# Chapter 1 Introduction

## 1. Thesis layout

This PhD thesis is compiled in the format of the article model, as approved by the North-West University. Thus, the main body of the thesis (methodology and experimental data) will be presented as various manuscripts that have either already been published in an international peer-review journal, or are currently in submission.

The *Introduction* chapter (Chapter 1) provides a general and concise orientation to the thesis and study, including the problem statement, study hypothesis, aims and study layout. A review of the relevant literature background of the study is provided in Chapter 2, and will be more comprehensive than that presented in the various manuscripts. Chapters 3, 4, 5 and 6 will contain the key findings of the project in the form of three already published and one "ready for submission" manuscripts. These manuscripts have been prepared for submission to a specific neuroscience journal that will be indicated at the start of each of these chapters. Already published manuscripts will be presented as they appear in the indicated journal, while the "ready for submission" manuscript is prepared in accordance with the house style of that particular journal. The latter will be as laid down in the "Instructions to the Author" provided by the publisher (refer to the respective web-links for each journal provided). Three of these manuscripts have been published (in *J Chromatography B* and *Neuropharmacology*), (in *Brain Behavior and Immunity)* and one as a "ready for submission" manuscript *(*in *Metabolic Brain Disease)*. A final chapter (Chapter 7), summarizes, discusses and concludes the study as a whole, incorporating all four manuscripts, as well as the data presented in the addenda. Addendum A contains additional method validation and other methodologies as well as additional results not included in the manuscripts. For the benefit of the reader, two published manuscripts describing work undertaken during the candidates MSc, and that were central in setting the course for the current PhD study, are provided in Addendums B and C. The latter two articles are intended for background information only and are not for examination purposes. The references for the manuscripts are provided

separately for each paper, whereas the bibliography for the sources referred to in the other chapters are presented at the end of the thesis.

## 2. Problem statement

Schizophrenia affects about 24 million people worldwide. More than 50% of persons with schizophrenia are not receiving appropriate care while 10% of patients will commit suicide (World Health Organization, 2012).

The underlying neurobiology of schizophrenia remains uncertain despite an intense research effort. This lack of understanding in many ways is responsible for our less than adequate management of the illness. Although second-generation antipsychotics, previously referred to as “atypical”, have been lauded for their comparable efficacy against the positive symptoms of schizophrenia, as well as improved negative symptom efficacy over earlier first-generation agents (Lidow *et al*., 1998), this has been challenged by the Clinical Antipsychotic Trials of Intervention Effectiveness (CATIE) study (Lieberman *et al*., 2005). This study suggests that first- and second-generation antipsychotic drugs are essentially 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). Moreover, most of the currently used new generation drugs remain poorly tolerated, especially concerning metabolic side effects that contribute to high rates of treatment discontinuation (Lieberman *et al*., 2005). Consequently there is not only a drive to learn more of the underlying cause of the illness, but a need to identify new drug targets and to develop new antipsychotic medications with an improved therapeutic outcome.

Animal models represent one of the foremost approaches with which to reach the above-mentioned goal. However, such models need to be well validated with respect to the human disorder they are attempting to model. Social isolation rearing (SIR) is one such model that demonstrates strong face, construct and predictive validity for schizophrenia (see (Geyer and Swerdlow, 2001; Weiss and Feldon, 2001; Ayhan *et al*., 2009 for review). Recently we established that SIR induces dopamine (DA) $D_1$ and glutamatergic N-methyl-D-aspartate (NMDA) receptor changes in the frontal cortex of rats. Furthermore, these receptor changes are differently affected by first or second generation antipsychotics (Toua *et al*., 2010; see Addendum B). This not only suggests a disturbance of $D_1$-NMDA receptor function underlying the cognitive disorders of schizophrenia, but may also explain how antipsychotic

drugs can selectively target these processes for improved response of negative symptoms (Toua *et al*., 2010; see Addendum B). There is also evidence that schizophrenia may be causally related to altered states of redox balance, especially increased oxidative stress (Akyol *et al*., 2002a, b; Ng *et al*., 2008). Indeed, new work from our laboratory using the SIR model has drawn the important association between disturbed markers of oxidative stress with behaviours related to that seen in patients with schizophrenia, namely reduced sensorimotor gating and disturbances in social interactive behaviours (Möller *et al*., 2011, see Addendum C). Targeting altered redox balance in schizophrenia may therefore represent an important new therapeutic target, although identifying the source of the apparent redox imbalance is critical before deciding on an appropriate pharmacological intervention strategy.

One possible source of increased oxidative stress may arise from altered tryptophan metabolism via the kynurenine pathway. Indeed a number of studies have demonstrated an imbalance in kynurenine metabolism in schizophrenia, particularly an increase in neurotoxic quinolinic acid (QA), a decrease in neuroprotective kynurenic acid (KYNA) and a resulting decrease in the neuroprotective ratio (Torrey *et al*., 1998; Miller *et al*., 2008; Myint *et al*., 2011). Important to note is that an imbalance in kynurenine metabolism may be evoked by a disturbance in the release of pro- and anti-inflammatory cytokines via modulation of indoleamine-2,3-dioxygenase (IDO), the key synthesizing enzyme in the kynurenine pathway (Myint *et al*., 2007b). Indeed, altered release of pro- and anti-inflammatory cytokines is also well-described in schizophrenia (Martinez-Gras *et al*., 2012; Miller *et al*., 2011), with evidence suggesting the presence of a pro-inflammatory state (Leonard *et al*., 2012).

A second source of increased oxidative stress may ensue via altered mitochondrial function (Kato *et al*., 2010). A disturbance in mitochondrial function is a well described abnormality in schizophrenia (Drzyzga *et al*., 2006; Djordjević *et al*., 2010; Myint *et al*., 2011; Miller *et al*., 2008; Schwarcz *et al*., 2001; Potvin *et al*., 2008; Prabakaran *et al*., 2004). Mitochondrial adenosine triphosphate (ATP) synthesis is frequently disrupted by damage to the respiratory chain, thereby contributing to cell death and dysfunction (Wallace *et al*., 2010). Defective mitochondrial ATP supply also leads to calcium dys-homeostasis, allowing cytosolic calcium levels to rise above the normal signalling range which in turns activates the generation of reactive oxygen species (ROS) (Mammucari *et al*., 2011). Oxidative damage, defective ATP synthesis and calcium dys-homeostasis frequently occur together, leading to a vicious cycle that culminates in cell damage.

Schizophrenia is closely related to neurotransmitter dysfunction, particularly with respect to dopamine (DA), serotonin (5-HT) and noradrenaline (NA) as well as their metabolites, homovanilic acid (HVA), 3,4- dihydroxyphenylacetic acid (Dopac), 5-hydroxyindoleacetic acid (5-HIAA) and 3-methoxy-4-hydroxyphenylethylene glycol, (MHPG). Perturbations in DA signalling is the best recognized, especially in lieu of the fact that all currently available antipsychotic drugs are potent DA $D_2$ receptor antagonists (Harvey *et al*., 1999). According to the DA hypothesis, schizophrenia is associated with frontal DA hypo-activity (indicating hypo-frontality) (Riehemann *et al*., 2001) and striatal DA hyper-activity (reviewed in Guillin *et al*., 2007a). In order to address construct validity, any new model or theory of schizophrenia should incorporate elements of the DA hypothesis in its presentation, e.g. effects on cortico-striatal DA release and/or metabolism.

Although various neurobiological targets may be responsible for elevated oxidative stress induced by SIR, this study will follow the cue provided by earlier work undertaken in our laboratory, viz. altered excitatory glutamatergic transmission and altered redox homeostasis (Toua *et al*., 2010; Möller *et al*., 2011, see Addendums B and C). Consequently, I will focus on the role of the glutamate-active components of the kynurenine pathway, pro-and anti-inflammatory cytokines, mitochondrial function, and regional brain monoamine levels, and how changes in these processes may be related to the expression of schizophrenia-like behaviours in rats subjected to SIR. Since more than 60% of brain kynurenine is contributed from the periphery (Heyes *et al*., 1997), and in order to relate my data to that described in patients with schizophrenia, I will investigate the effect of 8 weeks SIR on plasma tryptophan metabolites via the kynurenine pathway, focusing especially on disturbances that alter neuroprotective versus neurodegenerative pathways. Due to the association between altered kynurenine balance and immune function, I will also measure plasma levels of select groups of pro- and anti-inflammatory cytokines, including tumour necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), interleukin (IL)-4 and IL-6. Since altered mitochondrial function may also contribute to altered redox state previously observed in SIR animals, I will determine ATP as an indicator of mitochondrial function in the frontal cortex and striatum. Since all the above changes are expected to translate into changes in cortico-striatal monoamines responsible for the primary symptoms of schizophrenia, I will measure cortico-striatal levels of DA, 5-HT, NA and related metabolites, Dopac, HVA, 5-HIAA and MHPG under conditions of SIR. These biological changes will be related to concomitant changes in behavioural responses akin to schizophrenia, including deficits in social versus self-directed social interactive behaviours, locomotor activity, deficits in visual learning and memory, and deficits in sensory-motor gating. Finally, I will attempt to reverse the above bio-behavioural

changes following SIR with the novel second generation antipsychotic, clozapine and in so doing confer valuable predictive validity to these findings. A previous study in our laboratory has in fact found that both behavioural and redox changes induced by SIR can be reversed by clozapine (Möller *et al*., 2011, see Addendum C). Apart from the important aspect of predictive validity that clozapine response will introduce into the study, how it is able to modulate redox homeostasis is an enduring enigma that requires clarification. This study will also provide new information on this question.

The glutathione precursor, N-acetyl cysteine (NAC), has been found to prevent striatal oxidative stress in rats *in vivo* (Harvey *et al*., 2008), and to offer therapeutic benefits as an adjunctive treatment in schizophrenia (Berk *et al*., 2008a; Bulut *et al*., 2009). Not only is NAC an effective antioxidant, it also indirectly activates cysteine-glutamate antiporters to regulate glutamate levels (Chen *et al*., 2010), thus of relevance for the glutamate dysfunction theory of schizophrenia (Konradi and Heckers, 2003). I will attempt to establish whether NAC alone is able to offer similar efficacy to clozapine in reversing the bio-behavioural changes engendered by SIR. Moreover, I will establish whether it is able to bolster the response to clozapine, thus in line with the presented clinical evidence. In addition, NAC has also found clinical utility in treating other psychiatric conditions, such as depression and bipolar disorder (Berk *et al*., 2008b), as well as various anxiety disorders such as obsessive compulsive disorder (Lafleur *et al*., 2006), trichotillomania or hair pulling (Grant *et al*., 2009) and cocaine addiction (LaRowe *et al*., 2006). In line with this, I will also evaluate NAC's effect on SIR-induced alterations on cortico-striatal 5-HT, DA, NA and their related metabolites. These data may reveal more on how NAC is able to favourably affect the treatment of the aforementioned illnesses.

To summarise, the principal objectives of this study will therefore be (1) to establish a cause for the altered glutamate-redox balance observed in rats reared in isolation, as described in earlier work (Toua *et al*., 2010; Möller *et al*., 2011), (2) to establish whether these possible biological causes may be reversed by sub-chronic antipsychotic treatment, and (3) whether an antioxidant is effective in reversing the aforementioned changes, and possibly bolster the response to an antipsychotic. These objectives have to the best of my knowledge never been studied in a neurodevelopmental animal model, and are outlined in greater detail below.

## 3. Study hypothesis and aims

*Hypothesis:*

A. I hypothesize that rats subjected to SIR will present with various behavioural, mitochondrial, neurochemical and immune-inflammatory markers akin to that seen in patients with schizophrenia, including:

- Sustained deficits in visual learning and memory, social interaction, locomotor activity and sensory motor gating.
- Altered plasma kynurenine:KYNA balance indicative of increased neurodegeneration / reduced neuroprotection, as well as increased levels of the neurotoxin QA.
- Evidence of increased pro-inflammatory and reduced anti-inflammatory cytokines in plasma.
- Altered mitochondrial ATP levels in the frontal cortex and striatum of rats exposed to SIR indicative of mitochondrial dysfunction.
- Disturbed DA metabolism predictive of the DA hypothesis of schizophrenia, viz. frontal cortical hypo-dopaminergia and striatal hyper-dopaminergia, will be evident in SIR animals. Changes in 5-HT, DA and NA metabolism will be commensurate with various other symptomologies evident in schizophrenia, such as depression, cognitive deficits, feelings of persecution and obsessions, social withdrawal, alogia, decline in motivation and reduced emotional reactivity.

B. I propose that the bio-behavioural changes induced by SIR will demonstrate response to drug treatment as follows:

- All SIR induced behavioural, mitochondrial, immune-inflammatory and neurochemical changes will be reversed by sub-chronic clozapine treatment.
- Sub-chronic NAC treatment will demonstrate dose-dependent efficacy on selected behavioural, mitochondrial, immune-inflammatory and neurochemical parameters, but less effective than clozapine.
- I propose that the combination of clozapine and NAC following sub-chronic treatment will be equal or superior to that of clozapine alone in reversing the behavioural, mitochondrial, immune-inflammatory and neurochemical alterations induced by SIR.

*Study aims:*

- To develop a rapid and specific solid-phase extraction (SPE) -liquid chromatography electrospray ionization tandem mass spectrometry (LC-MS/MS) method that will me*et all* the required validation parameters, for assaying tryptophan, kynurenine, KYNA, 3-hydroxyanthranilic acid (3-OHAA), anthranilic acid and QA in rat plasma.
- *Face validity of SIR:* Studying the severity of 8 weeks SIR in rats on various psychomotor (prepulse inhibition (PPI) test), and psychosocial (social interaction test) parameters compared to that in socially reared control animals, with the additional intent of evaluating cognitive parameters of memory (viz. novel object recognition (NOR)).
- *Construct validity of SIR:* To investigate whether altered behaviours in SIR rats are associated with disordered plasma tryptophan metabolism, altered cortico-striatal ATP, DA, 5-HT, NA and related metabolite levels, as well as increased plasma pro-inflammatory activity and/or decreased anti-inflammatory activity, compared to socially reared control animals.
- *Predictive validity of SIR:* To determine whether any of the observed behavioural, mitochondrial, immune-inflammatory and neurochemical changes induced by SIR as described above can be reversed by sub-chronic treatment with clozapine. However, since the emphasis is on redox imbalance, an additional treatment outcome in this study will be to determine whether the above bio-behavioural changes can be reversed with the antioxidant and glutathione precursor, NAC. In addition, I will also assess whether the combination of NAC plus clozapine offers any advantage over clozapine treatment alone in reversing the behavioural, mitochondrial, immune-inflammatory and neurochemical changes induced by SIR.
- Reversal of all these changes with NAC treatment alone, and/or in combination with clozapine, will also provide novel evidence for construct validity of the SIR model.

## 4. Study layout

The thesis comprises four clearly defined studies that directly relate to the above aims, each being presented sequentially as published or submitted manuscripts as follows:

- The development and validation of a novel liquid chromatography tandem-mass spectrometry (LC-MS/MS) method for the assay of tryptophan and its kynurenine metabolites in rat plasma (published).
- A study where plasma tryptophan metabolism is studied in SIR and socially reared rats with or without sub-chronic clozapine treatment (published).
- Taking further the findings described in the aforementioned study, a bio-behavioural-neurochemical study in SIR vs. socially reared rats where changes in visual learning and memory, social interaction and sensory motor gating (as determined by %PPI), are determined with respect to concomitant changes in plasma levels of pro- and anti-inflammatory cytokines and tryptophan metabolism, as well as cortico-striatal changes in DA and mitochondrial function (ATP levels), and subsequent modulation by clozapine or NAC treatment (published).
- A neurochemical study in SIR vs. socially reared rats where changes in cortico-striatal 5-HT, DA and NA metabolism are studied following a dose-response challenge with NAC (“concept article”).

### *4.1. **Article 1:** LC-MS/MS method development for the assay of plasma tryptophan and its kynurenine metabolites in rat plasma*

Before the study could begin in earnest, it was incumbent on me to develop a novel method for the determination of tryptophan metabolites (viz. tryptophan, kynurenine, KYNA, anthranilic acid, 3-OHAA, QA and picolinic acid) in rat plasma using solid phase extraction (SPE) followed by reversed phase chromatography and mass spectrometry. This paper also includes an *in vivo* application study in rats as a prerequisite for validation of the analytical technique.

***4.2. Article 2:*** *Tryptophan metabolism in SIR vs. socially reared animals and response to sub-chronic clozapine treatment*

The study of tryptophan metabolism in SIR vs. socially reared rats, and its response to sub-chronic clozapine treatment, is outlined in figure 1. Six groups of animals were randomly separated at post natal day (PND) 21 into either SIR (3 groups) or socially reared (3 groups) animals and maintained under these housing conditions for a period of 8 weeks. Both SIR and socially reared animals then received either: no treatment, vehicle or clozapine intraperitoneally (i.p) at a dose of 5 mg/kg in 0.5 ml (Toua *et al*., 2010; Möller *et al*., 2011) for the last 11 days of the specified rearing condition. At the end of the 8 week housing period, the animals were sacrificed and plasma collected for the assay of tryptophan metabolites.

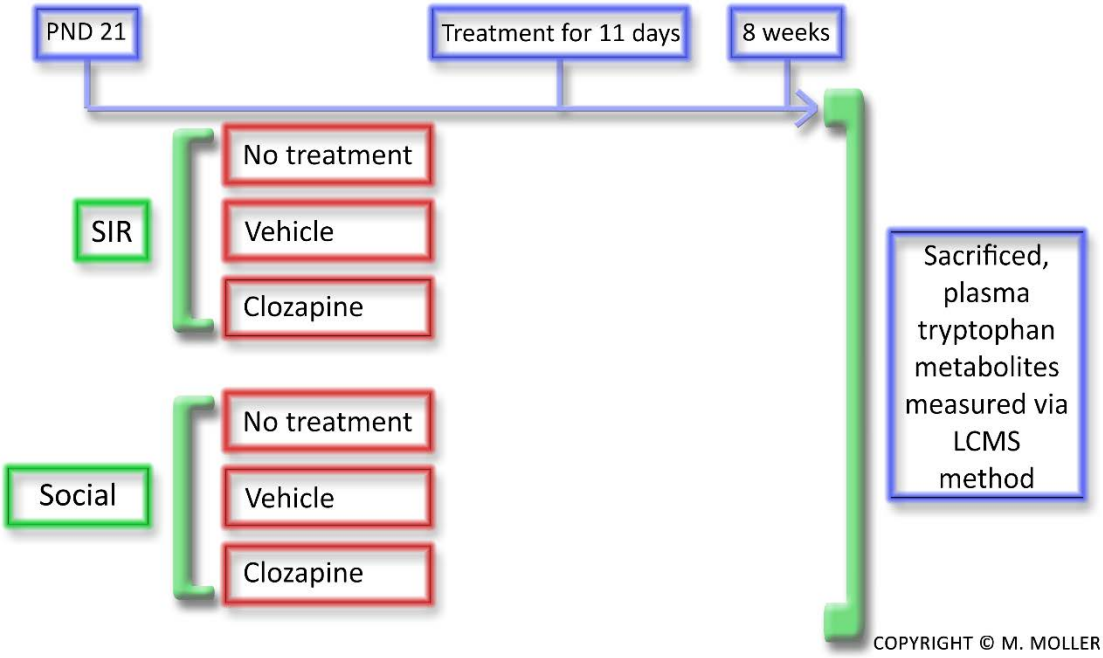


**Figure 1:** Study design for tryptophan metabolism in SIR vs. socially reared rats and following either: no treatment, vehicle treatment or clozapine, as described in article 2.

***4.3 Article 3:*** *Behavioural, immune-inflammatory, mitochondrial and neurochemical changes in SIR vs. socially-reared rats, and response to sub-chronic clozapine or NAC treatment*

This large multiple parameter study in SIR vs. socially reared rats, and response to sub-chronic clozapine, plus a dose-response analysis of NAC, are outlined in figure 2 & 3. Animals were randomly divided into two cohorts: a behavioural (14 groups) and immune-

inflammatory-neurochemical cohort (14 groups), as illustrated in figure 2 & 3 respectively. These two cohorts were then randomly allocated to either SIR (7 groups) or socially reared (7 groups) animals, receiving one of the following treatments: no treatment, vehicle and clozapine intraperitoneally (i.p) at a dose of 5 mg/kg in 0.5 ml (Toua *et al*., 2010; Möller *et al*., 2011) or NAC (50, 150 and 250 mg/kg i.p in 0.5 ml) (West *et al*., 2007; Dean *et al*., 2009; Ferreira *et al*., 2008). In this study, drug treatment took place during the last 14 days of the specified rearing condition. Following the dose-ranging study with NAC, the most effective dose of NAC was selected and used in a subsequent study where animals received a combination of clozapine and NAC (CLZ + NAC). After 8 weeks isolation or social rearing, the rats were tested with regard to social interactive behaviour (day 12 of treatment), visual learning and memory using the NOR test (day 13 of treatment), and %PPI (day 14 of treatment) (the behaviour cohort; figure 2). In the immune-inflammatory-neurochemical cohort (figure 3), the animals were sacrificed after the 8 week rearing condition, trunk blood was collected and the brain (frontal cortex and striatum) dissected for analysis of plasma pro- and anti-inflammatory cytokines and kynurenine metabolism, as well as cortico-striatal ATP and DA levels, respectively.

In order to contain the length and bulkiness of the paper, and to maintain a strong focus on schizophrenia, only the effects on DA are reported here. The effects of NAC on Dopac, HVA, 5HT, NA and metabolism are reported in article 4, and the effect of clozapine and CLZ + NAC on Dopac, HVA, 5-HT, NA and metabolism are relocated to Addendum A. The *Brain Behaviour and Immunity* referees also requested to reduce the amount of data presented in this manuscript, thus, all the socially reared (except the social reared, vehicle treatment group) and NAC dose response data will be presented as supplementary data. With regards to the kynurenine pathway, we will only present the kynurenine, KYNA, QA and neuroprotective ratio in the manuscript, relocating the tryptophan, anthranilic acid and 3-OHAA to Addendum A.

### ***4.4. Article 4:*** *Investigation into the effects of SIR on cortico-striatal DA, 5-HT and NA metabolism, and response to NAC treatment*

This study had exactly the same design and the same animals used in study 4.3. (figure 3), where cortico-striatal DA, HVA, 5-HT, 5-HIAA, NA and MHPG were analyzed in SIR vs. socially reared controls, and following a dose-response analysis with sub-chronic NAC treatment only. In order to allow more meaningful evaluation and interpretation of the effects of NAC on monoamines, DA was included here and in article 3. This apparent re-use of data

is declared in article 4 and was deemed necessary to allow for greater clarity and interpretation of findings described in this last “ready for submission” manuscript. A license for the re-use of data will be obtained from the publishers of article 3 and will be submitted with article 4. This manuscript has been written with the aim of providing new information on the effects of NAC on regional brain monoamine accumulation and metabolism, with a specific emphasis on its possible therapeutic utility in addressing certain co-morbid symptoms of schizophrenia, but also to reveal more of how NAC may be of value in treating other disorders, such as depression and anxiety disorders. All these findings will be brought together in the concluding chapter (Chapter 7) where data described in all four articles and the Addenda will be discussed and interpreted.

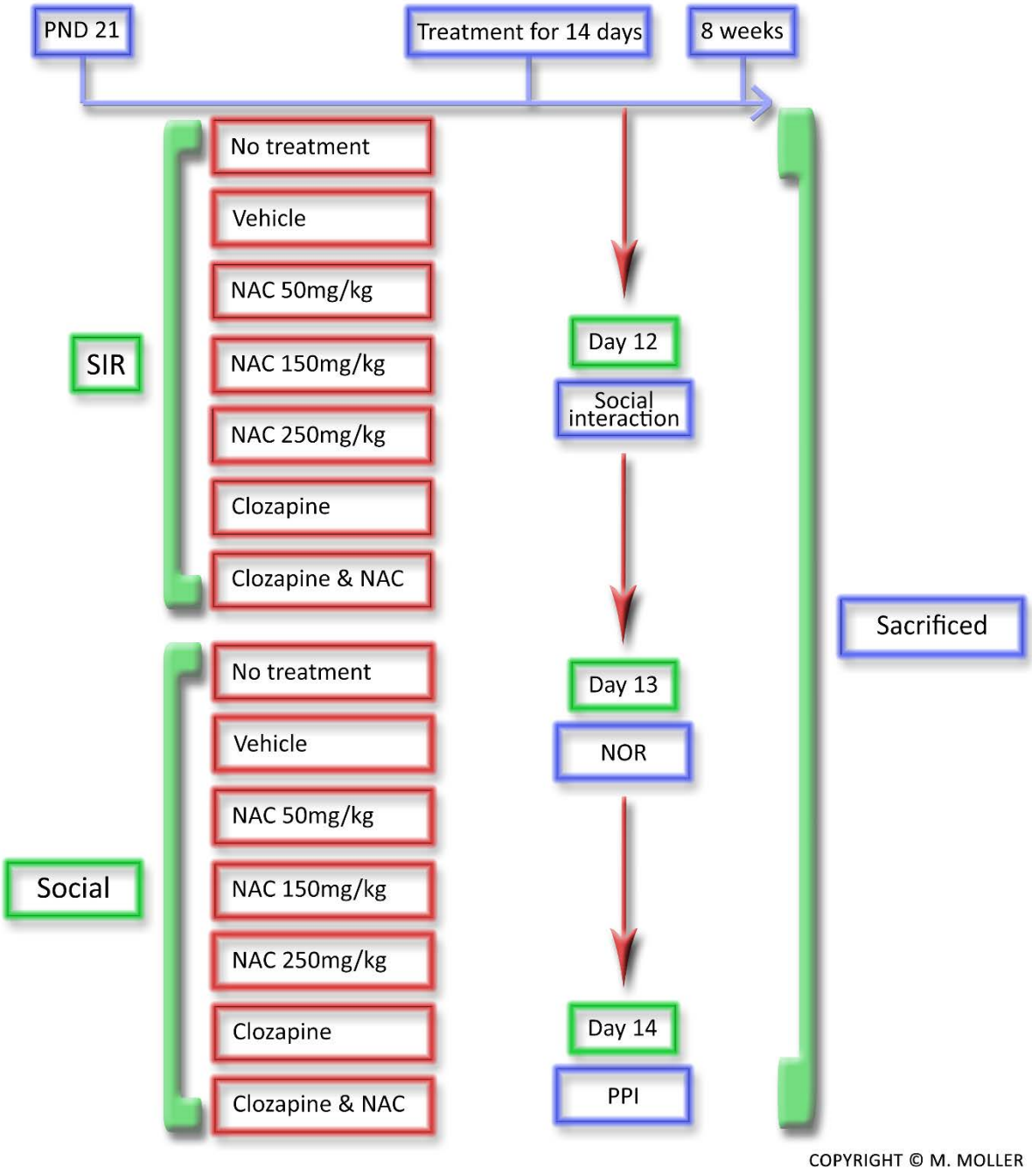


**Figure 2:** Behavioural cohort for the study described in 4.3, in SIR and socially reared rats, with selected treatments.


**Figure 3:** Immune-inflammatory-neurochemical cohort for studies described in 4.3 and 4.4 in SIR and socially reared rats, with selected treatments.