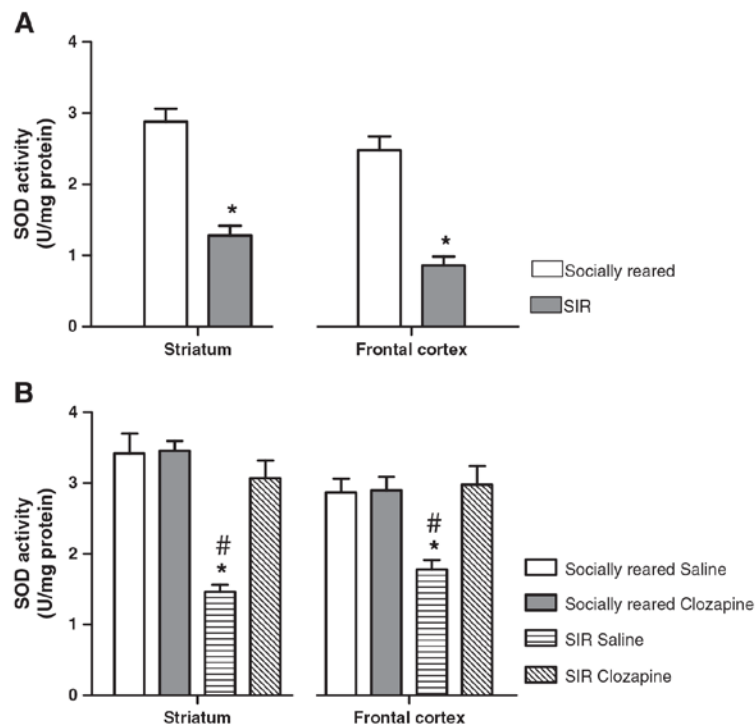


Figure 5 Superoxide dismutase activity (U/mg protein) in both the striatum and frontal cortex of the socially reared and SIR rats (n=10/group) in: (A) the non-treatment cohort (* $p < 0.0001$ vs. socially reared; Student's t-test) and (B) the treatment cohort (* $p < 0.0001$ vs. socially reared saline; # $p < 0.0001$ vs. SIR clozapine) (three-way ANOVA and Bonferroni post-hoc test).

deficits in %PPI (Geyer et al., 2001) and social interaction (Ferdman et al., 2007), as well as hyper-locomotor activity (Heidbreder et al., 2000; Levine et al., 2007). Specifically, we noted an elevation in self-directed behaviors in SIR rats, such as self grooming (Figs. 3F and 4F), as well as a decrease in various social interactive behaviors, including rearing, anogenital sniffing, approaching and socializing behaviors (Figs. 3A–D and 4A–D). We also noted an increase in locomotor activity in the OFT (Figs. 3E and 4E). Hyper-locomotor activity and elevated self grooming are indicative of increased hyperactivity and anxiety, respectively (Ferdman et al., 2007; Tandon et al., 2009; Koike et al., 2009), which like reduced social interactive behavior, share common underlying brain mechanisms and neurotransmitter involvement with schizophrenia (Lipska and Weinberger, 2000). To the best of our knowledge, this is the first paper to simultaneously study the effect of SIR on deficits in PPI and social interaction. However, a particularly novel observation in this study is that sub-chronic clozapine treatment reversed both PPI deficits and altered social interactive behaviors in SIR animals (Figs. 2 and 4 respectively), providing further convincing evidence of the face and predictive validity of SIR for schizophrenia. Importantly, the action of clozapine was a true effect of sub-chronic treatment and could not be reproduced by acute administration of the drug (Table 1).

Recent work in schizophrenia has provided evidence for increased oxidative stress (Chittiprol et al., 2010; Zhang et al.,

2009) as well as changes in glutamate function (Baier et al., 2009; Olney et al., 1999). There is also evidence to link altered NMDA receptor function to increased oxidative stress (Betzen et al., 2009; Fatokun et al., 2008). Recent pre-clinical work has brought to light evidence for altered frontal cortical NMDA receptor binding in rats subjected to SIR (Toua et al., 2010). However, whether such a disturbance may be a driving force for changes in redox state, as well as being associated with altered behavior, has not yet been studied. In the present study, in parallel with the above mentioned behavioral changes, we found significant deficits in frontal cortical and striatal SOD activity (Fig. 5) in SIR animals, as well as a decrease in oxidized (GSSG) versus reduced (GSH) glutathione balance in both frontal cortex and striatum (Fig. 6). Thus, SIR in rats is associated with an increase in reduced glutathione (GSH), possibly due to a decrease in glutathione peroxidase activity reminiscent of that observed in schizophrenia (Ng et al., 2008). Interestingly, an increase in GSH production is also suggestive of an increase in reductive stress (Rajasekaran et al., 2007). In addition, we observed significantly elevated levels of lipid peroxidation products (TBARS) in both the striatum and frontal cortex of SIR rats (Fig. 7), suggesting that SIR-induced changes in social and cognitive function are associated with increased oxidative stress and evidence for oxidative cell damage. Indeed, the brain has a high lipid and iron content and is particularly sensitive to ROS-mediated assault on poly-unsaturated fatty acids in cell membranes

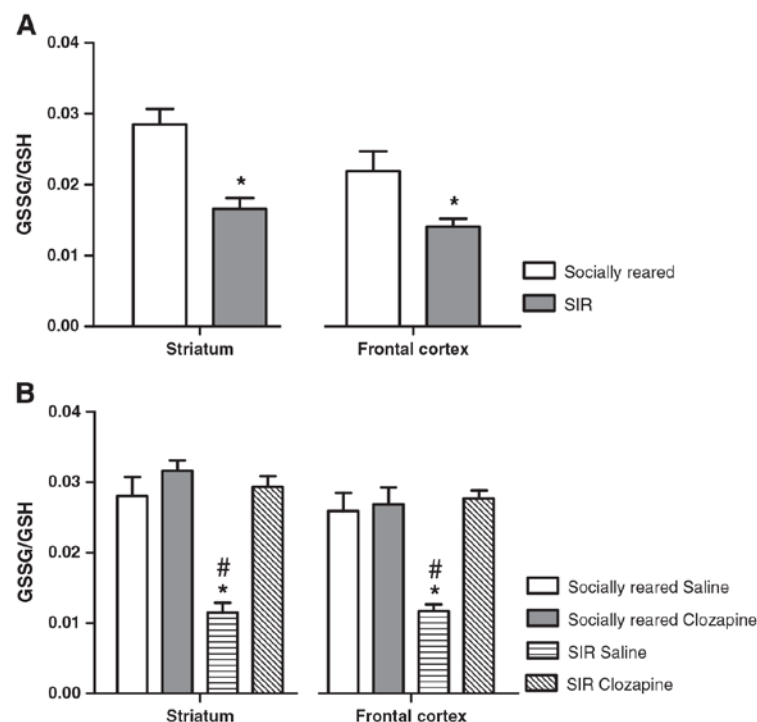


Figure 6 Oxidized/reduced glutathione ratio (GSSG/GSH) in the striatum and frontal cortex of the socially reared and SIR rats ($n=10$ /group) in: (A) the non-treatment cohort (* $p<0.003$ vs. socially reared; Student's t -test, refer to text for exact p values) and (B) the treatment cohort (* $p<0.0001$ vs. socially reared saline; # $p<0.0001$ vs. SIR clozapine) (three-way ANOVA and Bonferroni post-hoc test).

(Akyol, 2002). Decreased SOD activity, as noted in this study, can also contribute to superoxide-mediated DNA damage (Mahadik and Mukherjee, 1996). Decreased GSH peroxidation activity, possibly responsible for increased GSH in this study and which has been observed in the acute period of schizophrenia (Pavlović et al., 2002), may invoke increasing amounts of H_2O_2 to further compromise redox homeostasis.

Since SIR has been associated with increased frontal cortical NMDA receptor binding (Toua et al., 2010), and that earlier work in cell cultures has noted that NMDA receptor antagonism enhances [^3H]MK-801 specific binding (Hu et al., 1996) and/or up-regulates NMDA receptor mRNA (Hu et al., 1996), concurs that SIR may indeed evoke a state of frontal cortical *hypoglutamatergia*. In fact, NMDA receptor hypofunction has been associated with apoptosis (Olney et al., 1999; du Bois and Huang, 2007), which in itself may drive a diverse array of cellular redox disturbances (Castagne et al., 1999). Furthermore, NMDA antagonists not only induce schizophrenia-like symptoms (Olney et al., 1999), but they also have been found to increase redox imbalance and oxidative stress in rats (Radonjic et al., 2010). That the redox disturbances described in the current study occurred together with deficits in sensorimotor gating and social interactive behaviors provide support for an important association with schizophrenia. Moreover, sub-chronic treatment with clozapine not only reversed the aforementioned behavioral changes following SIR, but also reversed cortico-

striatal redox disturbances evident in these animals (Figs. 5–7). As with the behavioral changes, these effects were also unique to sub-chronic clozapine treatment, and could not be related to an acute effect of the drug (Table 1). PPI can be studied in humans and rats using almost identical procedures and stimuli (Bubeniková-Valešová et al., 2008). This suggests that the neurobiological mechanisms underlying deficits in PPI are similar across species. Our animal data therefore implies that changes in sensorimotor gating in humans may also be causally related to altered redox state.

Behavioral flexibility is mediated by the frontal lobes, but is dependent on a number of sub-cortical systems that interact with the prefrontal cortex (Bonilha et al., 2008), including the dorsal and ventral regions of the striatum, and the meso-cortico-limbic dopamine system (Tzschenke, 2001). Thus, altered frontal cortical glutamatergic activity (Adams and Moghaddam, 1998) and a hyperdopaminergic state in the mesolimbic system may be responsible for hyperlocomotor activity (Lipska and Weinberger, 2000) and excessive self grooming (Dall'Olio et al., 2000) observed in this study, and which in turn can be equated with the positive symptoms in schizophrenia (Bubeniková-Valešová et al., 2008; Tandon et al., 2009). The meso-accumbens dopaminergic system is also involved in the expression of social interaction behaviors (Louilot et al., 1986, reviewed by Seillier and Giuffrida, 2009), and by implication the decreased social interactive behaviors observed in SIR rats

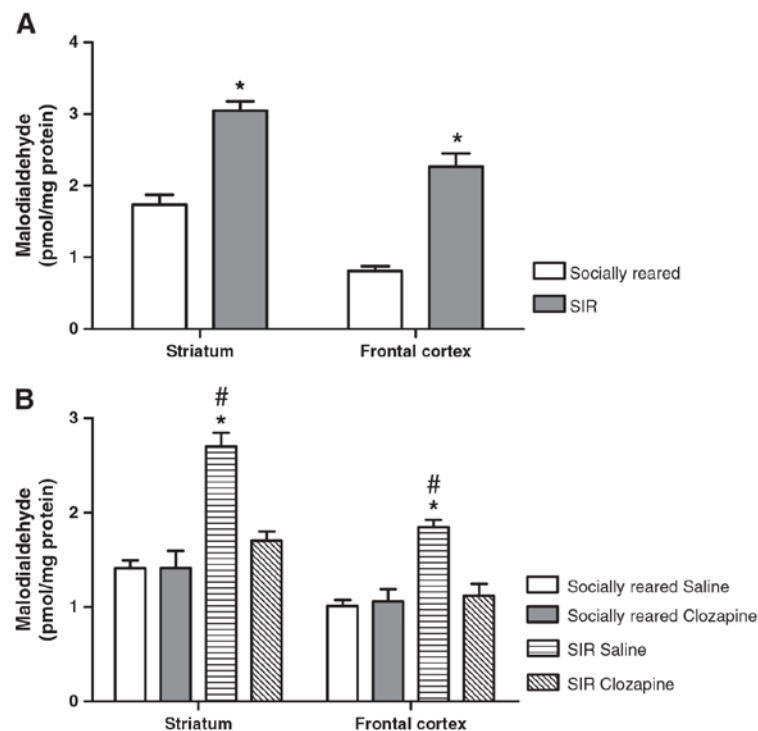


Figure 7 Concentration of malondialdehyde, a measurement of lipid peroxidation, in the striatum and frontal cortex of socially reared and SIR rats ($n=10/\text{group}$) in: (A) the non-treatment cohort (* $p<0.0001$ vs. socially reared; Student's t -test) and (B) the treatment cohort (* $p<0.0001$ vs. socially reared saline; # $p<0.0001$ vs. SIR clozapine) (three-way ANOVA and Bonferroni post-hoc test).

described here. It is also known that prefrontal dopamine release is strongly regulated by glutamate via NMDA receptors (Del Arco and Mora, 2001). Since SIR in rats induces opposing effects on D₁-NMDA receptor binding that is further modulated following clozapine treatment (Toua et al., 2010), it is of particular significance that in the current study clozapine was found to reverse deficits in PPI and social investigative behaviors in SIR animals, as well as altered cortico-striatal redox balance, thus strongly implicating a glutamate NMDA receptor mediated mechanism in SIR-induced behavior and redox changes. Indeed, while NMDA receptor activity is required for the expression of anti-oxidant enzymes (Fatokun et al., 2008), hypofunctioning or prolonged blockade of the NMDA receptor can evoke an increase in oxidative stress, leading to an increase in reactive oxygen and nitrogen species (RNS) *in vitro* (Wang et al., 2002) and *in vivo* (Wass et al., 2009). Moreover, a protracted state of NMDA receptor hypofunction can trigger neuronal injury throughout many corticolimbic brain regions (Olney et al., 1989; Corso et al., 1997). Since schizophrenia may be causally linked to a state of hypoglutamatergia, particularly in the cortico-striatal limbic regions (Hirsch et al., 1997), our data indicates that altered cortico-striatal redox state is a causal factor in the aberrant behavior seen in SIR rats, and possibly in schizophrenia. Indeed, we observed a significant effect of brain region in all redox parameters measured, thus

emphasizing the vulnerability of these areas to SIR-induced oxidative stress. Considering the evidence for oxidative stress in the striatum of SIR rats, disinhibition of pyramidal glutamatergic neurons in the sub-cortical brain regions and a resulting increase in glutamatergic activity (Konradi and Heckers, 2003 for review), may also explain the increase in striatal redox disequilibrium.

Of great interest is that, in addition to reversing SIR-induced decreased SOD activity and altered GSSG:GSH ratio, sub-chronic treatment with clozapine effectively reversed evidence of oxidative cell damage (increased lipid peroxidation). Thus, not only did clozapine display putative anti-oxidant properties, a particularly novel observation, but these data also re-emphasize its potential neuroprotective actions (e.g. Magliaro and Saldanha, 2009). Modulation of redox balance may occur via an indirect anti-oxidant effect. For example, it is well known that clozapine and similar second generation antipsychotics exert beneficial effects on striatal function by preventing excessive dopamine turnover in response to inappropriate D₂ receptor blockade (Harvey et al., 1999). Further, olanzapine, which is structurally similar to clozapine, can reverse haloperidol-induced striatal toxicity by reversing haloperidol-induced nitric oxide synthase inhibition, thereby re-establishing an important protective mechanism against excessive superoxide production (Nel and Harvey, 2003). Nitric oxide is an important downstream messenger of glutamate NMDA activation. Thus, by

Table 1 Behavioural and regional brain redox analysis in socially reared animals receiving either acute saline (n=10) or clozapine (5 mg/kg; n=10) treatment.

Parameter measured	Saline treatment Mean $\pm$ SEM	Clozapine treatment Mean $\pm$ SEM	p value
Behaviour			
Mean startle			
Block 1	147.90 $\pm$ 6.65	142.72 $\pm$ 2.30	p > 0.05
Block 2	129.72 $\pm$ 5.45	121.20 $\pm$ 3.19	p > 0.05
Block 3	124.28 $\pm$ 4.55	121.56 $\pm$ 2.28	p > 0.05
Block 4	122.78 $\pm$ 4.46	120.28 $\pm$ 1.83	p > 0.05
% PPI			
72 dB	46.09 $\pm$ 2.0	46.51 $\pm$ 2.96	p > 0.05
76 dB	52.35 $\pm$ 3.69	53.68 $\pm$ 3.22	p > 0.05
80 dB	62.92 $\pm$ 1.98	64.33 $\pm$ 1.41	p > 0.05
84 dB	69.54 $\pm$ 1.21	70.08 $\pm$ 1.62	p > 0.05
Social interaction			
Rearing (s)	22.39 $\pm$ 1.45	20.63 $\pm$ 2.20	p = 0.51
Anogenital sniffing (s)	26.82 $\pm$ 1.93	26.94 $\pm$ 1.63	p = 0.96
Approaching (s)	21.80 $\pm$ 1.60	23.00 $\pm$ 1.69	p = 0.61
Staying together (s)	112.70 $\pm$ 11.92	120.30 $\pm$ 7.02	p = 0.59
Self interaction			
Squares crossed	233.50 $\pm$ 18.26	242.90 $\pm$ 10.31	p = 0.66
Self grooming (s)	3.65 $\pm$ 0.52	3.29 $\pm$ 0.76	p = 0.70
Redox chemistry			
Lipid peroxidation (pmol/mg protein)			
Frontal cortex	1.11 $\pm$ 0.13	1.06 $\pm$ 0.21	p = 0.84
Striatum	1.51 $\pm$ 0.24	1.41 $\pm$ 0.26	p = 0.78
SOD (U/mg protein)			
Frontal cortex	2.87 $\pm$ 0.19	2.90 $\pm$ 0.19	p = 0.91
Striatum	3.42 $\pm$ 0.28	3.46 $\pm$ 0.14	p = 0.91
GSH/GSSG			
Frontal cortex	0.024 $\pm$ 0.002	0.025 $\pm$ 0.002	p = 0.76
Striatum	0.029 $\pm$ 0.003	0.029 $\pm$ 0.002	p = 0.92

conserving or bolstering NMDA-mediated events (Toua et al., 2010), or by presenting with agonist-like activity (Chen and Yang, 2002; Heresco-Levy, 2003), clozapine may successfully alter redox state in SIR rats.

In conclusion, early life adversity in the form of SIR evokes long-lasting bio-behavioral changes that become evident in adulthood. This study therefore provides further support for SIR as a model of schizophrenia, especially with respect to *simultaneously* documenting an array of aberrant behaviors akin to schizophrenia, namely locomotor hyperactivity, deficits in social and self-directed interactive behaviors, and impaired sensorimotor gating. Moreover, in line with recent clinical evidence in support of a role for altered oxidative status in schizophrenia, we have shown that the above SIR-induced behaviors are related to altered cortico-striatal redox imbalance, thus supporting a novel aspect of construct validity. Importantly, these bio-behavioral changes can be completely reversed by sub-chronic clozapine administration, thus ensuring the relevance of these data for schizophrenia.

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Contributors

M. Möller undertook all the behavioral, laboratory and statistical analyses, and wrote the first draught of the manuscript. BH Harvey designed, supervised and assisted in the interpretation of the study data, as well as finalized the manuscript for submission. JL du Preez supervised all aspects of laboratory analysis. R. Emsley advised on the study design and proof read the final manuscript.

Conflict of interest

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

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