

Chapter 7

Conclusion and recommendations

In this chapter the results from the project as a whole will be summarised and the key findings discussed. I will discuss how the findings from this study have advanced current knowledge and will provide suggestions and/or recommendations for future follow-up studies.

1. Introduction

Schizophrenia is a progressive degenerative illness that has been associated with poor long-term prognosis (Harvey *et al.*, 1999). The illness has been causally linked to environmental and neurodevelopmental factors (Weiss and Feldon, 2001), as well as dysfunctional redox balance (Mahadik and Mukherjee, 1996), mitochondrial dysfunction (Drzyzga *et al.*, 2006; Djordjević *et al.*, 2010), immune dysregulation (Martinez-Gras *et al.*, 2012) and a pro-inflammatory state (Leonard *et al.*, 2012), that in the end are regarded as the initiators of a host of neuroanatomical and monoamine changes that characterise the disease. Psychotic (positive) symptoms are the most distinctive feature of schizophrenia, although negative symptoms such as emotional flattening, social withdrawal and cognitive disturbances are the most treatment resistant manifestation of the illness (Keefe *et al.*, 2007; Marder and Fenton, 2004). These behavioural changes are underscored by a complex array of dopamine (DA), γ -amino butyric acid (GABA) and glutamate disturbances in the frontal cortex and sub-cortical regions of the brain (Reynolds, 2005). Despite the advances that have been made in recent years with regard to its treatment, especially with the introduction of the atypical antipsychotic drugs, treatment remains inadequate both in terms of efficacy and side effects (Harvey *et al.*, 1999). Consequently, there is a drive to better and quicker diagnosis of the illness in order to initiate treatment early, as well as a need to improve our understanding of schizophrenia, both of which will have lasting benefits for the effective management of the disorder. However, in order for this to be achieved, further clinical and pre-clinical research is needed. With regards to the latter, well-validated analogous animal models have a very

important part to play, especially in studying the bio-behavioural factors underlying schizophrenia, and to identify novel neurobiological targets for drug development and treatment of the illness.

Social isolation rearing (SIR) has been suggested to be a useful neurodevelopmental animal model of schizophrenia (Heidbreder *et al.*, 2000), having been found to have important face, construct and predictive validity for the human disorder, including presenting with deficits in sensorimotor gating and social interaction (Varty and Geyer, 1998; Möller *et al.*, 2011 - Addendum C), and reversal of these by antipsychotic drugs (Taylor *et al.*, 2009 for review; Möller *et al.*, 2011 – Addendum C). Recently our group noted that SIR is associated with changes in frontal cortical N-methyl-D-aspartate (NMDA) and D₁ receptor binding (Toua *et al.*, 2009 – Addendum B) and that SIR-induced deficits in social behaviour and sensorimotor gating are correlated with cortico-striatal oxidative stress (Möller *et al.*, 2011 – Addendum C). Like with schizophrenia, SIR therefore induces distinct changes in glutamate activity as well as redox balance, although the source of this disturbance remains unconfirmed. The model has also not been studied with respect to cognitive performance using visual recognition memory in the object recognition test, thus providing a more global view of its face validity. That schizophrenia is associated with redox, immune and mitochondrial dysfunction provides the construct for how this model can be further utilised to glean new information regarding the neurobiology of schizophrenia, viz. its association with a pro-inflammatory state (Leonard *et al.*, 2012), altered tryptophan metabolism via the kynurenine pathway (Myint *et al.*, 2011) as well as altered cortico-striatal adenosine triphosphate (ATP) (Djordjević *et al.*, 2010) and monoamine balance (DA, serotonin (5-HT), noradrenaline (NA); Harvey *et al.*, 1999). These aspects of schizophrenia have never before been studied in this animal model.

This study has therefore investigated the effect of SIR on peripheral pro – and anti-inflammatory cytokines, tryptophan metabolism via the kynurenine pathway, especially the role of QA and KYNA balance on neuroprotective balance, as well as cortico-striatal ATP and monoamine accumulation and metabolism. Moreover, the study related these biochemical changes to changes in social behaviours, visual recognition memory as well as sensory-motor gating; using the open field test (OFT), the object recognition test (ORT) and the prepulse inhibition test (PPI), respectively. Finally, in order to establish whether these bio-behavioural changes following SIR are altered by known treatments for schizophrenia, i.e. predictive validity, I studied whether sub-chronic treatment with the atypical antipsychotic, clozapine, could reverse the above changes. Since the anti-oxidant, glutathione precursor and glutamate modulator (Kerksick and Willoughby, 2005), N-acetylcysteine (NAC) has demonstrated therapeutic potential as an adjunctive treatment for schizophrenia (Berk *et al.*, 2008a; Lavoie *et al.*, 2008), I also evaluated whether the

aforementioned bio-behavioural changes could be reversed with NAC alone and in combination with clozapine (CLZ + NAC), thereby confirming the causal role for glutamate-redox dysfunction in SIR-induced immune-neurochemical and behavioural changes, and therefore strengthening the predictive and construct validity of the SIR model.

2. Summary of results

Since the results of the study are presented in separate sections in Chapter 3, 4, 5, 6, either as published or “in submission” manuscripts, and in Addendum A, a concise summary of all the results will be provided here. This study successfully achieved all the aims as outlined in Chapter 1, section 3, namely:

- The development and validation of a rapid, specific solid-phase extraction (SPE) – liquid chromatography electrospray ionization tandem mass spectrometry (LC-MS/MS) method. The method for assaying tryptophan, kynurenine, kynurenic acid (KYNA), 3-hydroxyanthranilic acid (3-OHAA), anthranilic acid and QA in rat plasma, presented with good linearity ($R^2 > 0.95$) and all the validation parameters were within acceptance range. A small application study in rats demonstrated its successful development and validation.
- *Face validity of SIR*: Rats reared in isolation (SIR) for 8 weeks showed marked deficits in psychomotor abilities (sensorimotor gating, as assessed by %PPI of the startle response), psychosocial (social interactive behaviours), as well as a significant increase in hyper-active / anxiety (self-directed behaviours) parameters compared to socially reared controls. In addition, SIR rats also presented with marked cognitive deficits, as presented in the form of deficits in visual recognition memory, compared to socially reared controls.
- *Construct validity of SIR*: SIR induced behavioural alterations was associated with disordered plasma kynurenine metabolism (indicative of a decrease in neuro-protective balance), increased pro-inflammatory cytokines (TNF- α , IFN- γ) as well as decreased anti-inflammatory cytokine (IL-4) and IL-6 (*dual action as pro-and anti-inflammatory cytokine*), compared to their socially reared controls.

In addition, SIR also induced deficits in frontal cortical ATP, DA, 3,4-dihydroxyphenylacetic acid (Dopac), homovanillic acid (HVA), 5-HT and hydroxyindoleacetic acid (5-HIAA) as well as elevated striatal ATP, DA, Dopac, HVA, 5-HT and 5-HIAA, along with cortico-striatal NA and 3-methoxy-4-

hydroxyphenylethylene glycol (MHPG) alterations, compared to their socially reared controls.

- *Predictive validity of SIR:* All of the observed behavioural (social interaction, PPI, object recognition memory), peripheral (kynurenine metabolism, cytokines) and neurochemical (ATP, DA, 5-HT, NA, Dopac, HVA, 5-HIAA, MHPG) changes induced by SIR as described above were reversed by sub-chronic treatment with clozapine. Moreover, NAC partially yet significantly reversed most of the SIR induced bio-behavioural alterations (except for elevations in plasma tryptophan, kynurenine and anthranilic acid, as well as striatal ATP and frontal cortical NA). In addition, the dose response study with NAC (50, 150, 250 mg/kg/day) indicated that NAC 150 mg/kg was the most appropriate dose to use for the combination treatment. Thus, an important observation was that CLZ + NAC 150 mg/kg was more effective than clozapine alone in reversing SIR induced changes in %PPI deficits, KYNA deficits, QA elevations, neuroprotective ratio deficits, IL-4 deficits, frontal cortical ATP, DA, Dopac, HVA, 5-HT and 5-HIAA deficits, and striatal DA, Dopac, HVA, 5-HT and 5-HIAA elevations.
- The fact that NAC treatment alone and in combination with clozapine reversed all these bio-behavioural changes provides novel evidence for construct validity of the SIR model as well.
- In addition, NAC's ability to reverse the alterations in cortico-striatal DA, Dopac, HVA, 5-HT, 5-HIAA, NA (striatal) and MHPG also emphasizes its possible therapeutic potential not only in schizophrenia but also in anxiety and depressive disorders.

3. Novel findings and conclusion

No single animal model can possibly replicate the overall symptomology of schizophrenia, which in itself is a heterogeneous, polygenetic disorder. However, this study has demonstrated that SIR in rats, which also emphasises early-life adverse environment exposure, can produce a range of reproducible, long-term changes in behaviour that are closely related to some of the typical symptoms and manifestations of schizophrenia. These behaviours include significant deficits in PPI, deficits in social interactive behaviours, and an increase in self-directed behaviours as well as deficits in visual recognition memory. The latter observation is novel and has, to the best of my knowledge, not been observed during simultaneous evaluation in the SIR model, and provides further evidence of the robust face

validity of the model. Moreover, in line with the efficacy of clozapine to treat both positive and negative symptoms of schizophrenia, all the above behaviours were effectively reversed following sub-chronic clozapine treatment, again a novel finding and evidence for the important predictive validity of the model. In addition, we also observed that NAC alone partially yet still significantly reversed these behavioural alterations, strengthening the predictive validity of this model even more. Importantly, in line with clinical evidence, I also found that the combination of CLZ + NAC completely reversed these changes, and also proved to be more effective than clozapine in certain parameters.

My study also provides the first robust evidence that SIR is associated with an inflammatory state, including a profound disruption of peripheral pro- and anti-inflammatory cytokines and tryptophan metabolites via the kynurenine pathway, as well as an overall decrease in neuroprotection. In addition, this study has provided novel evidence of cortico-striatal disruptions in mitochondrial function (ATP). Finally, these immune-inflammatory and redox disturbances occur simultaneously with altered accumulation and metabolism of cortico-striatal monoamines supportive of the DA hypothesis of schizophrenia, viz. frontal cortical hypo-dopaminergia (hypo-frontally) as well as striatal hyper-dopaminergia. Moreover, most of these changes relating to a pro-inflammatory state, altered cortico-striatal ATP and monoamines were reversed by sub-chronic clozapine (completely), NAC (partially) as well as the combination of CLZ + NAC (completely) treatment, thus providing novel predictive and construct validity for schizophrenia.

Thus, my results can be summarised as follows (see figure 1), (A) SIR as a post-natal stressor may cause mitochondrial dysfunction and increased reactive oxygen species (ROS) leading to (B) a peripheral pro-inflammatory response with (C) altered cytokine release (increased pro-inflammatory and decreased anti-inflammatory cytokines) which is responsible for (D) activation of indoleamine 2,3, dioxygenase (IDO), increasing the production of the neurotoxic quinolinic acid (QA) via the kynurenine pathway. The decrease in neuroprotective ratio in the kynurenine pathway along with increased QA causes (E), dysfunctional glutamate release. Glutamate-GABA pathways are affected and contribute to (F) altered cortico-striatal monoamine release, which in turns, is expressed as (G), positive-like (hyper-striatal) and negative-like (hypo-frontality) behavioural manifestations akin to schizophrenia.

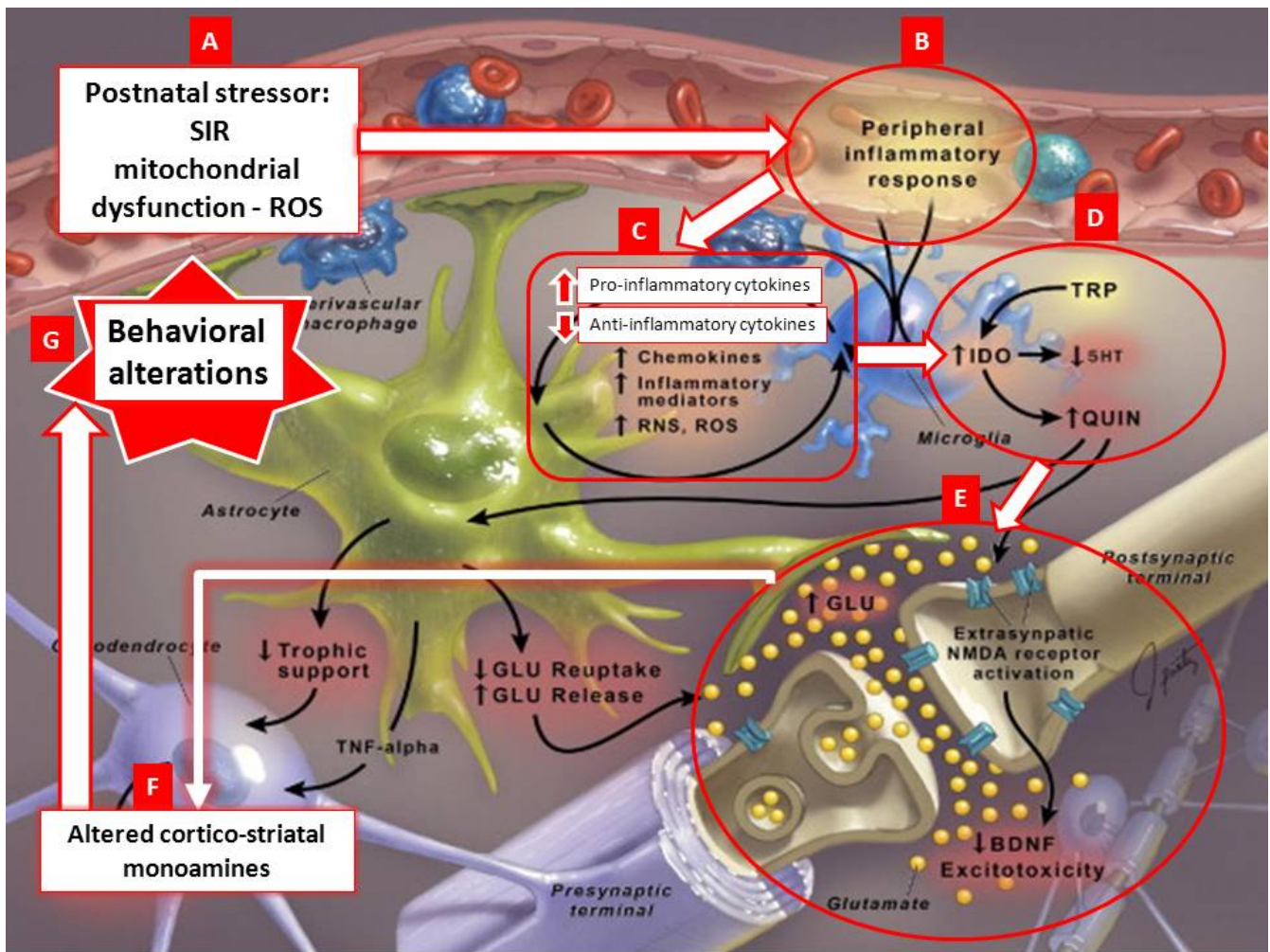


Figure 1: A simplified diagram, depicting the pro-inflammatory cascade observed in the SIR model and, by implication, in schizophrenia, causing numerous neurochemical alterations that could be related to the expression of positive and negative symptomatology (Adapted from: Miller *et al.*, 2009).

Moreover, clozapine, NAC and CLZ + NAC treatment reversed most of these bio-behavioural alterations in the SIR model, emphasizing the causal role of glutamate-redox abnormalities. However, the pathophysiology of schizophrenia is much more complex, and includes numerous brain regions, neurotransmitter pathways, environmental stressors and genetic predispositions; ultimately contributing to the symptomology. Clearly figure 1 is an over-simplified representation of our results using the SIR model. Nevertheless, it has provided new evidence that will assist in our understanding of the neurobiology and treatment of schizophrenia, and in identifying possible new biomarkers such as the tryptophan-kynurenine pathway.

An important observation of this study was that the combination treatment of CLZ + NAC was more effective than clozapine alone in reversing some of these bio-behavioural changes. Since Chapter 5 (article 3) indicates a commonality in neurobiological targets for

these two agents, it highlights a possible mechanism whereby NAC bolsters the anti-psychotic effects of clozapine, thus opening the window on new treatment strategies for schizophrenia.

Social isolation rearing, as an environmental stressor, therefore provides robust translational relevance to the core deficits in schizophrenia (positive and negative symptoms) as well as of its neurobiological underpinnings, i.e. a pro-inflammatory state (cytokines and related kynurenine metabolism), cortico-striatal mitochondrial and monoamine alterations. Thus, the model provides a useful and valid approach to investigating the neurodevelopmental aetiology of schizophrenia in order to learn more on this devastating illness and to identify longitudinal biomarkers of the illness. My results also suggest that SIR may serve as a valuable predictive screen for novel compounds with potential antipsychotic efficacy.

Finally, this study has identified numerous biomarkers that may assist in early onset schizophrenia, especially where a pro-inflammatory-state is presented that is amenable to early intervention with clozapine plus NAC. This will aid in preventing the development of long-term neurodevelopmental-neurodegenerative phenomena of schizophrenia and more favorably influence treatment outcome.

4. Recommendations for future studies

- In order to provide further insight into the underlying mechanisms involved in the neurodevelopment and aetiology of schizophrenia, evaluating another neurodevelopmental animal model, such as the immune activation model (Zuckerman *et al.*, 2003 and 2005), would be imperative. Penner and Brown (2007) examined medical records of over 12000 pregnant women and found that maternal infection increases the risk of schizophrenia in the offspring 2-fold. It will therefore be of considerable value to characterize maternal infection in an animal model, and its effects on behaviour and biology later in life.

In order to mimic these infections, pregnant rats are injected with a synthetic cytokine inducer such as influenza virus, poly (I:C) or lipopolysaccharide (LPS). The offspring of influenza and poly (I:C) challenged animals display a similar array of behavioural abnormalities described in SIR, including social interaction, PPI, open field latent inhibition and novel object recognition (Zuckerman *et al.*, 2003 and 2005; Meyer *et al.*, 2005; Wolff and Bilkey, 2010). Thus, evaluating these models with the same

study design as in my study by including the same bio-behavioural parameters will provide us with novel insight on the immune development of schizophrenia and therefore also a new approach to treating the illness.

- Another important aspect in treating schizophrenia is the duration of untreated psychosis (DUP) and its association with treatment resistance (Amminger *et al.*, 2002). Understanding the neurobiological basis for treatment resistance remains a crucial endpoint in schizophrenia research (Emsley *et al.*, 2012). Indeed, redox active molecules, such as nitric oxide, have been implicated (Emsley *et al.*, 2012). Although difficult to model DUP in an animal, studies investigating different durations of isolation rearing, e.g. 4, 8 and 12 weeks, and how these impact on the degree of bio-behavioural response in the animals, as well as response to antipsychotic treatment and/or an antioxidant such as NAC, would be a useful extension of the validity of the SIR model.
- With the establishment of SIR as a non-pharmacological animal model of schizophrenia in our laboratory, new experimental compounds with novel antipsychotic properties could be tested, for example the nitric oxide synthase-guanlyl cyclase inhibitor, methylene blue.
(<http://clinicaltrials.gov/show/NCT00214877>).
- Since volumetric brain reductions have been observed in schizophrenia patients (Wright *et al.*, 2000; Thompson *et al.*, 2001), reflecting not only neuronal atrophy but also loss of glia and interneurons, histological, immune-histochemical and stereological techniques could be used to quantify neurogenesis, cytomorphology, neuronal numbers and the volume of sub-cortical regions and frontal cortex in SIR versus socially reared animals.